Evaluating wild and commercial populations of *Bombus terrestris* ssp. *audax* (Harris, 1780): from genotype to phenotype.

A thesis submitted to the Maynooth University,

for the degree of Doctor of Philosophy

by

Sarah Jane Larragy B.A.

February 2023

Supervisor

Dr Jim Carolan,

Applied Proteomics Lab,

Dept. of Biology,

Maynooth University,

Co. Kildare

Head of Department

Prof Paul Moynagh,

Dept. of Biology,

Maynooth University,

Co. Kildare.

Table of contents

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Acknowledgements

The completion of my PhD research and the production of this thesis would not have been possible without the support of my colleagues, family and friends. They say it takes a village to raise a child; the same can certainly be said for a PhD student!

Firstly, I cannot thank my supervisor Jim Carolan enough for his dedication and belief in me throughout my PhD experience. From my first stint in the Applied Proteomics lab as an intern, I have always had so much admiration for your commitment to the education and wellbeing of your students. Receiving your encouragement to do a PhD will always feel like one of the best opportunities I have ever been offered, and I will always be grateful for it. Over the last 4+ years, you have been an endless well of support and advice, as well as a provider of much needed laughs and chats through all the blood, sweat and tears. I respect you so much not only as a scientist, but as a person and, somehow, I knew I came to the right place when I landed in 'the lab that helps people' (a.k.a. the lab that can't say no). I don't know what I did in a past life to deserve you as my supervisor and to become a member of the Applied Proteomics lab, but I certainly hope I do as well in this life. It really has been such an honour to work alongside you.

I also thank my co-supervisor Jane Stout who has been inspiring me since my TCD undergraduate days and her lectures in 'Entomology' and 'Plant and Animal Interactions'. I am so grateful to have had her on my supervisory team, for her knowledge and expertise as well as her good humour! I don't think I've ever had a meeting with either Jim or Jane end without a good laugh and my feelings of stress significantly eased. I also extend my deepest gratitude to Joe Colgan, my unofficial ex-situ supervisor! Thank you so much for taking me on and introducing me to the world of genomics and Bash. I will always be grateful for the hours you have spent training me up, running extra analysis, proof-reading papers and more. You have all been such role models for me and if I can be a fraction of the scientists and people you all are, I know I'll be doing well.

I thank all Applied Proteomics members, past and present. In particular, Dearbhlaith and Felipe – thank you for taking me under your wings before and after I started my PhD, for showing me the lay of the land and for adopting me into the Applied

Proteomics family. I also thank all fellow post-grads who have been in the Dept during my time for the much-needed tea-breaks, movie nights and, of course, the (in)famous summer and Christmas parties! I thank my student helpers, Emma Harris and Roisin Barrett, who helped me with the gruelling days of counting bees in the tropical temperatures of our bee room – I probably would not have survived that particular experiment without your help and good-humour! I also thank all of the staff in the Maynooth University Biology Department who have been the oil in the Department's engine – your constant work is so greatly appreciated.

Ride or die from day one, Merissa, you have been the lifejacket keeping me afloat these last four years with your outrageous sense of humour, extraordinary kindness, and never-failing support. We've experienced so much together be it a pandemic, angry bees on the loose, or discovering TikTok – you have brought so much light to my PhD experience. I can't think of anyone who I'd rather go through this process with, and it is going to be made all the more special that we will be crossing that PhD finish line together.

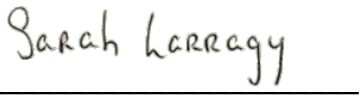
To my family - Mam, Dad, Anna, Uncle John, Nick, and my partner, Maurice - I do not know where I would be without your love, support, and belief in me. Through moments of doubt and anxiety, you have all played a role in picking me up and spurring me on. I could not have done this without you all so solidly in my corner. In particular, my parents - you both have always believed in me and encouraged me to reach for the stars. Since a very young age, you instilled in me the value of working hard, speaking up for my values and to be kind. You also helped me to find plastic boxes to house caterpillars in, provided me with endless David Attenborough DVDs, took me on outings to the zoo, butterfly farms and to see Fungi the dolphin. More than anything, you taught me to pursue my passions unapologetically. My gratitude to you both is endless, and I hope I have made you both proud. To my sister, Anna (who kindly offered to write this section of my acknowledgements for me...!) – I have always looked up to you. You are one of the most hardworking and bravest people I know. Thank you for being my number one fan, for always saying the right things and of course those little workbooks you made for me as kids – a thorough educational grounding! Your pep talks have never failed to motivate me and have been especially appreciated during my final weeks of thesis writing. I can now very proudly include my niece, Norah, who kindly waited until two days after my thesis submission to be

born. You may not be able to speak or walk yet, but you inspire in me so much hope for the future and I can't wait to get to know you more as you grow (and help convert you into a science/nature nerd). To Maurice - who is unwavering in his support of me - thank you for being so dependable, making me laugh when I don't want to and knowing (before I do) when I need to go take a nap. Of course, I also have to thank the pets (Leia, Bonnie and the late Max - the dogs; Ripley and Jones - the cats) for all of the cuddles and - perhaps mostly unintentional - moral support. I would also thank Lola the gecko, but she's not one for cuddling or, indeed, emotions at all.

I also thank my friends, who are always there to let me vent, make me laugh and generally help me take life a bit less seriously. Now that I am nearly finished with the PhD, I so look forward to actually seeing you all again!

Declaration

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

Signed 

Sarah Jane Larragy B.A.

Date **27 February 2023**

Abbreviations

% v/v	Percentage volume per volume
% w/v	Percentage weight per volume
µg	Microgram
µl	Microlitre
1D	One-dimensional
ACh	Acetylcholine
ACN	Acetonitrile
AMBIC	Ammonium bicarbonate
AMP	Antimicrobial peptide
APHAG	Autophagy
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BP	Biological process
CASP	Caspase
CASPA	Caspase A
CAT	Catalase
CC	Cellular component
CLIP	CLIP serine protease
cm	Centimetres
CTL	C-type lectin
dH_2O	Distilled H_2O
DNA	Deoxyribonucleic acid

DTT	Dithiothreitol
EHH	Extended haplotype homozygosity
FREP	Fibrinogen-like
F_{ST}	Fixation index
FUN	Functionally uncharacterised
g	g-force
g	Grams
GAL	Galectin
GNBP	Gram-negative binding/Beta-glucan recognition protein
GO	Gene ontology
GR	Gustatory receptor
GTP	Guanosine triphosphate
HPLC	High performance liquid chromotography
Hrs	Hours
IAA	Iodoacetamide
IAP	IAP repeat protein
IGG	Immunoglobulin
IMD	Immune deficiency
JAK/STAT	Janus kinase/ signal transducers and activators of transcription
JNK	Jun kinase
KEGG	Kyoto encyclopaedia of genes and genomes
KOALA	KEGG Orthology And Links Annotation
LC-MS/MS	Liquid chromatography mass spectrometry
LFQ	Label-free quantitation

LYS	Lysozyme
M	Moles per litre
m/z	Mass/charge ratio
MF	Molecular function
mg	Milligram
min	Minute(s)
ml	Millilitre
ML	MD-2-related lipid recognition
mm	Millimetre
mM	Millimoles per litre
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
MS	Mass spectrometry
nAChRs	Nicotinic acetylcholine receptors
NIMROD	Nimrod
nSL	Number of segregating sites by length
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PCA	Principal component analysis
PCR	Polymerise chain reaction
PGRP	Peptidoglycan recognition protein
Ppb	Parts per billion
Ppm	Parts per million
PPO	Prophenoloxidase

PRDX	Peroxidase
PRP	Pattern recognition proteins
R.H.	Relative humidity
REL	Relish
RNA	Ribonucleic acid
RNAi	RNA interference
s	Second(s)
SCR	Scavenger receptor
SDS	Sodium dodecyl sulfate
SG	Stress granule
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SPZ	Spätzle
SRPN	Serine protease inhibitor
SRRP	Small RNA regulatory pathway
SSDA	Statistically significant differentially abundant
STRING	Search tool for the retrieval of interacting genes
TCA	Tricarboxylic acid cycle
TEP	Thioester-containing protein
TFA	Trifluoroacetic acid
UPR	Unfolded protein response
WGS	Whole genome sequencing

Abstract

Bees, including bumblebees, are highly valued for the pollination services they provide to natural ecosystems and agricultural crops. However, many bee species are facing declines, likely a result of habitat loss, pesticide use and climate change. Additionally, the use of imported commercial bumblebee colonies for crop pollination poses several risks to wild pollinators, including competition, hybridisation and pathogen spillover. A stock-take is needed of wild bees on both genetic and functional levels to identify vulnerable populations, detect local adaptations and to prevent further pollinator losses. We examine wild Irish *B. terrestris* ssp. *audax* on genomic, proteomic, and behavioural levels with reference to British and commercial populations to deepen our understanding of the selective processes acting on wild and domesticated bumblebee populations. We find that wild Irish and British populations of *B. t. audax* are distinctive on genomic levels and exhibit differential signatures of selection. We also find putative evidence for genetic distinctions between wild and commercial populations. A genomic examination of canonical immune genes in wild, Irish bumblebees highlighted several genes undergoing positive, purifying and possibly balancing selection, possibly reflecting their functional diversity and indicating recent adaptation. We uncover distinctions in the proteomes of wild and commercial lineages of lab-reared worker bee fat bodies and brains, as well as in the proteomic responses of these organs to pesticide exposure and infection. Finally, distinctions in the growth dynamics of wild and commercial lineages of *B. t. audax* colonies were identified alongside differences in the bacterial and fungal gut microbiomes of lab-reared wild and commercial workers. Overall, the findings of this thesis provide novel insights into the genetic, physiological, and behavioural distinctions between wild and domesticated populations of *B. t. audax* which will likely have major implications for how we conserve valuable genetic resources and manage commercial bumblebee imports.

Chapter 1

General introduction

1.1 Bumblebees

Bees are insects recognised around the globe for their ecological, economic, and cultural importance. They are highly valued for the pollination services they provide to crops and wild plants as well as the production of economically and culturally valuable products such as honey (Tepedino, 1979; Garibaldi *et al.*, 2011; Sahle *et al.*, 2018; Schouten *et al.*, 2020). Bees belong to a highly successful order of insects: the Hymenoptera. This order contains bees, wasps, ants, and sawflies and all have defining characteristics such as two pairs of wings, constricted abdomens and modified ovipositors used for stinging and piercing (Richards and Davies, 2013). Bees are members of the superfamily of Apoidea and first appeared around 130 million years ago alongside the rise of angiosperms, or flowering plants (Michener and Grimaldi, 1988). Today, we see many life history strategies employed by bees, varying from highly social to completely solitary (Goulson, 2010a). However, the vast majority of bees depend on floral pollen and nectar during their entire life cycle (but see Figueroa *et al.*, 2021). Bumblebees (*Bombus* spp.) are common pollinating insects in temperate regions of the world and are well known for their characteristic hairy bodies and bright colour patterns (Goulson, 2010a; Falk, 2019). It is unknown when exactly the *Bombus* genus first appeared, although the first fossils date to between 38 – 26 million years ago (Zeuner *et al.*, 1976). It is theorised that bumblebees evolved in response to a global cooling event which selected for cold-adapted insects (Hines, 2008). Bumblebees make up approximately 1% of the 25,000 described species of bee on the planet, including both 'true' and cuckoo bumblebees (Williams, 1998; Cameron *et al.*, 2007).

1.2 Bumblebee lifecycle

One of the defining characteristics of bumblebees is their eusocial lifecycles. Eusociality, meaning "truly social" (Batra, 1966), is the term to describe organisms showing three traits: (1) cooperative brood care, (2) overlapping of generations and (3) division of labour (Sherman *et al.*, 1995). Bumblebees are considered primitively eusocial, compared with the highly complex colonies produced by honeybees (*Apis mellifera*). However, in many ways the bumblebee lifecycle is complex too, and many

aspects of the development and dynamics of their colonies and life stages remain unclear (Michener, 2000; Williams *et al.*, 2019).

Bumblebees have an annual life cycle which, as described by Sladen (1912), is largely comprised of the story of the *Bombus* gyne (pre-colony) or queen (once colony is initiated). The bumblebee gyne wakes from her winter diapause (Figure 1.1), spends some time warming up after months of inactivity and begins foraging on spring plants (Free and Butler, 1959; Alford, 1969). Emergence times vary between species, with *B. pratorum*, *B. terrestris* and *B. lucorum* gynes usually appearing first in February to March and *B. sylvarum* coming out as late as the summer months (Alford, 1975). Gynes begin looking for suitable nesting sites in the spring, the types of which also vary in preference among species (Svensson *et al.*, 2000; Liczner and Colla, 2019). *B. terrestris* is an underground nesting species and often takes over old rodent burrows to begin establishing a colony (Liczner and Colla, 2019). It can take up to several weeks for a gyne to find a suitable nest site (Free and Butler, 1959). Once she has located a suitable location, she spends time drying the colony out with her body heat while also dragging nearby materials into the nest and arranging them for insulation (Sladen, 1912; Goulson, 2010a). She also conducts orientation flights to memorise the location of her nest so as to navigate to and from (Sladen, 1912). Within the nesting cavity, the foundress uses the pollen and nectar she has foraged to make a round lump of pollen on which she will lay her eggs (Sladen, 1912). It is thought that between 8 - 16 eggs are laid in the first batch, which are quickly covered by a layer of wax and pollen by the queen (Goulson, 2010a), although the quantity of eggs laid in a cell and the number of cells made vary across species (Duchateau and Velthuis, 1988). The queen incubates this brood batch by pressing her ventral surface to the clump while pumping her abdomen muscles. This produces heat that is transferred to the developing brood, which have been measured to hold temperature of 30 - 32°C as a result (Heinrich, 1972; Alford, 1975).

Eggs take about 4 days to hatch into larva and develop into cocooned pupae about 10-14 days later; the new adult bees eclose as colourless callows about 2 weeks after this and develop adult colouration approximately 24 hours later (Alford, 1975; Goulson, 2010a). Once workers have emerged, the colony enters its eusocial phase and can grow almost exponentially after this, producing new batches of workers in shorter successions. Once emerged, workers begin foraging, participating in brood care and

maintaining the colony while the queen focusses on laying more brood clutches (Alford, 1975; O'Donnell *et al.,* 2000). Colonies of *B. terrestris* can have as many as 350 workers (Goulson, 2010a). Bumblebee workers are notable in their variation of size, with smaller workers tending to remain in the colony while larger workers tend to leave the colony to forage (Goulson *et al.*, 2002; Spaethe and Weidenmüller, 2002; Peat *et al.*, 2005). Workers can live for up to two months but lifespan is generally predicted to be much shorter for foragers than for colony-based nurse bees (Brian, 1952).

Over the course of the eusocial phase, there are two key turning points of the colony lifecycle. These are the switching point, when a queen ceases to lay diploid eggs and begins laying haploid eggs which result in male offspring (Duchateau and Velthuis, 1988), and the competition point, where workers compete, often aggressively (Bourke, 1994), with their queen to lay their own eggs (Duchateau and Velthuis, 1988; Cnaani *et al.*, 2002; Amsalem *et al.*, 2009). The switching point may be determined by the ratio of workers to larvae, as developing gynes need a higher amount of food over a longer developmental period than workers and so can only develop properly if there is sufficient food and brood care (Duchateau and Velthuis, 1988; Goulson, 2010a).

Both gynes and males leave their natal colony soon after they emerge, which is usually followed by the decline of the colony and the death of the current queen (Goulson, 2010a). Male bumblebees have been observed to use a number of tactics to locate a mate including territoriality, nest surveillance, hill-topping, scent marking and patrolling (Paxton, 2005; Goulson, 2010a). Gynes too are thought to produce pheromones from their head that attracts the attention of males (Free, 1971). Many studies suggest that most species of bumblebee seem to exhibit monandry (Estoup *et al.*, 1995; Schmid-Hempel and Schmid-Hempel, 2000). After mating, mated gynes will build up their reserves before finding a suitable hibernation location (Alford, 1975). While preferences vary between species, mated gynes will often choose hibernation sites composed of loose or disturbed soil so they can dig underneath easily (Goulson, 2002). There, she creates a small chamber where she shall stay throughout the Winter months and emerge from the following Spring (Alford, 1975).

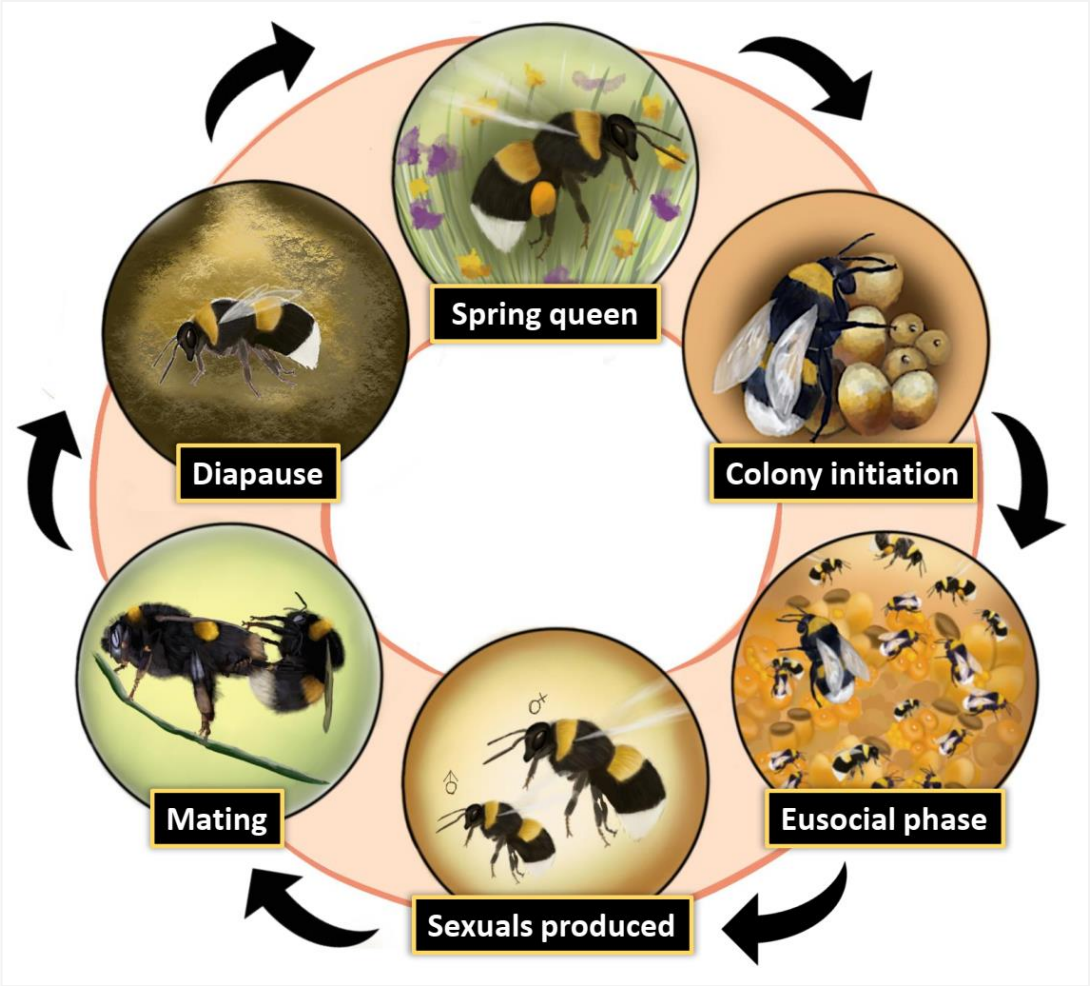


Figure 1.1 The annual lifecycle of *B. terrestris*. The *B. terrestris* queen emerges from diapause in Spring months when she will feed, build up her energy and search for a suitable nest site. Once found, she will lay her first egg clump and provide incubation and nutrition to her developing brood. Female worker bees are the first to emerge and these grow in numbers to form a fully functioning colony. The colony reaches a switch point, where the queen begins laying eggs that will become new gynes and males (reproductive individuals). Around the same time, the colony reaches its competition point, where the workers overthrow and often kill the queen. Reproductive individuals leave the colony to find emergent gynes and males from other colonies to mate with, while gynes will also build up their reserves before entering a Winter diapause (Alford, 1975; Goulson, 2010a).

1.3 Importance of pollination services

Pollination involves the transfer of male genetic material in the form of pollen to the receptive female organs of a plant, resulting in fertilisation and the production of seeds and/or fruit (Walker, 2020). The vast majority of angiosperm plants depend on animal pollination to some degree for successful reproduction (Ollerton *et al.*, 2011). Wild bees have been shown to provide significant benefit to crop and urban plant pollination

(Wilson, 1929; Winfree *et al.*, 2007; Winfree *et al.*, 2008; Jauker *et al.*, 2012; Lowenstein *et al.*, 2015; MacInnis and Forrest, 2019).

Bumblebees are one of many pollinator species that provide critically important pollination services around the world. Pollination is an ecosystem service i.e. a service provided by ecosystems that benefits humans (Begon and Townsend, 2020). Crop pollination in Europe is valued at nearly €15 billion per year, while in Ireland, these services alone had an estimated value of up to €59 million per year (Leonhardt *et al.*, 2013; Stout *et al.*, 2019). The contribution of pollinators to balanced human diets, food security and the economic value of agriculture is incontestable (Potts *et al.*, 2016), however pollinators are also essential for the health and functioning of ecosystems, which in turn provide many more ecosystem goods and services e.g., prevention of soil erosion, air and water purification, waste decomposition and renewal of soil (Daily, 2003).

Bees in general are excellent pollinators as they require pollen for their own nutrition and to feed the developing larva of the colony (Goulson, 2010a). In the case of honeybees and bumblebees, their social lifecycle means that they produce many individuals that perform foraging duties for the colony and so have high pollination capacities (Maia *et al.*, 2019). Bumblebees have certain adaptations that can make them particularly suited to pollination in certain scenarios. For example, bumblebees are adapted to temperate climates such as having dense hair on their bodies and often a large size (Goulson, 2010a). As a result, they can forage during rainy and sometimes even freezing temperatures (Corbet *et al.*, 1993; Heinrich and Vogt, 1993). Bumblebees can also buzz-pollinate, unlike honeybees. Buzz pollination is a method of releasing pollen from certain plant species by vibrating at a certain frequency (De Luca and Vallejo-Marin, 2013). Buzz-pollination is necessary for the pollination and improved yield of many fruit crops such as tomatoes, cranberries, blueberries and peppers (Van den Eijnde *et al.*, 1990; Morandin *et al.*, 2001; Guerra-Sanz, 2008; De Luca and Vallejo-Marin, 2013). Some bumblebees have longer tongues than honeybees and so are better suited to visit and extract nectar from flowers with long corollas such as red clover (Sladen, 1912).

1.4 Bumblebee declines

In parallel to many other insect population declines, wild pollinators are showing declines in both their population sizes and diversity of species across Europe (Kosior *et al.*, 2007; Nieto, 2014; Vogel, 2017; Mazed *et al.*, 2022). In Ireland, one third of our 102 bee species are threatened with extinction (FitzPatrick *et al.*, 2006). These declines have been attributed to four key stressors: habitat loss, climate change, pesticide use, and pathogen spread (Figure 1.2). Further risks posed towards wild pollinators are associated with the use of imported bumblebees, which will be discussed in Section 1.6.5.

1.4.1 Habitat loss

The intensification of agriculture and land use change is considered one of the most significant threats to ecosystems across the globe (Tilman, 1999). The second half of the 20th century saw major agricultural intensification which increased practices that supports monocultures of crops, use of fertilisers and pesticides (Raven and Wagner, 2021). Ancient grasslands and hay meadows were ploughed up to be replaced with fast growing grass varieties to increase productivity (Goulson *et al.*, 2008). As a result of these activities, 1984 saw less than 10% of the original grasslands that existed 50 years prior in the UK (Howard *et al.*, 2003). In Ireland, the main pressures and threats that face species are agriculture, urbanisation and the extraction and cultivation of resources (Wall *et al.*, 2020). In particular insect pollinator declines in Ireland are driven by the increasing demand for monocultures and the associated destruction of scrub and hedgerows, vital habitats for insects (Wall *et al.*, 2020). Other habitats in Ireland that provide food and nesting resources to bees such as grasslands, woodlands and peatlands have declined both in area and in quality (FitzPatrick *et al.*, 2015). Of all of Ireland's listed habitats, 85% were designated as having an 'unfavourable conservation status' (Wall *et al.*, 2020). Additionally, increased levels of fertiliser are associated with negative impacts on the diversity of species and richness of flower species (Berendse *et al.*, 1992; Bakker and Berendse, 1999; Kleijn *et al.*, 2009)

Declines in essential habitat area and quality impacts where bees can survive and reproduce. For example, many later emerging bumblebees exhibiting westward shifts in their distributions in Ireland, away from the midlands and East with a large

agricultural focus (FitzPatrick *et al.*, 2007). The range declines of these bumblebees correlate with those in Britain where rarer bumblebee species have moved to locations where there is much less agricultural pressure on habitats (FitzPatrick *et al.*, 2007). Farmers growing crops requiring pollination may feel the effects of this, as it has been shown that the further crops are from natural or semi-natural habitats, the fewer the visits to the crop from wild pollinators (Ricketts *et al.*, 2008). In addition, changes to floral and habitat resources could leave colonies isolated and increase the chances of inbreeding and lowered genetic diversity (Goulson *et al.*, 2008).

1.4.2 Climate change

Global climate change will likely have catastrophic impacts on many ecosystems, species and the services these provide (Sala *et al.*, 2000; Mooney *et al.*, 2009). Pollinators are vulnerable to climate change with some species likely more vulnerable than others (Fründ *et al.*, 2013). Changes in average temperatures and climate may alter the phenology of plants and pollinators (Parmesan, 2006). As many pollinators depend highly on floral resources during their lifecycles, any mismatch in the timing between pollinator emergence and flowering of essential plants could impact the involved species' fitness and, over evolutionary time, cause changes to ecosystem networks (Thomson, 2010; Petanidou *et al.*, 2014; Gérard *et al.*, 2020). In addition to changes in phenology, it is predicted that many bumblebee species will experience range shifts and shrinkage to remain in suitable climate conditions (Williams *et al.*, 2007; Rasmont *et al.*, 2015).

1.4.3 Pesticide use

Rising numbers in the human population and the associated demand for food contributes to increased pressure on growers to use chemical pesticides to protect their crops (Tilman *et al.*, 2001). These pesticides include a wide range of chemicals that target pests such as insects, weeds, fungi, rodents and molluscs (Aktar *et al.*, 2009). In agricultural settings, insecticides are used mainly to kill insect pest species such as aphids and whiteflies that feed on crop species (Bass *et al.*, 2015). Non-target organisms such as pollinators and bees can become exposed to these chemicals both directly after they are applied and indirectly, in the case of systemic pesticides, through consumption of treated plant nectar and pollen (Goulson, 2010a). Although

insecticides are known to impact many species of pollinator (Di Prisco *et al.*, 2013; Rundlöf *et al.*, 2015; Stanley *et al.*, 2015; Stanley and Raine, 2016; Czerwinski and Sadd, 2017), pesticide risk assessment have tended to be carried out on honeybees, the results of which do not always translate to other species (Thompson and Hunt, 1999).

Insecticides often have sublethal effects on non-target organisms. One well-known class of insecticide that is known to have sublethal impacts on bees are neonicotinoids. This insecticide class acts by binding to nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects (Tomizawa, 2013). Neonicotinoids compete with acetylcholine (ACh) to bind these receptors and once bound are mostly irreversible. They act systemically i.e. when applied to any part of the plant, the chemical is absorbed and can move through tissues (Tomizawa and Casida, 2005). Therefore, any insect feeding on treated plant material will be exposed to these insecticides, including non-target organisms such as pollinators feeding on plant pollen and nectar. Sublethal impacts of neonicotinoids on bees include disruptment to learning, navigation, foraging and colony growth (Laycock *et al.*, 2012; Whitehorn *et al.*, 2012; Stanley *et al.*, 2015; Stanley and Raine, 2016; Bantz *et al.*, 2018; Lämsä *et al.*, 2018; Muth and Leonard, 2019).

1.4.4 Pathogen spread

Bumblebees have a taxonomically diverse suite of pathogens and parasites (Rutrecht and Brown, 2008). These range from viruses, bacteria and fungi to nematodes and mites (Goulson, 2010a). The most well-studied bumblebee parasites are the protozoans *Crithidia bombi* and *Nosema bombi* (Otti and Schmid-Hempel, 2007; Yourth *et al.*, 2008; Cordes *et al.*, 2012). These parasites are known to have serious impacts on bumblebee fitness such as decreased levels of colony initiation by queens, reduced worker, gyne and/or male output (Brown *et al.*, 2003; Yourth and Schmid-Hempel, 2006; Otti and Schmid-Hempel, 2007; Yourth *et al.*, 2008).

Emerging diseases and pests are considered a major risk to wild bees (FitzPatrick *et al.*, 2015). In particular, pathogens spread by imported commercial colonies are thought to be a major risk to wild bees and this will be addressed further in Section 1.6.5.

In addition, managed honeybees are also thought to spread pathogens to wild bumblebees via pathogen spillover (Alger *et al.*, 2019). Over the last decade, there has been increased descriptions of honeybee viruses, such as Black queen cell virus, BQCV, and deformed wing virus, DWV (Peng *et al.*, 2011, Lucia *et al.*, 2014). Although many of these were described as honeybee specific pathogens, there have been incidences of these 'honeybee' viruses recorded in bumblebees (Fürst *et al.*, 2014). Additionally, honeybee viruses such as DWV and BQCV can readily replicate in *B. terrestris* bumblebee hosts but appear to have negligible impacts on mortality (Tehel *et al.*, 2020), contrary to other studies which saw impacts of honeybee viruses on bumblebee worker lifespan (Fürst *et al.*, 2014, Graystock *et al.*, 2015). The study by Tehel *et al.* (2020) indicates that although the same virus can infect numerous bee species, differences may exist in the pathogenicity of these viruses across host species. Bumblebees do not carry the *Varroa destructor* mite, a common honeybee brood parasite which acts as a vector for many of the aforementioned honeybee viruses (Morelle, 2014). Instead, they are thought to come into contact with these honeybee viruses through horizontal transmission at shared floral resources, but the exact mechanisms of this remain to be fully understood (McMahon *et al.*, 2015).

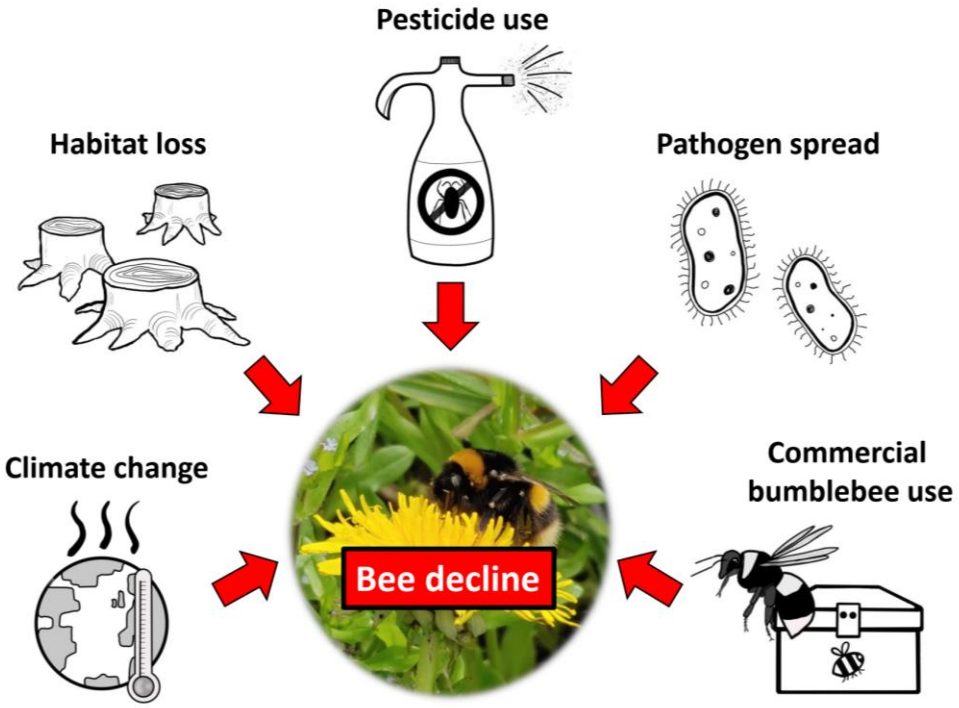


Figure 1.2 Factors thought to be contributing to bee decline. Factors thought to contribute to bee decline include habitat loss, climate change, use of pesticides, spread of pathogens and use of commercial bumblebees (Goulson *et al.*, 2008; Graystock *et al.*, 2013).

1.5 An overview of *Bombus terrestris* (L.)

In Europe the main domesticated bumblebee species used for commercially supplied pollination services is *Bombus terrestris* (Linnaeus, 1758) which comprises 9 distinct subspecies (Rasmont *et al.*, 2008). *B. terrestris* is among the most widely distributed species of bumblebee with a distribution (Figure 1.3) ranging from Scandinavia to Northern Africa, Britain and Ireland to the Altai and is found on almost all the Mediterranean islands (Estoup *et al.*, 1996; Rasmont *et al.*, 2008; Lecocq *et al.*, 2016). Mainland European populations of *B. terrestris*, *B. t. terrestris* and *B. t. dalmatinus*, are relatively homogenous in their genetic structure and have been from the very early days of bumblebee commercialisation (Estoup *et al.*, 1996). This suggests that the homogenous genetic structure of mainland European *B. terrestris* sub-species likely existed prior to any potential introgression resulting from the commercial bumblebee trade. Island subspecies of *B. terrestris* have been found to be significantly distinguished genetically, and often have quite distinctive morphologies, perhaps caused by increased genetic drift (Estoup *et al.*, 1996) or a result of phenotypic plasticity under different climatic conditions (Canale and Henry, 2010; Pauls *et al.*, 2013). Similarly, another distinct subspecies of *B. terrestris, B. t. audax*, is unique to Ireland and Britain (Rasmont *et al.*, 2008).

In terms of morphology, the subspecies *B. t. terrestris, B. t. dalmatinus* and *B. t. audax* differ slightly. *B. t. terrestris* and *B. t. dalmatinus* queens have white to whitish tails, and *B. t. audax* queens have a light-reddish or buff tail (Rasmont *et al.*, 2008). *B. t. terrestris* and *dalmatinus* have a broader yellow band on the thorax than *B. t. audax* (Rasmont *et al.*, 2008), and *B. t. dalmatinus*, found in a Mediterranean climate, has significantly shorter hairs than the British *B. t. audax* (Peat *et al.*, 2005). Although commercial *B. t. dalmatinus* workers are bigger in size than native British *B. t. audax* workers (Ings *et al.*, 2006) the differences are subtle, and it is difficult to reliably ID subspecies in the field (Goulson, 2010b).

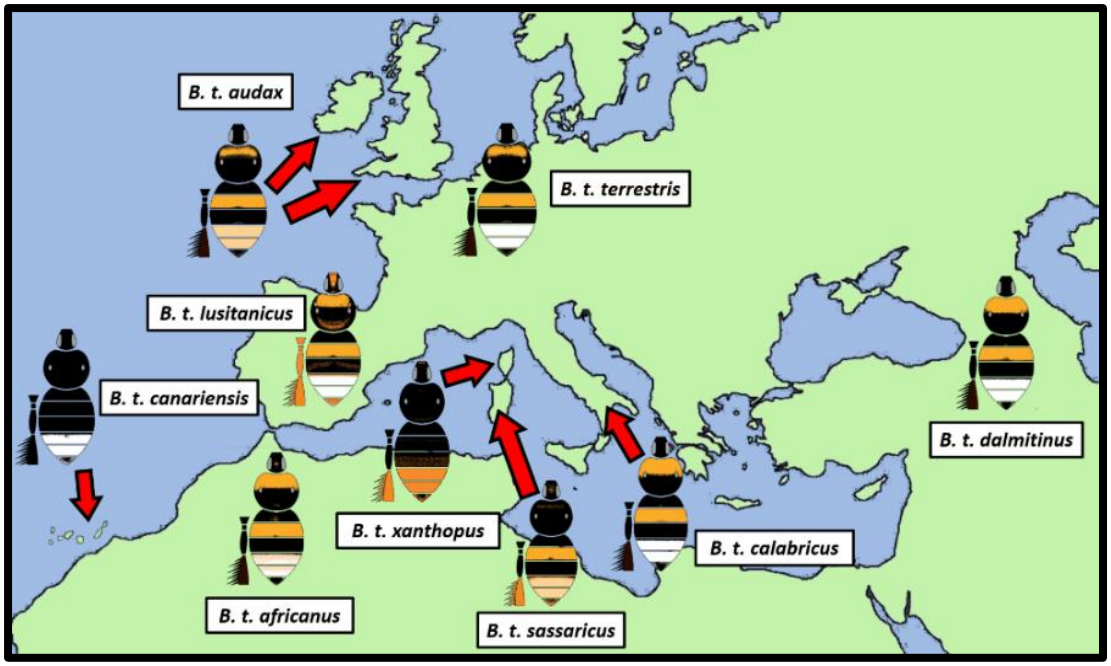


Figure 1.3 *B. terrestris* subspecies and their distributions across the Palaearctic. Distribution of the nine *B. terrestris* subspecies across Europe and North Africa, as defined in Rasmont *et al.* (2008). This figure is adapted from Figure 2 of Rasmont *et al.* (2008).

Using molecular markers, the Irish population of *B. terrestris* was found to be genetically distinct from the British population, despite both islands sharing the subspecies *B. t. audax* (Moreira *et al.*, 2015). Additionally, British populations showed more admixture with mainland Europe populations, suggesting that the English Channel is not as effective a gene flow barrier as the Irish sea (Moreira *et al.*, 2015). Furthermore, the commercial *B. terrestris* bumblebees used in this study, which were sourced from two different producers, were found to be genetically distinct from each other (Moreira *et al.*, 2015).

Prior to 2010, the stock of *B. terrestris* being imported into Ireland was a non-native subspecies of *B. terrestris*, assumed to be a mixture of *Bombus t. terrestris* and *B. t. dalmatinus* subspecies, as these are the subspecies found on mainland Europe (Moreira *et al.*, 2015). Responding to the growing concerns relating to the importation of non-native sub-species into Ireland and the UK, the commercial company Biobest began producing *B. t. audax* in the summer of 2010 (Biobest, 2011). Koppert followed suit in 2011 and began producing *B. t. audax* for its British and Irish customers (Koppert Biological Systems, 2011).

1.6 Domestication and commercial bumblebees

1.6.1 Domestication and domestication syndromes

Domestication, although a universally acknowledged process, is difficult to put a precise definition on (O'Connor, 1997). Décory (2019) puts forth the following definition of domestication: "Domestication of a group is a complex and multifactorial phenomenon affecting biological, behavioural and genetic processes in all individuals of this group and for several generations". Domestication has been described as a process with five increasing levels, with level one being the least domesticated and five being the most (Teletchea and Fontaine, 2014). Levels one, two and three are considered 'pre-domestication' stages, and levels four and five are considered true domestication (Bilio, 2007). Pre-domestication encompasses the process of organisms becoming acclimatised to captivity, where parts or the whole of their lifecycle can take place in captivity with wild inputs, whereas true domestication is where the entire lifecycle taking place in captivity without any wild inputs and the performance of selective breeding is carried out (Teletchea and Fontaine, 2014). This echoes Darwin's (1868) description of domestication as a process where "man selects varying individuals, sows their seeds, and again selects their varying offspring", a clear description of artificial selection. Darwin (1868) also mentions "unconscious selection", where the breeder is not aware of certain traits being selected for in the organisms in their domesticated setting. Although often seen in captive populations, groups of organisms do not need to be held in captivity and consciously put through a domestication process to become domesticated, as is seen for example in African mosquitos (Trpis and Hausermann, 1975).

Domestication often results in the organisms undergoing this process exhibiting an "abnormal character" when compared with their wild counterparts, and specifically exhibiting traits that do not confer the organisms themselves fitness benefits, but benefits to the humans domesticating them (Darwin, 1868). This is because the process of domestication usually results in long term changes in the genetics, physiology and/or behaviour of the group in question (Jensen, 2006; Wilkins *et al.*, 2014; Lecocq, 2018). The resulting traits culminating from these changes are referred to as a "domestication syndrome" (Lecocq, 2018). Domestication syndromes are well characterised in mammals. Common traits that arise through the domestication process

include depigmentation, floppy ears, docility, smaller brains and changes to reproductive cycles (as summarised in Wilkins *et al.*, 2014).

Although domestication has most often been associated with vertebrate species, this process has also been carried out on invertebrate species, and most acknowledged case of insect domestication is that of *Bombyx mori* (Gon and Price, 1984). *B. mori* is a very important species for the production of silk, producing over 609,332 tonnes of the textile annually (Chand *et al.*, 2022). Domestication of the silkworm began 7,500 years ago using Chinese populations of *B. mandarina* (Yang *et al.*, 2014). The domesticated *B. mori* silkworm depends entirely on humans to complete their lifecycle (Damodaram *et al.*, 2014; Lecocq, 2018). Through domestication, *B. mori* have acquired a combination of domestication traits and improvement traits. For example, the size of their bodies has grown and they can no longer fly (Lecocq, 2018). Traits such as higher silk production, larger cocoons and better feeding efficiency have developed through artificial or unconscious selection, whereas other traits such as camouflage and the ability to fly have been lost possibly due to the absence of selection pressures maintaining anti-predator behaviours (Goldsmith *et al.*, 2005; Yu *et al.*, 2011; Xiang *et al.*, 2018).

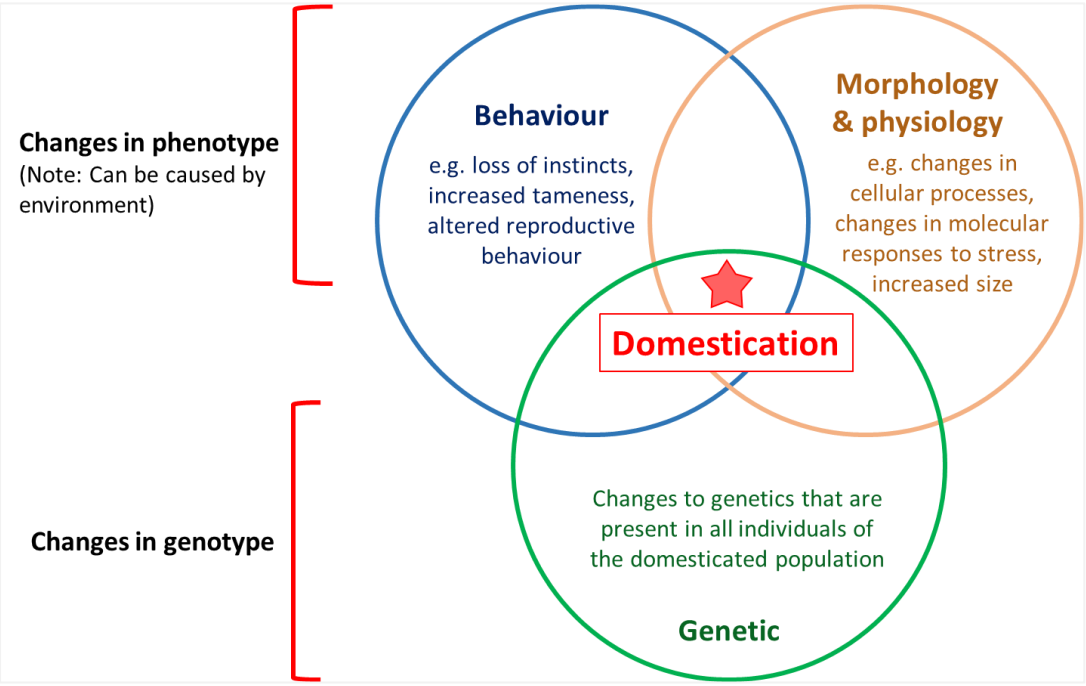


Figure 1.4 Categories of changes that occur during the domestication process that combine and form a domestication syndrome. This figure is adapted from "Illustration of the 3 Categories (3C) of Changes: Behaviour, Morphology/Physiology and Genetic" featured in Décory (2019).

Interestingly, many of the traits observed in the *B. mori* domestication syndrome echo those observed in domesticated mammals e.g., a greater tolerance to human handling, lowered aggression towards conspecifics that facilitates survival in dense living situations and depigmentation or loss of camouflage (Goldsmith *et al.*, 2005; Lecocq, 2018; Xiang *et al.*, 2018). Honeybees (*Apis mellifera* and *A. cerana*) have the longest domestication history and have undergone significant changes at the hands of this process which began at least 9000 years ago (Roffet-Salque *et al.*, 2015; Lecocq, 2018). Since their domestication, changes in behaviour have been observed such as, within at least the European groups, decreased aggression, improved honey yield, and less frequent swarming (Weber, 2013). Mass production of bumblebee colonies began much more recently in the 1980s due to many beneficial traits these species possess e.g., tolerance of temperate weather conditions and an ability to buzz-pollinate crops such as tomatoes and raspberries which honeybees cannot do.

Domestication is considered an irreversible process (Zeller and Göttert, 2019), as demonstrated by Kruska (2005) who found that domesticated mammals, which are normally characterised by a decrease in brain size, do not exhibit increased brain size on living several generations in wild settings. When a domesticated or captive species escapes into the wild and establishes itself there, it is therefore said to be feral. Feral populations can be endoferal i.e. descending directly from a domesticated population or exoferal i.e. descended from hybrids between a domesticated form and another (wild or domesticated) variety (Gressel, 2005). As discussed earlier, domesticated species have altered traits that are suited to a captive or domestic environment. When these species enter a wild environment, they are likely not to perform the same as truly wild populations that have been under natural selective pressures. This can potentially have several worrying impacts on the health and conservation of wild populations and ecosystems.

Feral populations may have traits linked to domestication that confer benefits to survival in the wild and so feral populations could impact natural populations through, for example, competition. Many domesticated plant species have been selected to be easier to grow e.g., no dormancy in annual crop species (Gepts, 2010) and so, once feral, may likely become weedy or invasive. Examples of plants descended from domesticated species that have become weed or invasive species include Artichoke thistle (*Cynara cardunculus* var. *scolymus)*, California wild radish (*Raphanus sativus*

X *R. raphanistrum*) and Weedy rye (*Secale cereale;* Ellstrand *et al.,* 2010). Alternatively, they could have traits linked to domestication that decrease their fitness in a wild setting, and these disadvantageous alleles could spread to wild populations through introgressive hybridisation (Randi, 2008).

1.6.2 Bumblebee domestication history

Bumblebee domestication and colony initiation has undergone a series of advancements and breakthroughs since it was first reported in the early 20th century (Sladen, 1912; Frison, 1927; Plowright and Jay, 1966; Röseler, 1985; Van den Eijnde *et al.*, 1990; Velthuis and Van Doorn, 2006). Now, this industry provides a viable commercial alternative to honeybee pollination for agriculture, which commenced in 1987. One of the early pioneers of the commercial bumblebee industry was Dr Roland de Jonghe who founded Biobest in Belgium in 1987 (International Pest Control, 2017). De Jonghe demonstrated that bumblebees from reared colonies were highly efficient pollinators of greenhouse tomatoes (*Solanum lycopersicum* L.), which up to this point were pollinated using manual vibrations, costing €10,000 per hectare per year, or hormonally (Velthuis and van Doorn, 2004; Velthuis and Van Doorn, 2006). The following year, Koppert Biological Systems, based in the Netherlands, began producing bumblebees alongside their biological control agent products and now, there are more than 30 producers of commercial bumblebees around the world (Velthuis and van Doorn, 2004). The principal species of bumblebee that have been commercialised include *Bombus terrestris*, *B. lucorum*, *B. ignites*, *B. impatiens* and *B. occidentalis* (Velthuis and Van Doorn, 2006). These species are "pollen-storers", meaning they store pollen near brood cells in separate pockets in contrast to "pocket-makers", which have cups of pollen attached directly to brood cells, which in turn allows the growing larvae to access food themselves (Sladen, 1912; Alford, 1975). This distinction means that "pollen-storers" are much easier to feed and rear in artificial conditions as they will accept pollen from any source, whereas pocket-makers only accept the pollen from the adjoined pockets of the brood cells (Sladen, 1912; Velthuis and Van Doorn, 2006).

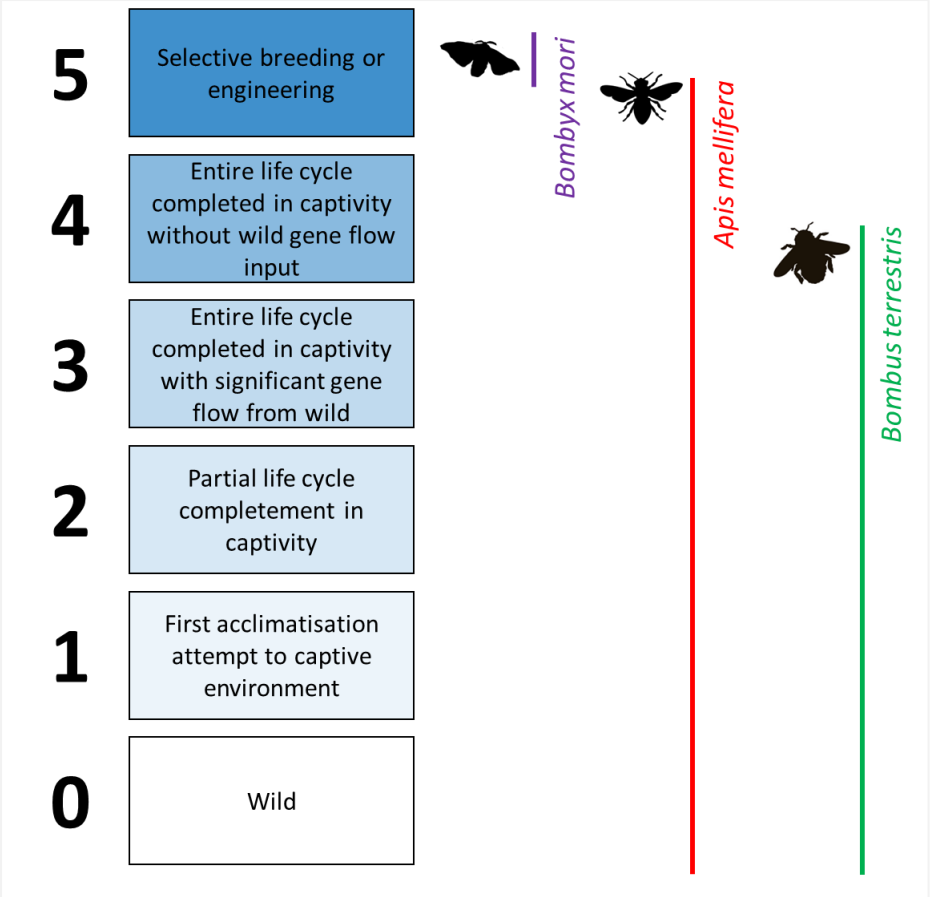


Figure 1.5 Levels of domestication. Levels of domestication and their description. Shown by the lines are where the species *Bombyx mori*, *Apis mellifera* and *Bombus terrestris* fall into the various stages of domestication. Diagram adapted from the figure captioned "Domestication process and insect domestication level" published in Lecocq (2018), using the levels of domestication as described in Teletchea and Fontaine (2014).

Since the production of commercial bumblebees began, the number of colonies exported internationally has continued to increase and in 2006, it was estimated that between one and two million *B. terrestris* hives were exported globally per annum (Velthuis and Van Doorn, 2006; Lecocq *et al.*, 2016). Today the major exporting countries of bumblebees are Israel, the Netherlands and Belgium with the chief importers being Mexico, China, Korea, Japan, Spain, Italy and Jordan (Goka *et al.*, 2001). Commercial importation of bumblebee colonies began in Britain in 1989 and it is estimated that 60,000 colonies are imported into the UK per year (Velthuis and Van Doorn, 2006; Goulson, 2010a).

Commercial bumblebee colonies are not at the extreme stage of domestication as seen in the silkworm *B. mori*, likely as they have only been bred and maintained in captivity in relatively recent years. The purpose of producing bumblebee colonies by companies

is to sell these colonies as a product to growers who can benefit from the natural behaviour of bumblebees i.e., foraging on plants to feed their colony so that, in the process, crops are pollinated. Therefore, it is likely we will not see bumblebee domestication advance to a stage where major abilities such as flight are compromised, as in silkworms. However, domestication has already reached a stage where the captive breeder stocks of bumblebees do not experience normal levels of gene flow from wild counterparts (Lecocq *et al.*, 2016). To our knowledge, it is unknown if or how frequently captive bumblebee stocks are supplemented with wild individuals in these artificial rearing facilities. As humans would control the majority of breeding in the captive bumblebee stocks, there is potential for human-directed selection, conscious or not, to cause change to drive evolutionary change in commercial bumblebee traits. There is evidence of domestication traits in commercially reared *B. terrestris* colonies sourced from mainland European subspecies population. For example, it is thought these populations have been selected for to skip or have shorter diapauses (Beekman *et al.*, 1999). Furthermore, it's possible that queens producing higher numbers of workers and reproductive individuals are being selected for, as there is evidence that commercial *B. t. dalmatinus* produce higher numbers of workers and sexuals than wild conspecifics (Gösterit and Baskar, 2016; Gösterit and Erkan, 2021). Domestication syndromes have not yet been investigated between wild and commercial populations of *B. t. audax* since its relatively recent appearance on the market.

1.6.3 Commercial bumblebee usage in Ireland

Data collected from surveys in 2007 and 2009 of commercial growers from both Northern and the Republic of Ireland have indicated that approximately 1,500 commercial colonies were imported into Ireland annually (Teagasc, unpublished). A survey in 2013 (DAFM, unpublished) of growers in the Republic of Ireland (ROI) alone, estimated that 2,000 colonies were imported that year. In addition to increasing imports, it seems that the number of colonies per grower also increased significantly from ~17 colonies per grower in 2007 to ~33 colonies per grower in 2013 (Figure 1.6; DAFM, unpublished; Teagasc, unpublished). This increase may in part reflect increased response rates from growers between the 2007 and 2009 surveys (58% and 62% respectively) and the ROI 2013 survey (estimated as 100%) and consequently the

survey in earlier years could have underestimated the true figure of bumblebee colony import numbers (Murray *et al.*, 2008; Teagasc, unpublished). However, the apparent trend from these data is that not only are more colonies being imported, but also individual growers seem to be intensifying their use of commercial bumblebee pollinators.

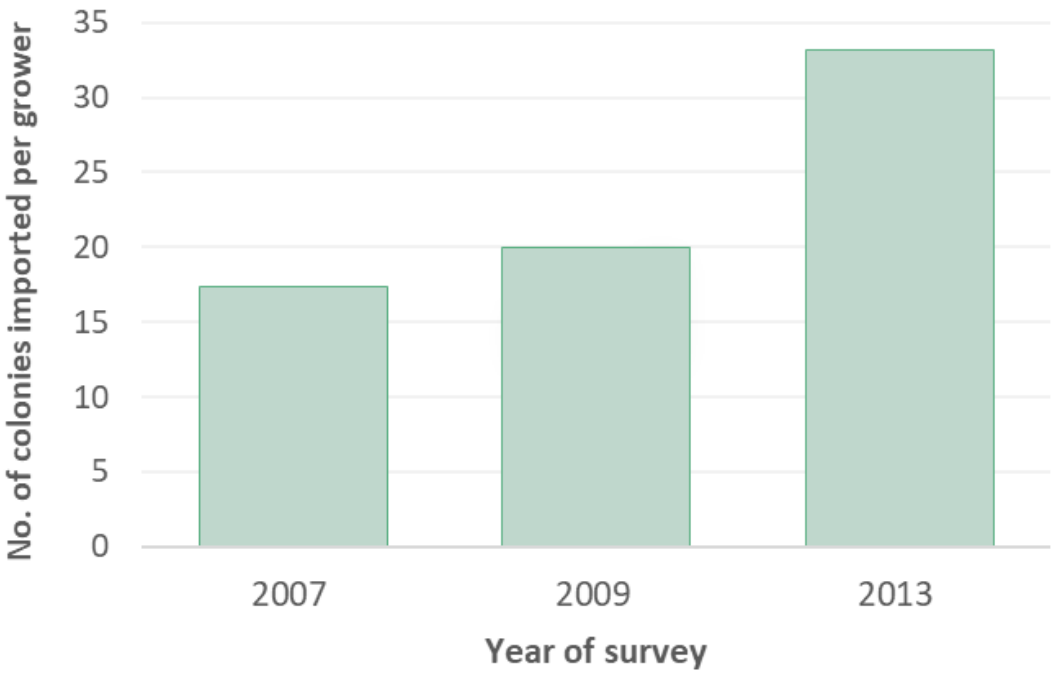


Figure 1.6 Results of Teagasc and Dept. of Agriculture grower surveys on quantities of commercial *B. terrestris* colonies imported. Results of Teagasc surveys (2007, 2009) and DAFM survey (2013) given to growers in across Ireland and in the Republic of Ireland respectively on the mean numbers of *B. terrestris* colonies imported per grower (DAFM, unpublished; Teagasc, unpublished).

Although importation of bumblebee colonies is on the increase, it is not clear how much of a benefit these colonies provide to crop pollination, particularly in open-field settings. In a Canada-based study, Whittington *et al.* (2004) found that up to 73% of pollen collected by commercial bumblebee workers (*B. impatiens* and *B. occidentalis*) came from plants other than the intended crop, located outside the greenhouses, a result supported by findings of an Irish study in 2008, which found that 28% of pollen collected by bumblebees in greenhouses did not originate from the target crop, and this increased to nearly 90% in field crops (Teagasc, 2012). Conversely, a study by Lye *et al.* (2011) found that, in the case of polytunnels, deployment of commercial

bumblebee colonies resulted in increased weight of raspberries, suggesting a clear benefit to profits via the use of imported bumblebee colonies.

In 2007, importation of commercial bumblebees was concentrated along the East coast of Ireland, where fruit production is also focussed, with Dublin-based growers importing greater than 71 colonies per grower, and growers in Wexford, Kilkenny, Waterford and Louth importing between 31-70 colonies per grower (Murray *et al.*, 2008). The principal and most consistent supplier to the Irish market is Koppert Biological Systems (DAFM, unpublished; Teagasc, unpublished). In 2013, nearly three quarters of growers imported bumblebee colonies from Koppert, 20% of growers sourced their bumblebees from Syngenta and less than 2% obtained colonies from Biobest (DAFM, unpublished). Internationally, over 95% of commercial *Bombus* colonies are used to pollinate greenhouse tomatoes and sweet peppers (Winter *et al.*, 2006). In contrast, survey data from 2013 showed that 60% of growers in Ireland import bumblebee colonies to pollinate strawberry crops (DAFM, unpublished), although from 2007-2013, there was an increase in the proportion of growers that utilised imported bumblebee colonies to pollinate apples and tomatoes (Figure 1.7).

1.6.4 B. terrestris as an exotic species

While *Bombus terrestris* is one of the most widely spread species of bumblebee in its native range, it also exists in many places outside this range due to deliberate and accidental introductions alike. In 1885 and 1906, queen bumblebees of many species were collected from the UK and brought to New Zealand to facilitate the pollination of red clover (*Trifolium pratense*), four of which became established there, including *Bombus terrestris* (Goulson and Hanley, 2004; Velthuis and van Doorn, 2004). These introductions were misguided, as these bumblebees actually "rob" the nectar of red clovers and are not effective pollinators as a result (Goulson, 2010a). *B. terrestris* arrived in Tasmania in 1992, though it is unknown whether this was an accidental or deliberate introduction (Semmens, 1993). Since then, its distribution has spread by ~10km per year and populations were found to be established throughout the island by 2005 (Buttermore, 1997; Stout and Goulson, 2000; Hingston *et al.*, 2006).

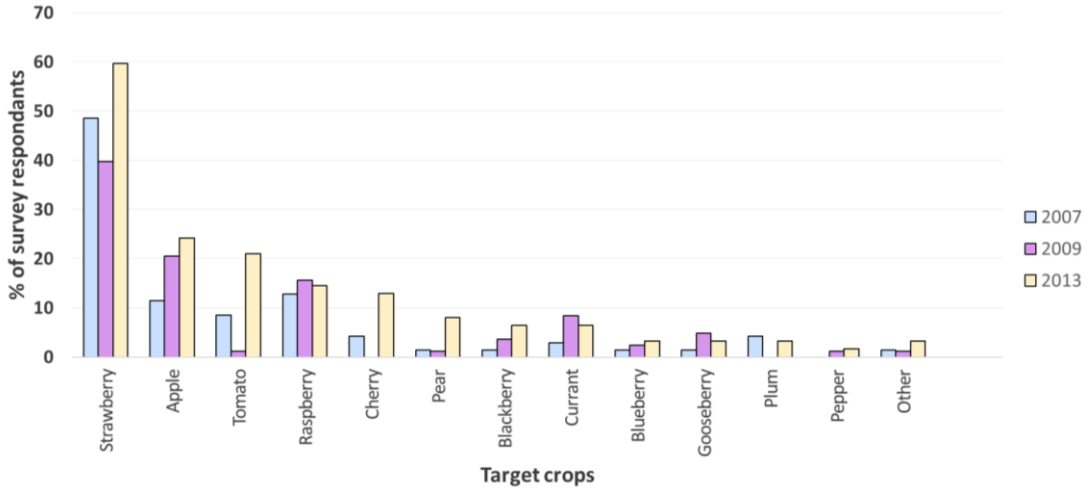


Figure 1.7 Survey results of crops grown by users of imported *B. terrestris* colonies. Results of Teagasc surveys (2007, 2009) and DAFM survey (2013) given to growers importing bumblebee colonies across Ireland and in the Republic of Ireland, respectively, on the types of crops they grow.

Japan has used imported *B. terrestris* colonies to pollinate greenhouse tomatoes in glasshouses since 1991, however it is thought that *B. terrestris* escaped from these greenhouses and were found established in the wild five years later (Goka *et al.*, 2001; Matsumura *et al.*, 2004). *B. terrestris* is now considered invasive in Japan, where it has caused the displacement of the native Japanese *B. hypocrita* in many locations (Matsumura *et al.*, 2004; Inoue *et al.*, 2008). *B. terrestris* colonies have been imported into Chile from Israel and Belgium from 1998 for greenhouse tomato pollination (Ruz, 2002). In 2001, an attempt was made to use these bumblebees for avocado pollination in Chile which led to their subsequent establishment in the wild, with reports of the species spreading as far as Argentina (Torretta *et al.*, 2006). Where honeybees have been established in places for much longer than bumblebees, the effects of competition on native pollinating insects by the introduction and spread of bumblebees may not be as significant (Goulson *et al.*, 2002). The introduction of exotic pollinators also poses a threat of pollinating and facilitating the spread of exotic and invasive weeds (Stout *et al.*, 2002).

1.6.5 Risks associated with the use of imported B. terrestris colonies

As a non-native, genetically distinct subspecies of *B. terrestris* was being imported into Ireland and the UK at least up until 2010, it is quite possible that this commercial stock of bumblebees established itself during and after this time. Escape of commercials poses significant threats to native wildlife, such as pathogen and parasite spill-over from commercials to wild pollinator populations, potential establishment of commercial bumblebees and the resulting competition with native species and hybridisation with native subspecies (Colla *et al.*, 2006; Goulson, 2010a). In Ireland, *Bombus* imports are usually used in greenhouses and polytunnels which require ventilation and thus facilitate the escape of bees (Murray *et al.*, 2008). Reproductive individuals (gynes and males) can escape from greenhouses and potentially establish themselves outside of their native range (Goulson *et al.*, 2002). Commercial bumblebee colonies are also used in field crops such as eating and cider apples where there are no barriers to escape.

Inadequate disposal of bumblebees, such as leaving the colonies *in situ* or outside to die, also enables the escape of commercial bees (Murray *et al.*, 2008). Surveys carried out in 2007, 2009 and 2013 highlighted that most growers in Ireland leave their commercial colonies *in situ* until they are dead (DAFM, unpublished; Teagasc, unpublished). Between 2007 and 2009, there was a significant increase in the number of growers disposing of their colonies correctly (i.e., destroying the colony) due to dissemination efforts from less than 5% to nearly 30%, however in 2013, only just over 20% of growers in the Republic of Ireland disposed of their colonies correctly (Teagasc, unpublished). In 2013, nearly a quarter of growers in the Republic of Ireland felt it was cruel to kill bees and this influenced their choice of disposal method (Teagasc, unpublished).

In a study investigating the mating preference of imported commercial *B. t. dalmatinus* in the UK, it was found that, while commercial *B. terrestris* preferred to mate with members of its own genetic stock, cross-mating events still took place between commercial *B. terrestris dalmatinus* gynes and males belonging to the British *B. terrestris audax* subspecies (Ings *et al.*, 2005). This suggests that hybridisation between subspecies is entirely possible and may have already taken place between populations of Irish and British *B. t. audax* since the importation of commercial, non-native sub-species of *B. terrestris* began in the late ‘80s (Goulson, 2010a). It is also

possible that establishment or hybridisation of commercial *B. terrestris* with native subspecies could explain the recent reports of winter active bumblebees in Britain (Stelzer *et al.*, 2010).

Using mitochondrial and microsatellite genetic markers, it was confirmed in Ireland that commercial bees could spread as far as 10km from the original site of importation (Teagasc, 2012). Furthermore, these genetic markers also confirmed that commercial *B. terrestris* can successfully mate and produce reproductive gynes and males; they also were shown to overwinter and produce workers the following Spring (Teagasc, 2012). At the very least, this suggests commercial *B. terrestris* could compete with native species and subspecies for hibernation and nesting sites. Reproductive commercial individuals could also have hybridised with the native *B. t. audax*, causing the loss of native, potentially beneficial alleles through introgression.

Assuming Biobest and Koppert may have sourced some of their initial stock of *B. t. audax* from Britain when developing these colonies for commercial use in 2010 and 2011 respectively, these commercial colonies may still be genetically distinct from Irish populations of *B. t. audax*. Additionally, commercial bumblebee lineages may be exposed to selection pressures derived from their breeding in commercial settings. The traits likely to be selected for, intentionally or not, are colony production of many workers and production of reproductive individuals in a captive setting. Assuming that captive stocks do not have subsequent wild individuals added to their population, internal physiological and cellular processes may have to change to adapt to life and reproduction in captivity. The goal of commercial companies is to produce suitable products for export. Once exported, the commercial colonies are placed in an environment that they have been evolutionary removed from by many generations. In order to effectively regulate bumblebee imports, it is crucial to determine the precise taxonomy and relatedness of both native and commercially-imported bumblebees, as was demonstrated by Williams *et al.* (2019) as well as investigate the effects of domestication on commercial bumble populations.

A comparison of nectar foraging ability and reproductive ability between British native *B. t. audax* and commercial *B. t. dalmatinus* was carried out by Ings *et al.* (2006). They found that not only were the commercial bees often superior nectar foragers to the native *B. t. audax* bees due to their increased size, they also produced

on average more gynes than the natives (Ings *et al.*, 2006). While it is difficult to provide evidence for competition between native and non-native species, increased size and foraging efficiency of the non-native *B. terrestris* subspecies could, if established or hybridised with *B. t. audax*, result in the out-competing of many native pollinator species.

Cases of pathogen spillover and disease transmission in invertebrates have not been as well documented as they have been for vertebrates, and particularly humans (Murray *et al.*, 2013). However, one of the most potentially significant threats posed by the importation of commercial pollination is the threat of pathogen spill-over (Goka *et al.*, 2001). In 2008, it was found that commercial *B. terrestris* colonies imported into Ireland, before production *of B. t. audax* began, carried *Nosema bombi* and *Crithidia bombi* (60% and 35% of colonies, respectively; Murray *et al.*, 2013). In total, 75% of the colonies were infected including one instance of *Apicystis bombi* infection.

Commercially produced non-native *B. t. dalmatinus* or *B. t. terrestris* colonies, and commercially produced native *B. t. audax* colonies from three producers were screened for pathogens by Graystock *et al.* (2013). Over three quarters of colonies were found to be infected or carried microbial parasites, a situation that would violate current import regulations. The parasites/pathogens found included *Crithidia bombi, Apicystis bombi* and *Nosema bombi* (bumblebee specialists), deformed wing virus and *N. ceranae* (infect both bumblebee and honeybees) and three additional honeybee pathogens including *Paenibacillus larvae* (found in pollen provided with colonies as food), which causes American foulbrood disease (Graystock *et al.*, 2013).

Pathogen spill-over from commercial bumblebees to wild bumblebees has been suggested to have contributed towards rapid drops in native bumblebee numbers in North America (Colla and Packer, 2008) and the incidence of *Crithidia bombi* and *Nosema bombii* in wild bumblebees increases near greenhouses using imported bumblebees (Colla *et al.*, 2006; Otterstatter and Thomson, 2008). In Ireland, very similar results were found to the Canada-based study by Colla *et al.* (2006) in spite of differences between cropping systems, with incidence of *C. bombi* observed to be highest within 2km of greenhouses using commercial bumblebees and a decline found in probability of bumblebees being infected with either *N. bombi* or *C. bombi* with increasing distance away from greenhouses using commercial bumblebees (250m to

10km away; Murray *et al.,* 2013). Evidence also exists for the introduction of *Apisistis bombi* into Argentina through the spread of *B. terrestris* bumblebees, which escaped from Chile in 2006 (Arbetman *et al.*, 2013).

1.6.6 Legislation in Ireland and the UK regarding the importation of commercial bumblebees

A risk assessment was carried out in Britain regarding the importation of commercial *B. terrestris,* concluding that the spread and establishment of non-native bumblebees was a likely risk and that it was highly probable that these bees could spread damaging organisms (NNSS, 2009). In the UK, it is currently illegal to import and allow the release of any animal that is "of a kind" non-native to the UK under section 14 of the Wildlife and Countryside Act 1981. According to DEFRA, this perspective applies to non-native commercial *B. terrestris* subspecies (Government of England, 1981; Natural England, 2018). Natural England now strongly advises that growers only import the native subspecies, *Bombus terrestris audax*, for commercial use (Natural England, 2018). However, it is still possible to obtain a license to release non-native *B. t. terrestris* and *B. t. dalmatinus* in greenhouses and polytunnels when the 'native' *B. t. audax* colonies are not available for purchase for either commercial or research purposes (Natural England, 2018). Non-native *B. terrestris* colonies are forbidden to be used in open fields and must be removed before the 70th day after their arrival / before September 30th and killed within two days of this removal.

While Ireland is a single island, there are two different jurisdictions that are responsible for regulating the importation of commercial bees. The Department of Agriculture, Environment and Rural Affairs (DAERA) regulates beekeeping and bee imports in Northern Ireland and The Department of Agriculture, Food and the Marine (DAFM) in the Republic. In the Republic of Ireland, under the European Communities (Birds and Natural Habitats) Regulations 2011 (Regulation 49) it is an offence for anyone to "[breed, reproduce or release] … or [allow]…escape from confinement" any animal (where the definition of animal includes both vertebrates and invertebrates of "any species, hybrid, subspecies, breed, race, strain, sport, variety, or other infraspecific taxon of such an animal") that is not "ordinarily resident", "not a regular visitor to the State" or which is not "of a kind that is domesticated or that is in the

normal course the subject of human husbandry" (S.I. No. 477/2011). Regulation 49 also provides prohibitions regarding the breeding, reproduction and release of mainly invasive animals listed in the Third Schedule, which includes animals such as the grey squirrel (*Sciurus carolinensis*) and zebra mussel (*Dreissena polymorpha*; S.I. No. 477/2011). Prior to Brexit and as members of the EU, bee imports in both the UK and Ireland followed EU rules and regulations outlined in the "Balai" Directive (Council Directive 92/65/EEC). Before bumblebees are exported, they must be certified that they are healthy/parasite-free in their country of origin and then are subject to checks once arrived at their destination. Bees must be imported from a location which does not have a "prohibition order associated with an occurrence of American foulbrood" or from an "environmentally isolated structure" with no occurrence of American foulbrood and that carries out inspections immediately before dispatch of colonies; bees also must not show any symptoms of American foulbrood infection (Council Directive 92/65/EEC). In addition, there are similar regulations requiring the area of origin of the bees to be free of the small hive beetle, *Tropilaelaps* mite and Fireblight (*Erwinia amylovora*; Council Directive 92/65/EEC). Bees and their packaging must be visually inspected for the presence of eggs, larva or adults of the small hive beetle, the *Tropilaelaps* mite and any other infestations. Notably, these are all pathogens and parasites mostly specific to honeybees. In the Republic of Ireland, importers of bees are required to notify the Horticulture and Plant Health Division of DAFM at least 24 hours before the arrival of the bees into the country. Similarly, in Northern Ireland, at least a full day's notice before arrival of the bees needs to be given in writing to DAERA's Quality Assurance Branch.

1.6.7 Guidelines for users of imported colonies

To address the risks posed by imported bumblebee colonies to pollinators in Ireland, I developed a document alongside the All-Ireland Pollinator Plan which aimed to empower and educate growers in Ireland using imported bumblebees (Figure 1.8; Appendix A1.1). This document included up-to-date information on the differences between wild and commercial *B. t. audax* bumblebees, why using commercial bumblebees could cause harm to wild pollinators with advice on how to mitigate these risks as well as encourage wild pollinators to a farm. We provided growers with information on the biology of bumblebees and how to tell if a colony is showing signs

of trouble or reaching a certain point in its lifecycle. We hope that these guidelines for users of imported bumblebees inform growers on the importance of careful management of imported colonies and empower them with the knowledge to monitor the quality and progression of colonies.

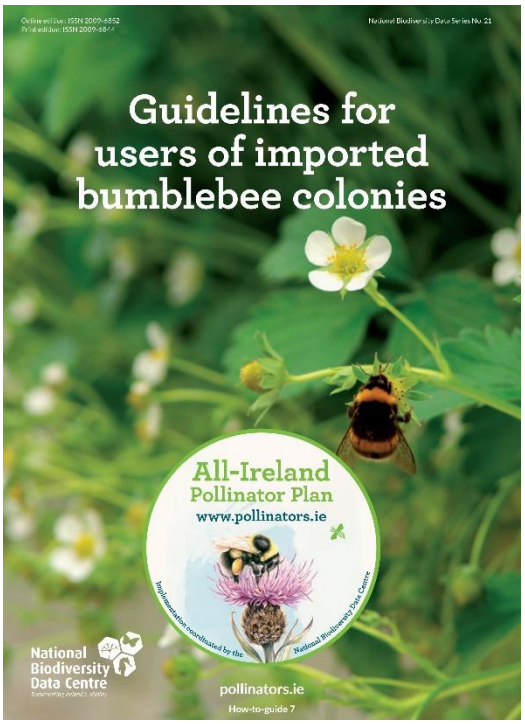


Figure 1.8 Cover of the All-Ireland Pollinator Plan "Guidelines for users of imported bumblebee colonies" publication. Guidelines aimed at growers in Ireland using commercial bumblebee colonies for crop pollination (Appendix A1.1). I wrote the main text of this publication with the help of Dr Úna FitzPatrick (National Biodiversity Data Centre), Dr James Carolan (Maynooth University), Prof Jane Stout (Trinity College Dublin), Dr Dara Stanley (Univeristy College Dublin), Dr Rachel Wisdom (DAFM) and Dr Michael Gaffney (Teagasc).

1.7 Important physiological systems of insects

To fully understand the possible risks and consequences of using commercial bumblebees, thorough research is required to investigate domestication of captive bumblebee populations and changes, both genetic and phenotypic, that may have occurred in these populations. This requires not only an evaluation of the observable traits that may manifest through domestication, but also an assessment of the molecular traits that may be being altered both directly and indirectly through practices such as artificial selection. In the following sections, I outline important physiological processes in bumblebees with a view to introduce experiments which will compare

organs central to these processes in wild and commercial bumblebees. In addition, I will address the importance and roles of the gut microbiome in bumblebees (Section 1.8), before discussion on the key molecular methods that I have used in my research to investigate selection and distinction in wild populations of *B. t. audax* bumblebees as well as phenotypic distinctions between wild and commercial bumblebees (Section 1.9).

1.7.1 The insect nervous system

Neurons and glial cells are the core main components of the insect nervous system. Neurons are made of a cell body, called a soma, and projections from this cell body called axons and dendrites (Klowden, 2013). Dendrites carry electrical excitation towards the soma, while axons conduct electrical excitation away from the soma (Nation, 2008). Axons of sensory neurons carry signals towards the central nervous system (CNS) while axons of motoneurons carry excitation towards muscle or glands. In vertebrates, glial cells make up to 90% of the brain cells, while in insects this proportion is much less (10-25%; Klowden, 2013). Glial cells support neurons via the provision of structural, nutritional and metabolic support (Freeman and Doherty, 2006).

The process by which cells within the nervous system communicate is facilitated by a neuron's ability to vary its electrical potential significantly. Once the dendrite in a neuron is stimulated and reaches its receptor potential, the axon of the neuron reaches it action potential and becomes depolarised along its length. To create the action potential, ion pumps in the membrane of the axon pump out cations (e.g., sodium or calcium) and take in anions (e.g., potassium). The action potential reaches the presynaptic membrane, ion channels allow calcium to enter the cell, synaptic vesicles fuse with the presynaptic membrane and release contained signalling molecules into the synaptic cleft (Klowden, 2013). These can include neurotransmitters, neuromodulators and neuropeptides. Acetylcholine and glutamate are well known excitatory neurotransmitters, while the γ-Aminobutyric acid (GABA) neurotransmitter is inhibitory (Hyman, 2005; Klowden, 2013). GABA is also known as a neuromodulator as it can influence the release of neurotransmitters of the same cell or impact the ability of the postsynaptic membrane to respond to neurotransmitters (Chanda and Xu-Friedman, 2010).

The brain and the ventral nerve cord are the main components of the insect central nervous system. The insect brain, or supraesophageal ganglion, is made up of a grouping of neurons and is found in the head, about the oesophagus (Klowden, 2013). There are three components to the insect brain: the protocerebrum, the deutocerebrum and tritocerebrum.

The protocerebrum is associated with visual systems such as the compound eyes and ocelli (Klowden, 2013; Lin *et al.*, 2013). It forms two lobes which house dense collections of neurosecretory cells in an area known as the pars intercerebralis (Klowden, 2013). Secreted material from these cells is transported to glands (the corpora cardiaca and corpora allata) for release (Klowden, 2013). The neurosecretory cells in the pars intercerebralis are important in the regulation of growth, reproduction and biorhythms (Pazaroviçi and Pener, 1978; Shiga and Numata, 2000; Matsui *et al.*, 2009). Also found within the protocerebrum are the mushroom bodies, known as the corpora pedunculata, which are made up of intrinsic neurons called Kenyon cells (Farris and Sinakevitch, 2003). Kenyon cells receive signals from sensory neurophils in the antennal lobes, tritocerebrum and are thought to be pivotal in the processing and storage of olfactory information and memory as well as facilitating the coordination between visual and locomotor systems (Davis, 1993; Skoulakis *et al.*, 1993; Fahrbach, 2006; Klowden, 2013). Mushroom bodies in honeybees also display a large degree for anatomical plasticity. Withers *et al.* (1993) found that honeybee foragers had larger neurophil volumes in their mushroom bodies than both non-foraging adult nurse workers and day-old workers.

The deutocerebrum forms the midsection of the insect brain and it innervates sensory receptors and antennal muscles in the antennal lobes and antennal mechanosensory and motor centres, respectively (Nation, 2008; Klowden, 2013). It is composed of many glomeruli in which receptor neurons synapse with interneurons which then synapse with neurons extending into the mushroom bodies of the protocerebrum (Klowden, 2013). In this way, the deutocerebrum forms a passageway between signals picked up by sensory receptors and the mushroom bodies, where these signals can be processed, interpreted and memories of them stored. The smallest section of the brain is the tritocerebrum connects the central nervous system to the circumesophageal connectives and visceral nervous system (Klowden, 2013). The tritocerebrum

innervates the stomatogastric nervous system which control the muscles of the insect foregut (Nation, 2008).

The central nervous system in insect is quite decentralised, meaning ganglia are located throughout the body and these are sufficient to control certain activities with no input from the brain e.g., walking (Schilling and Cruse, 2020). The visceral nervous system innervates the digestive tract, glands and the heart and plays an important role in digestive processes (Klowden, 2013). Thoracic ganglia are largely responsible for the activity of legs and wings (Nation, 2008; Klowden, 2013).

1.7.2 The insect fat body

Insects possess a unique organ called fat body; that exists in a loosely organised tissue just inside the cuticle which is present in the insect head, thorax but mainly in the insect abdomen (Nation, 2008). This organ has many roles and has been compared to both the vertebrate liver and adipose tissue in function (Klowden, 2013). During insect development, the fat body originates from cell clusters that form from embryonic mesodermal tissue, which then proliferate and generate the dorsal, ventral and lateral fat body domains (Hoshizaki *et al.*, 1994; Hoshizaki, 1998). The main cells of the fat body are trophocytes, also known as adipocytes, which can exhibit differentiation depending on their location and the needs of the insect (Klowden, 2013). Trophocytes house vacuoles containing lipid droplets, free and bound glycogen and protein granules (Klowden, 2013). Across insects, there are several less common fat body cell types including haemoglobin cells, urocytes, mycetocytes and chromatocytes, however bumblebee fat bodies have been shown to just have adipocytes, described above, and oenocytes, which are involved in detoxification, the processing of lipids and have close association with the insect cuticle (Martins and Ramalho-Ortigao, 2012)

The fat body is responsible for the accumulation and storage of reserves such as lipids and carbohydrates, as well as their utilisation (Hoshizaki, 1998; Arrese and Soulages, 2010). Insects are using up energy constantly and depend on stored reserves that were accumulated in times of increased nutrient intake. These reserves are essential for insects to survive diapause, to undergo developmental processes and to fuel intense periods of activity e.g., when flying (Beenakkers *et al.*, 1984; Ziegler and Van

Antwerpen, 2006; Hahn and Denlinger, 2007). The core energy reserves stored in animal cells are triglyceride and glycogen, which store fatty acids and glucose respectively (Arrese and Soulages, 2010). Storage of glucose and fatty acids also facilitate the production of eicosanoids and pheromones (Lockey, 1988; Stanley, 2006). Triglycerides often contribute to over half the dry weight of the fat body, and are stored as lipid droplets within fat body cells (Hoshizaki, 1998). Both fatty acids and dietary carbohydrates can be converted to triglycerides. Triglycerides are particularly important reserves due to their higher caloric content and release of H_2O upon β-oxidation (Downer and Matthews, 1976; Athenstaedt and Daum, 2006). Although what exactly triggers lipid synthesis in insects is not well defined, the Target of Rapamycin (TOR) signalling pathway is thought to play a role (Hoshizaki, 1998). While glucose is stored as glycogen in the fat body, it is in the form of trehalose when circulating in the haemolymph. The levels of trehalose in the haemolymph influence the total glycogen stored in fat body cells (Hoshizaki, 1998).

The release of energy reserves is usually stimulated by starvation or high activity levels (Arrese and Soulages, 2010). The fat body can sense changes in nutrient status of the insect by deploying amino acid transporters. In *Drosophila*, a gene named *slimfast*, encodes an amino acid transporter, and when this gene is downregulated within the fat body, it causes a phenotype similar to one created by poor nutrition (Colombani *et al.,* 2003). The mobilisation of reserves are regulated by the adipokinetic hormone (AKH) protein family which is important in regulating the provision of energy to tissues, particularly trehalose to flight muscles (Kaufmann and Brown, 2008). The insulin/insulin-like growth factor (IGF) pathway (IIS) maintains energy homeostasis through the regulation of carbohydrate storage. Also, the IIS pathway and the Target of Rapamycin (TOR) signalling pathway together control protein synthesis and autophagy (Hoshizaki, 1998; Li *et al.*, 2019).

In addition, the fat body is centrally involved in the formation of haemolymph components. In *Calpodes* larva, 90% of the haemolymph proteome is synthesised by the fat body (Hoshizaki, 1998). The form and distribution of the fat body means that most fat body cells are in immediate contact with the haemolymph and so can efficiency exchange metabolites and proteins. Fat bodies produce vitellogenin which are taken up by maturing oocytes as they develop (Roy *et al.*, 2018). In honeybees, *A. mellifera*, the vitellogenin produced by fat bodies also regulates juvenile hormone

signalling (JH) and caste-specific behaviours such as foraging (Nilsen *et al.*, 2011). In addition, the insect fat body plays a key role in the innate immune system. Recognition of pathogens triggers intracellular signalling cascades, such as the Toll and IMD pathways, in fat body cells, which result in the synthesis of anti-microbial peptides (AMPs) and molecules that regulate the immune response (Charroux and Royet, 2010; Choi and Hyun, 2012; Azeez *et al.*, 2014). The innate immune system will be discussed in more detail in the following section.

1.7.3 Insect immunity

Insects to do not have an adaptive immune system, in which specialised cells and antibodies in the immune system respond to specific pathogens and "remember" them if infection reoccurs (Janeway *et al.*, 2001). Instead, they depend on several traits to avoid infection, and in the cases where pathogens or parasites overcome these barriers to infection, insects have an innate immune system. The first physical barrier insects use to ward off infection is their external cuticle or exoskeleton alongside the cuticular lining of their digestive, tracheal and reproductive systems. The insect cuticle is composed of chitin microfibrils that are embedded in protein which contributes greatly to both the mechanical and biological resistance of the exoskeleton to infection (St. Leger, 1995; Nation, 2008). Social insects also exhibit social immunity. This is an adaptation to living in highly-dense and highly-related communities which can increase the chance of pathogen infection and transmission (Schmid-Hempel, 1998; Barribeau and Schmid-Hempel, 2013). These behaviours include both individual level and colony-level actions that can prevent contamination and infection. Nests of social insects are regularly cleaned of contaminants and waste; individuals groom themselves and, in some species like honeybees, each other (Cremer, 2019).

The innate immune response is composed of two arms: the cellular and humoral responses. Cellular responses are comprised of processes such as phagocytosis and encapsulation orchestrated by haemocytes (Gillespie *et al.*, 1997; Strand, 2008). There are at least eight kinds of haemocytes known in insects, however, in terms of bumblebees not much is known about the specific roles and characteristics of each type and their role within the immune system (Ghoneim, 2019). Melanisation, another cellular process, is the process where phenoloxidase acts on phenols such as tyrosine to generate quinones. Quinones then undergo autopolymerisation and produce

melanin, which creates a toxic and suffocating layer around encapsulated infectious organisms (Chase *et al.*, 2000).

The humoral response is initiated when specific pathogen associated molecular patterns (PAMPs) are recognised by host pattern recognition proteins (PRPs). PAMPs include pathogen related proteins such as components of pathogenic membranes and cell walls e.g., β-1,3-glucans, lipopolysaccharides and peptidoglycans (Steiner, 2004). The recognition of a PAMP by a PRP usually results in a serine protease cascade (Gorman and Paskewitz, 2001). Through a signalling cascade, the presence of an infective agent is communicated to cells in the fat body. There are four key intracellular signalling pathways which can be activated in the humoral response: the Toll, IMD, JNK and JAK/STAT pathway, which all take place in the insect fat body cells (Agaisse and Perrimon, 2004; Evans *et al.*, 2006). These pathways then usually culminate in the expression of genes encoding effector molecular such as antimicrobial peptides (AMPs; Nation, 2008).

When Gram-positive bacterial and fungal pathogens are detected by host PRPs, a serine protease cascade occurs and results in the cleavage of the haemolymph protein Späetzle (Ligoxygakis *et al.*, 2002). Späetzle then binds Toll, the transmembrane signal transducing receptor on fat body cells, which then activates the intracellular Toll pathway. Components such as MyD88, Tube and Pelle are known to be involved in this serine kinase cascade in *Drosophila* (Imler and Hoffmann, 2001). A kinase then phosphorylates the Cactus (IκB) – DIF (dorsal immune factor) complex, which causes the release of Dorsal (an NF-κB) and its transport to the nucleus (Ferrandon *et al.*, 2004). Dorsal, or DIF in *Drosophila*, acts as a transcription factor and causes expression of genes encoding AMPs. In *Drosophila*, the Toll pathway activates expression of *Drosomycin* (Meng *et al.*, 2018)

Gram-negative and some Gram-positive bacteria activate the Immune Deficiency (IMD) pathway. A similar process happens in that the PAMPs, such as Gram-negative peptidoglycan containing diaminopimelic acid, are likely recognised by host PRPs which then activate a serine proteinase cascade that in turn activates the transmembrane IMD receptor (Kaneko and Silverman, 2005). Some of these PRPs e.g., PGRP-LE may be localised near or at the fat body cell membrane and so could act with the IMD receptor to activate the intracellular signalling response (Kaneko *et*

al., 2006). Like the Toll pathway, the intracellular IMD pathway involved a series of kinase reactions, resulting in the localisation of Relish to the nucleus. Like DIF, Relish is an NF-κB-like transcription factor that bind DNA and induces expression of genes encoding major AMPs such as diptericin in *Drosophila* (Myllymäki *et al.*, 2014). In honeybees, the IMD pathway appears to be highly conserved with orthologues for all major participant proteins present (Evans *et al.*, 2006). The IMD also has a downstream branch called the JNK pathway. This pathway is thought to regulate expression of AMP genes through both up- and down-regulation (Wojda *et al.*, 2004).

The Janus kinase–signal transducer and activator of transcription pathway, or JAK/STAT pathway for short, is involved in processes such as immunity, cell death and division alongside its role in immune functioning (Rawlings *et al.*, 2004). The signal transducer and activator of transcription (STAT) protein family is involved in regulating immediate responding genes (Aaronson and Horvath, 2002). However, these proteins are unable to act as transcription factors unless they are activated through tyrosine phosphorylation (Aaronson and Horvath, 2002). Janus kinases are receptor associated proteins in the cytoplasm and have the required tyrosine kinase activity to activate STAT proteins (Shuai and Liu, 2003). In the insect immune system, this pathway is essential in coordinating host immune responses through, for example, maintaining levels of haemocyte proliferation and degradation (Makki *et al.*, 2010) and by producing AMPs to target viral infections (Kingsolver *et al.*, 2013)

Immune stimulation has shown in bumblebees to cause an increase in food consumption (Tyler *et al.*, 2006). An activated immune system is therefore costly and has been shown to be associated with trade-offs. Honeybees and bumblebees both exhibit reduced learning when their immune systems were activated using non-pathogenic elicitors (Mallon *et al.*, 2003; Alghamdi *et al.*, 2008). Bumblebees show impaired flight when infected with heat-killed *Paenibacillus larvae*, whereas honeybee flight is not affected, suggesting bumblebees suffer a higher cost when their immune response is activated (Riessberger-Gallé *et al.*, 2015). Barribeau and Schmid-Hempel (2013) found that *B. terrestris* infected with different strains of the trypanosome, *C. bombi*, elicited different responses. Bumblebees have been shown to exhibit evidence of a trade-off between eliciting a general immune response compared with a pathogen-specific immune response (Mallon *et al.*, 2003).

In contrast to the general consensus that insect depend solely on an innate immune system, there is some evidence that insects may have some level of adaptive immunity. One gene, Down syndrome cell adhesion molecule (DSCAM), is a possible candidate involved in such an immune response. The *Dscam* gene in *Anopheles gambiae* mosquitoes has been shown to produce over 30,000 alternative splice forms and produces specific splicing patterns depending on the type of infection (Dong *et al.*, 2006). When *Dscam* is silenced, the mosquito host's susceptibility to infections is increased (Dong *et al.*, 2006). While *Dscam* resembles antibodies in these characteristics, it does not produce one highly specific product but instead a suite of effectors with varying levels of specificities (Ziauddin and Schneider, 2012). Furthermore, there is also evidence that insect immune systems can be primed. By infecting them with non-pathogenic *E. coli*, hornworm (*Manduca sexta*) caterpillars were more resilient to subsequent infections with a virulent pathogen *Photorhabdus luminescens* through an upregulation of PRPs and AMPs (Cooper and Eliefthérianos, 2017). Future research will hopefully shed light on the specificities of Dscam and other immune effectors, e.g., AMPs, in invertebrates and their potential to influence subsequent immune responses and host resilience.

1.8 Gut microbiomes

In addition to associations with microbial pathogens, many organisms have commensal and symbiotic microbes in organs such as the digestive tract (Walter and Ley, 2011; Krishnan *et al.*, 2014; Clayton *et al.*, 2018; Jang and Kikuchi, 2020). The evolutionary success of insects is credited partly to their relationships with symbiotic microbes (Engel and Moran, 2013). Symbiotic microorganisms provide insect and invertebrate hosts with protective (Johnson, 2015; Kwong *et al.*, 2017; Van Arnam *et al.*, 2018), nutritional (Douglas, 1998; Nikoh *et al.*, 2014; Smith *et al.*, 2017; Raymann and Moran, 2018) and developmental benefits (Chouaia *et al.*, 2012; Lee *et al.*, 2017; Paludo *et al.*, 2018; Negroni *et al.*, 2020). Furthermore, in *D. melanogaster*, the components of the gut microbiome can impact learning and memory (DeNieu *et al.*, 2019).

Microbial communities of bumblebees and other bee species have similar associations with health and fitness. Honeybees and bumblebees host a characteristic gut microbial

community that is made up of several core genera including *Gilliamella, Snodgrassella, Lactobacillus* and *Bifidobacterium* (Martinson *et al.*, 2011; Hammer *et al.*, 2022), however strain diversity within these select few communities can be high (Engel *et al.*, 2014; Kwong *et al.*, 2014; Ellegaard *et al.*, 2015). Sociality facilitates the transmission of microbes to newly emerged adult bees which is key for the development of a typical bee gut microbiome (Martinson *et al.*, 2012; Koch *et al.*, 2013). This is called vertical transmission and is likely encouraged by behaviours such as trophallaxis among social bees. Rare or non-core microbiota can be acquired through horizontal transmission i.e. from environmental sources (Newbold *et al.*, 2015). Of course, sociality can be associated with increased risk of pathogen spread via the same behaviours (Otterstatter and Thomson, 2007). The mechanisms by which bumblebees differentiate and regulate harmful and helpful microbes are unknown (Engel *et al.*, 2016).

Many factors can affect gut microbial communities including age (Tarpey *et al.*, 2015; Hammer *et al.*, 2022), diet (Billiet *et al.*, 2016; Harris *et al.*, 2019), seasonal weather (Castelli *et al.* 2022), exposure to agrochemicals (Kakumanu *et al.*, 2016; Cullen *et al.*, 2023) and antibiotic treatment (Raymann *et al.*, 2017). Bee gut microbial communities can influence bee health and fitness. Gut symbionts can influence weight gain in honeybees likely via lowering of the gut pH through production of short-chain fatty acids (Zheng *et al.*, 2017). It has also been shown that *Gillimella apicola* in honeybees metabolise toxic sugars found in certain nectars (Zheng *et al.*, 2016). *Gilliamella* has also been shown to degrade pectin, a sugar found in pollen walls (Engel *et al.*, 2012). Presence of key microbiota groups in bumblebees can protect against parasites such as *Crithida bombi* (Koch and Schmid-Hempel, 2011). For both social and solitary bees, learning and memory are important in navigation, gathering resources and avoiding predation (Collett *et al.*, 2006; Klein *et al.*, 2017; Nicholls and Hempel de Ibarra, 2017; Howard, 2021). Interestingly, the symbiotic bacterial species *Lactobacillus apis* has been shown to improve memory capabilities when supplemented to *B. terrestris* diets (Li *et al.*, 2021), however other studies have not found this link between memory and gut microbiome in bumblebees (Leger and McFrederick, 2020). In addition, *Zygosaccharomyces*, a common bee symbiont, which aids bee development through the production of sterols (Paludo *et al.*, 2018), has been linked to microcolony survival after pesticide exposure (Rutkowski *et al.*, 2022)

1.9 Molecular methods to investigate distinctions in *B. t. audax* populations

1.9.1 Genomics

The complete genome of *B. terrestris* (Bter_1.0) was first sequenced by Sadd *et al.* (2015). The genome of *B. terrestris* is an estimated length of 433Mb (Stolle *et al.*, 2011), although estimates have ranged from 274 Mb to 625 Mb (Gadau *et al.*, 2001; Wilfert *et al.*, 2006). More recently in February 2022, another genome became available for *B. terrestris* (iyBomTerr1.2) through the Wellcome Institute Darwin Tree of Life project (Darwin Tree of Life Project Consortium, 2022)

The initial sequencing of the *B. terrestris* genome, alongside the *B. impatiens* genome, revealed many novel insights into the evolution of these species' genomes and bumblebees generally (Sadd *et al.*, 2015). *B. terrestris* and *B. impatiens* genomes display large levels of synteny and contain many similar genes associated with development, also found across all Hymenoptera. There are also many highly similar genes shared between these bumblebee species and honeybees e.g., those involved in haplo-diploid sex determination, endocrinology, behaviour, neurophysiology and venom components (Sadd *et al.*, 2015). Also, highly similar between the bumblebees sequenced and *A. mellifera* were genes involved in RNAi and RNA editing. However, distinctions were found in the miRNAs between the *Bombus* and *Apis* species: each group had several unique miRNAs and changes to highly conserved miRNAs. MiRNAs are linked to key traits underlying social behaviour such as caste determination (Collins, 2014; Collins *et al.*, 2017) and so these distinctions may be responsible for distinctions between bees in their levels of eusociality (Sadd *et al.*, 2015). Interestingly, the bumblebee genomes were found to have experienced an expansion in gustatory receptors (GR) in bumblebees, with the *B. terrestris* genome containing 15 genes for GRs while *A. mellifera* contains just three (Sadd *et al.*, 2015). This finding suggests that the bumblebee lineage may have distinctions in their chemosensory abilities compared with other bee and hymenopteran species and may be underly differences in their ecology e.g., diverse nest building habits (Sadd *et al.*, 2015). All components of the classical innate immune pathway are represented in bumblebee genomes (Sadd *et al.*, 2015; Sun *et al.*, 2020). Genes involved in immune and detoxifying processes are depauperate in bumblebees, as in *A. mellifera* (Sadd *et al.*, 2015; Sun *et al.*, 2020). Indeed, the depauperate immune gene set of bees is thought

to precede the evolution of sociality in bees, as similar repertoires were found in a solitary leafcutting bee, *Megachile rotundata* (Barribeau and Schmid-Hempel, 2013).

Population genetics has already been used to evaluate many parameters essential to conservation e.g., distinctness or relatedness of populations, evidence of bottleneck or hybridisation events and the structure within populations (Nielsen *et al.*, 1997; Luikart *et al.*, 1998; Hedrick *et al.*, 2006; McLean *et al.*, 2008; McDevitt *et al.*, 2009; Hayden *et al.*, 2010). In Ireland, genetic research on Irish bees has until this point been performed mainly using microsatellite data and mitochondrial genes (e.g., Moreira *et al.*, 2015; Hassett *et al.*, 2018). Population genomics can answer questions that many population genetics approaches cannot. In population genetics, neutral markers are often used to gain insight into genetic variation and inbreeding within populations. Genomics increases the volume of neutral loci available for analyses, which consequently improves the accuracy and power of many downstream tests, such as those for inbreeding (Allendorf *et al.*, 2010). The approach of using just neutral markers in genetic analyses impedes any attempt to investigate how selection is affecting these parameters and adaptation within a population (Ouborg *et al.*, 2010). Genomics utilises both neutral and selectively important genetic variation to answer these questions which, as stated by Ellegren and Sheldon (2008), may lead to insights into "the genetic basis of those traits affecting fitness that are key to natural selection".

1.9.2 Label-free mass spectrometry based proteomics

Proteomics is the global analysis of proteomes i.e. the entire complement of proteins in an organ or tissue of an organism which are expressed by the genome at a given point in time (Wilkins *et al.*, 1996). Proteins are involved in all cellular processes and so when the proteins of one system are compared to another, it may be possible to identify differences in cellular or physiological functioning (Cox and Mann, 2007). Proteomics can provide data on the functional culmination of genome expression alongside host regulation of expression – taking into account, therefore, translational activity, posttranslational modifications and protein degradation (Cox and Mann, 2007). Proteomics is particularly advantageous over transcriptomics, the quantification of a complete set of transcripts in a cell, for these reasons and because several studies comparing have found that a sizeable percentage of proteins do not coexist alongside their corresponding transcripts (Maziarz *et al.*, 2005; Mack *et al.*,

2006). For example, Maziarz *et al.* (2005) carried out a side-by-side proteomic and transcriptomic analyses on murine pancreatic islet αTC-1 cells and found that about 15% of proteins were not represented by mRNA transcripts. However, transcriptomics can provide a cheaper alternative to proteomics (Tan *et al.*, 2009) as well as give information on the role of non-coding DNA sequences (Giuliani *et al.*, 2022) and the "transcriptional structure" of genes e.g., transcription start sites, mRNA splicing patterns and additional post-transcriptional modifications (Wang *et al.*, 2009).

Proteomics has been made possible largely through advancements in bioinformatics and mass spectrometry to analyse mixtures of proteins (Valcu and Kempenaers, 2015). Previously, targeted approaches were the only way to quantify specific proteins which required the use of antibodies or epitope-tags (Walther and Mann, 2010). Now, liquid-chromatography alongside tandem mass spectrometry (LC-MS/MS) – based proteomics is a widely used approach to analyse protein samples (Gillet *et al.*, 2016) due to its high sensitivity and specificity and its potential to analyse multi-analyte samples (van den Ouweland and Kema, 2012). To quantitatively analyse mixtures of proteins, the classical approach was to separate and compare proteins on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by MS or MS/MS to identify and quantify proteins (Issaq and Veenstra, 2008; Zhu *et al.*, 2009). However, gel-based methods were biased against proteins with certain properties e.g., high molecular weights or hydrophobic (Garbis *et al.*, 2005; Hart and Gaskell, 2005).

In contrast to gel-based approaches, bottom-up, high throughput or 'shotgun' approaches allow the MS equipment to carry out highly efficient analyses on a mixture of peptides which allows researchers to examine all proteins in an organism, tissue or cellular component under specific conditions or treatments (Nesvizhskii and Aebersold, 2005). This approach requires protein samples to undergo a digestion by proteolytic enzymes such as trypsin prior to mass spectrometry. High-performance liquid chromatography (HPLC) allows peptide sequences to be separated before entering the MS, which uses a fragment ion spectrum to identify the sequence of the peptides that can then be assigned to their original proteins through protein interference (Nesvizhskii and Aebersold, 2005; Gillet *et al.*, 2016). Several approaches of stable isotope labelling have been developed for this type of 'shotgun' quantitative proteomics. This involves using isotope labelled compounds identical to their natural versions in all but mass as tags (Zhu *et al.*, 2009). Control and treated protein samples

are labelled with light and heavy stable isotopes, combined, digested and then run through LC-MS/MS (Issaq and Veenstra, 2008). There are limitations associated with this approach including high expense, time-demand and the potential for incomplete labelling (Zhu *et al.*, 2009). Label-free quantitative proteomics instead analyses control and treated protein samples separately on LC-MS/MS and peak intensities of peptides are used to quantify these proteins (Patel *et al.*, 2009). This approach is significantly less expensive and faster than isotope-labelling methods (Zhu *et al.*, 2009).

Proteomics gives an opportunity to link genotype to the final protein-driven phenotype (Feder and Walser, 2005; Karr, 2008). As a result, it is becoming a very important tool in evolutionary ecology as natural selection acts primarily on the phenotypic level as species adapt (Khaitovich *et al.*, 2004). Furthermore, proteomics has the potential to identify proteins involved in the molecular processes that govern cellular, physiological and behavioural phenotypes in ecologically important organisms (Gorman and Paskewitz, 2001; Valcu and Kempenaers, 2015). In bumblebees alone, proteomic methods have provided insight into bumblebee proteomic responses to mating and diapause (Colgan *et al.*, 2019) and pesticide exposure (Cullen *et al.*, 2023) as well as paved the way for thorough characterisations of the bumblebee fat body, haemolymph (Larkin, 2018) and venom (Van Vaerenbergh *et al.*, 2015). These studies provide valuable information on important bumblebee physiological processes such as immunity and detoxification as well as elucidating bumblebee-specific proteins and pathways. Furthering our understanding of bumblebee biology and their physiological responses to threats likely causing their decline is essential to the conservation of these important pollinators.

1.10 Overview of thesis objectives

The aim of my thesis is to investigate selection and identify distinctions between wild and commercial populations of *B. t. audax* on genetic, proteomic, and behavioural levels. Understanding intraspecies variation in wild bumblebee populations as well as elucidating the impact of domestication on the traits of captive-reared commercial bumblebees is key for appropriate conservation, identification of vulnerable wild populations and appropriate risk assessment.

First, as described in Chapter 2, we use population genomics to compare two wild populations of *B. t. audax* which are currently considered to be the same subspecies. We use whole genome sequencing to sequence the genomes of Irish *B. t. audax* male bumblebees and compare these to previously sequenced genome data for a British population of *B. t. audax*. We investigate the genetic structure of these two populations alongside genomes of continental European subspecies and commercial *B. t. audax* representatives. We also look for signatures of recent selection in each population and identify alleles that distinguish populations. In Chapter 3, we take a closer look at the genome data from the Irish population and investigate the canonical immune gene set of this population. Canonical immune genes are those involved in conserved innate immune pathways across the animal kingdom. We use nucleotide diversity analyses and extended haplotype homozygosity to identify immune genes undergoing selection and those under constrained or diversifying evolution. This will provide insight into immune gene products that are potentially important and adapting to the pathogenic threats facing them in their natural environments. We also investigate the presence of high impact SNPs in immune gene sequences, indicating potential redundancy of certain immune genes. In Chapter 4, we investigate the proteomes of two key organs, the brain and fat bodies of wild-caught, Irish, and commercial *B. t. audax* workers (reared under the same procedures) and their responses to two key stressors (1) pesticide exposure and (2) infection with Gram-negative bacteria. We use label-free quantitative (LFQ) mass spectrometry to analyse the proteomes of these samples and investigate distinctions and similarities in the proteomes of wild and commercial bumblebees. This will provide insight into the potential effects of natural selection and domestication or artificial selection in bumblebees on their physiology. Finally, in Chapter 5, we evaluate distinctions between wild and commercial bumblebees in their colony rearing dynamics and growth to see if commercial bumblebees show higher success rates in captive-rearing conditions and exhibit evidence of domestication syndromes i.e., traits that have evolved or been selected for by humans in a captive setting. This will not only further our understanding of the impacts of domestication on captive bumblebee populations, but it will also provide information on the risks associated with commercial colony use in terms of competition with wild populations of pollinators.

It is anticipated that this research will provide novel and highly informative insights into the population structure and selective processes that are present within both wild and captive populations of *B. t. audax*. Findings of this research will also be essential to inform policy, risk assessments and management strategies regarding the use of commercial bumblebees in Ireland. These steps are essential if we are to protect locally adapted populations from the risks posed by imported colony use. Methods outlined in this thesis also provide the framework to assess other populations of ecologically important and potentially vulnerable pollinators.

1.11 Thesis hypotheses

Populations evolve through genetic drift and natural selection to the environment in which they live. Barriers to gene flow, such as geographic isolation and captive rearing of bumblebees, is likely to create distinctive populations. My thesis applies this theory to wild and captive populations of bumblebee. We hypothesise that wild Irish, wild British, and commercial populations of *B. t. audax* are distinctive populations, likely subjected to unique selective forces driven by the environments in which they live. Thus follows our second main hypothesis – that wild and commercial bumblebees show distinctions on the phenotypic level, which will be explored on behavioural and proteomic levels.

We take caution to hypothesise any further, as the framework of scientific knowledge required to put forth meaningful hypotheses is not very common in molecular bumblebee studies, and so, particularly in my molecular-based studies, I take a discovery-driven approach. Genomics and proteomics offer an opportunity to carry out unbiased and large-scale assessments that do not require a clear hypothesis to test. Considering this, I prefer to be led by the results of my genomic, proteomic, and behavioural experiments and, with these findings at the core, generate focused and informed hypotheses that will inspire future research.

Chapter 2

A genomic evaluation of population structure and selection in Irish and British *Bombus terrestris* ssp. *audax* populations

2.1 Introduction

Understanding intraspecific variation is of paramount importance for bee conservation. Bees are small flying insects that can disperse both passively and actively (Osborne *et al.*, 2002). Consequently, many bee species have wide distributions that span across seas and landmasses (Ranta, 1982). Once separated by barriers to gene flow and exposed to differential selective pressures, subpopulations can develop along unique evolutionary trajectories which result in distinctive groups (Allmon, 1992; Thorpe *et al.*, 2010; Amor *et al.*, 2014). These populations may also be adapted to local environmental conditions such as the climate, pathogens and human activities in the area (Parker *et al.*, 2010; Jackson *et al.*, 2020; Kelemen and Rehan, 2021; Hart *et al.*, 2022). However, adaptive alleles can be lost, for example, through bottleneck events or hybridisation with non-native populations and this could decrease the fitness of the subpopulation and disrupt the ecological balance within these localities. Bees are already facing declines in numbers and diversity across many regions in the world due to intensification of agriculture, agrochemical use and climate change (Ferrier *et al.*, 2016; Hallmann *et al.*, 2017; Zattara and Aizen, 2019). This is greatly concerning as bees are critical for ecosystem functioning and food production (Klein *et al.*, 2007; Ollerton *et al.*, 2011). With this in mind, it is essential that distinctive subpopulations and subspecies of bees are conserved as the loss of genetic diversity may greatly exacerbate bee declines by reducing the capacity for adaptation and increasing levels of inbreeding (Reed and Frankham, 2003). It follows that the evaluation of potentially unique and vulnerable bee subpopulations, such as those found on islands (Francisco *et al.*, 2016), is crucial for the execution of targeted and effective conservation actions.

Evaluation of the genetic diversity and adaptive capacities of pollinator populations is essential in preventing further declines of these species. One such wild pollinator species, *Bombus terrestris* L. (the earth bumblebee), provides an excellent opportunity to investigate genetic diversity and adaptation (Rasmont *et al.*, 2008). Firstly, it is a widespread generalist pollinator (Raine and Chittka, 2007) and likely forms a key component of many pollination networks (Memmott *et al.*, 2004). In addition, it is one of the main model bumblebee species for both molecular and ecological studies alike (Baer and Schmid-Hempel, 2006; Leadbeater and Chittka, 2007; Raine and Chittka,

2007; Barribeau and Schmid-Hempel, 2013; Sadd *et al.,* 2015; Colgan *et al.,* 2019), meaning there is a vast knowledge base surrounding its ecology, physiology and genetics. It is also one of the main species used by commercial companies which rear and export bumblebee colonies around the world for supplemental crop pollination services (Velthuis and Van Doorn, 2006). Finally, *B. terrestris* has a wide distribution across the Palaearctic and, already, nine subspecies have been recognised across its range, including several endemic island subspecies in the Canaries and Sardinia (Estoup *et al.,* 1996; Rasmont *et al.,* 2008). Many of these subspecies show several key distinctions on the morphological, behavioural, and genetic levels (Estoup *et al.,* 1996; Rasmont *et al.,* 2008; Ings *et al.,* 2009). One such subspecies is *B. t. audax*, known as the buff-tailed bumblebee, found in Ireland and Britain. This subspecies is characterised by a reddish or buff tail and tends to have smaller workers than the continental subspecies *B. t. dalmatinus* (Ings *et al.,* 2006; Rasmont *et al.,* 2008). However, there is only one study that has evaluated British and Irish *B. t. audax* populations on a genetic level, which found evidence that Irish and British populations were genetically divergent (Moreira *et al.,* 2015). Indeed, many studies have shown that Ireland has distinct subpopulations of species found across Britain and Europe, including a genetically distinct honeybee population (Carlsson *et al.,* 2014; Pedreschi *et al.,* 2014; Hassett *et al.,* 2018). This is not surprising, as many island populations have high incidences of endemic species and subsets of species found on parent landmasses (Whittaker, 2007).

Despite evidence for distinctive *B. t. audax* populations, growers in Ireland and Britain both import commercial *B. t. audax* colonies for crop pollination services, as they are still considered on the whole to be of the native subspecies (Appendix A1.1; Biobest, 2011; Koppert Biological Systems, 2011). It is unknown how genetically similar the commercial *B. t. audax* stocks are to wild populations. Commercial populations of *B. t. audax* consist of closed populations which may experience elevated levels of genetic drift as well as artificial selective processes that have altered their genomes in comparison to wild population (Beekman *et al.*, 1999; Beekman *et al.,* 2000; Lecocq, 2018). Traits linked to domestication have been found in other commercial *B. terrestris* populations such as altered colony growth compared to wild conspecifics (Gösterit and Baskar, 2016). If genetically dissimilar populations of *B. terrestris* are imported, there is a risk of hybridisation (Seabra *et al.,* 2019; Cejas *et al.,* 2020).

Therefore, an evaluation of the genetics of wild populations of *B. t. audax* will provide a foundation of knowledge required to further investigate commercial *B. t. audax* and allow for evidence-based management strategies for imported bumblebee colonies.

So far, Irish bee genetics research has been mainly utilising microsatellites and mitochondrial genes (Davis *et al.,* 2010; Moreira *et al.,* 2015; Hassett *et al.,* 2018) and until this study no other Irish population of bumblebee has been analysed on a genomic-wide level. WGS provides an incomparable platform to analyse an enormous diversity of genetic variants within a population, far outside the scope of traditional population genetics methods (Fuentes-Pardo and Ruzzante, 2017; Lozier and Zayed, 2017). Genomic techniques can delineate a range of conservation parameters including gene flow, genetic diversity, hybridisation or genetic bottlenecks which can be used to inform and enhance conservation actions (Allendorf *et al.,* 2010, Lozier and Zayed, 2017). Genomic studies on bumblebees have already provided important insights into the genomic structure and evolution of these insects. For example, Sadd *et al.* (2015) sequenced and analysed genomes from *B. terrestris* and *B. impatiens* and found that, like honeybees, bumblebees have a reduced set of immune and detoxification genes. Genomic data has also provided data on the rates of evolution in *Bombus* populations at different elevations (Lin *et al.,* 2019). Furthermore, a recent study evaluating a British population of *B. terrestris* by Colgan *et al.* (2022) was the first to carry out a population genomics study of a wild bumblebee population. This study found both high levels of genetic diversity and strong evidence for recent selection acting on genes implicated in detoxification and resistance to insecticides, demonstrating the power of population genomics to evaluate pollinator populations.

In our study, we build on the methods and findings of Colgan *et al.* (2022) and perform a population genomics study on two populations instead of one i.e. comparing their British *B. terrestris* population and an Irish *B. terrestris* population. As there have been many previous reports of distinctive populations of fauna in Ireland (Sleeman, 2014), we hypothesise that Ireland hosts a genetically distinct population of *B. terrestris*, as was found by Moreira *et al.* (2015). To evaluate this hypothesis, we use individual-based whole genome sequencing (WGS) rather than microsatellite data as used by previous researchers (Davis *et al.,* 2010; Moreira *et al.,* 2015; Hassett *et al.,* 2018) to generate a genomic dataset of an Irish population of *B. terrestris* alongside mainland European and commercial *B. terrestris* representatives and compare to the

British *B. terrestris* population using population structure analyses. We also investigate differential signatures of recent selection between populations using population comparison analyses (e.g., F_{ST} and XP-nSL). Recent selection is considered microevolutionary and can be on the timescale of decades or even ongoing (Mills and Mathieson, 2022) and has been described as selection which has impacted the genetic variability of current populations (Nielsen, 2007). Population comparisons of recent signatures of selection may indicate differences in selective pressures acting within subpopulations. This differs from the Colgan *et al.,* (2022) study which investigates signatures of selection in just one (British) population. We anticipate that the findings of this study will highlight the potential of population genomics to identify distinctive, locally adapted and potentially vulnerable pollinator populations and will pave the way for future genomic assessments of threatened and data-deficient bee species and populations. Furthermore, this study will generate evidence that will influence sustainable management practices and policies regarding commercial bumblebee use in Ireland and to promote the conservation of our key pollinating species.

2.2 Methods

2.2.1 Sample Collection

To perform a population genomics analysis on Irish and British bumblebees, we first carried out whole genome sequencing on 33 wild-caught, male *B. terrestris* from Ireland. Collections of Irish *B. terrestris* samples took place from June until September 2018, from a total of 27 sites around Ireland (Table 2.1, Figure 2.1) and were stored dry and frozen until DNA extractions could take place. In addition to these samples, a further 17 *B. terrestris* samples were sequenced by Novogene and included in this study to compare natural *B. t. audax* populations to both commercial *B. t. audax* and wild mainland European subspecies. These additional samples included male *B. t. audax* sourced from commercial colonies of two suppliers, Koppert Biological Systems (n = 6) and Biobest (n = 5), male *B. t. terrestris* collected in Germany (n = 3) and male *B. t. dalmatinus* collected in Turkey (n = 3), which were stored in ethanol until DNA extraction (Table A2.1). We then accessed previously sequenced, wild-caught, male *B. terrestris* raw sequence genomes from Britain to carry out a

comparison between these two populations (n = 51; available from the NCBI BioProject PRJNA628944; Colgan *et al.*, 2022).

2.2.2 DNA extraction, species confirmation and sequencing

To extract genomic DNA from bumblebee samples, the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) was used according to the protocol for animal tissues. Certain modifications to the protocol were included and were as follows: to each sample, 40µl of Qiagen Proteinase K was added and this was followed by a 3-hour incubation step at 56°C where samples were vortexed beforehand and once an hour throughout. Sample quality and yield was assessed using agarose gel electrophoresis and a NanoDrop™ Spectrophotometer (ThermoFisher Scientific).

As *B. terrestris* male colouration patterns can be difficult to distinguish from males of several similar looking species, primarily *B. lucorum, B. magnus* and *B. cryptarum* (Scholl and Obrecht 1983; Urbanova *et al.* 2001), we carried out a PCR and restriction fragment length polymorphism reaction (RFLP) to confirm that all our samples were *B. terrestris.* Furthermore, DNA extractions from heads can contain PCR-inhibiting eye-pigment (Boncristiani *et al.*, 2011), we subjected back-up elutions of DNA samples to a cleaning step prior to RFLP testing. First, back-up DNA elutions for each sample were diluted 1 in 10. Using reagents provided by the GenElute PCR clean-up kit (Sigma-Aldrich), diluted samples were cleaned according to the accompanying protocol. All cleaned sample aliquots were stored at –20 °C until PCR. In each 20µL PCR reaction mixture there was 8.48µL of ddH2O, 0.4µL dNTPs (10mM each), 4µL of 5X Green GoTaq® Reaction Buffer (Promega), 1.8µL of MgCl2 (25mM; Promega), 0.6µL of Tanaka forward (5'-ATAATTTTTTTTATAGTTATA-3') and reverse (5'-GATATTAATCCTAAAAAATGTTGAGG-3') primers (10uM), 0.12µL of GoTaq® G2 DNA Polymerase Taq (5u/µl; Promega) and 4µL of template DNA from the cleaned samples. PCRs were carried out as follows: initial denaturation at 95°C for 1 min; 30 cycles of 95°C for 1 min, 45°C for 45 s, 60°C for 1 min, with a final extension of 72°C for 4 min. RFLPs were carried out with 15µL of PCR product, 2.8µL ddH2O, 2µL of NEB Buffer 2 (10x; Promega), 0.1µL of EcoN1 (1.5U/µL; Promega) and 0.1µL Hinf (1.0U/ul; Promega). RFLP reaction tubes were incubated at 37°C for at least 6 hours and then ran on gel electrophoresis alongside *B. terrestris* reference PCR RFLP product to confirm species ID for all samples as being *B.*

terrestris. Samples were sent to Novogene (Cambridge, UK) for library preparation and whole genome sequencing. PCR-free libraries were generated using NEBNext Ultra I DNA library kit and pair-ended sequencing (150bp) was performed on an Illumina NovaSeq6000 platform.

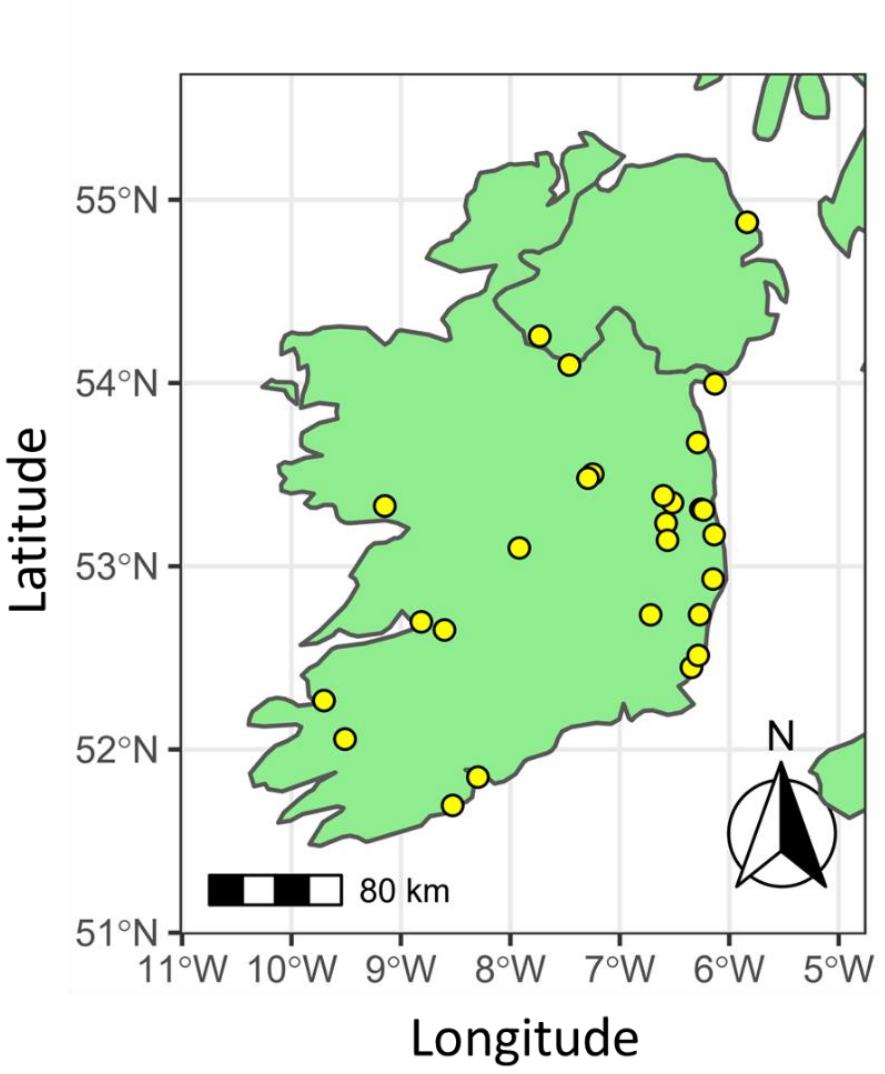


Figure 2.1 Map of collection sites of Irish *Bombus terrestris* samples which underwent whole genome sequencing. Irish *B. terrestris* sample collection sites (n = 27) shown on map are represented by yellow circles at given geographical co-ordinate positions.

Table 2.1 *Bombus terrestris* sample collection information. Sample codes for male *B. terrestris* with location and coordinates of sample collection sites.

Sample Code	Collection site	Latitude	Longitude
2018 Bter MU 1	Co. Carlow, Ireland	52.7353	-6.7176
2018 Bter MU 3	Co. Wicklow, Ireland	52.9305	-6.1479
2018 Bter MU 6	Co. Offaly, Ireland	53.1003	-7.9177
2018 Bter MU 7	Co. Wexford, Ireland	52.4486	-6.3447
2018 Bter MU 8	Co. Galway, Ireland	53.3294	-9.1469
2018 Bter MU 9	Co. Galway, Ireland	53.3294	-9.1469
2018 Bter MU 10	Co. Kildare, Ireland	53.3470	-6.5188
2018 Bter MU 12	Co. Cork, Ireland	51.8496	-8.2962
2018 Bter MU 13	Sabanci Univ, Turkey	40.8918	29.3743
2018 Bter MU 14	Naumburg, Germany	51.1477	11.8244
2018 Bter MU 15	Tongrube, Germany	51.4596	11.7237
2018 Bter MU 16	Garten, Germany	51.5174	10.9312
2018 Bter MU 17	Co. Cavan, Ireland	54.0983	-7.4604
2018 Bter MU 18	Co. Antrim, Ireland	54.8775	-5.8364
2018 Bter MU 19	Co. Antrim, Ireland	54.8775	-5.8364
2018 Bter MU 20	Co. Kildare, Ireland	53.2360	-6.5774
2018 Bter MU 21	Co. Kildare, Ireland	53.2360	-6.5774
2018 Bter MU 22	Co. Westmeath, Ireland	53.5034	-7.2470
2018 Bter MU 23	Co. Meath, Ireland	53.6755	-6.2877
2018 Bter MU 25	Co. Kerry, Ireland	52.0569	-9.5107
2018 Bter MU 26	Co. Kerry, Ireland	52.0569	-9.5107
2018 Bter MU 27	Co. Cork, Ireland	51.6952	-8.5270
2018 Bter MU 28	Co. Wexford, Ireland	52.5136	-6.2831
2018 Bter MU 29	Co. Wicklow, Ireland	53.1731	-6.1378
2018 Bter MU 31	Koppert Biological Systems		
2018 Bter MU 32	Co. Limerick, Ireland	52.6527	-8.6016
2018 Bter MU 33	Co. Louth, Ireland	53.9962	-6.1310
2018 Bter MU 35	Co. Fermanagh, Ireland	54.2561	-7.7310
2018 Bter MU 36	Co. Kildare, Ireland	53.3851	-6.6031
2018 Bter MU 38	Co. Fermanagh, Ireland	54.8775	-5.8364
2018 Bter MU 39	Co. Westmeath, Ireland	53.4809	-7.2907
2018 Bter MU 40	Co. Wicklow, Ireland	53.1419	-6.5639
2018 Bter MU 41	Co. Dublin, Ireland	53.3120	-6.2590
2018 Bter MU 42	Co. Kerry, Ireland	52.2674	-9.7032
2018 Bter MU 43	Co. Kerry, Ireland	52.2674	-9.7032
2018 Bter MU 44	Co. Dublin, Ireland	53.3069	-6.2353
2018 Bter MU 45	Co. Wexford, Ireland	52.7363	-6.2706
2018 Bter MU 46	Co. Clare, Ireland	52.6970	-8.8138
2018 Bter MU 47	Koppert Biological Systems		
2018 Bter MU 48	Koppert Biological Systems		
2018 Bter MU 49	Biobest		
2018 Bter MU 50	Biobest		
2018 Bter MU 51	Istanbul, Turkey	40.8918	29.3743
2018 Bter MU 52	Istanbul, Turkey	40.8918	29.3743
2019 Bter MU 53	Koppert Biological Systems		
2019 Bter MU 54	Koppert Biological Systems		
2019 Bter MU 55	Koppert Biological Systems		
2019 Bter MU 56	Biobest		
2019 Bter MU 57	Biobest		
2019 Bter MU 58	Biobest		

2.2.3 Quality assessment, filtering, and alignment

We assessed the quality of all sample raw reads using FastQC (v.0.11.9; Andrews, 2010). Sequences were then filtered using FastP (v.0.23.2; Chen *et al.*, 2018) to remove sequences matching Illumina adapters, those with more than 10% ambiguous bases, those which had 40% of bases with Phred scores of 20 or less and those that were less than 50 bases long. All filtered reads were aligned against the most recent *B. terrestris* reference genome assembly (iyBomTerr1.2, GCA_910591885.1) using bwa-mem2 (v.2.0pre2; Vasimuddin *et al.*, 2019), SAMtools (v1.16.1; Li *et al.*, 2009) and samblaster (v.0.1.26; Faust and Hall, 2014).

B. terrestris male sequences from Colgan *et al.* (2022) had more variable raw coverage estimates ($\bar{x}$ = 5.3X; s = 3.6X; min = 0.8X; max = 18.9X), calculated with genome size estimate of 392Mb, than the samples sequenced as part of this study ($\bar{x}$ = 10.6X; s = 1.6X; min= 8X; max = 15X). Across all samples, there was a mean of 11.2 million reads per sample (s = 5.1 million reads) with a mean estimated raw coverage of 7.9X (s =3.8X). Reads were of high quality, with 95.74% of reads having a Phred quality (Q) score of ≥ 20 and 90.21% of reads a Q score of ≥ 30. Variant calling identified 8.68 million SNPs.

2.2.4 Variant calling and filtering of single nucleotide polymorphisms

Freebayes (v.1.3.6; Garrison and Marth, 2012) was used to call variants on the resulting alignment files using the parameters: --ploidy 2 –report-genotype-likelihood-max –use-mapping-quality –genotype-qualities –use-best-n-alleles 4 –haplotype-length 0 –min-base-quality 3 –min-mapping-quality 1 –min-alternate-frac 0.25 –min-coverage 1. VCFtools (v.0.1.17; Danecek *et al.*, 2011) was used to filter calls by removing indels and remove sites which had quality scores (QUAL) of less than 20 or a maximum mean depth more than 100pb. The percentage of missing calls in each individual sample was then calculated using vcftools (parameter – imiss). Two datasets were created as filtering of SNPs can greatly reduce the size of available SNPs to use, which is not optimal for certain analyses, e.g., F_{ST} and XP-nSL, while population structure analyses are more accurate with higher numbers of individuals represented. We generated (1) a dataset where samples with >15% of missing calls alongside samples with high levels of heterozygous calls were removed (referred to as DS1 henceforth) and (2) a dataset where samples with >10% of missing calls and

samples with high levels of heterozygous calls were removed (DS2). Post filtering, the DS1 dataset had 536,466 SNPs representing 89 individuals, and was used in population structure analyses while the DS2 dataset had 1,131,226 SNPs from 75 individuals and was used in F_{ST} and XP-nSL analyses.

VCF files were generated for each dataset. As our samples originated from haploid bumblebee individuals, the VCF files for DS1 and DS2 were filtered to keep only homozygous, biallelic calls found in at least two individuals found on the 18 linkage groups (representing chromosomes) outlined in the *B. terrestris* BomTerr1.2 reference genome assembly. Chromosomes were renamed in the gene annotation file to match NCBI gene annotations using the annotate function of Bcftools with the –rename-chrs parameter. SNPs were annotated with SnpEff (v.4; Cingolani *et al.*, 2012) using gene annotation information from NCBI *Bombus terrestris* Annotation Release 103.

2.2.5 Population structure analysis and nucleotide diversity

To assess the population structure in our dataset we carried out two main analyses. A principal component analysis was performed using an R package called SNPrelate (v1.30.1; Zheng *et al.,* 2020) on pruned SNPs. Finally, an ADMIXTURE analysis was performed on the same larger dataset (DS1) on pruned SNPs. To calculate the most likely *K* value for our population, ADMIXTURE was performed with K = 1 – 20 and with cross-validation (--cv).

2.2.6 Comparisons of recent signatures of selection

To detect genetic differentiation between populations of SNPs, Fixation Indices (F_{ST}) were calculated per SNP which measured the level of genetic differentiation between two populations at a particular genomic site (Wright, 1942). Popgenome (v.2.7.5; Pfeifer *et al.,* 2014) was used to calculate F_{ST} values per SNP. For all SNPs within coding regions, the SNP with the highest F_{ST} value per gene was extracted and used in downstream analyses. F_{ST} values were calculated for Irish and British population comparisons. We also performed preliminary F_{ST} comparisons of wild populations and the commercial individuals. F_{ST} values over 0.25 were indicative of divergence between populations at a particular SNP site, as described by Hartl and Clark (1997).

A Manhattan plot was generated to visualise SNPs in the top 1% of F_{ST} values in the Irish – British comparison.

We also performed a cross-population test for extended haplotype homozygosity (XP-nSL) that compares haplotype patterns between two populations to identify regions showing evidence of 'hard' or 'soft' selective sweeps (Szpiech *et al.*, 2021). XP-nSL scores were calculated for each SNP in the genome, which were subsequently normalised against background genome estimates. This analysis also filtered out SNPs with a minor allele frequency of less than 0.05. XP-nSL scores were generated, similarly to F_{ST}, for Irish – British population comparisons. Preliminary XP-nSL comparisons of wild populations and the commercial individuals were also carried out. A SNP site with an |XP-nSL| score greater than 2 was considered to show evidence of divergence between populations (Szpiech *et al.*, 2021).

For outputs of both analyses comparing Irish and British populations, SNPs were ranked by highest values and the genes that included the top 5% most significantly divergent SNPs were subset and assessed by GO analysis. The topmost genes in each analysis were searched for associated function on UniProt (UniProt, 2023). Also, shared genes showing distinctions in F_{ST} ($F_{ST} > 0.25$) and XP-nSL (|XP-nSL| > 2) were assessed using a Venn analysis (https://bioinformatics.psb.ugent.be/webtools/Venn/) across all comparisons (Irish – British, Irish – commercial, and British – commercial).

2.2.7 Gene ontology term enrichment analysis

To identify possible links between genes showing distinctive signatures of selection between populations, a Gene Ontology (GO) term enrichment analysis was performed on F_{ST} and XP-nSL results. As there is a low-resolution of GO terms assigned to *B. terrestris* genes, *Drosophila melanogaster* GO terms were obtained from Ensembl Metazoa Biomart (Kinsella *et al.*, 2011) and these were assigned to bumblebee homologues. A rank-based, Kolmogorov-Smirnov (KS) test ($p < 0.05$; node size = 20) was performed on the top 5% of SNPs in the outputs from the F_{ST} and XP-nSL analyses comparing Irish and British population to find enriched GO terms in each GO category ('biological process', 'cellular components' and 'molecular function'. A Fisher's exact test ($p < 0.05$; node size = 20) was also carried out on overlapping genes across comparisons within F_{ST} and XP-nSL analysis. The topGO package (v.2.48.0;

Alexa and Rahnenführer, 2009) in R was used to perform this test, which was used with the "weight01" algorithm.

2.3 Results

2.3.1 Population structure

To identify distinctions between populations of *B. terrestris* examined in this study, we compared genome-wide SNPs using a principal component analysis (PCA) and ADMIXTURE analysis. The PCA, which is used to visualise genetic structure of populations showed considerable distinction between wild-caught Irish and British *B. t. audax* populations, separated primarily across the PC1 axis (Figure 2.2). Commercial and mainland European samples of *B. terrestris* grouped more closely with the British *B. t. audax* population, however there were distinctions within this non-Irish group along the PC2 axis. Commercial *B. t. audax* samples sourced from Koppert Biological Systems clustered with *B. terrestris* samples from Turkey, Germany and one British sample. Commercial *B. t. audax* samples sourced from Biobest seemed to be more distantly related to the wild British *B. t. audax* population than the commercial bumblebees sourced from Koppert Biological Systems, although two British individuals also cluster with these. In the Irish group, there appears to be a subset of individuals that are slightly more closely related to the non-Irish group than seen in others in the Irish population which disperse slightly across the PC1 axis.

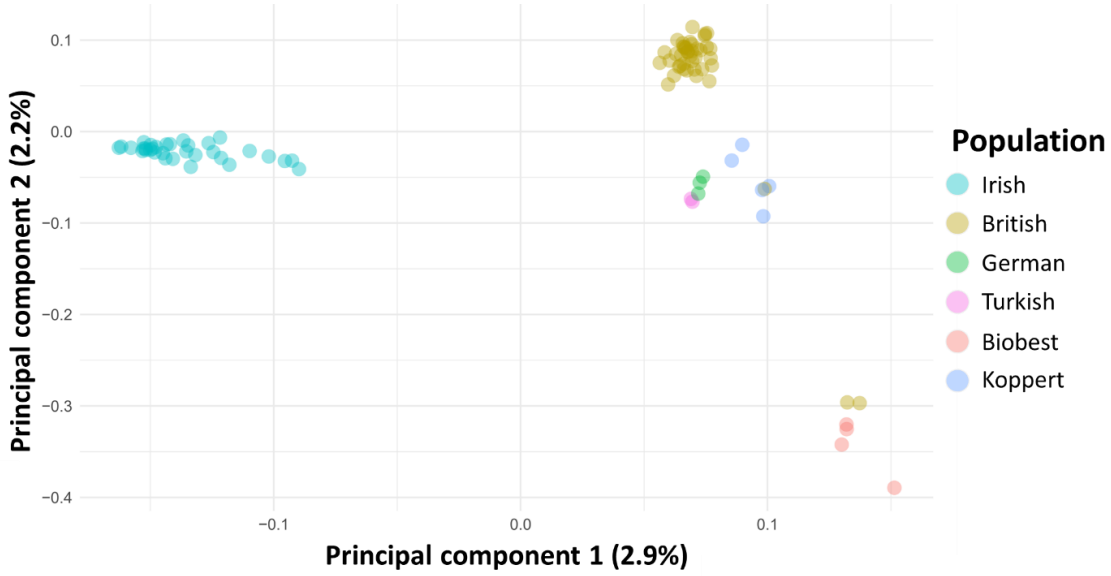


Figure 2.2 Principal component analysis on the genotype calls of wild and commercial populations of *B. terrestris*. Principle component analyses carried out on genome-wide, high-quality SNPs from 89 individuals. Each point refers to an individual male sample, while colours represent each population. Axes represent the first (PC1) and second principal components (PC2) which, in total, account for 5.1% of the variation in the dataset.

ADMIXTURE analyses supported the results of the PCA, which found most support for K = 2, providing evidence for two populations, largely grouping samples collected from Ireland into one population and all other samples into a second population (Figure 2.3, Table A2.2). All Irish samples had over 60% ancestry to Cluster 1. Of the 33 Irish samples, 22 had < 1% Cluster 2 ancestry, while 11 samples had between 1.5 – 36 % Cluster 2 ancestry. All British had over 89% Cluster 2 ancestry, with many showing almost complete Cluster 2 ancestry. All commercial, German and Turkish representatives were entirely of Cluster 2 ancestry.

2.3.2 F_{ST} analyses comparing differential selection signatures in Irish, British and commercial groups

To assess differential selection and specific distinctions between Irish and British populations, F_{ST} values were calculated per SNP. The mean F_{ST} value for distinction between Irish and British populations was 0.23, indicating genetic distinction between these populations. There were 3,756 SNPs with F_{ST} values over 0.25, which according

to (Hartl *et al.*, 1997) indicates high levels of distinction between populations at these sites (Figure 2.5; Table A2.3a).

The top 20 SNPs as ranked by F_{ST} values when comparing Irish and British populations (Table 2.2) contained many genes whose products had roles in nervous system development (e.g., *connectin* (LOC100643941), *receptor-type tyrosine-protein phosphatase* (LOC100646666), *tyrosine-protein kinase transmembrane receptor Ror* (LOC100645050), *transcription factor collier* (LOC100650834) and *protein split ends* (LOC100642196)) and neuronal signalling (e.g., *potassium voltge-gated channel protein Shab* (LOC100646670), *protein unc-13* (LOC100650524) and *bestrophin* (LOC100649883)). The results of a GO term enrichment analysis on genes containing SNPs within the top 5% signatures of differential selection between Irish and British populations, enrichment was found in terms relating to morphogenesis, pattern formation and generation of nervous system components such as neurons and glial cells (Table 2.2). These terms included "embryonic development via the syncytial blastoderm", "dorsal/ventral pattern formation", "neuron development", "gliogenesis" and "axonogenesis". Furthermore, terms relating to the regulation of certain behaviours and processes affecting behaviours were also included e.g., "olfactory behaviour", "regulation of locomotion" and "circadian sleep/wake cycle process" (Table 2.3; Table A2.5a).

1

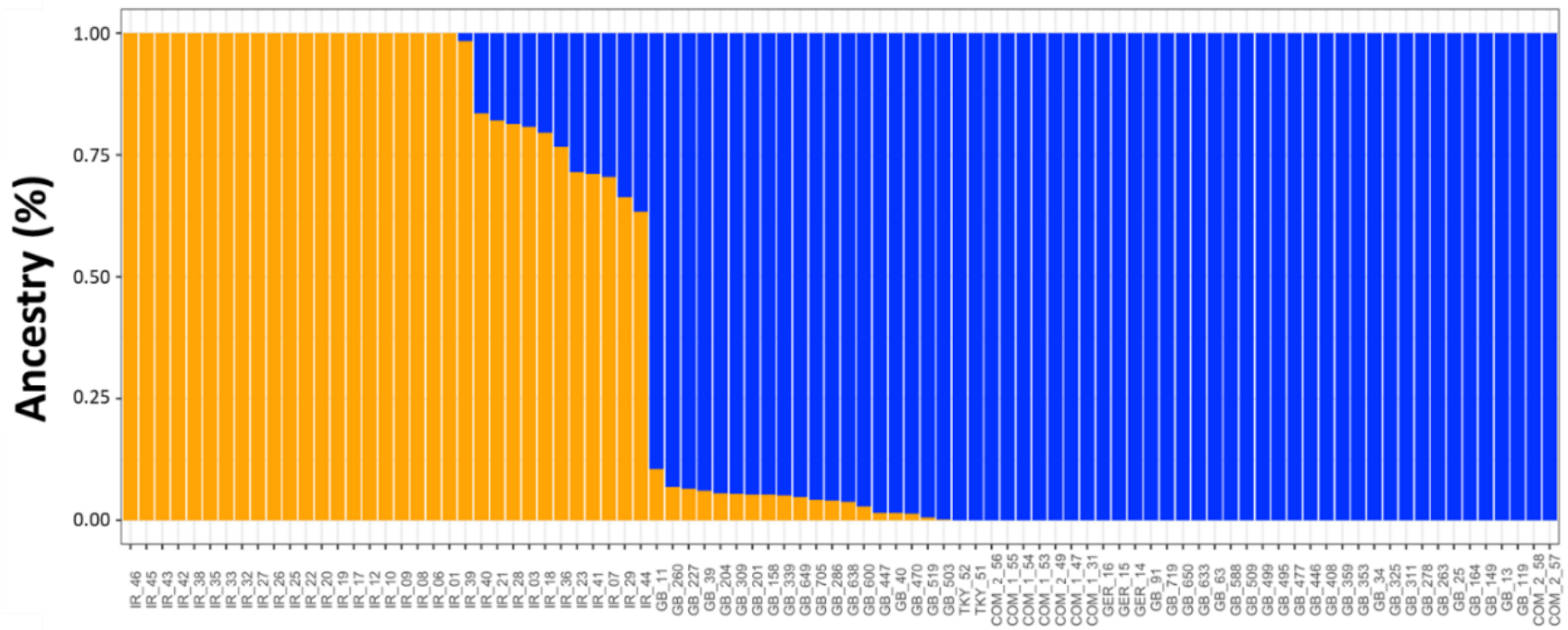


Figure 2.3 Population structure of Irish and non-Irish populations with evidence for admixture. Results of admixture analysis for $K = 2$ where support is shown for distinctive Irish and non-Irish populations in pruned SNPs from 89 individuals. Each bar represents a single individual with percentage ancestry donated by colour (orange = Cluster 1, blue = Cluster). Prefixes of sample names indicate their origin i.e. Ireland ("Ir"), Britain ("GB"), Turkey ("TKY"), Germany ("GER") or commercially-sourced colonies ("COM").

When Irish bees were compared with commercial samples as a complementary analysis (although this population was comparatively much smaller than both Irish and British sample sizes), a mean F_{ST} value of 0.297 was calculated, with 5,136 SNPs showing high levels of distinction ($F_{ST} > 0.25$; Table A2.3b). The Irish – commercial comparative F_{ST} results revealed two SNPs showing complete distinction ($F_{ST} = 1$) between the two populations when top SNPs per gene were extracted; these were located in genes for a *neuronal acetylcholine receptor subunit* (LOC100649796) and *mucin protein* (LOC100650591). Other genes included in the top 20 SNPs of the Irish and commercial analysis were linked to processes such as nervous system development and signalling (*protein unc-13* (LOC100650524), *fibroblast growth factor* (LOC100643345), *transcription factor collier* (LOC100650834), *connectin* (LOC100643941) and *protein couch potato* (LOC100645197)). Many of these also appeared in the top 20 SNPs ranked by F_{ST} scores in the British and Irish F_{ST} analysis (*protein unc-13* (LOC100650524), *transcription factor collier* (LOC100650834) and *connectin* (LOC100643941)). All other genes in the top 20 of the British and Irish population F_{ST} analysis had an $F_{ST} > 0.4$ in the Irish – commercial comparison, with six of these having F_{ST} values > 0.8.

There were 1,369 shared genes with SNPs exhibiting elevated levels of genetic differentiation ($F_{ST} > 0.25$) between the F_{ST} analysis of the Irish – British comparison and the Irish – commercial comparison only (Figure 2.4; Table A2.3d). A GO term enrichment analysis found enrichment in biological processes such as ‘anion transport’, ‘sensory perception of sound’, ‘Golgi organization’, ‘developmental pigmentation’ and ‘synaptic vesicle transport’ (Table A2.5d).

Commercial and British population comparisons resulted in a smaller mean F_{ST} value than the previous pairwise comparisons ($\bar{x} = 0.18$) and just 2,847 SNPs showing high levels of distinction ($F_{ST} > 0.25$) indicating less differentiation between these groups (Table A2.3c). In the F_{ST} analysis of British and commercial groups, genes containing SNPs with F_{ST} values in the top 20 of all SNPs included several with gene functions relating to proteolysis (a *disintegrin and metalloproteinase with thrombospondin motifs 9* (LOC100650487), *calpain-C* (LOC100652040), *tensin-1* (LOC100644032), *venom dipeptidyl peptidase 4* (LOC100646982), nervous system functioning and development *(phospholipid-transporting ATPase ID* (LOC100647024), *octopamine receptor beta-3R* (LOC100650229), *collagen alpha-1(XVIII) chain*

(LOC100649852), *laminin* (LOC100651805) and *lachesin* (LOC100651189)) as well as the genes *protein timeless* (LOC100646408), involved in the entrainment of circadian rhythms, and *TLD domain-containing protein* (LOC100646293), linked to defence response to Gram-negative bacteria.

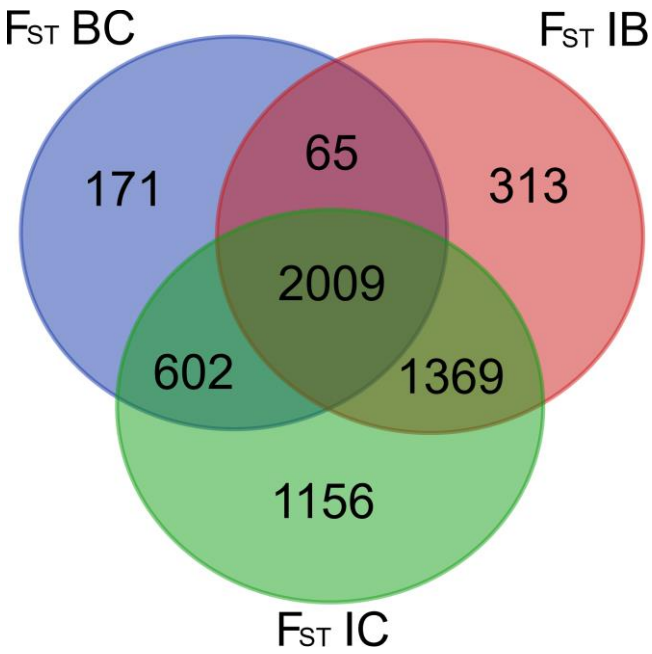


Figure 2.4 Venn diagram of overlapping SNPs showing elevated levels of distinction, as determined by F_{ST} values, in each population comparison. Venn analysis showing unique and shared numbers of SNPs exhibiting differential signatures of selection (F_{ST} > 0.2) in the F_{ST} analysis of Irish – British, Irish – commercial and British – commercial population comparisons.

There was also overlap of two genes between the top 20 genes as ranked by their SNP F_{ST} values in the Irish – commercial comparison and the British – commercial comparison; these were *phospholipid-transporting ATPase ID* (LOC100647024) and *collagen alpha-1(XVIII) chain* (LOC100649852). Both Irish – British and British – commercial F_{ST} outputs had *venom dipeptidyl peptidase 4* (LOC100646982) and *TLD-domain containing protein* (LOC100646293) represented among the SNPs with the 20 highest F_{ST} values. There were 602 shared genes with SNPs exhibiting signatures of differential selection between the F_{ST} analysis results of the Irish – commercial comparison and the British – commercial comparison only (Table A2.3d). These common genes showed enrichment for terms such as 'germarium-derived oocyte fate determination', 'dicarboxylic acid metabolic process', 'lipid homeostasis' and

'tricarboxylic acid cycle' (Table A2.5c). Between Irish – British and British – commercial F_{ST} analyses only, 65 genes with SNPs showing signatures of differential selection were shared. GO terms such as 'protein modification by small protein conjugation', 'histone H4 acetylation' and 'phospholipid biosynthetic process' were associated with this shared group (Table 2.5b).

Common genes showing distinction in all three pairwise comparisons (n = 2,009; Figure 2.4; Table A2.3d) were enriched for similar biological processes to those mentioned previously e.g., 'axon guidance', 'cell differentiation', 'synapse organisation', 'brain development', 'animal organ development', alongside other processes such as 'male courtship behaviour', 'feeding behaviour', 'chemosensory behaviour' and 'response to wounding' (Table A2.5e).

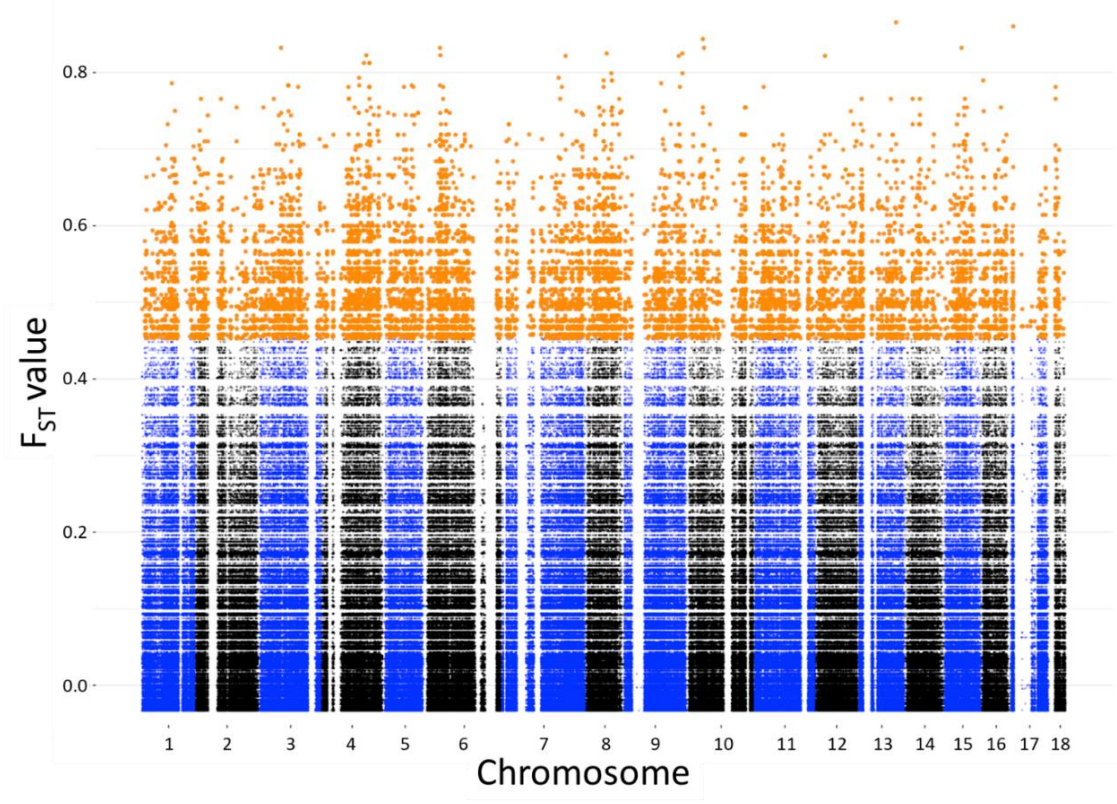


Figure 2.5 F_{ST} values for SNPs measuring distinction between Irish and British populations. Manhattan plot of F_{ST} values per SNP between Irish and British populations, represented by data points, in each *B. terrestris* chromosomal linkage group (1 – 18). Points in orange represent SNPs in the top 1% of F_{ST} values comparing Irish and British populations.

2.3.3 XP-nSL analyses comparing positive selection signatures in Irish, British and commercial groups

A cross-population extended haplotype homozygosity (XP-nSL) analysis was used to examine differences in recent selection between our samples *B. terrestris* populations and provide information on which population in a comparison was exhibiting specific selection signatures.

The XP-nSL analysis of the Irish and British population resulted in a total of 1,105 genes with |XP-nSL| values of over 2 and therefore exhibiting signatures of positive selection (Table A2.4a). Of these, 557 genes were showing selection in the Irish population (XP-nSL < -2), and 548 were showing selection in the British population (XP-nSL > 2). The Irish group and British group had similar mean XP-nSL values (|XP-nSL| of 1.14 and 1.16, respectively). The top 10 genes showing signatures of positive selection in the Irish population (Table 2.4) included genes highlighted in the F_{ST} analysis comparing Irish and British populations such as *protein unc-13* (LOC100650524) and *venom dipeptidyl peptidase 4* (LOC100646982). The Irish population showed strongest selection in SNPs within genes such as *venom dipeptidyl peptidase 4* (LOC100646982), *acetylcholine receptor subunit* (LOC100648987) and a *glutamate receptor* (LOC100643486). Many genes showing highest selection signatures in the Irish population encoded for products with roles in synaptic transmission and nervous system development. Genes such as cadherin (LOC100651590), UPF0585 protein C16orf13 (LOC105666912) and two WD repeat-containing proteins (LOC100650754, LOC100651445) were represented among the SNPs with highest XP-nSL values showing distinction in the British population. The roles of gene products represented by genes showing highest selection signatures in the British populations were involved in embryonic/larval development and synaptic transmission. The GO term enrichment analysis on genes containing SNPs with the top 5% of XP-nSL values in the Irish – British comparison found enrichment in terms such as “adult behavior”, “nervous system development”, “locomotory behavior”, “taxis” and “anatomical structure morphogenesis” (Table 2.3; Table A2.6a).

Table 2.2 Top 20 SNPs (as ranked by F_{ST} value) in the Irish – British comparative F_{ST} analysis. Results of top 20 highest F_{ST} values and their corresponding SNPs (chromosome, SNP position and variant type) and the gene name and description of where the SNP is found.

Chromosome	SNP position	Variant type	F_{ST} value	Gene name	Gene description
NC_063281.1	11735711	Intron	0.87	LOC100643501	Atrial natriuretic peptide-converting enzyme
NC_063285.1	825990	Intron	0.86	LOC100650996	Lachesin
NC_063278.1	4883716	3’ UTR	0.84	LOC100646670	Potassium voltage-gated channel protein Shab
NC_063271.1	6883448	Intron	0.83	LOC100642196	Protein split ends
NC_063283.1	5322077	Intron	0.83	LOC100644380	Syndecan
NC_063274.1	4149211	Intron	0.83	LOC100650524	Protein unc-13 homolog B
NC_063277.1	17829797	Intron	0.82	LOC100646666	Receptor-type tyrosine-protein phosphatase kappa
NC_063277.1	16728686	Intron	0.82	LOC100643941	Connectin
NC_063275.1	19315403	Intron	0.82	LOC100646417	Uncharacterized LOC100646417
NC_063280.1	2823037	Intron	0.82	LOC100649702	Uncharacterized LOC100649702
NC_063276.1	7840455	Intron	0.8	LOC100645050	Tyrosine-protein kinase transmembrane receptor Ror
NC_063272.1	11365996	Intron	0.79	LOC100646174	Nuclear receptor coactivator 6
NC_063275.1	17189024	Intron	0.79	LOC100649883	Uncharacterized protein LOC100649883
NC_063284.1	537089	Intron	0.79	LOC100650792	Polypyrimidine tract-binding protein 2
NC_063276.1	5493552	Intron	0.79	LOC100646982	Venom dipeptidyl peptidase 4
NC_063277.1	11373450	3’ UTR	0.79	LOC100648213	Protein jim lovell
NC_063269.1	9305414	Intron	0.79	LOC100650834	Transcription factor collier
NC_063273.1	8867341	Intron	0.78	LOC100646293	Nuclear receptor coactivator 7
NC_063286.1	2081417	Intron	0.78	LOC100651423	Cystinosin
NC_063277.1	17074791	5’ UTR	0.78	LOC100644262	FAD-linked sulfhydryl oxidase ALR

A total of 1,085 genes exhibiting potential signatures of positive selection (|XP-nSL| > 2) were found in the XP-nSL analysis comparing Irish and commercial groups (Table A2.4b), with 626 genes showing selection in the Irish group (XP-nSL < -2) and 459 genes in the commercial group (XP-nSL > 2). Overall mean |XP-nSL| values for Irish and commercial populations were 1.17 and 1.07 respectively. A total of 189 genes highlighted as undergoing positive selection in the Irish population of the Irish – British XP-nSL analysis were also among those also potentially showing positive selection in the Irish group of the Irish – commercial comparison (Figure 2.6; Table A2.4d). These overlapping genes showed enrichment in GO terms such as "synaptic target recognition", "multicellular organismal homeostasis", "regulation of response to stress" and "mushroom body development" (Table A2.6b). Genes *protein unc-13* and *acetylcholine receptor subunit* were also among the top 10 genes undergoing positive selection in the Irish group of the Irish – commercial comparison. Other genes within the top ten included *lachesin, cysteine sulfinic acid decarboxylase* and a homolog of the *Drosophila melanogaster* gene *JhI-26*. In terms of the commercial group, when analysed against the Irish population there were several genes involved in nervous system functioning (*neuroligin-1* (LOC100651474), *phospholipid-transporting ATPase ID* (LOC100647024), small conductance calcium-activated potassium channel protein (LOC100646717)).

XP-nSL analysis of British and commercial groups found 2,137 genes with an |XP-nSL| value greater than 2 (Table A2.3c). Of all genes showing potential signatures of selection, 988 of these had XP-nSL of < -2, showing selection in the British group, while 1,149 genes showed potential selection in the commercial population (XP-nSL > 2). British and commercial groups had overall mean |XP-nSL| values of 1.4 and 1.47 respectively. The top 10 genes showing positive selection in the British group when compared to the commercial group in the XP-nSL analysis included *choline O-acetyltransferase* (LOC100650308), *dopamine receptor 1* (LOC100642586) and a homolog of *D. melanogaster* gene *bedraggled* (LOC100647131). The commercial group had highest potential signatures of positive selection in genes such as *peroxidasin* (LOC100643921), *gustatory receptor for sugar taste 64f* (LOC100647322) and *protein sickie* (LOC100643922). There were 309 common genes showing positive selection in commercial groups in both the XP-nSL analysis of Irish – commercial populations and British – commercial populations (Figure 2.6;

Table A2.4e). When these shared genes were analysed for GO term enrichment, terms such as 'tricarboxylic acid cycle', 'lipid homeostasis', 'response to toxic substance' and 'synaptic target recognition' were found enriched (Table A2.6c). When the XP-nSL analysis results of Irish – British populations and British – commercial populations were compared, there were 164 common genes showing positive selection in the British population (Figure 2.6; Table A2.4f). GO term enrichment analysis in these common genes found enrichment in terms such as 'membrane biogenesis', 'neuromuscular junction development', 'adult locomotory behaviour', 'appendage development' and 'memory' (Table A2.6d).

Table 2.3 Results of Gene Ontology (GO) term enrichment analysis on genes containing SNPs showing high signatures of selection between Irish and British populations. Results of ranked KS Gene Ontology (GO) term enrichment analysis ($p < 0.05$) on the top 5% of SNPs showing the highest signatures of differential selection, as determined by both F_{ST} and XP-nSL value ranks, between Irish and British populations of *B. t. audax*. Top ten GO terms are shown for in the "biological process" category.

Enrichment in SNPs ranked by F_{ST}		**Enrichment in SNPs ranked by XP-nSL**	
GO ID	**GO term**	**GO ID**	**GO term**
GO:0050808	Synapse organization	GO:0007610	Behavior
GO:0098609	Cell-cell adhesion	GO:0032501	Multicellular organismal process
GO:0007606	Sensory perception of chemical stimulus	GO:0030534	Adult behavior
GO:0001736	Establishment of planar polarity	GO:0007399	Nervous system development
GO:0007411	Axon guidance	GO:0048869	Cellular developmental process
GO:0007186	G protein-coupled receptor signaling pathway	GO:0007626	Locomotory behavior
GO:0003008	System process	GO:0030154	Cell differentiation
GO:0042391	Regulation of membrane potential	GO:0042330	Taxis
GO:0009887	Animal organ morphogenesis	GO:0009653	Anatomical structure morphogenesis
GO:0007268	Chemical synaptic transmission	GO:0003008	System process

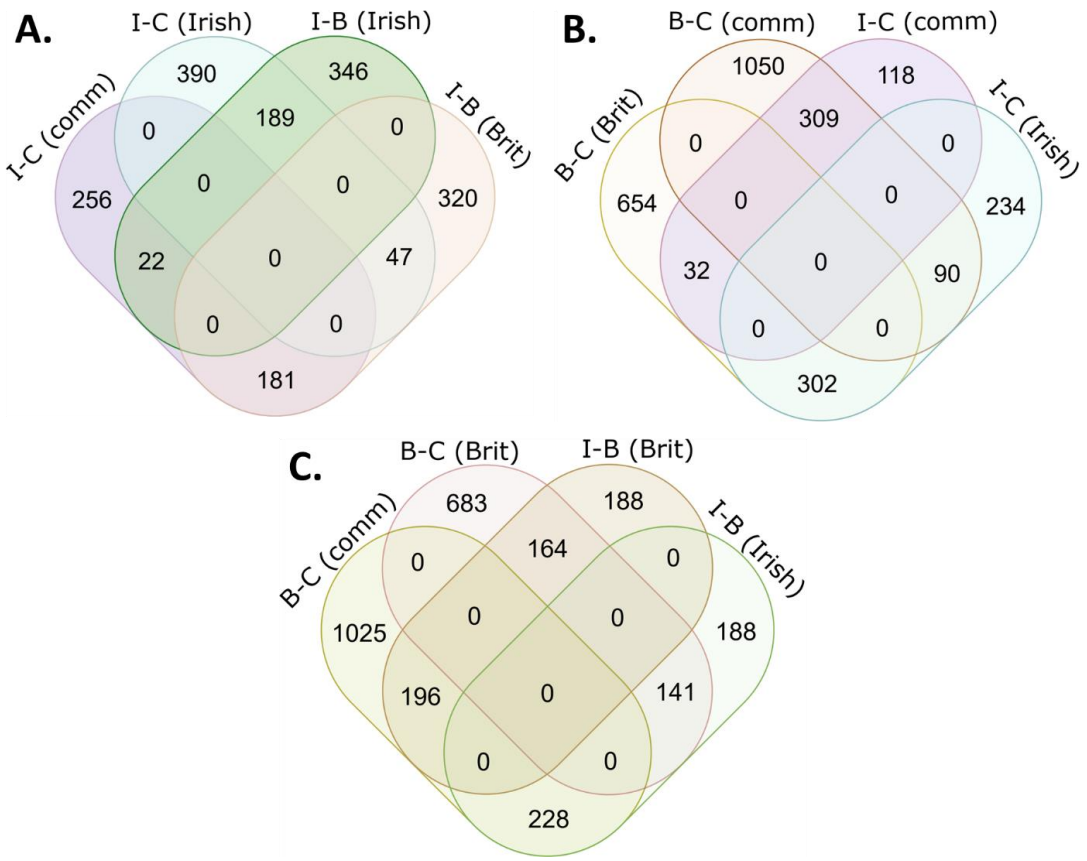


Figure 2.6 Venn diagram of overlapping genes showing differential signatures of positive selection, as determined by XP-nSL values, in each population comparison. Venn analysis showing unique and shared numbers of genes exhibiting differential signatures of selection (|XP-nSL| > 2) in the XP-nSL analysis between (A) Irish – commercial and Irish – British, (B) Irish – commercial and British – commercial and (C) British – commercial and Irish – British comparisons. The genes showing distinctions in a specific population of each XP-nSL comparison are indicated in brackets.

Table 2.4 Top SNPs (as ranked by XP-nSL value) in the Irish – British comparative XP-nSL analysis. Results of top 10 highest XP-nSL values in the Irish (green) and British (yellow) populations with corresponding SNPs information (chromosome, SNP position and variant type) and the gene name and description of where the SNP is found.

Chromosome name	SNP position	Variant type	XP-nSL	Gene ID	Gene description
NC_063276.1	5503916	Intron	-4.01	LOC100646982	Venom dipeptidyl peptidase 4
NC_063278.1	17716166	Intron	-3.84	LOC100648987	Acetylcholine receptor subunit alpha-like 1
NC_063272.1	12809798	Intron	-3.66	LOC100642241	Uncharacterized protein LOC100642241
NC_063274.1	4390848	Intron	-3.62	LOC100650524	Protein unc-13 homolog B
NC_063275.1	24157322	Intron	-3.62	LOC100643486	Glutamate receptor ionotropic, NMDA 2B
NC_063274.1	3815589	Intron	-3.56	LOC125385223	Uncharacterized LOC125385223
NC_063272.1	14518804	Missense	-3.54	LOC125385041	Terminal nucleotidyltransferase 5A-like
NC_063279.1	2919137	Intron	-3.51	LOC100645566	Uncharacterized protein LOC100645566
NC_063275.1	23415323	Intron	-3.45	LOC100650962	F-actin-methionine sulfoxide oxidase Mical
NC_063272.1	14480575	Intron	-3.44	LOC100644394	POU domain, class 2, transcription factor 1-like
NC_063278.1	14396566	Intron	3.07	LOC100651590	Cadherin-87A
NC_063269.1	262050	Missense	3.04	LOC105666912	UPF0585 protein c16orf13
NC_063269.1	271883	Intron	3.02	LOC100650754	WD repeat-containing protein 7
NC_063275.1	13340118	Intron	2.99	LOC100651445	WD repeat-containing protein 47
NC_063269.1	3735688	Synonymous	2.98	LOC100649721	Monoacylglycerol lipase ABHD12
NC_063269.1	3730914	3’ UTR	2.97	LOC100649485	Uncharacterized protein LOC100649485
NC_063280.1	8518965	Intron	2.95	LOC100645212	Intersectin-1
NC_063269.1	3728850	Synonymous	2.94	LOC100649609	Mitochondrial import inner membrane tranSLocase
NC_063278.1	10384576	Synonymous	2.94	LOC100648585	Stromal cell-derived factor 2-like protein 1
NC_063271.1	2931857	5’ UTR	2.94	LOC105667155	Tubulin polyglutamylase TTLL4-like

2.4 Discussion

B. terrestris (L.) is a species of bumblebee that is distributed across the Palaearctic and has shown many instances of differentiation in genetics, morphology and behaviour across its range (Widmer *et al.*, 1998; Rasmont *et al.*, 2008). Within their local environments, subpopulations of bumblebees likely face many drivers of selection such as competition for resources, predation and infection as well as pressure from human-related changes to their environments (Alford, 1975; Schmid-Hempel, 1995; Goulson *et al.*, 2008; Colgan *et al.*, 2022). Depending on the degree and length of genetic isolation from other subpopulations and the location-specific set of selective forces, populations may become distinctive and exhibit signatures of selection that reflect adaptation to environmental changes or stressors. It is more important than ever to investigate intraspecific variation and selection in pollinator communities as there have been notable declines in pollinator populations across Europe and North America thought to be caused by pesticide use, habitat loss, pathogen spread and climate change (FitzPatrick *et al.*, 2007; Kosior *et al.*, 2007; Colla and Packer, 2008; Goulson, 2010). Also, in order to assess risks associated with the use of commercial *B. terrestris* for crop pollination services on wild pollinator populations, it is essential to understand the genetic variance and structure of wild *B. terrestris* populations within which commercial varieties of *B. terrestris* are likely to mix.

In this study, we use a population genomics approach to examine genetic structure between an Irish and British population of *B. t. audax*. For comparison, we also sequenced and included commercially sourced *B. terrestris* classified as ssp. *audax* and mainland European *B. terrestris* ssp. genomes. This study presents clear evidence for strong genetic differentiation between Irish and British populations of *B. t. audax*. We also find evidence that both British and Irish populations have distinctive signatures of selection that have likely been driven by location specific pressures that appear to have targeted genes associated with the nervous system and developmental processes. In addition, we find that commercial *B. t. audax* bumblebees may also have genetic distinctions that differentiate them from both wild populations of *B. t. audax*, while being closer in relatedness to British bumblebees than Irish. This study emphasises the clear necessity of research exploring intraspecific variation of important pollinating species and the extent and impact of distinctions between wild

and domesticated bumblebees. Furthermore, we believe the results of this study will have implications on policy and management surrounding the use of commercial bumblebee colonies.

2.4.1 A distinctive Irish population of B. t. audax and possible evidence of hybridisation

While both Irish (see Figure A3.1) and British bumblebees (Colgan *et al.* 2022) have some indication of weak substructure within their respective populations, the findings of this study supports the existence of a genetically distinct population of *B. t. audax* in Ireland and a British *B. t. audax* population which is more closely related to mainland European populations of *B. terrestris*. The PCA analysis visualises a low percentage variance value as it is intraspecific population comparison, where many of the alleles carried among the populations are generally polymorphic. Similar low percentage variance values represented by PCAs are found in several intraspecific population studies (e.g., Fernandez-Fournier *et al.*, 2021, Jones *et al.,* 2023). In spite of the low percentage variance represented, we see clear distinctions in our dataset between Irish and non-Irish populations. Evidence found by Moreira *et al.* (2015) for genetic distinction between Irish and British populations of *B. terrestris* in addition to high-levels of admixture between British and mainland European *B. terrestris* subpopulations consolidates our findings. In addition, identity-by-state analysis, which assesses the likelihood of two individuals being siblings by measuring shared alleles, as carried out in the Chapter 2 study and by Colgan *et al.* (2022) confirms that distinctions are not due to related individuals in either population.

It is perhaps no surprise that we found evidence of a relatively isolated and distinctive population of *B. terrestris* in Ireland, as the island has previously been described by Sleeman (2014) as a 'focus for genetic diversity' and hosts many distinctive populations of several other insect species found elsewhere in Europe, including the honeybee *A. mellifera* (Hassett *et al.*, 2018), a caddisfly species (*Plectrocnemia conspersa*; Wilcock *et al.,* 2001) and the hairy wood ant (*Formica lugubris;* Mäki-Petäys and Breen, 2007), as well as several amphibian (Rowe *et al.*, 2006; Teacher *et al.*, 2009) and mammalian species (Randi *et al.,* 2003; Hamill *et al.,* 2006; Finnegan *et al.,* 2008; O'Meara *et al.,* 2012). Our findings, alongside these studies, suggest that the Irish population of *B. terrestris* may be sufficiently genetically isolated from other

European populations by the physical barrier of the Irish Sea and this may have contributed to its differentiation (Moreira *et al.*, 2015; Hassett *et al.*, 2018). Conservation of genetic resources and variability is essential for the maintenance of wild and captive population viability. For example, lowered genetic variability can depress a population's overall fitness or resistance to infection (Lacy, 1997). Certain genetic resources may also hold alleles that confer adaptive advantages, e.g., drought resistance, that can be beneficial when expressed in economically important species as seen in food crops (Reynolds *et al.*, 2007). Further population studies should be carried out on this population and other Irish pollinator species to explore the existence of unique adaptations in these populations to their native environments as well as to identify additional distinctive populations and assess the genetic 'health' of potentially endangered native pollinators.

Our analysis finds that the origin of the commercial *B. t. audax* stock remains unclear. Commercial bumblebees classified as *B. t. audax* were more closely related to British populations than Irish, suggesting initial stocks of commercial bumblebees may have been caught from Britain. However, we observed that individuals sourced from Koppert Biological Systems grouped more closely with other mainland European subspecies than the British samples. Individuals from sourced from Biobest colonies showed much more distinction from British, German, and Turkish samples than Koppert Biological Systems. Conditions of rearing facilities are likely variable, although exact practices and management of captive bumblebees in commercial rearing facilities are classified and therefore unknown to us. It's possible that differences in rearing practices and conditions have driven differentiation between captive bred populations. Interestingly, two British samples group with Biobest individuals and one with Koppert Biological Systems samples, which could indicate accidental collection of escaped males from commercial colonies being used nearby where sampling took place. Although we bear in mind the small sample sizes for both sets of commercial bumblebees, it is concerning that neither group of commercial bumblebees show a strong genetic resemblance to either wild population of *B. t. audax*, particularly the Irish, nor each other, despite being classified as same subspecies.

In some Irish samples, we found evidence of admixture with non-Irish groups. This may be a result of hybridisation with migrating British bees (although this is unlikely;

see Moreira *et al.,* 2015), imported *B. terrestris*, either the previously imported *B. t. dalmatinus* subspecies (Murray *et al.*, 2013) or the currently imported *B. t. audax* subspecies (Biobest). Interestingly, many of the Irish samples that showed admixture were from counties in the East of Ireland, where commercial colony imports are concentrated and fruit production is primarily carried out (Murray *et al.*, 2008). This suggests that commercial reproductive individuals may have escaped, cross-mated with wild conspecifics resulting in wild-established, hybrid colonies. This would not be surprising, as escape of bumblebees from commercial colonies, even when used in greenhouse settings, has been widely documented in countries such as Israel (Dafni *et al.*, 2010), Chile (Schmid-Hempel *et al.*, 2014) and Japan (Matsumura *et al.*, 2004; Inoue *et al.*, 2008). Various subspecies of *B. terrestris* have also shown the capacity to cross mate (Ings *et al.*, 2005) and there are several documented cases of hybridisation between commercially imported *B. terrestris* and wild populations (Kraus *et al.*, 2011; Seabra *et al.*, 2019; Cejas *et al.*, 2020), including Ireland (Teagasc, 2012).

Hybridisation has the potential to have positive or negative impacts on the general fitness and adaptive ability of a population (Allendorf *et al.*, 2001). Hybridisation has often been considered to have a creative potential, particularly by botanists, by introducing adaptive alleles into certain populations (Warschefsky *et al.*, 2014; Sreeman *et al.*, 2018). However, any hybridisation-related change to a population's fitness, even if positive, may have serious consequences on the ecological balance of the ecosystems in which they exist. In plants, hybridisation between and within species has been shown to stimulate invasiveness (Ellstrand and Schierenbeck, 2000). For example, the hybrid weedy sunflower *Helianthus annuus ssp. texanus*, has higher fitness in terms of seed production than either parent subspecies and was more resistant to herbivory (Whitney *et al.*, 2006). Generally, hybridisation between native and introduced, non-native populations is often considered to impact the conservation status of the native species (Rosinger *et al.*, 2021). Interbreeding and introgression is thought to increase the likelihood of extinction of several indigenous populations (Rhymer and Simberloff, 1996; Levin, 2002) through mixing and outbreeding depression. For example, hybridisation with introduced varieties of mallard duck species caused the decline of the endangered Hawaiian duck, *Anas wyvilliana* (Griffin *et al.*, 1989; Fowler *et al.*, 2009). Outbreeding depression, defined as the introduction

of maladaptive alleles into one population as a result of introgression with another, genetically distinctive population (Rhymer and Simberloff 1996 as cited in Svärdso, 1970), has been observed in many plant species (Montalvo and Ellstrand, 2001; Lázaro and Traveset, 2006) but also in insects (Peer and Taborsky, 2005) and mammals (Marshall and Spalton, 2000).

In the case of commercial bumblebees, it is possible that, through domestication and artificial selection, alleles key to fitness and survival in natural settings have been lost (Lecocq, 2018). If commercial and wild populations hybridise, these maladaptive alleles could decrease the fitness of wild *B. terrestris* population or, conversely, introduce captivity-linked alleles that increase the competitive ability of wild *B. t. audax* e.g., increased reproductive output. Certain population characteristics may help predict the likelihood of a 'hybrid vigour' or an 'outbreeding depression' outcome of hybridisation, such as population size, genetic diversity and genetic distance (Edmands and Timmerman, 2003, Lohr and Haag, 2015). Considering this, further population genomic research into wild and captive *B. terrestris* populations may help predict the potential risks and impacts of hybridisation between these populations.

2.4.2 Differential signatures of selection in geographically separated populations of wild B. t. audax

Animal populations are shaped by the environment in which they live. Currently, wild species are experiencing ever increasing changes to their environments caused by human-related activities such as agriculture and urbanisation (Grimm *et al.*, 2008; Dudley and Alexander, 2017). Through investigation and comparison of the patterns left by selection and adaptation in the genomes of populations, we can gain insight into degree of selection pressures acting on populations (Vatsiou *et al.*, 2016; Abondio *et al.*, 2022). By looking at the specific genes within regions showing selection signatures, we may also identify potential biological processes undergoing selection. We find strong evidence that Irish and British populations of *B. t. audax* are experiencing differential positive selection processes which have resulted in distinctive patterns in their genomes. Overall, genes containing SNPs with highly distinctive frequencies between Irish and British populations were involved in morphogenesis, functioning and development of the nervous system and behaviour. Single population analyses on signatures of selection in just the British population,

performed by Colgan *et al.* (2022), also found selection generally acting on processes such as morphogenesis, nervous system development and functioning. Irish *B. t. audax* genomes showed consistent signatures of positive selection in genes relating to synaptic transmission, homeostasis and mushroom body development when compared to either the British or commercial groups. The British population also had consistent signatures of selection in genes associated to synaptic endocytosis and phospholipid membranes. In the Irish population, signatures of positive selection were found in several genes encoding proteins involved in neurotransmission. For example, among the top genes showing positive selection in the Irish population compared with the British was an acetylcholine receptor and glutamate receptor. Acetylcholine and glutamate are two key excitatory neurotransmitters that act within the insect brain and are detected by acetylcholine receptors and glutamate receptors, respectively, expressed in insect neurons (Albert and Lingle, 1993; Tomizawa and Casida, 2001). Glutamate and cholinergic neurotransmission has been shown to be key for olfactory learning and formation of memories in insects (Locatelli *et al.*, 2005; Gauthier and Grünewald, 2012; Cartereau *et al.*, 2022). Positive selection in genes involved in such processes may reflect selective pressures exerted on wild Irish bumblebees in their environments. For example, the mode of action of many insecticides is to selectively target and disrupt neurotransmission in insects (Zhao *et al.*, 2004; Barbara *et al.*, 2005; Vehovszky *et al.*, 2015) and so changes in the genes encoding neurotransmitter receptors in the Irish population could indicate an adaptation against abiotic stressors such as pesticide use. Furthermore, differences between Irish and British selection signatures may be a result in differences in land use and management on these islands. For example, the UK has higher levels of arable land than Ireland, whereas Ireland has proportionally more grassland in comparison to the UK (Eurostat, 2023).

Other genes displaying signatures of positive selection in both the Irish and British populations encode for proteins with functions relating to development. As processes relating to embryo and larval development are usually under purifying selection due to their essential nature (e.g., Lawrie *et al.*, 2013), it was surprising to see strong signatures of differential selection between Irish and British *B. t. audax* in genes relating to nervous system development, axis-formation and morphogenesis. Normal development of anatomical structures in embryonic and larval stages are vital to the establishment of functioning organs and physiological processes that are essential to

adult survival (Tadkowski and McCann, 1980; Campos and Hartenstein, 1985; Singhania and Grueber, 2014; Wang *et al.*, 2015). In particular, the properly developed nervous system is required for many essential processes and behaviours such as coordination of movements, learning and memory and the detection and ability to respond to stimuli, to name a few (Nation, 2008; Klowden, 2013). Our findings indicate there are differences in the selective processes acting on genes which are involved in fundamental physiological and developmental processes in Irish and British populations. Future research should explore the potential drivers of selection in each population. It should also be explored whether differential selection in Irish and British *B. t. audax* populations has resulted in phenotypic difference, particularly in nervous system development and functioning.

2.4.3 Selection within commercial B. terrestris populations

When Irish and British populations were compared to commercial bumblebees, there were several genes showing consistent signatures of selection in the commercial group. The association of these genes to lipid homeostasis and the TCA cycle suggest this population may be facing selective pressures that are acting on processes associated with energy utilisation and storage (Klowden, 2013; Alberts *et al.*, 2015). Furthermore, genes showing signatures of differential selection in wild and commercial populations were also linked to reproductive processes such as oocyte cell fate determination and oocyte karyosome formation. Although further research is required to determine the true origin of genetic distinctions in commercial *B. t. audax* compared with wild populations, it is possible that artificial or unconscious selection in captive environments has altered processes associated with nutrition, energy utilisation, storage of resources and reproduction (Lecocq, 2018). Interestingly, domesticated chickens have been found to show differential expression of proteins relating to the TCA cycle and fatty acid oxidation, suggesting that these key pathways have become altered through the domestication process and selection for traits of commercial interest e.g., increased muscle growth (Schmidt *et al.*, 2023). Domesticated populations of *B. t. dalmatinus* also show increased colony growth and reproductive success when compared to their wild counterparts (Gösterit and Baskar, 2016). Admittedly the sample size of the commercial bumblebee evaluated in this study was much smaller than the Irish and British groups, and we therefore take

caution in our interpretation of the results from the wild to commercial comparisons and encourage future research to further investigate the differences in selective processes between wild and commercial bumblebee populations and the consequences of these processes on bumblebee physiology and behaviour.

2.4.5 Implications of our findings

We anticipate that the identification of a distinct *B. t. audax* population in Ireland will provide the basis for its protection as a genetic resource. Although *B. terrestris* is not an endangered species, all bees in Ireland and across Europe are facing declines, thought to be a result of pesticide use, pathogen spread from managed bees, habitat loss and climate change (FitzPatrick *et al.,* 2007; Kosior *et al.,* 2007; Goulson *et al.,* 2008). Preserving genetic variability within our pollinator populations is essential if we experience further bee declines which will lead to an even greater dependence on commercially produced bees for pollination services. Our findings also show the importance of taking caution when generalising about biodiversity of Ireland and Britain, as Ireland holds many genetically distinctive populations (Teacher *et al.,* 2009; O'Meara *et al.*, 2012; Sleeman, 2014; Hassett *et al.,* 2018).

As suggested by Rhymer and Simberloff (1996), there is a need for accurate taxonomic descriptions between subpopulations which can interbreed. We believe that the evidence provided by our study indicates a possible need for taxonomic re-evaluation and clarification of wild and commercial populations currently classified as *B. t. audax* particularly on the island of Ireland. However, there is longstanding debate and ambiguity on how we classify subpopulations, for example, as separate subspecies (Wilson and Brown, 1953; Ebach and Williams, 2009; Burbrink *et al.,* 2022). Patten (2009) defined subspecies as "a collection of populations occupying a distinct breeding range and diagnosably distinct from other such populations", where populations are not reproductively isolated from one another. Patten (2015) then adds that the term subspecies refers to "heritable geographic variation in phenotype", with the "75% rule" (where group A must have ≥75% of its group outside the 99% variation range of group B to be defined as a subspecies), being the most common statistical threshold used to support subspecies classification (Patten and Unitt, 2002). Morphological phenotypic distinctions are one of the requirements for subspecies

classification (Mousseau and Sikes, 2011). Indeed, the currently accepted nine *B. terrestris* subspecies were originally classified on the basis of differential morphology (Rasmont *et al.*, 2008). Further studies into possible subtle morphological distinctions between populations of *B. t. audax* may provide foundation to the possible existence of distinct subspecies as opposed to genetically and geographical distinct populations of the same subspecies. However, Moretti *et al.* (2008) find support for cryptic *Fusarium subglutinans* subspecies using non-morphological phenotypes (in this case, beauvericin production by the fungus *Fusarium subglutinans*). As methods continue to develop to link genotypes to phenotypes and the use of non-morphological phenotypes are investigated and discussed in the context of subspecies identification and classification, it may be possible to classify more genetically distinct populations as subspecies.

In terms of *B. t. audax*, these future developments and research foci may help clarify future discussions regarding how imported bumblebee colonies be managed in Ireland and Britain. Where there is a high risk of hybridisation between commercial bumblebees and distinctive, wild bumblebee populations, as seems to be the case in Ireland, preventative actions should be taken, such as the use of queen excluders and proper destruction of colonies at the end of the crop flowering period, to limit the risks of hybridisation, pathogen spread and competition with wild pollinator communities.

2.4.6 Conclusion

In this study, we identified a genetically distinct population of *B. t. audax* in Ireland. We also find differential signatures of selection between Irish and British populations, suggesting that these populations are exposed to location-specific selective pressures that could have impacts on fitness related traits. Tentative evidence exists of distinctive signatures of selection occurring in commercial bumblebees, which may be explained by domestication-related selective processes. We hope our findings allow for evidence-based policy decisions and recommendations to protect genetically distinctive populations of *B. terrestris* from hybridisation with non-native varieties of commercial bumblebees. Appropriate management of commercial bumblebees will not only protect wild *B. terrestris* populations from admixture, but will also protect all wild pollinator species from pathogen spread and competition (Goulson, 2010; Graystock *et al.*, 2013). This is a vital step that needs to be taken to prevent further

declines in numbers and biodiversity of ecologically and economically essential insect pollinators.

Chapter 3

Signatures of adaptation, constraints, and potential redundancy in the canonical immune genes of a key pollinator

The following chapter forms the basis of a paper that has been published in Genome Biology and Evolution (https://doi.org/10.1093/gbe/evad039).

3.4 Introduction

Pathogens threaten all living organisms, leading to the evolution of host mechanisms to resist, tolerate or avoid pathogen interaction (Barreiro and Quintana-Murci, 2010; Balloux and van Dorp, 2017). Insects, in particular, have evolved several effective traits to prevent infection, including avoidance and hygienic behaviours, physical barriers, such as a tough exoskeleton, and chemical defences (Siva-Jothy *et al.*, 2005). However, if a pathogen can overcome these defences and successfully invade their host, the last barrier to the establishment of infection is the insect innate immune system (Tsakas and Marmaras, 2010). The insect immune system is comprised of two arms: the cellular response, coordinated by haemocytes, which implements phagocytosis, encapsulation, and melanisation (Mavrouli *et al.*, 2005; Strand, 2008) and the humoral response, a protein-derived defence involved in the recognition of and response to pathogen-associated molecular patterns. The recognition of such molecules activates pathway cascades, such as the Toll pathway which is activated on host recognition of Gram-positive (Rutschmann *et al.*, 2002) and fungal invaders (Roh *et al.*, 2009). These cascades stimulate the production of effector molecules, such as antimicrobial peptides that target and eliminate invaders (Ali Mohammadie Kojour *et al.*, 2020).

Genes involved in the immune system are often subject to strong selective pressures to defend against and co-evolve along with pathogens while also being able to distinguish pathogens from beneficial foreign products (e.g., ingested food), as well as symbiotic microbes (Royet *et al.*, 2011; Nyholm and Graf, 2012). Similarly, components of the immune system have evolved to recognise and differentiate between self and non-self to protect the host from its own defences (Cooper and Eleftherianos, 2017). The strong selective pressures placed on immune genes are reflected in the routine identification of these genes as fast evolving across diverse taxa through comparative genomic scans (Roux *et al.*, 2014; Shultz and Sackton, 2019). However, there are different types of selection, which often produce characteristic patterns in the host genome. For example, positive selection produces genomic regions of reduced diversity through “selective sweeps”, whereby selected beneficial alleles, as well as neighbouring sites, increase in frequency within a population to the point of fixation (Nielsen *et al.*, 2005). Recent studies on insects

have applied population genetic approaches to investigate the genetic variability of immune genes, as well as to determine the different forms of selection acting upon them (Palmer *et al.*, 2018; Tan *et al.*, 2021). However, with a few exceptions, studies employing genome-wide population genomic techniques to better understand how selection has acted on different functional classes of the immune system are still lacking, especially for non-model organisms within the field of genetics (but see Tan *et al.* 2021).

For many ecologically and commercially beneficial insect species, such as pollinators, it is vitally important to understand the selective pressures acting on their immune genes as pathogens and associated disease are one of several factors suggested to contribute to population declines (Goulson,, 2010; Wagner, 2020). Pathogen spread is hypothesised to be exacerbated by the use of commercially reared bumblebees. These bumblebee colonies, usually *B. terrestris* or *B. impatiens*, are reared in commercial facilities and exported around the globe to provide pollination services to crops, such as tomatoes (Velthuis and Van Doorn, 2006). However, they are known to often carry bumblebee and honeybee pathogens (Graystock *et al.,* 2013; Murray *et al.,* 2013) and there is evidence supporting pathogen spread from commercial bumblebees to wild populations of many bee species (Colla *et al.*, 2006; Murray *et al.*, 2013). Declines in pollinator populations, including bumblebees (FitzPatrick *et al.,* 2007; Kosior *et al.*, 2007; Cameron *et al.*, 2011), have led to concern about the sustainability of pollination services provided by insects which greatly contribute to global economies, food security and ecosystem functioning (Aizen *et al.*, 2009; Stout *et al.*, 2019). Our understanding of the capacity of wild bee populations to respond to ongoing environmental threats has been greatly informed by recent studies using population genomics, which have highlighted the genetic variation harboured in a population, as well as the targets of recent positive selection acting throughout the genome (Heraghty *et al.*, 2020; Harpur and Rehan, 2021; Colgan *et al.*, 2022; Hjort *et al.*, 2022), including immune genes (Kent *et al.*, 2018). In addition, studies have shown that the worldwide spread of the *Varroa* mite, a brood parasite, have placed strong selective pressures on managed *A. mellifera* honeybees and have driven the emergence of *Varroa* resistance traits in in these populations (Le Conte *et al.*, 2020; van Alphen and Fernhout, 2020). Even in Ireland, where *Varroa* was first identified in 1998, tolerance to the Varroa mite has been observed 14 years later (McMullen, 2018). However, despite the

importance of hymenopteran insects such as bees, wasps, and ants, focussed population genetic studies on their immune genes have yet to be performed. Addressing this knowledge gap is essential in understanding how these genes are evolving in response to the risks currently facing many valuable insects in their natural environments.

A valuable study system to examine how recent selection has shaped immune gene evolution is the earth or buff-tailed bumblebee species, *Bombus terrestris*. Bumblebees (*Bombus* spp.), in general, are a particularly beneficial group of pollinators for a variety of commercial crops due to their variation in tongue length, ability to buzz pollinate, and tolerance of temperate climates (Velthuis and Van Doorn, 2006). Bumblebees are also a classical system for studying insect immunity as they are social insects that live in thermoregulated, densely packed nests of closely related individuals, providing prime conditions for pathogen spread among nest mates (Shykoff and Schmid-Hempel, 1991). This fact is further reflected by bumblebees serving as hosts for a taxonomically diverse group of pathogens, including viruses, bacteria, fungi, protozoans, nematodes, insect parasitoids and social parasites (Rutrecht and Brown, 2008). Bumblebees, like other holometabolous insects, also have morphologically and behaviourally distinct life stages that face different pathogenic threats and risks (Schmid-Hempel, 1998). Similarly, being social insects, bumblebees have caste differentiation in the female sex with the evolution of queen and worker castes, which exhibit different behaviours and perform different colony-level tasks that likely influence their risk of pathogen exposure and infection (Colgan *et al.*, 2011). For example, gynes and queens have a much longer lifespan than the short-lived worker caste and so likely have an increased total exposure to pathogens over their lifetime. This may require them to have greater protections against infections facilitated by social behaviours (Cremer *et al.*, 2007) or mating (Colgan, 2019). At the genetic level, bees have a decreased set of canonical immune genes in comparison to model organisms, such as *Drosophila melanogaster*, suggesting that bees may have a reduced immune capacity to combat infection (Evans *et al.*, 2006; Barribeau *et al.*, 2015). While reduced, the set of canonical immune genes found in bees still represents each of the core canonical immune gene groups, just with fewer representatives in each group (Evans *et al.*, 2006, Barribeau *et al.*, 2015, Sadd *et al.*, 2015). Finally, like other Hymenoptera, sex determination is haplodiploid, with

females developing from fertilised, diploid eggs and males from unfertilised, haploid eggs (Cook and Crozier, 1995). The haploid status of the male means if a deleterious allele is inherited, they do not have an extra gene copy to rescue function (Hartl and Brown, 1970). Therefore, males automatically express deleterious alleles that they carry and, therefore, selection acts strongly to remove these from a population (Joseph and Kirkpatrick, 2004). Additionally, haploid individuals may carry alleles that are predicted to impact gene function and, simultaneously, exhibit no obvious impediment on fitness. The presence of these alleles in what appear to be robust, healthy individuals may highlight genes with reduced, redundant, or non-essential roles in important biological processes.

In this study, we performed whole-genome resequencing of a natural population of *B. terrestris*, to understand the genetic variation maintained in their canonical immune genes and the selection pressures acting on them. We sampled male bumblebees across the island of Ireland and applied a population genomics-based approach to examine patterns of positive selection through estimating genome-wide levels of nucleotide diversity and extended haplotype homozygosity to provide evidence for recent selective sweeps acting on canonical immune genes (Barribeau *et al.*, 2015; Sadd *et al.*, 2015). Furthermore, we investigated evidence of evolutionary constraints through the characteristic of immune genes evolving strong purifying selection. Lastly, to understand potential non-functional or redundant components of the bumblebee immune system, we investigated the presence and frequency of putative loss of function alleles carried by wild-caught adult males.

3.2 Methods

3.2.1 Sample collection

To assess signatures of selection acting on canonical immune genes, we sampled 33 *B. terrestris* males foraging in natural settings across 27 sites from across the island of Ireland between June and September 2018 (Table A3.1). Field-based identifications of *B. terrestris* males were based on the standard morphology of this species (Falk, 2019) and the lack of an aculeus, which is found only in females. All collected samples were frozen on the same day and stored at -20°C until DNA extractions.

3.2.2 DNA extraction, species confirmation, library preparation and sequencing

Genomic DNA was extracted from the heads of each bee using the Dneasy Blood and Tissue Kit (Qiagen, Valencia, CA), following the protocol for animal tissues. Some modifications to the protocol were applied and included the following: 40µl of Qiagen Proteinase K was added to each sample and samples were vortexed prior to and every hour during a 3-hour incubation step at 56°C. DNA samples were eluted in 150µl Qiagen Buffer AE and assessed for quality and quantity using agarose gel electrophoresis and a NanoDrop™ Spectrophotometer (ThermoFisher Scientific). Extracted DNA samples were then sent for further quality assessment, genomic library preparation and subsequent sequencing by a commercial company (Novogene, Cambridge, UK). PCR-free genomic libraries were produced using the NEBNext Ultra I DNA library kit and pair-end sequencing (150bp) was performed on an Illumina NovaSeq6000 sequencing platform. Sequencing generated an average of 13.4 million paired-end (PE) reads per individual (Table A3.1), with a range from 10.6 to 19.8 million PE reads and a standard deviation of 1.64 million PE reads. Based on an estimated genome size of 248Mb, the mean predicted raw coverage of each sample was 16.2X (min: 12.76X; max: 23.95X; Table A3.1).

3.2.3 Quality assessment, filtering, and data alignment

Using FastQC (v.0.11.9; Andrews, 2010), we assessed raw reads for overall base quality. Raw sequencing quality was high as the mean Phred score across all samples was greater than 30 per base position. Using fastp (v.0.22.0; Chen *et al.*, 2018), we removed sequences with over 10% ambiguous bases and those with more than 40% of bases with Phred scores less than 20. In addition, we used fastp to identify and remove Illumina adapter sequences. Post-filtering, we retained sequences that were equal to or more than 50 bases in length.

3.2.4 Variant calling and filtering of single nucleotide polymorphisms

Our variant calling and filtering approach followed that of Colgan *et al.* (2022). In brief, to identify polymorphic sites within our population, we aligned filtered reads against the *B. terrestris* reference genome assembly (Bter_1.0, GCF_000214255.1) using bowtie2 (v.2.4.4; Langmead and Salzberg, 2012). Using these alignment files,

variants were called using Freebayes (v.1.3.5; Garrison and Marth, 2012) with the following parameters: --ploidy 2 –report-genotype-likelihood-max --use-mapping-quality –genotype-qualities –use-best-n-alleles 4 –haplotype-length 0 –min-base-quality 3 –min-mapping-quality 1 –min-alternate-frac 0.25 –min-coverage 1). We analysed the samples as potential diploids to allow for the identification of low complexity sites, putative copy number variants and misalignments, which may be called as heterozygous and can be removed from downstream analyses. We used VCFtools (v.0.1.17; Danecek *et al.,* 2011) to filter out low-quality variant sites with a quality (QUAL) score of <20, variants in low complexity, repetitive regions with a max mean depth of >100 bp, insertions and deletions ("indel") variants and sites with missing data (--max-missing 1.0). As the sequenced bees were haploid, and therefore hemizygous, any sites called as heterozygous were removed. To reduce the complexity of the datasets and to facilitate downstream analyses, sites with more than two alleles (i.e., multiallelic sites) and rare alleles occurring only once across all 33 individuals were removed using VCFtools (v.0.1.17; Danecek *et al.,* 2011). Lastly, we subsetted single nucleotide polymorphisms (SNPs) found only on the 18 linkage groups (representative of chromosomes) of the *B. terrestris* Bter_1.0 reference genome assembly. We annotated all retained high-quality SNPs with variant type data using snpEff (v.5.0e; Cingolani *et al.,* 2012).

3.2.5 Assessment of population structure

To determine evidence of population substructure in our dataset, we use three approaches. First, we performed a principal component analysis (PCA) using a pruned dataset from called genotypes using the R package SNPRelate (v.1.20.1; Zheng, 2013). Second, using this pruned dataset, we performed an identity-by-state analysis using SNPRelate to determine relatedness amongst individuals. Third, we used a cluster-based approach to estimate co-ancestry amongst sampled individuals using ADMIXTURE (v.1.3.0; Alexander *et al.,* 2009) with K = 1-20 and cross-validation (--cv) to identify the most probable K populations in our dataset. All three analyses suggested that our sampled bumblebees originate from a panmictic, single population, displaying only weak substructure (Figure A3.1).

3.2.6 Sliding windows-based estimation of nucleotide diversity and Tajima's D

We used PopGenome (v.2.7.5; Pfeifer *et al.*, 2014) on the high-quality SNPs to calculate mean nucleotide diversity ($\boldsymbol{\pi}$) and Tajima's D using 100kb sliding windows to identify genomic regions with significantly elevated or reduced measures of diversity in comparison to the rest of the genome. As the reference genome assembly contains regions of ambiguous bases, which can falsely present as regions of low diversity, we first identified and removed such regions. For this approach, we used bedtools (v.2.30.0; Quinlan, 2014) to generate window-specific FASTA files containing the nucleotide sequences present in each window. We then calculated the percentage of ambiguous bases in each window using seqtk comp (v1.3-r106; Available at: https://github.com/lh3/seqtk). We removed windows that contained over 10% of ambiguous bases (n = 417), and therefore, represent likely gaps in the genome assembly. Post-filtering, we estimated nucleotide diversity and Tajima's D for each of the remaining windows (n = 3,894). We then performed a z-score transformation on nucleotide diversity estimates and calculated corresponding *p* values to determine regions of significantly elevated or reduced nucleotide diversity, which may represent signatures of positive and balancing selection, respectively. Lastly, we extracted the gene coordinates from the *B. terrestris* gene feature file (gff3) obtained from Ensembl Metazoa (Howe *et al.*, 2021) for the bumblebee canonical immune genes (Sadd *et al.*, 2015) and used bedtools intersect (v.2.30.0; Quinlan, 2014) to identify the presence of such genes in windows of elevated or reduced nucleotide diversity.

As a complementary measure, we performed a gene-level analysis using PopGenome. We calculated the number of segregating sites and length-corrected nucleotide diversity for each gene in the *B. terrestris* genome. Based on these measures, we extracted canonical immune genes with zero diversity (i.e., where there was no evidence of SNPs). We further assessed genes that lacked diversity by calculating the number of reads covering each gene per sample using samtools depth (v.1.10; Li *et al.*, 2009) finding no significant difference in read coverage for each gene of interest compared to the genome-wide mean (t-test; $p < 0.05$). Lastly, for canonical immune genes that lacked genetic variation, we examined individual alignment (BAM) files using the Integrated Genome Viewer (IGV; v.2.13.2; Robinson *et al.*, 2011) to confirm the lack of called SNPs.

3.2.7 Extended haplotype homozygosity tests of recent positive selection

To complement the nucleotide diversity analyses, we performed a haplotype-based analysis to identify regions of extended haplotype homozygosity (EHH), which can include products of "hard" or "soft" selective sweeps. We used selscan (v.2.0; Szpiech, 2021) to calculate nS_L (number of segregating sites by length) scores for each polymorphic site in our dataset. nS_L is calculated using the number of segregating sites and the length of regions of EHH (Ferrer-Admetlla *et al.*, 2014). We used selscan 'norm' under default settings to normalise our nS_L results against the background genome. We calculated absolute nS_L ($|nS_L|$) scores to explore patterns attributed to positive selection in line with approaches used in other studies (Colgan *et al.*, 2022). For each gene on the *B. terrestris* linkage groups, we identified the SNP with the highest $|nS_L|$ score. We then searched for canonical immune genes that had an $|nS_L|$ score ≥ 2, a threshold for positive selection (Ferrer-Admetlla *et al.*, 2014).

3.2.8 Copy number variation analysis of canonical immune genes

For the purposes of identifying putative duplicated or deleted regions of the genome, we followed an approach developed by Colgan *et al.* (2022). We first used lumpySV (v.0.2.13; Layer *et al.*, 2014), a software tool that uses read pair information to determine evidence for the presence of copy number variation. Under the author recommendations, we first realigned our filtered reads against the reference genome assembly using bwa (v.0.7.17-r1188; Li and Durbin, 2009). From the resultant alignment file, we extracted discordant and split-paired reads using samtools (v.1.10; Li *et al.*, 2009). We next extracted genomic coordinates for each putative duplicated site and retained only sites greater than 500bp in length but also less than 100,000bp. Neighbouring sites, which overlapped by at least 1bp, were sorted and merged using bedtools (v.2.28.0; Quinlan, 2014). For each putative duplicated site, we next calculated the percentage of ambiguous bases within each and removed sites where the percentage of ambiguous bases was above 10%. To determine if duplicated sites had elevated read depth compared to other parts of the genome, we used bedtools (intersectBed) and samtools (depth) to calculate the number of reads aligned to each base within each putative duplicated region. We then calculated the median read depth for each genomic region and each individual using R (v.3.6.3). After this, we

compared the median read depth per site against the genome-wide median read depth retaining sites whereby read depth was more than three standard deviations from the genome-wide median. For putative deleted sites, we examined overlap of putative sites with canonical immune genes. We generated individual BAM files per site with bedtools intersectBed and calculated read depth per individual bee. Using these calculations, we examined loci with zero read depth suggestive of a deletion in that individual. We only retained deletions found in at least two individuals. To identify copy number variation in immune genes, we parsed both the list of putative duplicated and deleted sites for canonical immune genes.

3.2.9 Gene Ontology term enrichment analysis

We performed Gene Ontology (GO) term enrichment analysis on canonical immune genes with signatures of recent selection using topGO (v.2.38.1; Alexa and Rahnenführer, 2009) using scripts previously developed by Colgan *et al.* (2019). Given the low-resolution GO terms assigned to *B. terrestris* genes, we assigned GO terms from *D. melanogaster* obtained from Ensembl Metazoa Biomart (Kinsella *et al.*, 2011) to their bumblebee homologues. We performed a Fisher's exact test ($p < 0.05$; node size = 50) for immune genes in genomic regions of significantly low nucleotide diversity. In this analysis, we used the "weight01" algorithm for topGO and ran the analysis separately for each GO category ('biological process', BP; 'molecular function', MF; and 'cellular component', CC).

3.3 Results

3.3.1 Genetic diversity within and across bumblebee canonical immune genes

As a provisional step to examine different selective pressures acting on canonical immune genes, we assessed the genetic diversity found within the sequenced genomes of the 33 wild-sampled *B. terrestris* males. We identified 1,103,300 million high-quality biallelic SNPs with a sliding window-based analysis (window size: 100kb) estimating mean genome-wide nucleotide diversity ($\boldsymbol{\pi}$) as 1.71 x 10^{-3} (Table A3.2a), similar to diversity estimates calculated for a British population of *B. terrestris* (1.51 x 10^{-3}; Colgan *et al.,* 2022). In terms of the canonical immune genes that were analysed (n = 166 genes found on chromosomal scaffolds), we found

no significant difference in terms of mean nucleotide diversity between them and either all other non-immune genes (Wilcoxon rank sum test; W = 789815, $p = 0.098$) or using the same number of size-matched non-immune genes (Wilcoxon rank sum test: W = 13668, $p = 0.397$). The most abundant variant type found in the immune genes was intronic (n = 8,442 SNPs, 66% of total SNPs found in or within 5kb of a canonical immune gene), which was also the most abundant SNP found genome-wide (Table 3.1). We found immune genes have significantly less intronic SNPs compared to non-immune genes ($x^2_{df=1}=541.6$, $p < 2e^{-16}$) but significantly more SNPs in untranslated ($x^2_{df=1}=51.7$, $p < 6.4e^{-10}$) and coding ($x^2_{df=1}=146.9$, $p < 2e^{-16}$) regions.

Table 3.1 Functional annotation of polymorphic sites found in the immune genes of a wild-caught population of *Bombus terrestris*. The total number of genome-wide SNPs, the numbers of immune and non-immune SNPs are shown for variant categories, including SNPs found in intronic regions, 5kb up or downstream of a gene, within 5' or 3' untranslated regions (UTRs) and coding regions.

Variant category	Total no. of SNPs	Total no. of SNPs located in immune genes	Total no. of SNPs located in non-immune genes
Intronic region	627,882	8,442	619,440
5kb Upstream/Downstream of a gene	209,505	3,038	206,467
Untranslated region (UTR)	34,078	641	33,437
Coding region (CDS)	34,428	726	33,702

3.3.2 Canonical immune genes in genomic regions of reduced or elevated diversity

As positive selection can produce genomic regions of reduced diversity generated by "hard" selective sweeps, we assessed genome-wide nucleotide diversity in 100kb sliding windows. We found 77 windows with significantly reduced diversity (z-score < -1.96; $p < 0.05$), within which we found 23 canonical immune genes (Table A3.2a), which was not more than expected by random chance ($x^2_{df=1}=0.88$; $p = 0.35$). Members of several immune gene families were identified in these low diversity regions including: the small RNA regulatory pathway (SRRP: LOC100642865, LOC100644688, LOC100647119, LOC100647746, LOC100648299); autophagy-related genes (APHAG: LOC100642312, LOC100642580, LOC100650799); inhibitors of apoptosis (IAP: LOC100647366, LOC100647488), the IMD pathway

(IMD_PATH; LOC100631093, LOC100645550) and C-type lectins (CTL: LOC100644559, LOC100645018; Figure 3.3; Table A3.2a). Gene Ontology (GO) term enrichment analysis for these immune genes identified the top five most significantly enriched biological processes-associated terms (Fisher's exact test; $p < 0.05$) as "defence response to other organism", "cellular catabolic process", "regulation of defence response", "immune response" and "regulation of immune system process" (Table A3.3).

In these low diversity regions, we also found three canonical immune genes that lacked evidence of polymorphic sites. We further identified five immune genes in other parts of the genome with no SNPs (i.e., no genetic variation amongst sampled bees; Table A3.2b). These genes included *FAS-associated factor 1* (LOC100645550), *ubiquitin-like-conjugating enzyme ATG3* (LOC100642312), *caspase-1* (LOC100645000), *TGF-beta activated kinase 1* (LOC100631093), *plasminogen activator inhibitor 1 RNA-binding protein* (LOC100649628), *32 kDa beta-galactoside-binding lectin* (LOC100649028), baculoviral IAP repeat-containing protein 5 (LOC100647478) and *trypsin-3* (LOC100642942).

Given that many immune genes, especially those involved in pathogen recognition, may evolve under balancing selection and therefore have higher than expected polymorphisms, we investigated the presence of immune genes in regions of significantly elevated nucleotide diversity compared to the background genome. We found 127 genomic regions with elevated nucleotide diversity (z-score > 1.96; $p < 0.05$), which included five canonical immune genes (Figure 3.3): two autophagy (APHAG) gene family members (LOC100647690, LOC100644291); *down syndrome cell adhesion molecule-like protein Dscam2* (immunoglobulin or IGG family; LOC100649765); *ecdysteroid-regulated 16 kDa protein* (MD-2-related lipid recognition or ML family; LOC100643802); and *GTP-binding nuclear protein Ran* (SRRP; LOC100646748). We found that there were significantly more windows with elevated diversity than expected by random chance (Binomial test: $p < 0.0006$).

3.3.3 Recent positive selection acting on autophagy and scavenger receptor proteins

To identify immune genes with evidence of recent positive selection due to "soft" selective sweeps, whereby selection acts on standing genetic variation, we investigated genes with absolute nSL (|nSL|) scores greater than two, an indicator of recent selection (Ferrer-Admetlla *et al.*, 2014; Table A3.4a). We identified 38 canonical immune genes with evidence of positive selection (Figure 1A; Table A3.4b) associated with immune gene groups involved in immune signalling (n = 9 genes total: CLIP serine proteases (n = 5); spätzle proteins (n = 4); and Toll and Toll pathway (n = 4)) and pathogen recognition genes (n = 11 genes total: scavenger receptor proteins (SCR; n = 5); C-type lectins (n = 4); immunoglobulins (n = 2); and ML family member (n = 1); Figure 3.1). Signatures of positive selection were also identified for genes involved in detoxification (peroxidases (n = 5)), immune regulation (serine protease inhibitors (n = 3), small RNA regulatory pathway (n = 1)), autophagy (n = 1), and a thioester containing protein (n = 1).

The strongest evidence for positive selection was found for the *autophagy-related protein 16* gene (|nSL| = 4.299), which had across the genome-wide SNPs, the 17th highest |nSL| score (Table A3.4). The immune gene group with the strongest signatures of selection was the SCR family, which had five genes with signatures of strong positive selection (each containing SNPs within the top 1% of genome-wide |nSL| scores (|nSL| $\geq$ 2.51)), which was greater than expected by random chance (Fisher's Exact test; $p < 0.05$). Across all immune genes, however, we did not find significant differences (Wilcoxon rank sum test: W = 698704, $p = 0.179$) in the mean |nSL| scores assigned to immune genes (mean |nSL| =1.6) compared to the rest of the genome (mean |nSL| for non-immune genes = 1.5). Similarly, we found no specific immune-associated processes under strong recent selection, with a GO term enrichment analysis for immune genes ranked by |nSL| score (KS test; $p > 0.05$).

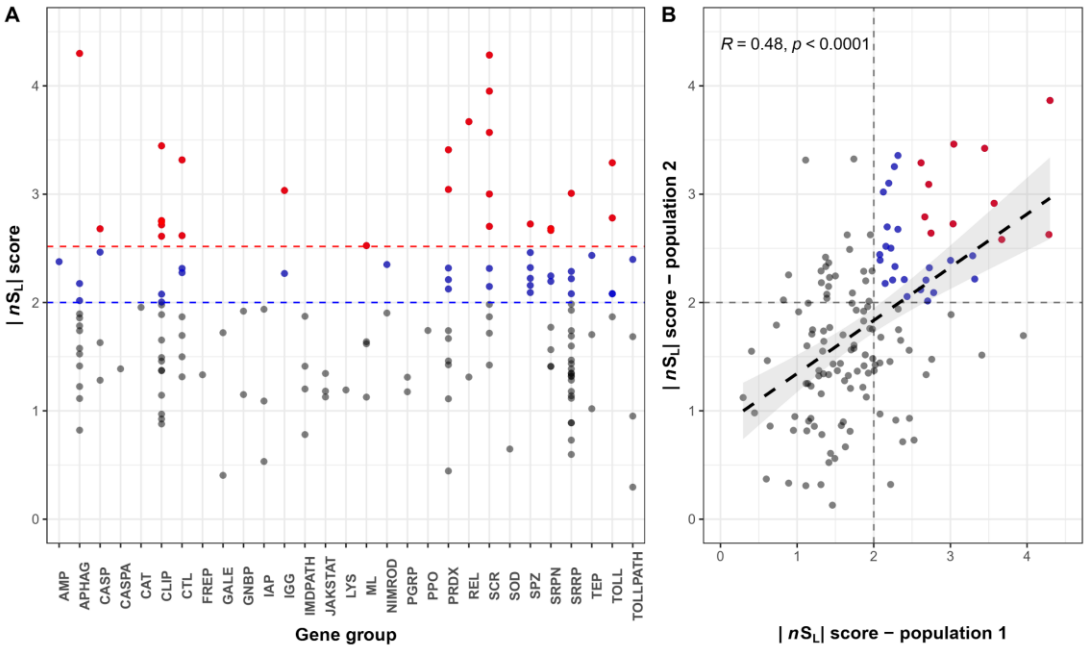


Figure 3.1 Signatures of positive selection vary across canonical immune genes. (A) Scatterplot displaying the top $|nS_L|$ scores assigned to each canonical immune gene with such genes categorised by gene group (x-axis). Dashed horizontal lines indicate thresholds of positive selection ($|nS_L| = 2$; blue line) and those within the top 1% of genome-wide SNPs ($|nS_L| = 2.51$; red line). (B) The correlation of absolute $|nS_L|$ scores calculated for the present dataset consisting of *B. terrestris* males sampled in Ireland ("population 1") and publicly available scores for a British population ("population 2") performed by Colgan *et al. (*2022) highlighting conservation in signatures of positive selection in geographically isolated populations. Grey dashed vertical and horizontal lines indicate thresholds of selection ($|nS_L|$ score ≥ 2) in each population while red dots represent canonical immune genes with signatures of recent positive selection found in both populations. Thick, black-dashed line indicates a best fit regression line complete with 95% confidence intervals. For both plots, genes with a top $|nS_L|$ score ≥ 2 are coloured blue, while those with a top $|nS_L|$ score ≥ 2.51 are coloured red. Gene groups adapted from Sadd *et al. (*2015): AMP = Antimicrobial peptides; APHAG = Autophagy; CASP = Caspase; CASPA = Caspase A; CAT = Catalase; CLIP = CLIP serine protease; CTL = C-type lectin; FREP = Fibrinogen-like; GALE = Galectin; GNBP = Gram-negative binding protein/Beta-glucan recognition protein; IAP = IAP repeat; IGG = Immunoglobulin, IMDPATH = Imd pathway; JAKSTAT = JAK/STAT pathway; LYS = Lysozyme; ML = MD-2-related lipid recognition; NIMROD = Nimrod; PGRP = Peptidoglycan recognition protein; PPO = Prophenoloxidase; PRDX = Peroxidase; REL = Relish; SCR = Scavenger; SOD = Superoxide dismutase; SPZ = Spätzle; SRPN = Serine protease inhibitor; SRRP = Small RNA regulatory pathway; TEP = Thioester-containing protein; TOLL = Toll genes; TOLLPATH = Toll pathway.

To determine if canonical immune genes are experiencing similar forms of selection in other populations, we compared the $|nS_L|$ scores assigned to such genes in our dataset with those previously published by Colgan *et al.* (2022), which involved analysis of male *B. terrestris* sampled across the island of Great Britain. We found a significant positive correlation (Pearson correlation coefficient (R) = 0.48, $p < 0.05$;

Figure 1B) in terms of $|nS_L|$ scores assigned to immune genes in both datasets, highlighting strong positive selection acting on one-fifth of immune genes (n = 34/151 genes with $|nS_L| \geq 2$) in two geographically separated populations. Despite this overlap, this number of genes was not more than expected by random chance ($x^2_{df=1} = 0.95$, $p = 0.33$).

3.3.4 Potential loss of function SNPs in immune genes

As haploid males automatically express the alleles they carry, we investigated the presence and frequency of potential loss of function polymorphic sites within our wild-sampled population, which may highlight non-functional or potentially non-essential immune genes. Across all polymorphic sites, we identified 286 SNPs, affecting potentially 247 genes, that were annotated as being putatively high impact (Table 3.2) whereby the variant was predicted to impact transcription or produce truncated proteins during translation.

Table 3.2 Predicted loss of function or high impact variants in the genomes of wild-caught male bumblebees. The total number of high impact SNPs categorised by snpEff-annotated variant type across the entire genome and among the canonical immune genes from a population of *Bombus terrestris*.

High impact variant	Total no. of SNPs in genome	Total no. of SNPs in canonical immune genes
Stop gained	103	3
Splice donor variant & intron variant	81	1
Splice acceptor variant & intron variant	48	1
Stop lost	38	0
Start lost	11	0
Stop lost & splice region variant	3	0
Stop gained & splice region variant	2	0

The gaining of an early stop codon ('Stop gained') was the most common predicted high-impact variant, with 103 occurrences detected. We found four canonical immune genes with high impact SNPs (Figure 3.2A), including predicted early stop codons in

a serine protease LOC100652157 (the variant found in 36% of all sampled males) and the gene *transmembrane protease serine 9* (LOC100652036; found in 24% of all sampled males). We also detected a splice donor variant within an intronic region in the gene *serine proteinase stubble* (LOC100648752; detected in 24% of all males). The final gene, *antichymotrypsin 2* LOC100648602, a member of the serine protease inhibitor gene family, contained a rare splice acceptor variant (present in 6% of sampled males) and an early stop codon variant (present in 18% of sampled males). For these four genes, pairwise protein sequence similarity was lower and more variable compared to other canonical immune genes shared with the closely-related honeybee *A. mellifera* (Figure 3.2A). A homology-based analysis across a phylogenetically broader set of bee species identified these four genes as taxonomically restricted to members of the Apidae (Figure 3.2B).

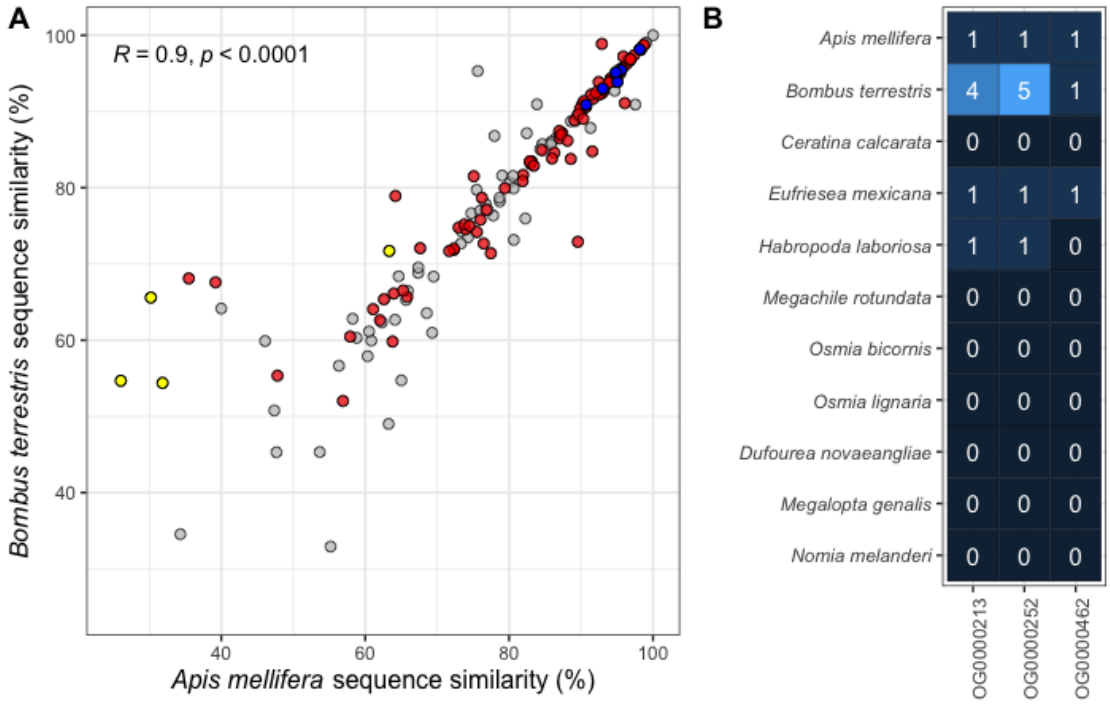


Figure 3.2 Comparative genomics of potential non-essential canonical immune genes. (A) For the four canonical immune genes with predicted high impact SNPs in wild-caught bumblebees, protein sequence similarity was compared between homologues of *B. terrestris* and the closely related honeybee *Apis mellifera*. Each dot represents individual homologues with coloured dots indicating genes that are single copy orthologues (red), those that lack polymorphism in the wild-sampled population (blue) or are genes with predicted high impact SNPs (yellow). The strength (Pearson Correlation Coefficient (R)) and significance (*p* value) of correlation between sequence similarity is provided. (B) Homology-based analysis resolved the four genes of interest into three orthogroups. For two out of three orthogroups, *B. terrestris* had multiple gene copies (designated by the number inside each box, as well as change in colour) while for each orthogroup, the presence of homologues was largely restricted to other members of the Apidae (*Apis mellifera*, *Eufriesea mexicana*, and *Habropoda laboriosa*), with the exception of *Ceratina calcarata* and missing in members of other bee families (Megachilidae: *Megachile rotundata*, *Osmia bicornis*, *Osmia lignaria*) and Halictidae (*Dufourea novaeangliae*, *Megalopta genalis*, *Nomia melanderi*).

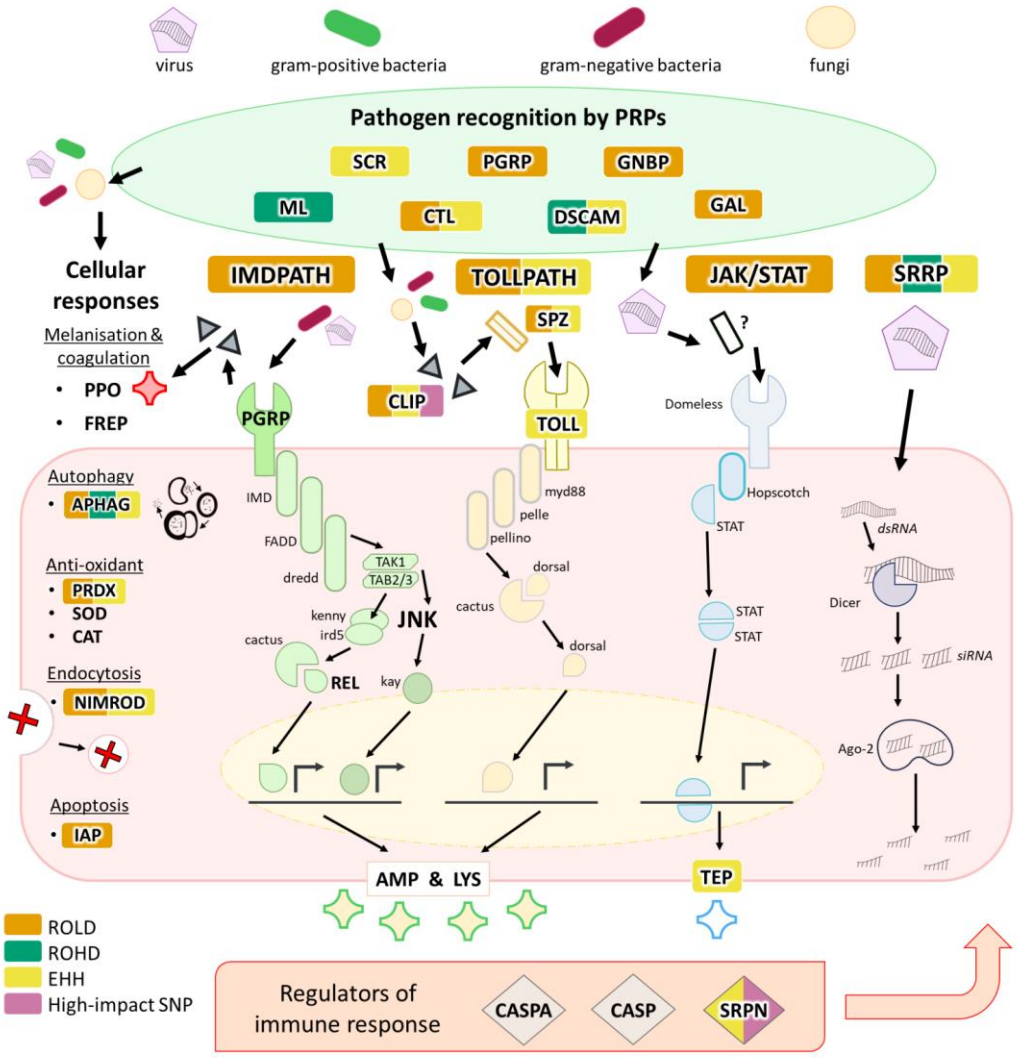


Figure 3.3 Signatures of selection acting on canonical immune genes and associated immune pathways of *Bombus terrestris*. Overview of genes and associated pathways of the bumblebee immune system with genes coloured to reflect whether they display one or more of the following characteristics: found in regions of low genetic diversity (ROLD; orange); found in regions of high genetic diversity (ROHD, green); evidence of extended haplotype homozygosity (EHH; yellow); or contain a predicted high-impact SNP (purple). Diagram displays the IMD/JNK, Toll, JAK/STAT, SRRP pathways and cellular responses of the bumblebee immune response including all functional immune gene groups outlined by Sadd *et al. (*2015): AMP = Antimicrobial peptides; APHAG = Autophagy; CASP = Caspase; CASPA = Caspase A; CAT = Catalase; CLIP = CLIP serine protease; CTL = C-type lectin; FREP = Fibrinogen-like; GAL = Galectin; GNBP = Gram-negative binding protein/Beta-glucan recognition protein; IAP = IAP repeat; IGG = Immunoglobulin, IMDPATH = Imd pathway; JAKSTAT = JAK/STAT pathway; LYS = Lysozyme; ML = MD-2-related lipid recognition; NIMROD = Nimrod; PGRP = Peptidoglycan recognition protein; PPO = Prophenoloxidase; PRDX = Peroxidase; REL = Relish; SCR = Scavenger; SOD = Superoxide dismutase; SPZ = Spätzle; SRPN = Serine protease inhibitor; SRRP = Small RNA regulatory pathway; TEP = Thioester-containing protein; TOLL = Toll genes; TOLLPATH = Toll pathway.

We found evidence of 74 putative duplicated regions (Table A3.5) across the wild-collected bumblebees based on read alignment information and elevated read depth (three standard deviations from genome-wide median), including evidence of a putative duplicated exon in the detoxification enzyme *catalase* (LOC100651198), which was found in half of sampled males (n = 17) and may impact function.

3.4 Discussion

Insect pollinators, such as bumblebees, face increasingly challenging environments, with pathogens among the key proposed contributors to their documented decline. Despite this, we know comparatively little about the types of recent selective forces specifically shaping the immune system in bumblebees. To address this, we used population genomics to identify patterns of selection acting on canonical immune genes in a natural population of the key bumblebee pollinator, *B. terrestris*. The population of *B. terrestris* sequenced in this study had similar genomic nucleotide diversity to a British population of *B. terrestris* (Colgan *et al.*, 2022). We found that within and across functional categories of immune genes, different selective pressures are acting, shaping the diversity of roles and activities that such genes play in the immune response. We found evidence of adaptation with strong signatures of recent selection acting on pathogen recognition receptors, essential for the detection of diverse pathogenic threats, the coordination of the immune response, as well as distinguishing self from non-self. We also found evidence of putative evolutionary constraints acting on bumblebee immunity with certain immune genes lacking polymorphic sites, suggesting that such genes are evolving under strong purifying selection. Lastly, we find potential redundancy or non-essential components of the bumblebee immune gene repertoire through identification of putative loss of function SNPs in the genomes of wild-caught haploid males. Our study provides novel insight into the evolution of the genes underlying the hymenopteran immune defence and the capacity and putative constraints of bumblebees to respond to ongoing and emerging pathogenic threats.

3.4.1 Signatures of positive selection acting on genes involved in pathogen recognition and viral defence

The insect immune system represents the final obstacle to the establishment of pathogen infection and disease onset. Bumblebees, like other insects, face a taxonomically diverse range of pathogenic threats ranging from viruses to macroparasites (Schmid-Hempel, 1998) which likely require different components of the immune system for detection and generation of appropriate responses. The co-evolutionary nature of host-parasite interactions means that both parties can exert selective pressures on each other, which can result in rapid changes at the genomic level (Roux *et al.*, 2014; Shultz and Sackton, 2019). In the present study, we found strong signatures of recent positive selection acting on members of the scavenger receptor proteins (SCRs), a family of cell surface proteins that bind to a variety of ligands, such as pathogen associated molecular patterns, and play a fundamental role in pathogen recognition (Krieger, 1997). The SCR family consists of 17 genes in the *B. terrestris* genome (Barribeau *et al.*, 2015; Sadd *et al.*, 2015). SCR family members have been shown to have elevated gene expression in response to infection by common parasites, such as the trypanosome, *Crithidia bombi* (Riddell *et al.*, 2014) and the nematode *Sphaerularia bombi* (Colgan *et al.*, 2020). Evidence of recent adaptive selection acting on SCRs has been described in other insects, such as the fruit fly *D. melanogaster* (Lazzaro, 2005), suggestive of the important role of SCR genes in innate immunity and highlighting them as potentially conserved targets of positive selection across taxonomically diverse insect groups. The exact role of SCRs in bees remains largely unknown, although studies in *A. mellifera* show increased expression of a SCR protein in bees with a higher resistance to deformed wing virus (DWV) when compared to individuals susceptible to the virus (Weaver *et al.*, 2021). The importance of recognition proteins is further highlighted by the identification of genes coding for ligands and receptors associated with the Toll signalling pathway also with signatures of recent positive selection (Figure 3.3). Although we may expect genes involved in intracellular signalling to evolve under purifying selection in order to maintain function, selection acting on genes coding for receptors of the Toll signalling cascade may allow for adaptation to novel or re-emergent threats. Consequently, initiators and ligands of the Toll pathway, such as CLIP serine proteases and spätzle proteins, have been previously reported to have accelerated rates of evolution at the macroevolutionary scale across *Apis* and *Bombus* species (Barribeau *et al.*, 2015) as

well as in *Drosophila* species (Jiggins and Kim, 2007; Levin and Malik, 2017; Wang *et al.*, 2022), highlighting the importance of such genes to contribute to the immune response. Our results support previous findings of positive selection acting on initiators and receptors of these pathways suggestive that such genes may help bumblebees cope with pathogens in their natural landscape.

A particular group of pathogens that have received attention of late are viruses, which can cause extensive morbidity and increased mortality within at-risk pollinator populations (Manley *et al.*, 2015). Similarly, viruses have raised concerns from an ecological perspective given that shared resources between commercial and wild pollinators can lead to increased prevalence and exposure in wild populations (Fürst *et al.*, 2014). Within regions of potential hard sweeps, we find genes involved in antiviral defence, such as three ATP-dependent RNA helicases and Dcr-1, as well as enrichment of the GO term 'helicase activity' suggestive of strong selection acting on these genes. While our analysis did not involve a screen for viruses, accelerated rates of evolution have been described for classic antiviral RNAi pathway genes (Dcr2, R2D2 and Ago2) in wild populations of several fruit fly species (Obbard *et al.*, 2006). However, given the essential functions such genes play in cellular defence, RNA metabolism and degradation, purifying selection may also contribute to the low nucleotide diversity found in these genes (Jin and Xie, 2007; Xing *et al.*, 2019). Roles in bee immunity have been investigated at the molecular level for RNA helicase Dbp2 and Dcr-1, which change in expression in response to viral and other parasitic infections in *A. mellifera* (Huang *et al.*, 2016; Pizzorno *et al.*, 2021). Our data also shows the likely importance of several types of recognition genes in *B. terrestris* and that they may be facing selective pressures from pathogens in their environment.

In comparison to regions of low diversity, we found less canonical immune genes in regions of significantly elevated nucleotide diversity. The relatively low number detected (n=5; Figures 3.1, 3.3) in such regions is surprising as many immune genes are predicted to have evolved under balancing selection, whereby numerous alleles are maintained at higher-than-expected frequencies in a population and can occur under different scenarios, such as heterozygote advantage and negative frequency selection (Unckless and Lazzaro, 2016; Chapman *et al.*, 2019). However, balancing selection can be difficult to detect and distinguish from other types of selection that produce

similar genomic patterns e.g., incomplete sweeps caused by positive selection (Fijarczyk and Babik, 2015). Similarly, some immune genes also overlap with previously documented crossing over events in *B. terrestris* (Liu *et al.* 2017), suggesting that recombination also contributes or may be the primary cause of the elevated genetic diversity that we observe. While we detected signatures of recent positive selection acting on pathogen recognition genes, such genes are also often highly polymorphic as they are under selection to detect existing, remerging and novel variants of co-evolving pathogens (Morlais *et al.*, 2004; Sommer, 2005; Early *et al.*, 2017). Similarly, not only do recognition receptors need to differentiate between self and non-self to prevent attacks on an individual's own tissue, they also need to identify beneficial or symbiotic microbes from harmful ones (Pan *et al.*, 2018). Similarly, we find certain autophagy genes in these regions, which may also be polymorphic to allow for the targeting of diverse and foreign compounds intended for degradation (Ying and Feng, 2019). Future studies on determining the extent of balancing selection acting on immune genes will benefit from intersexual (haploid vs. diploid), cross-population and cross-species comparisons to identify conserved regions of elevated diversity, such as trans-species polymorphism, consistent with genomic signatures of long-standing balancing selection.

3.4.2 Evolutionary constraints on immune gene evolution

Comparative genomic studies on genes of the immune system have routinely identified such genes to evolve under positive selection to respond to evolutionary changes in co-evolving pathogens. While in the present study, we identify genes with signatures of positive selection, suggestive of recent adaptive responses, immune genes may also face other evolutionary pressures that place constraints on the adaptation of functional diversity. Purifying selection is a powerful force that acts to preserve gene function through the removal of deleterious alleles from a population (Nielsen *et al.*, 2005). This form of selection acts particularly strongly on bumblebee males, given their haploid nature. In our analysis, we find several canonical immune genes that lack genetic variation and may be the product of purifying selection. This is supported by our finding of a number of these genes exhibiting signatures of episodic purifying selection (Table A3.6). Our sampling of wild-caught adult males across the island of Ireland suggests that such low diversity in our contemporary

population does not hinder development or survival in the natural landscape. Such low diversity may also be the result of low recombination, which is particularly true for two genes, *FAS-associated factor 1* and *ubiquitin-like-conjugating enzyme ATG3*, found on chromosome one, which were previously reported by Colgan *et al.* (2022) as located in an extended region of extremely low genetic diversity. Colgan *et al.* (2022) found that this region of low genetic diversity was also found in *A. mellifera* and *B. impatiens*, suggestive of a long-standing process enforcing low nucleotide diversity in this region across social bees. Recombination rates across genomes are often not uniform (Kong *et al.*, 2002) and can be influenced by genome architecture, environmental conditions, and selective forces (Chinnici, 1971; Shaw, 1972; Stapley *et al.*, 2017; Winbush and Singh, 2021). The location of genes in these potential 'coldspot' regions or of regions with low recombination rates may place evolutionary constraints on the ability of these genes to respond to ongoing environmental challenges highlighting potential limitations on the ability of immune components to adapt to future pathogenic threats.

3.4.3 What is driving selection in Bombus canonical immune genes?

Given the strong selective pressures placed by pathogens on host immune systems, recent selection signatures in many of these canonical immune genes may be in response to increasing pathogen threats and their detrimental effects on bumblebee condition and fitness (Otti and Schmid-Hempel, 2007; Yourth *et al.*, 2008; Colgan *et al.*, 2020). Indeed, these threats are becoming increasingly serious and, as mentioned previously, considered a key factor contributing to pollinator decline (Goulson, 2010b). In particular, the increasing use of commercial pollinators has been shown to cause pathogen spillover to wild populations which may have been previously naive to particular pathogens and/or specific strains (Colla *et al.*, 2006; Otterstatter and Thomson, 2008). While adaptation to pathogens may be the most parsimonious reason for our identified patterns, such selection signatures may also be the result of other processes or targets of selection. Many canonical immune genes have pleiotropic effects with their products being expressed at different points in the insect life-cycle, contributing to essential roles in development, reproduction and metabolism such as the Toll-like receptors, which play central roles in both embryonic pattern formation and immunity (Zou *et al.*, 2006; Valanne *et al.*, 2011). Such genes may therefore be

selected due to a function unrelated to immunity. Similarly, immune genes may not be the direct targets of selection but hitchhike through physical linkage with neighbouring sites under selection (Nielsen *et al.*, 2005). For several canonical immune genes, we find strong signatures of extended haplotype homozygosity, which is predicted to break down with physical distance from the target of selection, especially within bees, which have high recombination rates (Beye *et al.*, 2006) suggesting that these genes are at least located in regions exposed to recent selection. Similarly, given the documented role of many selected genes in immune function (e.g., SCRs, Zhang *et al.*, 2021) we may predict that such genes are evolving in response to pathogen-driven selection but further experimental work would be required to determine this link.

3.4.4 Potential redundancy in certain immune genes of a wild-caught B. terrestris population

Despite the importance of the immune system, studies in other taxa have suggested that certain components of the immune system may be non-essential and evolve under relaxed selection (Nish and Medzhitov, 2011). In our study, we found potential loss of function SNPs in three CLIP serine proteases and one serine protease inhibitor (serpin) present in the genomes of wild-caught males. Similar to canonical immune genes that lack polymorphism, the presence of loss of function alleles in wild-caught adult haploids would suggest that these alleles may not negatively impact development or survival in carriers. CLIP serine proteases perform diverse immune roles, including the triggering of the immune pathways, such as the Toll pathway and the prophenoloxidase cascade (Kanost and Gorman, 2008; An *et al.*, 2013) while in contrast, serpins act as regulators of such protease cascades (Shakeel *et al.*, 2019). While bees, in general, are documented to have a depleted canonical immune gene set compared to other insects (Evans *et al.*, 2006), *B. terrestris* has an expanded gene set of both serine proteases and serpins compared to *A. mellifera* with many such genes evolving through tandem gene duplications (Barribeau *et al.*, 2015). Duplication events are common but are usually quickly purged from the genome. However, retained copies may also diverge through adaptive or neutral processes (Hastings *et al.*, 2009). In the latter scenario, random accumulation of mutations through relaxed selection may occur, disrupting function, and potentially leading to pseudogenisation. While we found no evidence of copy number variation for such genes in our

population dataset nor did we specifically test for signatures of relaxed episodic selection, we do find evidence of greater divergence in terms of predicted protein sequence similarity for such genes compared to other immune genes shared with *A. mellifera* (Figure 3.2). This result in combination with the presence of putative loss of function alleles, suggest that the additional copies may be evolving under relaxed selection, resulting in the genes becoming functionally divergent and potentially non-essential from an immune perspective, which may explain why purifying selection has not removed these potential loss of function SNPs from our wild-caught population. However, functional studies on this population of bumblebees are needed to evaluate if and how potential loss of function SNPs impact protein function and to what extent. Furthermore, it should be explored whether possible loss of function SNPs in immune genes have divergent consequences on fitness across bumblebee sexes and castes, especially considering observed differences in immunity between males and females (Baer and Schmid-Hempel, 2006).

In the current analysis, we investigated genomic patterns of selection through nucleotide diversity and extended haplotype homozygosity, which can provide insight into targets of recent positive and purifying selection. The use of haploid males for population genomic analyses, which has been used in previous studies (Colgan *et al.*, 2022), likely provides higher resolution in patterns of selection due to lack of requirement for phasing. However, to fully understand how much genetic variation is maintained in bumblebee populations, and how selection is shaping their genome, we should also examine variation in females, which given their diploid nature will provide additional insights into the genetic variation found within individuals in wild populations. Similarly, while bees have been characterised as having a depauperate canonical immune gene set (Evans *et al.* 2006; Barribeau *et al.* 2015), more recent studies suggest additional genes function in the hymenopteran immune system (e.g., Doublet *et al.*, 2017; Möllmann and Colgan, 2021); such genes were not examined in our present study given our restriction to the canonical immune gene set and associated gene groups detailed by Sadd *et al.* (2015). Furthermore, our analysis was restricted to canonical immune genes located on linkage groups, representative of chromosomes, accounting for approximately 90% of the original genes described by Sadd *et al.* (2015). The generation of new bumblebee assemblies, such as those generated by the Darwin Tree of Life (Darwin Tree of Life Project Consortium, 2022) or European

Reference Genome Atlas (Formenti *et al.*, 2022), may hopefully allow for the assessment of the entire expanded canonical immune gene set in future studies.

3.4.5 Conclusion

Advances in population genomics approaches are providing us with valuable new insights into many aspects of population biology and evolution, including the adaptive abilities of organisms to respond to their natural environments. Genomics is a powerful tool that can assess huge amounts of genetic markers and yields the resolution required to understand how the fundamental process of natural selection is working in wild populations of ecologically important species. Evaluating how various kinds of selective pressures are acting in a population will help biologists decipher evolutionary history, local adaptation, and resilience to threats, such as climate change and habitat fragmentation, of important keystone species. In this study, we used population genomics to investigate how evolution is shaping the immune system of a key bumblebee species. Bumblebees, like many other insect species, are experiencing dramatic declines across Europe and North America driven by intensification of agriculture, habitat loss, use of pesticides, climate change and pathogen spread. As we know, the latter threat can place the immune systems of their hosts under strong selective pressures. However, we know little about the kinds of selection wild pollinator host immune systems are experiencing. Considering this, we aimed to shed light on the selective processes acting on canonical immune genes in a wild-caught population of the earth bumblebee, *B. terrestris* and expect that our findings will develop our understanding of pollinator-pathogen dynamics, an essential feat if we are to move towards saving these vital species from potentially one of the key causes of their decline.

Overall, we found that canonical genes of a wild-caught bumblebee immune system are experiencing different kinds of selection pressures, which reflects the functional complexity of these important physiological systems and indicates how certain genes may be responding to changing environmental pressures. We found evidence that certain elements of the bumblebee immune genome are undergoing adaptive change that may increase this population's ability to defend against the parasites and pathogens they are exposed to. Furthermore, we found other canonical immune genes

that appear to be under constrained evolution, presumably reflecting their roles in essential cellular, developmental, and physiological processes, and, additionally, genes that may be redundant in bumblebee immunity or otherwise. To conclude, we show the potential of population genomics to inform us on the adaptive abilities of a species in a natural environment, which may prove a highly valuable tool to identify pollinator populations vulnerable or robust to increasing pathogenic threats. We anticipate our findings will also motivate future characterisations of wild bumblebee immune systems as well as assessments of the immunogenetic variation found in wild bumblebees and other insect pollinators. Additionally, we believe these population genomics approaches could be used in the future to evaluate the genomes of wild and commercially imported *B. terrestris* bumblebees and their capabilities to deal with immune threats. This information would inform changes to the management of and policy surrounding bumblebee imports. Overall, this study helps cultivate a deeper understanding of the dynamics between bumblebees and their pathogens, an area that requires thorough investigation if we are to fully comprehend, and potentially mitigate, the negative impacts pathogen spread is thought to be having on wild pollinating insect populations.

Chapter 4

A comparative analysis of wild and commercial *B. terrestris* brain and fat body proteomes and their proteomic responses to acute clothianidin exposure and *E. coli* infection

4.1 Introduction

Evolution can impact the molecular responses and the resilience of organisms to the stressors they face in their environment. These changes are most often described in wild organisms that adapt to their native environments and conditions therein. However, domestication is another process that can drive rapid evolutionary changes in organisms (Jensen, 2006). Domestication is often observed to create changes in behavioural traits and morphology of species, creating a "domestication syndrome". Common traits observed in domesticated populations, which are predominantly mammalian, include docility, floppy ears, changes in colouration, changes to teeth and more frequent oestrous cycles (Larson and Fuller, 2014; Raghanti, 2019). As morphology and behaviour are often altered through domestication, it is no wonder we also find evidence that domestication can drive change in molecular processes and gene expression. Many studies have identified clear molecular changes in domesticated populations when compared with wild counterparts. For example, wild and domesticated sea bass (*Dicentrarchus labrax*) eggs show distinctions in several protein components, including the presence of a nucleoside diphosphate kinase in the domesticated variety that correlated positively with egg quality (Crespel *et al.*, 2008). Research into domestication varieties of cotton have also found key shifts in protein expression (Hu *et al.*, 2013), as well as the evolution of novel molecular pathways to regulate reactive oxygen species levels in domesticated varieties that may have assisted with the development of the domestic trait of longer spinnable fibres (Hovav *et al.*, 2008). The domestication of the silkworm *Bombyx mori* resulted in a change in the amino acid sequence of the SPI51 gene, a protease inhibitor, that increased antifungal activity (Zhang *et al.*, 2021).

Bees, such as honeybees and bumblebees, have also been subjected to domestication processes. Commercial facilities rear bumblebee colonies in captivity and export them around the world for pollination services (Velthuis and Van Doorn, 2006). However, as highlighted by Lecocq (2018), research into potential differences between wild and domesticated populations of bumblebees and characterisations of possible domestic traits in captive-bred bumblebee populations are still lacking. While truly wild populations of honeybees are thought to be virtually non-existent (Lecocq, 2018; but see Hassett *et al.,* 2018), the wild populations of all commercially-reared bumblebee species still exist in their natural distributions (Velthuis and Van Doorn, 2006).

Commercially reared colonies are imported to areas within and outside their natural distributions and, while they provide beneficia pollination services to crops (e.g., Lye *et al.,* 2011), they can also have potentially harmful impacts on wild pollinators.

When used with no restrictions to escape, commercial bumblebees can compete with wild insect pollinators for foraging resources and, if new queens escape into surrounding environments, nesting sites (Goulson, 2003; Ings *et al.,* 2006). It has also been widely documented that commercial bumblebees can spread pathogens to many species of wild bee (Colla *et al.,* 2006; Graystock *et al.,* 2013; Murray *et al.,* 2013). Finally, hybridisation with wild conspecifics may threaten the adaptive traits of wild populations of *B. terrestris* (Cejas *et al.,* 2020). For example, some domesticated insect species have lost many wild-adapted traits. *Bombyx mori* is an extreme example of this as it has lost the ability to fly (Dai *et al.*, 2019). Reintroductions of domesticated species within the distribution of their wild counterparts could therefore spread maladaptive alleles to wild populations through introgressive hybridisation (see Barilani *et al.,* 2007).

Bumblebees are becoming important model species for research into social insect immunity (Evans *et al.,* 2006) and insect neurobiology (Rother *et al.,* 2021). However, much research evaluating bumblebee physiological processes and responses to stressors such as infection or pesticide exposure is being carried out on research colonies sourced from commercial suppliers, due to their quality and accessibility (e.g., Riddell *et al.*, 2014; Botías *et al.*, 2021; Costa *et al.*, 2022). If these samples are exhibiting altered characteristics due to artificial or unconscious selection, then caution must be taken when extrapolating results of research using commercial bumblebees to wild bumblebee populations that are still evolving under natural environmental conditions. This is especially important considering the conservation interest in wild bees and bumblebees experiencing declines across Europe and North America as a result of pesticide use, habitat destruction and pathogen spread (Goulson *et al.,* 2008; Grixti *et al.,* 2009; Potts *et al.,* 2010; Arbetman *et al.,* 2017).

Through recent whole genome sequencing of the buff-tailed bumblebee *Bombus terrestris* (Sadd *et al.,* 2015), we are now provided with the foundations necessary to carry out high-throughput analysis of protein expression spatially and temporally in this species. Proteomics, the study of the full complement of proteins associated with

a specific biological component, provides the opportunity to gain deep insight into expressional changes associated with domesticated species. Just as the advancement of genomics has revolutionised research into evolution and population biology of many non-model organisms, so too has mass-spectrometry-based proteomics revolutionised our approaches to characterising and understanding essential biological processes of virtually any system (Cox and Mann, 2007; Karr, 2008). While genomics is restricted to analysing patterns and signatures within the molecular sequences of DNA, proteomics evaluates the functional culmination of gene expression i.e. the full protein complement of a cell type, tissue, organ or organism (Karr, 2008). Proteomics can also identify post-translational modifications of these important gene products (Cox and Mann, 2007). Proteomics has now been used to investigate protein composition in various bumblebee tissues and fluids e.g., haemolymph (Colgan *et al.*, 2019), digestive tracts (Cullen *et al.*, 2023) and venom (Van Vaerenbergh *et al.*, 2015). These studies have provided novel information on physiological processes, responses to stressors and adaptations in these insects. These advantages of proteomic approaches may enable a deeper understanding of the expressional differences between wild and domesticated bumblebee populations.

Given the potential of proteomics to shed light on key physiological processes in these important pollinators, we wanted to investigate differences in the proteomes between wild and domesticated *B. terrestris* workers. In Chapter 2, we show genetic distinctions between wild, Irish, and commercial *B. terrestris audax* in genes linked to the nervous system and neurodevelopment alongside elevated distinctions in commercial bumblebee genomes in genes linked to the TCA cycle and lipid homeostasis. Furthermore, as shown in Chapter 3, the immune systems of wild bumblebees are under a variety of selection pressures and exhibiting signatures of adaptation in canonical immune genes, such as scavenger receptor genes. For these reasons, it was decided to investigate the proteomes of two key organs in wild and commercial bumblebee workers, reared in the same conditions, to identify whether these bees exhibit functional as well as genetic distinctions. The selected organs, the brain and fat body, will allow insight into two essential and topical physiological systems i.e. the nervous and the innate immune system. Bees, like all insects, have a central nervous system that is made up of a brain and a ventral nerve cord (Klowden, 2013). The brain is involved in a number of highly important processes key to bee

fitness, such as sensory perception of olfactory and visual patterns, control of flight, coordination of limbs, memory and learning (Klowden, 2013). The fat body is an organ exclusive to insects that performs a dynamic set of functions involving energy storage and the utilisation of these resources. It plays a central role in intermediary metabolic processes, and its organisation as loose tissue maximises its contact with the surrounding haemolymph with which is supplies with metabolites and haemolymph specific proteins (Hoshizaki, 1998; Arrese and Soulages, 2010). The fat body is also an endocrine organ, generates key immune molecules such as antimicrobial peptides (AMPs) and carries out detoxification functions (Charroux and Royet, 2010; Li *et al.,* 2019).

We also wanted to investigate wild and commercial bumblebee responses to threats such as pesticide exposure and pathogen infection. Clothianidin is a neonicotinoid insecticide developed to have high toxicity in Hemiptera, Thysanoptera and many other insect pests. It works by binding to insect nicotinic receptors, ion-channels in the insect central nervous system, with strong affinity (Uneme, 2011). However, in spite of pollinating insects not being a conscious target of these pesticides, they can be impacted by these when they are used on crops they forage on or when residues remain in their environment (Di Prisco *et al.,* 2013). Often the effects of neonicotinoid exposure to bees are sub-lethal, meaning that while there may be no instances of direct mortality as a result of pesticide exposure, there may be impacts on functions that are important to colony fitness and success (Gogoi *et al.,* 1997; Franklin *et al.,* 2004; Baron *et al.,* 2017; Banks *et al.,* 2020; Tison *et al.,* 2020). As insecticides have been associated with pollinator declines and are a threat that wild bees may be adapting to cope with, we decided to compare the proteomic responses of the brain between wild and commercial bumblebee workers to acute clothianidin exposure.

Although the Gram-negative bacteria *Escherichia coli* is not a natural pathogen of bumblebees, we chose to infect wild and commercial workers with this bacteria to illicit a 'naïve' immune response in these bees, rather than comparing differences in innate immune responses that have been shaped by specific pathogens through coevolutionary pressures (Tellier *et al.,* 2014). We also chose to use a non-pathogenic strain for the infections as non-pathogenic strains of *E. coli* can induce a general immune reaction (including signalling, effector and regulation pathways) in insects (Eleftherianos *et al.,* 2006; Freitak *et al.,* 2007; Larkin, 2018). Specialised pathogenic

invaders can also block host immune responses (Eleftherianos *et al.,* 2007; Eleftherianos *et al.,* 2009; Castillo *at al.,* 2011). Therefore, comparing responses of wild and commercial lines of worker fat bodies to this type of infection may shed light on fundamental distinctions in general immune responses to an infection that most insects, bar the tsetse fly (*Glossina morsitans*) and the pea aphid (*Acyrthosiphon pisum*), are able to efficiently clear (McKean and Nunney, 2001; Grenier *et al.,* 2006; Haine *et al.,* 2008; Weiss *et al.,* 2008; Mukherjee *et al.,* 2010).

In this experiment, we aimed to use label-free mass spectrometry to:

- Investigate proteomic differences between (a) the brains, and (b) the fat bodies of wild and commercial lines of *B. t. audax* bumblebee workers, and
- Assess distinctions between wild and commercial bumblebees in proteomic responses to (a) an acute exposure to a field-relevant dose of clothianidin, and (b) an *E. coli* infection.

Evaluating changes caused by domestication in key pollinating bee species is essential for numerous reasons. First, we can understand how domestication changes key physiological systems and responses in bumblebees as a result of artificial or unconscious selection, as well as release of natural selective pressures. Second, it is crucial that we understand the differences between wild and domesticated species to assess threats posed by re-introduced or even feral populations of domesticated varieties to wild pollinator species.

4.2 Methods

Unless where stated otherwise, all chemicals used in the experiments outlined in this chapter were of analytical, molecular, and proteomic grade and obtained from Sigma-Aldrich Co. Ltd. (Arklow, Ireland).

4.2.1 Bumblebee rearing and worker bee collection

Bumblebee workers for the following proteomic experiments were sourced from lab-reared colonies of wild Irish and commercial lines *B. terrestris*. For details on how these colonies were reared, see section 5.2.

4.2.2 Pesticide exposures

(a) Pesticide and control solution preparation

A 500ppm stock of Clothianidin (stock 1; Fluka Analytical) was prepared by combining 5mg of Clothianidin to 10ml acetone. From this stock, a 1 ppm stock (stock 2) was created by diluting by a factor of 500, taking 20µl of stock 1 and combining with 9980µl of acetone. From stock 2, a 1/ 100 dilution was carried out but with 9900µl of 50% (w/v) sucrose and 100µl of stock 2 to form a 10ppb solution of clothianidin with a <1% concentration of acetone (the max concentration as outlined by OECD guidelines (OECD, 2017). The procedural control was prepared with 0ng/ml of clothianidin and with <1% acetone using 50% (w/v) sucrose.

(b) Pesticide exposures

Commercial and wild lab-reared worker bees aged 13-18 days old and with unique identifying tags were placed into separate boxes for control and treatment, as shown in Figure 4.1, and provided with 50% (w/v) sucrose (BioXtra >99.5%, Sigma Aldrich) feeders. Bees were allowed to acclimatise in the dark at 23°C (+/- 1°C) and 70% R.H. (+/- 10%) for approximately 24hrs.

Sucrose feeders were removed from boxes the following day, one hour prior to clothianidin exposures to starve bees. After this starvation period, each bee was placed into a single plastic chamber and a droplet of either 35µl of control solution or 35µl of 10ppb clothianidin solution (a clothianidin dose of 0.35 ng) were pipetted onto the base of the container. Each bee was observed closely during this period and the time at which the bee completely drank the provided solution was recorded. After drinking the solution, the bee was replaced into its original housing and provided with 50% (w/v) sucrose. Once all bees had been exposed and had been transferred back to their original boxes, they were monitored periodically (15 mins, 30 mins, 45 mins, 1 hour

and 20-24 hours post exposure) and number of bees with symptoms such as mortality, spasms, paralysis, or no symptoms were recorded (although no bees exhibited any symptoms post-exposure). At approximately 24 hours post exposure, brain dissections from each bee took place.

(c) Brain dissection and collection for proteomic analysis

Lysis buffer (6M urea, 2M thiourea with added protease inhibitor cocktail tablet (Roche Diagnostics), pH 8.0) was mixed approximately 30 mins prior to brain dissections. An aliquot of 300µl of lysis buffer was dispensed into 1.5 ml tubes (one tube per brain sample). Tubes containing lysis buffer were placed onto ice.

Bees, aged between 15-20 days old, were removed from their treatment box one at a time and immobilised using CO_2 gas. The abdomen and legs were removed from the thorax, leaving the head and thorax attached. From the head, the antennae, tongue, and mandibles were cut off using a sterile scissors. Hair on the top and front of head was trimmed. Using a sterile blade, the exoskeleton from the ocelli to the clypeus was carefully sliced and removed from the head, exposing the surface of the brain. The exoskeleton of the eyes was then removed with the blade and sterile tweezers. Using sterile tweezers, the exposed brain was extracted from the remaining head exoskeleton and placed into a chilled 1.5ml containing 300µl of lysis buffer.

Each sample containing a brain and lysis buffer was homogenised using a motorised pestle. Then, all samples were placed into a sonication bath and sonicated 3 times for 10 secs. The samples were placed into a microcentrifuge and spun at 9000 x *g* for 5 mins. Supernatant from each sample was transferred to a new 1.5ml tube. After this, all samples were stored at -70°C until further processing could take place.

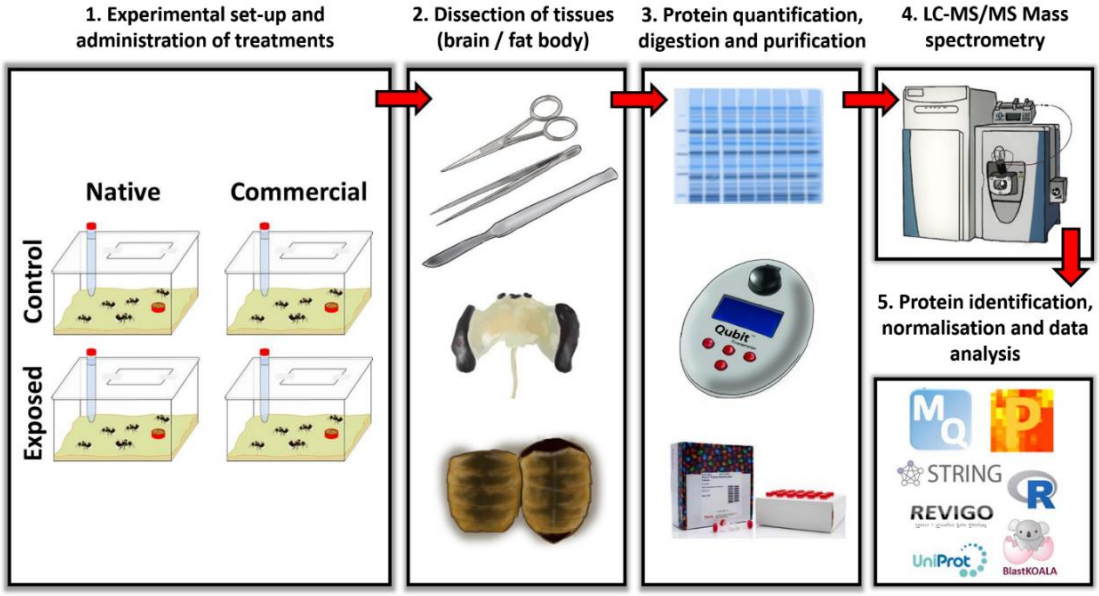


Figure 4.1 Workflow of proteomic experiments. Wild and commercial workers were set up in separate boxes according to treatments, these treatments were administered, and tissues (brain or fat body) were dissected. For each sample, protein was extracted, quantified, and assessed for quality before being digested and purified. Samples were run on LC-MS/MS Mass spectrometry. Data produced by mass spectrometry was then handled and analysed by several programmes.

4.2.3 E. coli infections

(a) E. coli prep

The use of live microorganisms is preferred over the use of pathogen associated molecular markers (PAMPs), e.g., peptidoglycan, for studying insect immune responses as live infection produces a higher immune response than PAMP infection (Charles and Killian, 2015). A non-pathogenic strain of *Escherichia coli* (K12) was aseptically spread on nutrient agar and maintained. A sterile loop was used to inoculate a nutrient broth with a single *E. coli* colony. The culture was then placed in a shaker (200rpm) and grown overnight at 37°C until stationary phase was reached. After incubation, aliquots of *E. coli* cells taken from the prepared liquid culture and were placed in a centrifuge. Cells were spun at 2,000 x *g* for 1 minute. The supernatant was discarded, and the pellet was resuspended in sterile Ringer's solution (NaCl, KCl, $CaCl_2$, $NaHCO_3$, Merck). This centrifugation and wash step was repeated two more times. At this point, a spectrophotometer (Eppendorf BioPhotometer) was used to estimate the cell density of the cultures. The final OD600 of the culture in Ringer's

solution was adjusted to reach 1.0 (which corresponds to a concentration of ~8 x 10^8 cells/ml). Finally, cell counts were confirmed using a standard light microscope and Neubauer haemocytometer.

(b) Infection of bees

Wild and commercial worker bees tagged with unique identifiers (Thorne) aged between 13-18 days old were removed from 3 – 4 lab-reared colonies each and distributed among boxes designated for the following treatments (Figure 4.1):

- Wild control (Ringer),
- Wild *E. coli* infected,
- Commercial control (Ringer),
- Commercial *E. coli* infected.

Boxes were kept at 23 °C (+/-1°C) and 70% (+/- 10%) R.H. and workers were provided with 50% (w/v) sucrose (BioXtra >99.5%, Sigma Aldrich) feeders and one 15ml falcon tube cap of pollen (Biobest, Agralan Ltd.). Workers were allowed to acclimatise for approximately 24hrs prior to infection.

After the acclimatisation period, each bee was taken from its assigned box and anesthetised using CO_2 gas. The bee was then secured on a wax plate with tape and pins with ventral side facing up. Once secured, the abdomen was stretched using tweezers to reveal the internal membranes between sterna. A 2µl droplet of each treatment was pipetted out onto parafilm. Using a syringe (Myjector® U-40 Insulin, Terumo®), the treatment solution, either Ringer's solution (control) or *E. coli* (treatment), a dose of approximately 1.6 x 10^7 *E. coli* cells, was taken up into the tip of the syringe and injected into the worker's abdomen between the 3rd and 4th sterna, taking care not to insert the syringe into the abdomen beyond a 2mm depth. After injection of either Ringer's or *E. coli* solution, bees were returned to their original treatment-assigned housings. Fat body dissections took place 24 hours later.

(c) Fat body dissection and collection for proteomic analysis

Lysis buffer (6M urea, 2M thiourea with added protease inhibitor cocktail tablet, pH 8.0) was prepared prior to all organ dissections for proteomic analysis. The lysis buffer

was mixed for approximately 30 minutes. After this time, 300µl of the lysis buffer was added to 1.5 ml tubes (one tube per fat body sample). All tubes containing lysis buffer were placed on ice and allowed to chill before dissections began.

Bees were removed from their treatment boxes, immobilised using CO_2 and their heads and thorax were removed using scissors. The abdomen was pinned on a wax plate using insect mounting pins. Once pinned, ovaries, digestive tract, muscle, and tracheal tissue were removed and discarded from the abdomen using a dissection microscope. Using sterile dissection needles and tweezers, fat body was collected from the inside of both dorsal and ventral cuticles. Fat body cells from each bee were placed into an individual 1.5ml containing 300µl of lysis buffer.

Each fat body sample was homogenised for approximately 15 seconds using a motorised pestle and then placed into a sonication bath where samples were sonicated 3 times for 10 secs to prevent proteins in samples from getting too hot and denaturing. Sample homogenates were then centrifuged at 9000 x *g* for 5 mins in order to pellet any cellular remnants. The supernatant from each sample was retained and stored in a -70°C freezer until required.

4.2.4 Mass spectrometry preparation

(a) *2D clean-up*

Protein quantification was conducted using the Qubit Protein Assay Kit (Invitrogen) and Qubit Fluorometer (ThermoFisher Scientific). Approximately 100µg of protein was removed from each sample to a new 1.5ml tube and purified using a 2D Clean Up Kit (GE HealthCare Life Sciences) following the manufacturer's instructions. In brief, 300µl of precipitant solution was added to the sample, vortexed, incubated on ice for 15 minutes followed by the addition of 300µl of co-precipitant. The protein, precipitant and co-precipitant mix were then vortexed and centrifuged at 13,000 x *g* for 5 mins. The supernatant from each 1.5ml tube was removed without disturbing the pellet formed in the previous step. The centrifugation step was repeated on all samples after removing supernatant to gather any remaining supernatant. A pipette was used to remove any visible supernatant after this step. In each of the 1.5ml tubes, 25µl of distilled H_2O was pipetted onto the pellet. All tubes were vortexed to disperse the

pellets. After this, 1ml of wash buffer (stored at -20°C) and 5µl of wash additive were added to each tube and vortexed until the pellets in each had fully dispersed. Each sample tube with H_2O, wash buffer and wash additive were incubated at -20°C for at least 30 mins and vortexed for 30s every 10 mins.

Samples were centrifuged at 13,000 x *g* for 5 mins. The supernatant was discarded from each tube and all tubes were placed upside down on tissue paper for no longer than 5 minutes to allow pellet to air dry. 50µl of resuspension buffer (6M urea, 2M thiourea, 0.1M Tris-HCl, pH 8.0) was then added to each sample and vortex until pellet had dissolved. Each sample was placed into a sonication bath and sonicated for 10s three times. All sample tubes were then placed into the microcentrifuge for a final spin at maximum speed (at least 12,000 x *g* to pellet any insoluble materials in the tubes). From each sample tube, the supernatant was removed and placed into a fresh, labelled 1.5ml tube. 10µl each of these 2D-cleaned samples were aliquoted into separate 1.5ml tubes to be used and requantified using the QUBIT method. One-dimensional, sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDSPAGE) was performed to assess protein quality and consistency using: Ammonium persulphate, Ultrapure Protogel® (National Diagnostics), 4X Protogel® stacking buffer (National Diagnostics), 4x ProtoFLOWGel resolving buffer (Flowgen Bioscience), tetramethyl ethylenediamine (TEMED) solution (National Diagnostics) and Ultrapure 10x 0.25M tris/1.92M glycine/1% SDS buffer (National Diagnostics).

For protein digestion, 20µl of each 2D-cleaned sample was used. These aliquots were stored at -70°C until protein digestion could take place. The remaining 20µl of each sample was also kept at -70°C for long term storage.

(b) Protein digestion

To begin, 25ml of 50mM of Ammonium Bicarbonate (AMBIC) was made up in 18MΩ-cm dH_2O (purified with Millipore Milli-Q apparatus) on the same day as digest. A 0.5M Dithiothreitol (DTT) solution was made up in 500µl of the prepared 50mM AMBIC solution. A 500µl 0.55M of Iodoacetamide (IAA) solution was also made using the prepared 50mM AMBIC solution and the solution was wrapped in tinfoil to prevent exposure to light. A 100µl ProteaseMAX stock solution of 1% (w/v) was made up in 50mM AMBIC. Finally, 0.5µg/µl of trypsin was made up by resuspending a

sequencing grade trypsin vial (20µg; Promega) with 40µl of trypsin resuspension buffer (Promega). All prepared solutions were stored on ice immediately prior to the protein digestion protocol.

To 20µl of each 2D-cleaned protein sample, 105µl of the 50µl AMBIC solution was added. Afterwards, 1µl of DTT was added to each sample tube and sample tubes were incubated at 56°C for 20 mins to reduce proteins. Samples were vortexed at the start of and at 10 minutes into this incubation step. After the incubation and when samples had cooled down, 2.7µl of IAA was added to each sample and incubated at room temperature in the dark for 15 mins to alkylate proteins. After incubation, 1µl of ProteaseMax (Promega) and 1µl of 0.5µg/µl trypsin were added to each of the samples and incubated overnight at 37°C to digest sample proteins.

(c) C18 clean up and purification for MS

All digested samples were removed from the 37°C incubator and allowed to cool at room temperature for 5 – 10 minutes. During this time, stock solutions of trifluoroacetic acid (TFA) and acetonitrile (ACN) and a sample buffer (2% TFA in 20% ACN), activation solution (50% ACN, 50% ddH_2O), an equilibration solution (also used as a wash buffer; 0.5% TFA in 5% ACN) and an elution buffer (70% ACN) were made up. Pierce^{TM} C18 spin columns (ThermoFisher Scientific) were also prepared during this time by removing the caps and tapping the column and settling the resin inside. C18 columns were then placed in 1.5ml tubes. The lids of 2ml tubes were cut off for later steps to prepare these as receiver tubes required in later steps. The amount of 2ml receiver tubes prepared was determined by the number of samples multiplied by two.

1µl of TFA (100%) was added to each sample to stop the digestion process and samples were allowed to incubate at room temperature for 5 mins. Acidified samples were then placed into a microcentrifuge and spun at 13,000 x *g* for 10 mins. The supernatant of each sample was removed and placed into a fresh, labelled 1.5 ml tube and these were used in the following C18 procedure.

150µl of Sample Buffer 1 was added to each sample. To the C18 columns in the 1.5ml tubes, 250µl of the Activation Solution was added to the resin by pipetting along the walls of the column to collect any remaining displaced resin. The resulting flow-

through was pipetted back onto each column and all columns were placed in a microcentrifuge and spun at 1,500 x *g* for 1 min. The flow-through was discarded after the spin and a further 250µl of Activation Solution was added to columns before an additional spin at the same settings. This step was repeated once more for a total of 3 spins. 200µl of Equilibration Solution was then added to the columns and centrifuged at 1500 x *g* for 1min. Flow-through was discarded and this step was repeated once more. Columns were placed in the first set of 2ml receiver tubes and 170µl of each sample and Sample Buffer 1 solution was added to a prepared column. Columns were then spun at 1,500 x *g* for 1 min. Flow-through from each column was pipetted back onto the column itself and spun at the same settings again. This was repeated once more for a total of 3 spins to ensure sample had completely binding to the resin.

Columns were transferred to the second set of 2ml receiver tubes. 200µl of Wash buffer was added to each column. Columns were placed into a microcentrifuge and spun at 1,500x g for 1 min. Flow-through was discarded. A further addition of 200µl of Wash buffer was added to each column and all columns were spun again at 1,500 x *g* for 1 min. This step was repeated once more for a total of three spins. Columns were then placed into fresh, labelled 1.5ml tubes. 30µl of Elution buffer was added directly onto the resin in each column. Columns were reinserted into the microcentrifuge and spun at 1500 x *g* for 1 min. This step was repeated twice more for a total eluant volume of 90µl. Columns were discarded and the eluted samples in 1.5ml tubes were placed into a SpeedyVac concentrator (ThermoFisher Scientific Savant DNA 120) and spun at medium heat until dry (about 1.5 hrs). Dried samples were stored at 4°C until they could be run on mass spec.

Once samples were ready to be ran on the mass spectrometer, the volume to add to each dried sample to have an approximate protein concentration of 0.5µg/µl was calculated for each sample. The calculated volume of QE buffer (2% (w/v) acetonitrile and 0.05% (v/v) TFA) was added to each sample. Samples were then placed into a sonication bath and were sonicated for 5 mins at 16,000 x *g* to resuspend proteins. The supernatant of each sample was then removed and pipetted into a fresh, labelled 1.5ml tube.

4.2.5 Mass spectrometry

From each prepared sample, 1µg was loaded onto a QExactive (ThermoFisher Scientific) high resolution mass spectrometer which was connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system to perform mass spectrometry analysis. A gradient of 2 – 40% acetonitrile was used to separate peptides on a Biobasic C18 Picofrit column (100mm length, 75mm ID). For both fat body and brain samples, a 120 min reverse-phase gradient was used with a flow-rate of 250nL uant 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 *Bombus terrestris* genome (Sadd *et al.* 2015) obtained from the National Centre for Biotechnology Information (NCBI) repository (17,508 entries, downloaded September 2018).

4.2.6 Protein annotation and statistical analysis

Perseus v.1.6.1.1 was used to perform data processing, visualisation, and statistical analyses of all proteins. The quantitative measurement of proteins used in these analyses were the normalised LFQ intensity values. Rows of data of the inputted matrix were filtered to remove peptide entries only identified by site, reverse hit peptides and potential contaminants. Empty columns were also removed and the values in the resulting matrix were transformed by $\log_2(x)$. Rows were annotated by their treatment group i.e., commercial, wild, exposed, control. Rows were filtered again to keep those with valid values in a minimum of four in each group (n=4). Using an imputation, missing values were replaced with values from a normal distribution. A PCA was performed on the matrix to visually assess degrees of variation between each treatment group. Two samples t-tests were carried out between appropriate treatment groups to identify statistically significant differentially abundant proteins (SSDA; $p < 0.05$).

For both experiments, the following comparisons were performed using t-tests to assess the effect of the treatment:

(1) wild exposed/infected – wild control,
(2) commercial exposed/infected – commercial control.

Similarly, to compare wild and commercial worker organ proteomes in both exposed and control states to identify general distinctions between wild and commercial groups and distinctions in their responses to the treatments administered, we compared the following using t-tests:

(1) wild exposed/infected – commercial exposed/infected,
(2) wild control – commercial control.

Volcano plots were generated for each of these t-tests in Perseus plotting negative log p-values (y-axis) against log_2 fold-change values (x-axis). A hierarchical clustering analyses was performed on the mean Z-score normalised intensity values of SSDA proteins in each treatment group using Euclidean distance.

To assess SSDA proteins in each comparison and those highlighted by hierarchical clustering for processes represented among them, several analyses were carried out. The Search Tool for the Retrieval of INteracting Genes/Proteins (STRING; v11.5; Szklarczyk *et al.*, 2019) was used first to map SSDA proteins and proteins within clusters. All SSDA proteins were searched using the corresponding *A. mellifera* Uniprot codes. The search was run at medium confidence of 0.4 and a protein network was created for each SSDA protein group in each comparison. STRING provided significant enrichment terms for each protein group. Non-redundant enrichment terms were extracted manually by examining enrichment terms and overlapping proteins. Enrichment terms that had more than 1000 proteins associated with them usually indicated very general processes e.g., metabolism, and so were also excluded. To visualise differences in gene ontology terms between groups of SSDA proteins, a REVIGO analysis (Supek *et al.*, 2011) was performed on non-redundant STRING GO term lists and the corresponding false discovery rate (FDR) value. The plots were exported to R and the strength values (Log_{10}(observed proteins / expected proteins)) of each GO term result were inputted. In addition, FDR values were multiplied by -1 in R so that smaller (more significant) values were visualised as larger circles on the plot.

To complement the enrichment analyses carried out by STRING, a topGO (v2.48.0; Alexa and Rahnenführer, 2009) analysis was performed on the same protein groups in RStudio (R v4.2.1; R Core Team, 2021) using scripts developed by Colgan *et al.* (2022). *Drosophila melanogaster* GO terms were sourced from Ensembl Biomart (Kinsella *et al.*, 2011) and assigned to *B. terrestris* homolog genes. A Fisher's exact test was then carried out ($p < 0.05$; node size =20) on each protein group using the algorithm 'weight01'. TopGO was ran separately for each category of gene ontology terms (i.e., 'biological process', 'cellular component' and 'molecular function'. Furthermore, to identify Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways that were enriched among protein groups, FASTA files containing the corresponding protein sequence of each protein within a group were searched on BLAST Koala (v2.2; Kanehisa *et al.*, 2016).

4.3 Results

4.3.1 Proteomic comparison of commercial and wild B. terrestris brains and responses to acute clothianidin exposure

(a) Protein identification and statistical analysis

Four replicates from the brains of each treatment group were analysed by LC-MS/MS and label free quantitative proteomics. Prior to any filtering, 3,384 proteins were identified across all brain protein samples. After filtering for the removal of peptides identified only by site, reverse hits, and contaminants as well as $\log_2$ transformation of LFQ intensity values and removal of proteins which were not present in all four replicates of at least one treatment group, 1,816 proteins remained.

(b) SSDA proteins and variability across groups

T-tests performed in Perseus identified the statistically significant SSDA proteins in relevant treatment group comparisons (Figures 4.5, 4.7; Table A4.1a - d). Four t-tests were performed on clothianidin exposed commercial workers (CC), control commercial workers CC), clothianidin exposed wild workers (WE), and control wild workers (WC) as detailed in Table 4.1. The PCA shown in Figure 4.2 illustrates a high degree of variation across all treatment groups with no clear grouping of the samples according to treatment.

(c) Hierarchical clustering

A hierarchical clustering analysis was performed on samples to gain insight into the overall differences in increased or decreased protein abundances across treatment groups. This analysis resulted in eight clusters that groups proteins with similar expression profiles across each of the four treatment groups (Figure 4.3; Table A4.1e). Clusters B and F contained proteins that changed in expression between wild and commercial worker brains regardless of treatment. Cluster B contained 11 proteins which are increased in abundance in the wild brains with respect to commercial brains. These were associated with mitochondrial transmembrane transport (sideroflexin and mitochondrial import inner membrane translocase), regulation of actin cytoskeleton

(moesin/ezrin/radixin and twinfilin) and nuclear export (exportin). Also in this cluster is a cytochrome c oxidase subunit, involved in the electron transport chain, and a eukaryotic translation initiation factor, involved in translation, and a glypican which is involved in developmental processes and signalling regulation.

Table 4.1 Numbers of SSDA proteins found by pairwise t-tests in clothianidin exposure experiment. T-tests were performed on the groups listed in column one, where group one is tested against group two. Columns two and three show the total numbers of proteins found to be SSDA and increased in abundance in each respective group.

Comparison (Group 1 – Group 2)	*# SSDA proteins increased in Group 1*	*# SSDA proteins increased in Group 2*
Commercial exposed (CE) – comm. control (CC)	33	74
Commercial exposed (CE) – wild exposed (WE)	25	38
Commercial control (CC) – wild control (WC)	69	44
Wild exposed (WE) – wild control (WC)	17	31

Cluster F, containing 19 proteins, had the opposite trend, with proteins in this cluster increasing in abundance in commercial bumblebee brains and decreasing in wild samples. These proteins were enriched for the GO term ‘protein dephosphorylation processes’ (Table A4.4n), likely due to the presence of proteins such as glycerol-3-phosphate phosphatase, protein-tyrosine-phosphatase and sex-regulated protein janus in this cluster. There are also three tubulin proteins included in this cluster, as well as proteins related to detoxification (catalase), amino acid metabolism (maleylacetoacetate isomerase) and RNA binding (28 kDa heat- and acid-stable phosphoprotein and pentatricopeptide repeat-containing protein).

Cluster G shows proteins that decrease in expression due to clothianidin exposure. This cluster contains 19 proteins enriched for ‘cell redox homeostasis’ (thioredoxin and glutaredoxin) and ‘transition metal ion homeostasis’ (copper transport protein ATOX1 and cytochrome c oxidase copper chaperone; Table A4.4o). Additionally, two nucleolin proteins, proteins associated with the nucleolus, are in this cluster. Otherwise, the functions associated with proteins in this cluster are broad e.g., memory formation, RNA binding, translational initiation and mitochondrial electron transport.

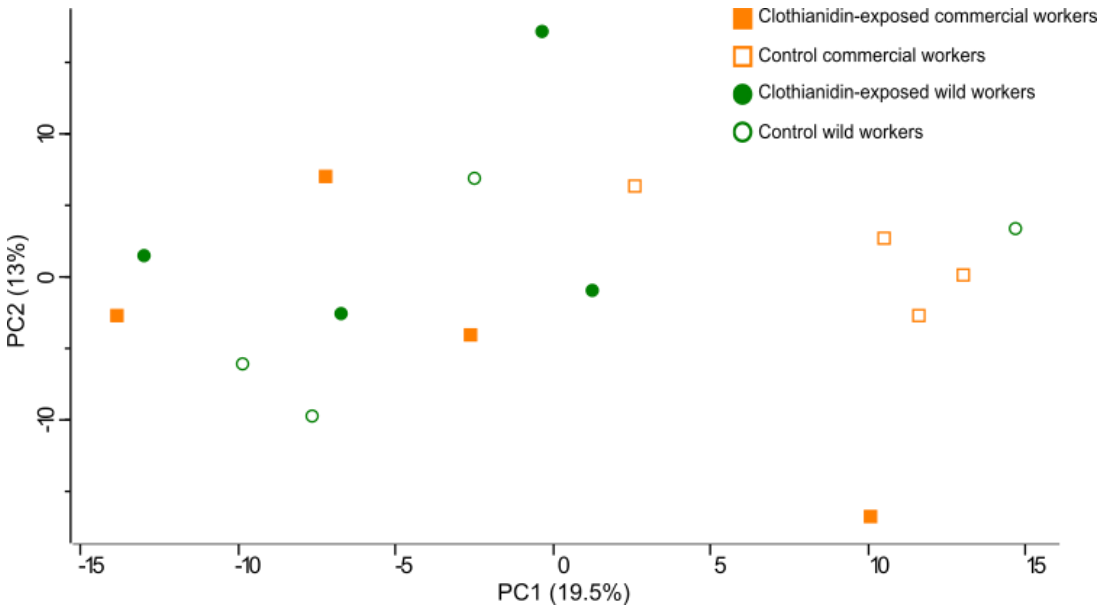
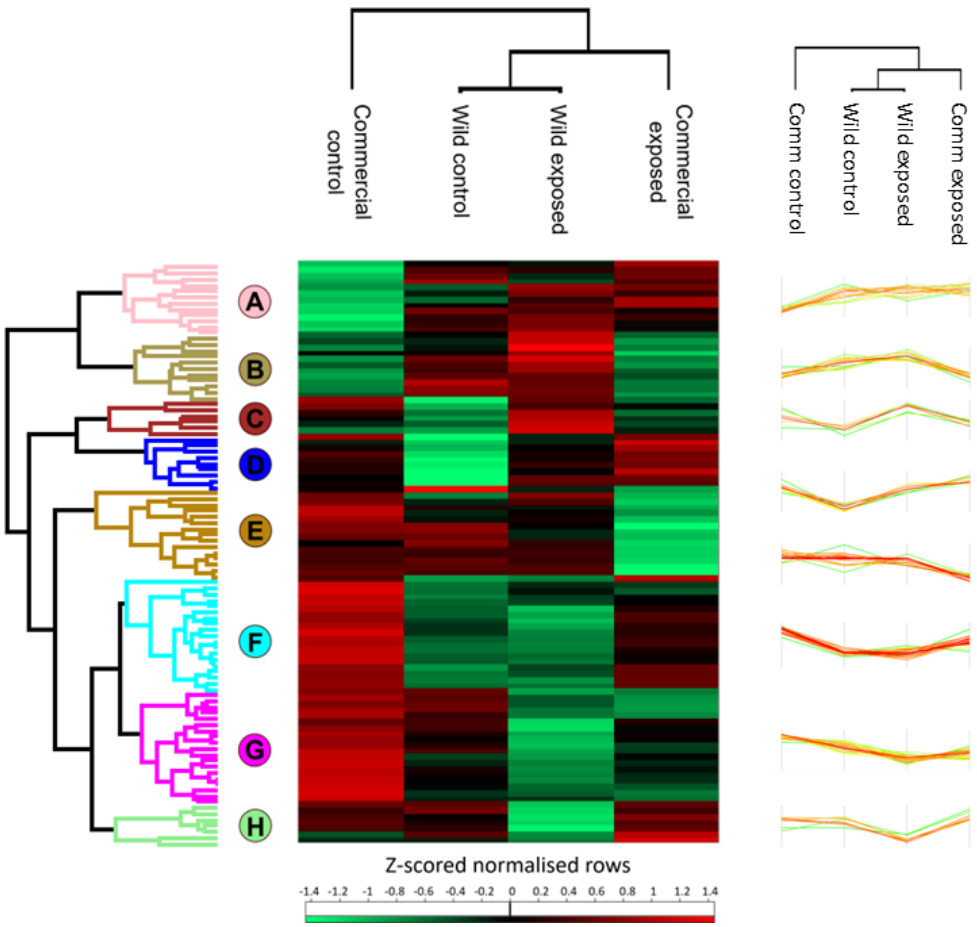


Figure 4.2 Principal component analysis of all brain samples in the clothianidin exposure experiment. PCA of wild (green datapoints) and commercial (orange) exposed (filled) and control (unfilled) samples. The x and y axis show the directions in which the samples vary in protein composition and explain 32.5% of the variance within the entire dataset.

Several groups show just a single treatment group out of the four total groups differing in expression (A, D, E, H). Cluster A, with 11 proteins, shows a decrease in just commercial control brains. Enrichment analysis resulted in no associated GO terms, however at least four proteins in this cluster are associated with transmembrane transport and two are linked to GTPase activity and GTPase activator binding. Other processes highlighted by proteins in this cluster include autophagy, chromatin binding and autophagy. Cluster D contains 9 proteins, and these proteins are decreased in abundance in wild control brains only. Several of these proteins were linked to melanin metabolic processes (phenoloxidase and spermatogenesis-associated protein 20). Two proteins in this cluster were also linked to visual signal transduction (mycosubtilin synthase and a glycine receptor). Cluster E includes 15 proteins decreased in abundance in commercial clothianidin exposed bees only. Some of these proteins were associated with 'cell part morphogenesis' (e.g., cytoplasmic dynein 1 light intermediate chain; Table 4.4m). Also included in this cluster were a programmed cell death protein and a superoxide dismutase, both with potential functions relating to innate immunity. There were also two metal binding proteins (UPF0587 protein C1orf123 and uridine diphosphate glucose pyrophosphatase) in Cluster E.



Cluster	GO terms (Biological processes)
A	-
B	(BP) Mitochondrial transmembrane transport, regulation of actin cytoskeleton organisation, nuclear export
C	(BP) Glycerophospholipid biosynthetic process
D	(BP) Melanin metabolic process
E	(BP) Cell part morphogenesis
F	(BP) Protein dephosphorylation
G	(BP) Cell redox homeostasis, transition metal ion homeostasis
H	(BP) Epithelial cell morphogenesis

Figure 4.3 Hierarchical clustering of wild and commercial *B. terrestris* bumblebee brains acutely exposed to clothianidin and wild and commercial control brains. Hierarchical clustering of the z-scored normalised mean intensity values for 98 SSDA proteins of wild and commercial brains response to clothianidin exposure. Analysis revealed eight clusters labelled and coloured accordingly. Alongside these clusters are treatment expression profiles and a table showing TopGO gene ontology terms associated with each cluster (Table A4.4 a - h).

Cluster H holds 7 proteins decreased in abundance in wild clothianidin exposed bees that were linked to 'epithelial cell morphogenesis' (e.g., myosin regulatory light chain sqh; Table A4.4p). Two proteins in this cluster also have oxidoreductase activity (dehydrogenase/reductase SDR family member and quinone oxidoreductase). Others in this cluster have potential functions such as gene regulation and subcellular trafficking. Cluster C (6 proteins) contains proteins that are decreased in abundance in the wild control and commercial exposed. Proteins in this cluster were linked to 'glycerophospholipid biosynthetic processes' (choline/ethanolamine kinase and 1-acyl-sn-glycerol-3-phosphate acyltransferase; Table A4.4k). Also included in this cluster is a choline transporter, involved in choline import to the neuron.

(d) Overlapping proteins

Venn diagram analysis found that three proteins were consistently increased in abundance in commercial bumblebee brains when compared with wild brains (Figure 4.4A; Table A4.2a). These proteins were mycosubtilin synthase, glutathione S-transferase and glycerol-3-phosphate phosphatase. Also examined were overlapping proteins in the clothianidin exposed vs control SSDA group comparisons (Figure 4.4B; Table A4.2b). In response to clothianidin exposure, one protein was increased in abundance in both commercial and wild bees – chromobox protein. Consistently decreased in abundance in response to clothianidin exposure were nucleolin, ubiquitin-40S ribosomal protein and RNA-binding protein. Also, a choline ethanolamine kinase was decreased in abundance in commercial exposed bees while increased in wild clothianidin exposed brains.

(e) Wild B. terrestris brain proteome response to acute clothianidin exposure

Student's t-tests found that when clothianidin-exposed and control wild worker brain proteomes were compared, there were 48 total SSDA proteins, with 17 proteins being significantly increased in abundance in clothianidin-exposed wild worker brains, and 31 proteins significantly increased in abundance in control wild worker brains (Figure 4.4A).

The top ten most differentially expressed proteins increased in clothianidin-exposed wild workers (Table 4.2) were involved in DNA binding and transcription (chromobox

protein and DNA-directed RNA polymerase), mRNA processing and splicing (U6 snRNA-associated protein and proteasome activator complex protein), translation (eukaryotic translation initiation factor), membrane biosynthesis and metabolism (choline/ethanolamine kinase, 1-acyl-sn-glycerol-3-phosphate acyltransferase and high-affinity choline transporter) and melanisation (phenoloxidase). GO enrichment using TopGO on all SSDA increased in abundance in this group supported these findings and revealed an enrichment in 'melanin metabolic process' (Table A4.4a), reflecting the presence of proteins mycosubtilin synthase and cysteine sulfinic acid decarboxylase.

In the top ten proteins increased in the wild control brains, processes such as gene regulation (protein SET, nucleolin and nuclear pore glycoprotein p62), cytoskeleton related processes (myosin regulatory light chain protein and intersectin), enzyme repair (ATP-dependent (S)-NAD(P)H-hydrate dehydratase) and the ubiquitin-proteosome pathway (Ubiquitin-conjugating enzyme) are highlighted among proteins. There were also proteins involved in the homeostatic regulation of sleep (FUN protein LOC100652008), electron transfer activity (NADH dehydrogenase iron-sulfur protein) and inhibition of apoptosis (Apoptosis inhibitor). Using TopGO, it was found that all 31 SSDA increased in abundance proteins in wild control brains had enrichment in biological processes such as 'wound healing' and 'dendrite development' and in molecular functions such as 'translation initiation factor activity' (Table A4.4b). A STRING analyses found enrichment of the same group of proteins in 'ribonucleoprotein complex' and 'eukaryotic 43s preinitiation complex' (Table A4.3a).

While a Blast KOALA analysis of both sets of SSDA proteins found no specific pathways enriched in either group of this comparison, overall a higher percentage of proteins relating to lipid, amino acid, carbohydrate and energy metabolic pathways were increased in the wild workers exposed to clothianidin, while in the control wild workers, a larger proportion of proteins associated with genetic information processing pathways, cellular processes and environmental information processing were found (Figure 4.5 A - B).

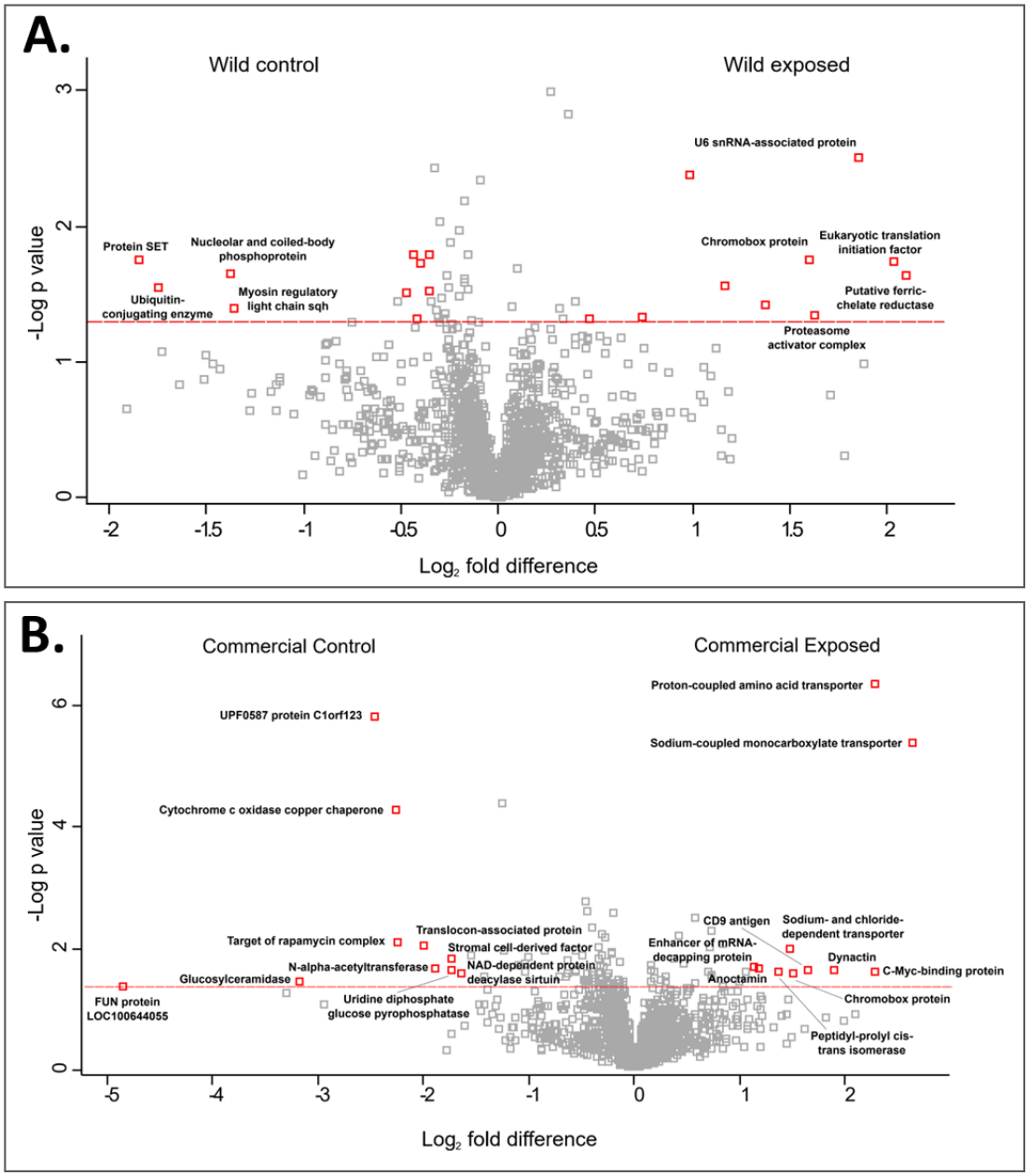


Figure 4.4 Differential abundance of proteins in (A) wild clothianidin-exposed brains compared with wild control brains and in (B) commercial clothianidin-exposed brains compared with commercial control brains. The -$\log_{10}$ *p* value and fold change ($\log_2$ mean LFQ intensity difference) are represented on the y- and x-axis respectively. All proteins above the red dashed line, indicating the cut-off point for significance ($p < 0.05$), are statistically significant differentially abundance proteins. Datapoints highlighted with red squares represent the top 10 most differentially abundant proteins in each group. Only those proteins with a relative fold change greater than 2 were annotated.

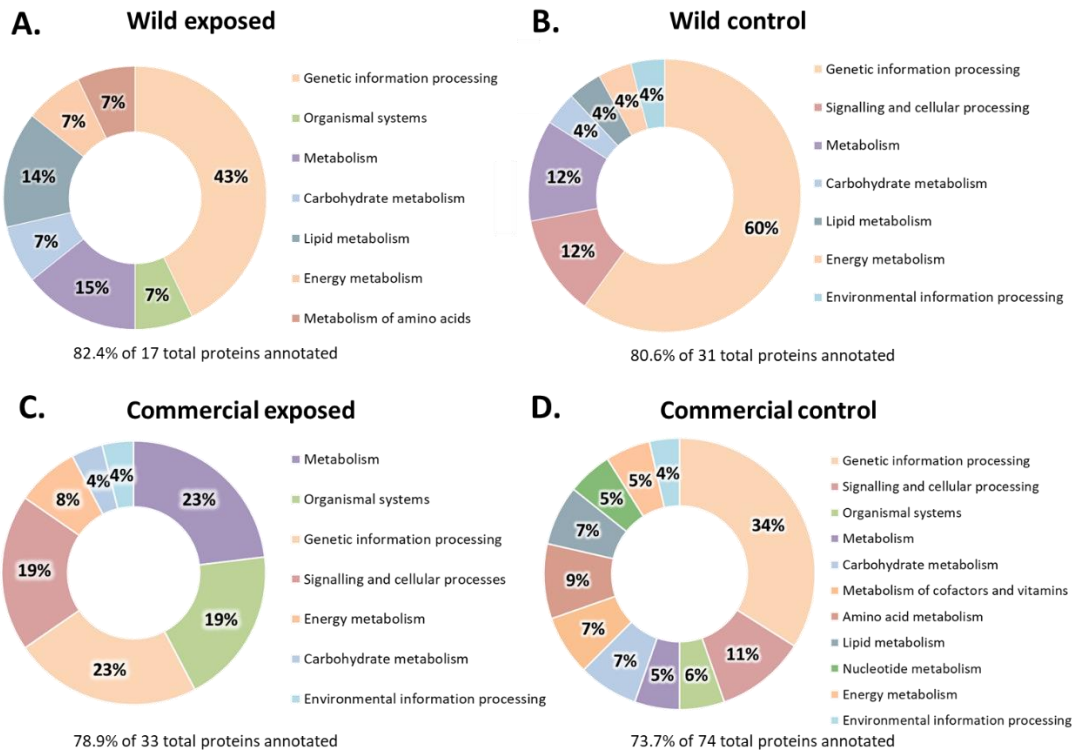


Figure 4.5 KEGG pathways enriched in all SSDA proteins increased in abundance within each group in the exposed – control comparisons. Relative proportions (%) of proteins annotated with various KEGG pathway categories in SSDA proteins increased in abundance (with % of annotated SSDA proteins) in **(A)** wild clothianidin exposed and **(B)** wild control brains in the WE – WC comparison and **(C)** commercial clothianidin exposed and **(D)** commercial control brains in the CE – CC comparison.

(f) Commercial B. terrestris brain proteome response to acute clothianidin exposure

When clothianidin exposed commercial *B. terrestris* brain proteomes were compared to control commercial *B. terrestris* brains using a Student's t-test, 33 SSDA proteins were found to be increased in abundance in the clothianidin-exposed group and 74 increased in the control commercial brains (Figure 4.4B; Table A4.1b).

In the top ten differentially abundant proteins (Table 4.2) increased in abundance in the clothianidin-exposed commercial brain proteomes, half were linked to transmembrane activity and transport (sodium-coupled monocarboxylate transporter, proton-coupled amino acid transporter, CD9 antigen protein, sodium- and chloride-dependent transporter and anoctamin-8). Other processes represented among the top ten proteins increased in abundance in clothianidin exposed commercial workers were gene regulation (c-myc-binding protein and chromobox protein), mRNA degradation (enhancer of mRNA-decapping protein), regulation of microtubule activities

Table 4.2 Top 10 SSDA proteins in clothianidin-exposed and control brain proteome comparisons. The fold change, accession number and name of the top 10 SSDA proteins increased and decreased in abundance in wild and commercial brain proteomes in response to clothianidin exposure, ranked from highest intensity to lowest. Relative fold change reflects the mean protein LFQ intensity difference between exposed and control brain proteomes. Fold changes highlighted in yellow represent proteins increased in abundance in *B. terrestris* brain in response to clothianidin and those in purple represent proteins increased in abundance in the control samples.

	Wild clothianidin exposed – Wild control			Commercial clothianidin exposed – Commercial control		
	Fold change	**Accession no.**	**Protein name**	**Fold change**	**Accession no.**	**Protein name**
↑	2.11	XP_012163761.1	Putative ferric-chelate reductase	6.21	XP_003399925.2	Sodium-coupled monocarboxylate transporter
	2.04	XP_003393124.1	Eukaryotic translation initiation factor	4.87	XP_003394540.1	Proton-coupled amino acid transporter
	1.86	XP_003396461.1	U6 snRNA-associated protein	4.85	XP_003392987.1	C-Myc-binding protein
	1.64	XP_003400167.1	Proteasome activator complex protein	3.7	XP_012163494.1	Dynactin
	1.61	XP_003396731.1	Chromobox protein	3.13	XP_003401526.1	CD9 antigen protein
	1.38	XP_020721640.1	Choline/ethanolamine kinase	2.83	XP_003396731.1	Chromobox protein
	1.18	XP_003394354.1	1-acyl-sn-glycerol-3-phosphate acyltransferase	2.77	XP_003402418.1	Sodium- and chloride-dependent transporter XTRP3
	1	XP_020721684.1	High-affinity choline transporter	2.58	XP_012165944.2	Peptidyl-prolyl cis-trans isomerase FKBP8
	0.75	XP_003399854.1	DNA-directed RNA polymerase	2.25	XP_012165593.1	Anoctamin-8
	0.48	XP_003400548.1	Phenoloxidase	2.18	XP_003394044.1	Enhancer of mRNA-decapping protein
↓	3.58	XP_003402230.1	Protein SET	29	XP_020718843.1	Uncharacterized protein LOC100644055
	3.34	XP_003395063.1	Ubiquitin-conjugating enzyme	9.13	XP_003402892.1	Glucosylceramidase
	2.58	XP_012168000.2	Nucleolar and coiled-body phosphoprotein	5.54	XP_020720608.1	UPF0587 protein C1orf123
	2.55	XP_003396242.1	Myosin regulatory light chain	4.8	XP_003400457.1	Cytochrome c oxidase copper chaperone
	1.42	XP_012167732.1	Uncharacterized protein LOC100652008	4.77	XP_003395791.1	Target of rapamycin complex protein
	1.38	XP_003396300.1	Nuclear pore glycoprotein p62	4	XP_003393009.1	Translocon-associated protein
	1.35	XP_020721783.1	Intersectin-1	3.72	XP_003396013.1	N-alpha-acetyltransferase
	1.33	XP_012173712.1	Apoptosis inhibitor	3.35	XP_012174873.1	Stromal cell-derived factor
	1.32	XP_003403335.1	NADH dehydrogenase iron-sulfur protein	3.33	XP_003395627.1	Uridine diphosphate glucose pyrophosphatase
	1.27	XP_012170866.1	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	3.13	XP_003400675.1	NAD-dependent protein deacylase sirtuin

(dynactin) and autophagy (peptidyl-prolyl cis-trans isomerase FKBP8). Other notable SSDA proteins increased within clothianidin-exposed commercial brains were three cytochrome P450 proteins, involved in detoxification. TopGO analysis of all 33 SSDA proteins increased in abundance in this group found enrichment in 'synapse organisation', 'axon development' and 'ribonucleotide binding' (Table A4.4c). Additionally, STRING enrichment analysis associated keywords of 'iron', 'heme' and 'monooxygenase' with this group (Table A4.3b).

Of the proteins with increased abundance in commercial control brains (Table 4.2), there were a broad range of processes and functions associated with the top ten most differentially abundant proteins. Two proteins were involved in electron transport processes (FUN protein LOC100644055 and cytochrome c oxidase copper chaperone). Three proteins were involved in protein regulation and processing including the regulation of proteins in the endoplasmic reticulum (translocon-associated protein), the binding of misfolded proteins (stromal cell-derived factor) and acetylation of proteins (N-alpha-acetyltransferase). Another protein represented in this group, NAD-dependent protein deacylase sirtuin, is involved in diacylation of target proteins and is also linked to anti-viral activity. Among other SSDA proteins outside of the top ten were prosaposin, a protein linked to membrane lipid metabolism, heat shock protein 75 kDa, linked to redox homeostasis, target of rapamycin Lst8, involved in cell growth, and several proteins linked to ubiquitination processes. When all 74 proteins in this group were analysed using TopGO, terms such as 'cellular oxidant detoxification', 'ribonucleotide biosynthetic process', and 'unfolded protein binding' were found to be enriched (Table A4.4d). STRING found enrichment in these proteins for 'endoplasmic reticulum' and 'glutathione metabolism' (Table A4.3c). No specific KEGG pathways were found to be very enriched for this SSDA protein group when assessed with Blast KOALA.

Both groups of SSDA proteins, those that were increased in abundance in clothianidin commercial worker brains and those in commercial control workers, were assessed using Blast KOALA and it was found that while there were higher proportions among proteins in general metabolic processes, organismal systems and energy metabolism in clothianidin exposed commercial brains, genetic information processing was linked to higher proportions of proteins with increased abundances in control commercial brains (Figure 4.5 C - D).

(g) Proteomic differences between wild and commercial B. terrestris brains acutely exposed to clothianidin

Student's t-tests revealed that there were, in total, 63 SSDA proteins between commercial and wild clothianidin exposed *B. terrestris* brains. There were 25 SSDA proteins increased in abundance in commercial exposed brains and 38 SSDA proteins increased in wild exposed brains (Figure 4.6A; Table A4.1c).

The top ten proteins with increased abundances in commercial exposed brains compared with wild exposed brains included those involved in RNA binding (28 kDa heat- and acid-stable phosphoprotein), oxidation-reduction (dehydrogenase/reductase SDR protein), membrane transporters (CD63 antigen protein) and the TOR pathway (regulator complex protein LAMTOR4; Table 4.3). Although not in the top ten, other notable proteins increased in the commercial exposed group were glutathione S-transferase and mycosubtilin synthase. GO terms generated by TopGO found enrichment among the SSDA proteins increased in abundance in the commercial exposed proteomes in processes such as 'mitotic cytokinesis' and 'alpha-amino acid metabolic process' (Table A4.4e). Cellular component enriched terms included 'actin filament', 'vesicle membrane' and 'lysosome'. STRING enrichment analysis only showed enrichment generally in the KEGG entry 'metabolic pathways' (Table A4.3d).

Four proteins (carbonic anhydrase, uridine diphosphate glucose pyrophosphatase, translocon-associated protein and UPF0587 protein C1orf123) within in the top ten proteins with increased abundance in wild exposed brains were also found to be decreased in commercial exposed brains when compared to the commercial control brains (Table 4.3). The other six proteins within the top ten proteins increased in abundance in the wild exposed brains were involved with translation (eukaryotic translation initiation factor), neural signalling (glypican-4 and pro-corazonin), gene regulation (zinc finger protein on ecdysone puffs) and glutathione metabolism (glutamate-cysteine ligase regulatory protein). Proteins increased in abundance in wild exposed brains but outside of the top ten included alcohol dehydrogenase, involved in oxidoreductase activity, and several proteins involved in phospholipid biosynthesis (e.g., ethanolamine kinase, high-affinity choline transporter and phosphatidylinositide. phosphatase). Wild exposed SSDA proteins showed

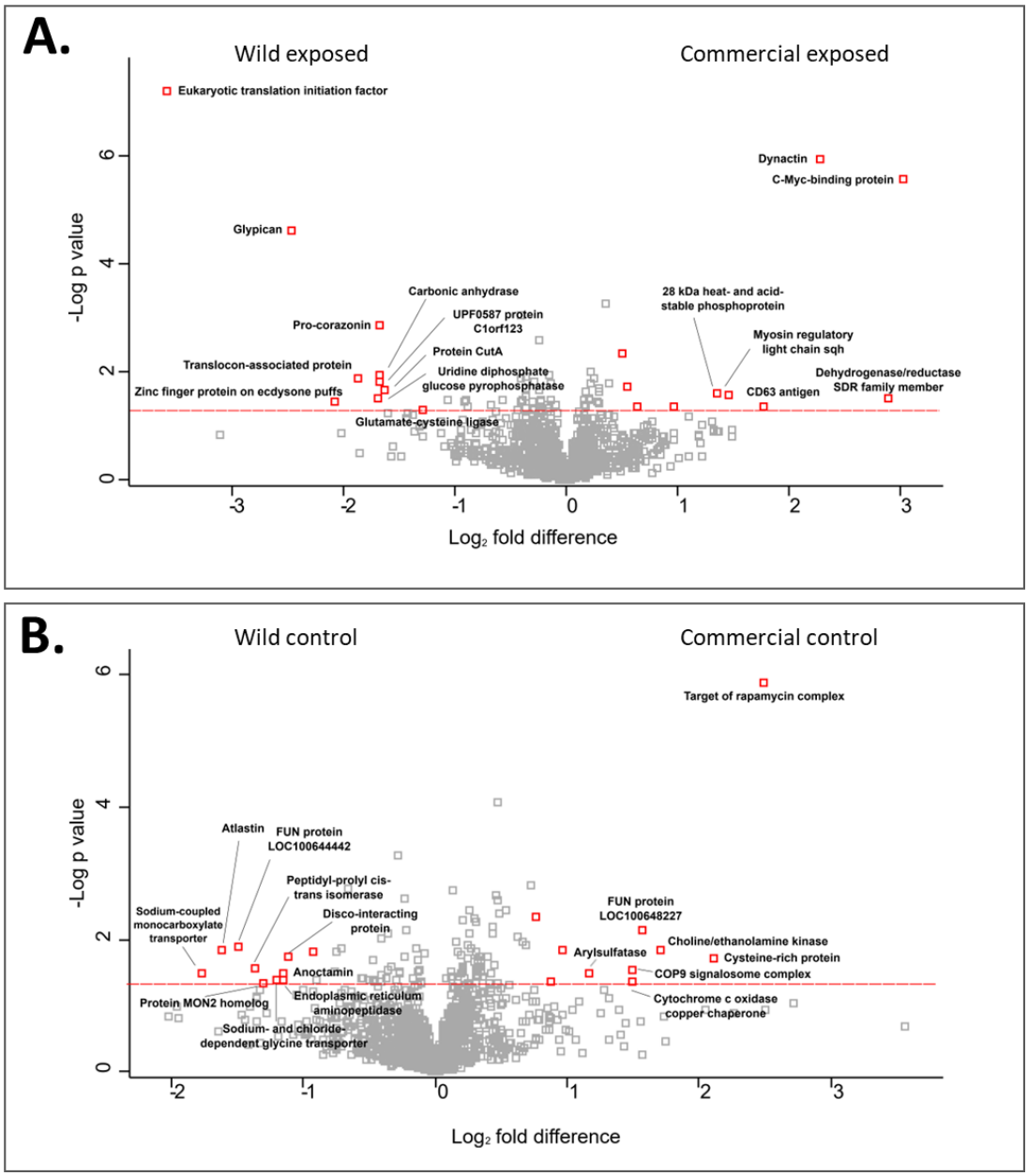


Figure 4.6 Differential abundance of proteins in (A) commercial clothianidin-exposed brains compared with wild clothianidin-exposed brains and in (B) commercial control brains compared with wild control brains. The -$\log_{10}$ *p* value and fold change ($\log_2$ mean LFQ intensity difference) are represented on the y- and x-axis respectively. All proteins above the red dashed line, indicating the cut-off point for significance ($p < 0.05$), are statistically significant differentially abundance proteins. Datapoints highlighted with red squares represent the top 10 most differentially abundant proteins in each group. Only those proteins with a relative fold change greater than 2 were annotated.

enrichment in 'long-term memory', 'cytoplasmic translation', 'mitochondrial transport' and 'mushroom body development', according to TopGO analysis (Table

A4.4f). STRING enrichment analysis revealed enrichment in the 'ribosome' KEGG entry (Table A4.3e). While neither group of all SSDA proteins increased in abundance in commercial exposed brains nor wild exposed brains showed enrichment in specific KEGG pathways according to a Blast KOALA analysis, overall, the commercial exposed group had a higher proportion of proteins associated with amino acid metabolism and cellular processes while the wild exposed group had a higher percentage linked to genetic information processing (Figure 4.7 A - B).

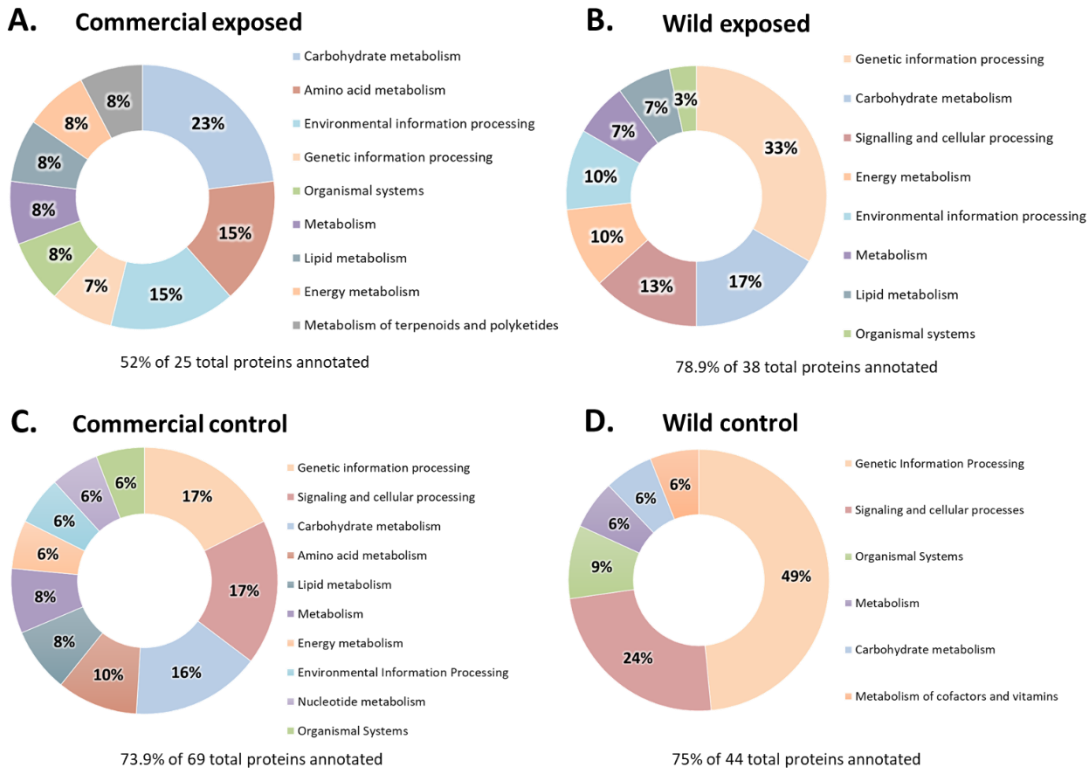


Figure 4.7 KEGG pathways enriched in all SSDA proteins increased in abundance within each group in the commercial – wild comparisons. Relative proportions (%) of proteins annotated with various KEGG pathway categories in SSDA proteins increased in abundance (with % of annotated SSDA proteins) in **(A)** commercial clothianidin exposed and **(B)** wild clothianidin exposed brains in the CE – WE comparison and **(C)** commercial control and **(D)** wild control brains in the CC – WC comparison.

Table 4.3 Top 10 SSDA proteins in wild and commercial *B. terrestris* brain proteome comparisons. The fold change, accession number and name of the top 10 SSDA proteins increased and decreased in abundance in clothianidin-exposed wild and commercial brains and control wild and commercial brains, ranked from highest intensity to lowest. Relative fold change reflects the mean protein LFQ intensity difference between exposed and control brain proteomes. Fold changes highlighted in yellow represent proteins increased in abundance in *B. terrestris* brain in commercial brains and those in purple represent proteins increased in abundance in the wild brains.

	Commercial clothianidin exposed – Wild clothianidin exposed			Commercial control – Wild control		
	Fold change	**Accession no.**	**Protein name**	**Fold change**	**Accession no.**	**Protein name**
↑	8.16	XP_003392987.1	C-Myc-binding protein	5.61	XP_003395791.1	Target of rapamycin complex
	7.43	XP_012174511.1	Dehydrogenase/reductase SDR family member 11	4.3	XP_003395120.1	Cysteine-rich protein
	4.89	XP_012163494.1	Dynactin	3.26	XP_020721640.1	Choline/ethanolamine kinase
	3.42	XP_003399497.1	CD63 antigen	2.97	XP_003401118.1	Uncharacterized protein LOC100648227
	2.77	XP_003396242.1	Myosin regulatory light chain sqh	2.81	XP_003393306.1	COP9 signalosome complex
	2.57	XP_003400743.1	28 kDa heat- and acid-stable phosphoprotein	2.81	XP_003400457.1	Cytochrome c oxidase copper chaperone
	1.95	XP_012171337.1	Filamin-A	2.23	XP_003401982.1	Arylsulfatase B
	1.57	XP_020718419.1	Glycine-rich cell wall structural protein	1.96	XP_003399854.1	DNA-directed RNA polymerase II
	1.47	XP_003400780.2	Ragulator complex protein LAMTOR4	1.84	XP_003402955.1	E3 ubiquitin-protein ligase RNF126-A
	1.43	XP_012170866.1	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	1.7	XP_012165421.1	Mycosubtilin synthase
↓	11.91	XP_003394753.1	Eukaryotic translation initiation factor	3.42	XP_003399925.2	Sodium-coupled monocarboxylate transporter
	5.46	XP_003397886.1	Glypican-4	3.07	XP_012164268.1	Atlastin
	4.22	XP_020719521.1	Zinc finger protein on ecdysone puffs	2.83	XP_003396724.1	Uncharacterized protein LOC100644442
	3.65	XP_003393009.1	Translocon-associated protein	2.57	XP_012165944.2	Peptidyl-prolyl cis-trans isomerase FKBP8
	3.22	XP_003395627.1	Uridine diphosphate glucose pyrophosphatase	2.48	XP_012171816.1	Protein MON2
	3.18	XP_020720608.1	UPF0587 protein C1orf123	2.3	XP_003395922.1	Sodium- and chloride-dependent glycine transporter
	3.17	XP_003395501.1	Carbonic anhydrase	2.23	XP_012165593.1	Anoctamin
	3.17	XP_003393262.1	Pro-corazonin	2.22	XP_003393363.1	Endoplasmic reticulum aminopeptidase
	3.07	XP_012171808.1	Protein CutA	2.17	XP_020719701.1	Disco-interacting protein
	2.44	XP_003402709.1	Glutamate-cysteine ligase regulatory protein	1.91	XP_003395799.1	Replication protein A 70 kda DNA-binding

(h) Proteomic differences between commercial and wild control brain samples

When control commercial and wild brain proteomes were compared using Student's t-tests, 113 SSDA proteins were identified, 69 proteins increased in abundance in commercial controls and 44 in wild controls (Figure 4.6B; Table A4.1d).

Among the top ten proteins increased in abundance in commercial controls in this analysis (Table 4.3), four proteins (target of rapamycin complex, choline/ethanolamine kinase, cytochrome c oxidase copper chaperone and E3 ubiquitin-protein ligase RNF126-A) were also represented among the increased in abundance proteins in the control samples in the CE - CC comparison (Section 4.3.1(f)). Processes and structures represented among the top ten proteins in the commercial controls include metal-binding (cysteine-rich protein), membrane biosynthesis (choline/ethanolamine kinase), gene regulation and expression (FUN protein LOC100648227 and DNA-directed RNA polymerase II) and ubiquitination (E3 ubiquitin-protein ligase RNF126-A). STRING GO term enrichment analysis found SSDA proteins increased in commercial control brain samples in the CC – WC comparison to be enriched for 'cell redox homeostasis', 'structural constituent of the cytoskeleton', 'sulfur compound metabolic process' and 'mitochondrion' (Figure 4.8; Table A4.3f). TopGO analysis found similar enrichment in 'disulfide oxidoreductase activity' and 'structural constituent of cytoskeleton (Table A4.4g).

Five proteins in the top ten increased in abundance in the wild control group (Sodium-coupled monocarboxylate transporter, peptidyl-prolyl cis-trans isomerase FKBP8, anoctamin, endoplasmic reticulum aminopeptidase and disco-interacting protein) in this analysis were also found among the SSDA proteins increased in abundance in the exposed samples in the commercial exposed –control comparison (Table 4.3). Proteins within the top ten increased in abundance in wild control samples were involved in processes including neural signalling (sodium- and chloride-dependent glycine transporter), protein transport to endosomes (protein MON2), cell membrane associated proteins (FUN protein LOC1006444420, sodium-coupled monocarboxylate transporter, anoctamin and disco-interacting proteins) and endoplasmic reticulum-related activities (atlastin and endoplasmic reticulum aminopeptidase). Both TopGO and STRING analysis of wild control SSDA proteins revealed involvement in 'protein targeting to the mitochondrion' (Figure 4.8; Table A4.3g; Table A4.4h).

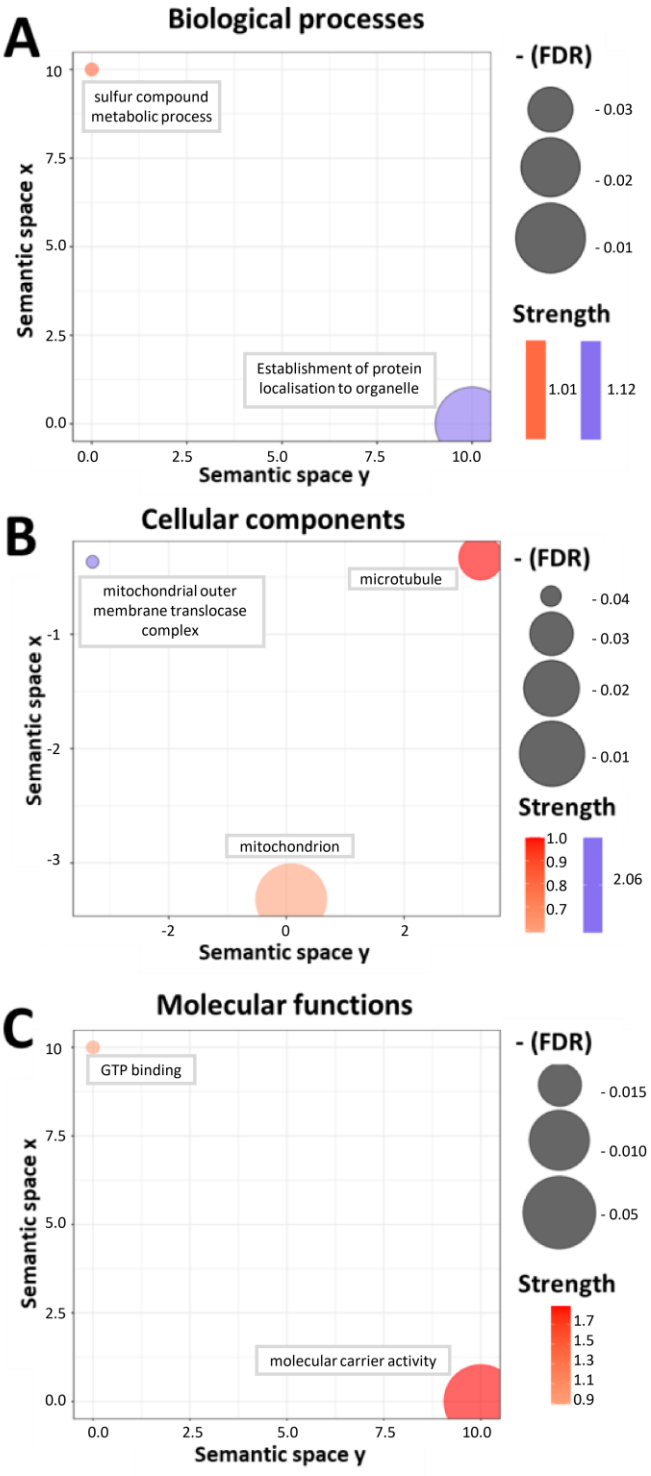


Figure 4.8 Revigo plots showing STRING GO terms associated with up and decreased in abundance SSDA proteins in the commercial and wild control brains. GO terms associated with SSDA proteins increased in abundance (represented by red circles) and decreased in abundance (blue circles) in commercial control brains when compared with wild control brains. Size of circles represent the false discovery rate (FDR) multiplied by -1. The intensity of the colours reflects the strength of the enrichment effect is (Log10(observed / expected). Separate plots have been made for each GO term category: (A) Biological processes (B) Cellular components and (C) Molecular functions.

SSDA proteins increased in abundance in commercial controls did not show major enrichment in any KEGG pathways according to Blast KOALA results. Blast KOALA showed that SSDA proteins increased in abundance in commercial controls had a larger proportion of proteins involved with carbohydrate metabolism and amino acid metabolism than wild controls, while proteins significantly increased in abundance in wild control brains had a higher proportion involved in genetic information processing and signalling and cellular processes (Figure 4.7C - D).

4.3.2 Proteomic comparison of commercial and wild B. terrestris fat bodies in context of E. coli infection

(a) Protein identification and statistical analysis

Four fat body samples were run on LC-MS/MS and 2,455 proteins were found in total. After filtering, carried out as described in section 4.3.1 (a), the remaining proteins totalled 1,362 and were used in downstream analyses.

(b) SSDA proteins and variability across groups

To identify the total number of SSDA proteins in each group comparison, student's t-tests were performed. Four student's t-tests were performed on the fat body proteomes of *E. coli* infected commercial workers, ringer control commercial workers, *E. coli* infected wild workers, and ringer control wild workers (Figures 4.13 and 4.15; TableA4.5 a-d). Each group comparison and the resulting SSDA proteins is in Table 4.4. The PCA, performed to visualise variability among treatment groups, didn't show clear distinction between *E. coli* infected and ringer control fat bodies but clearly differentiated between all wild and commercial fat bodies, indicating that their fat body proteomes are in general very distinctive (Figure 4.9).

Table 4.4 Numbers of SSDA proteins found by pairwise t-tests in *E. coli* infection experiment. T-tests were performed on the groups listed in column one, where group one is tested against group two. Columns two and three show the total numbers of proteins found to be SSDA and increased in abundance in each respective group.

Comparison (Group 1 – Group 2)	*# of SSDA proteins increased in abundance in Group 1*	*# of SSDA proteins increased in abundance in Group 2*
Commercial *E. coli* (CE) – comm. ringer (CR)	15	13
Commercial *E. coli* (CE)– wild *E. coli* (WE)	86	125
Commercial ringer (CR) – wild ringer (WR)	107	266
Wild *E. coli* (WE) – wild ringer (WR)	10	9

(c) Overlapping proteins

Both wild and commercial bees with *E. coli* infection had, within their top ten significantly differential proteins, increased in abundance levels of leukocyte elastase inhibitor (a serpin) and mycosubtilin synthase (associated with free biogenic amine regulation, visual signal transduction and cuticle formation), suggesting these are part of a conserved *Bombus* response to *E. coli* infection (Figure 4.10A). Protein takeout was also shared in commercial and wild proteins decreased in abundance in response to *E. coli* infection (Table A4.6a).

In terms of proteins shared when wild and commercial groups were compared, 39 proteins were consistently increased in abundance in commercial fat bodies while 90 proteins were increased in abundance in wild fat bodies, regardless of treatment (Figure 4.10B; Table A4.6b**)**. A STRING analysis on each significant protein group found proteins increased in abundance in the commercial bees were involved in 'cell redox homeostasis', 'carboxylic acid metabolic processes' and 'alpha-amino acid processes' (Table A4.7k). Those increased in abundance in wild fat bodies were enriched for 'translation', 'protein folding', 'establishment of protein localisation' and also 'carboxylic acid metabolic processes' (Table A4.7l). These processes likely reflect the fundamental differences between wild and commercial fat body proteomes.

When these shared proteins are excluded from the SSDA protein lists produced by the *E. coli*-exposed commercial and wild fat bodies comparison to show the likely

differences between wild and commercial responses to *E. coli* infection, we see that the proteins increased in abundance in commercial bumblebee fat bodies exposed to *E. coli* are more related to nuclear export, protein localisation and mRNA processing (Table A4.7m). Wild fat bodies infected with *E. coli* (with shared proteins excluded) show enrichment in 'cytoplasmic stress granule' when STRING analysis was run on corresponding *Drosophila melanogaster* UniProt codes using kmeans clustering (Table A4.7n).

The same process (i.e., exclusion of overlapping proteins and assessing enrichment among unique SSDA proteins) was repeated to examine SSDA proteins between commercial and wild ringer control fat bodies. When the remaining SSDA proteins are analysed with STRING, enrichment terms for proteins increased in abundance in commercial Ringer samples include 'muscle contraction', 'cell development', 'actin cytoskeleton organisation', 'ATP metabolic process' and 'cellular respiration' (Table A4.7o). With SSDA proteins increased in abundance in wild ringer control fat body samples, enrichment was found in processes such as protein folding, proteasomal protein catabolic process, endoplasmic reticulum to Golgi vesicle-mediated transport and translation (Table A4.7p).

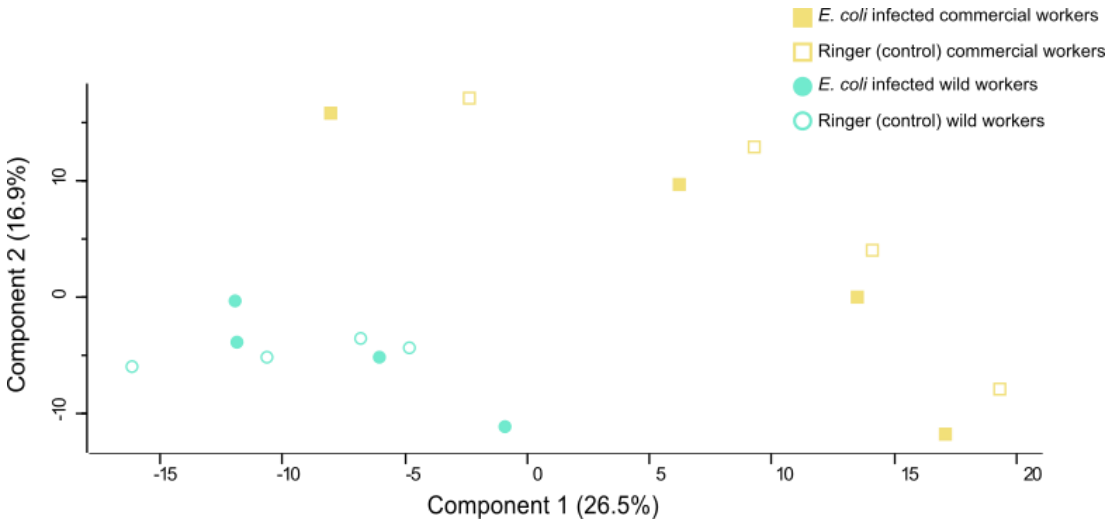


Figure 4.9 Principal component analysis of all samples in *E. coli* infection experiment. Filled shapes represent those *B. terrestris* worker fat bodies exposed to *E. coli*, empty shapes represent experimental control fat bodies injected with ringer only, wild and commercial fat body samples are shown by blue and yellow datapoints respectively. The x and y axis show the directions in which the samples vary in protein composition and explain 43.4% of the variance within the entire dataset.

(d) Hierarchical clustering

Hierarchical clustering determined six clusters showing the expression profiles of SSDA proteins across each treatment group (Figure 4.11; Table A4.5e). Cluster A contains just two proteins that are increased in abundance in wild ringer and commercial *E. coli* infected bees. These two proteins were DNA-directed RNA polymerase II and vacuolar protein sorting-associated protein.

Cluster E contains five proteins that are elevated in response to *E. coli* infection in both wild and commercial fat bodies. These proteins showed enrichment in 'regulation of secondary metabolic process' and 'melanin metabolic process' (Table A4.7i; Table A4.8m). Two proteins in this cluster were **s**erine/threonine-protein phosphatases (**s**erine/threonine-protein phosphatase 6 catalytic protein and calcineurin). Leukocyte elastase inhibitor is a serine protease inhibitor implicated in the insect innate immune response. The remaining proteins in this cluster (cytochrome b5 and mycosubtilin synthase) are involved in heme binding and metal ion binding. Mycosubtilin synthase is also linked to visual systems and cuticle formation.

All other clusters (B, C, D, and F) displayed expressional changes between wild and commercial fat bodies, regardless of infection. Clusters B, C and D show elevated protein abundances in the wild groups compared with commercial groups, while Cluster F shows the opposite trend.

There are 16 proteins in Cluster B. According to TopGO, there is enrichment in this cluster for peptide metabolic process (Table A4.8j). Several proteins in this cluster have functions relating to proteolysis and proteasome (26S proteasome non-ATPase regulatory subunit, COP9 signalosome complex, Phospholipase a-2-activating protein, mitochondrial intermediate peptidase and probable peroxisomal acyl-coenzyme) and RNA binding (lysine—tRNA ligase and poly(rC)-binding protein). Protein takeout is also included here, involved in multicellular organism reproduction and circadian rhythms. Cluster C, with 147 proteins, shows enrichment in circadian rhythm (proteins annotated with this GO term were mainly proteasome subunits), protein folding, translational initiation, fatty acid oxidation, proteasome complex and peroxisome (Table A4.7g; Table A4.8k). Several immune related genes were also included here including pathogen recognition proteins (peptidoglycan-recognition protein and MD-2-related lipid-recognition protein), anti-viral proteins

(staphylococcal nuclease domain-containing protein) and antioxidant proteins (catalase). Cluster D contains 69 proteins that, from TopGO analysis, show enrichment in negative regulation of neuron death, regulation of lipid storage, endoplasmic reticulum to Golgi vesicle-mediated transport, ribosomal small subunit biogenesis and positive regulation of innate immune response (Table A4.8l). STRING analysis also found enrichment in terms such as 'translation', likely owing to the large amount of 40S and 60S ribosomal proteins making up half of the proteins in this cluster (Table A4.7h).

Cluster F includes 97 proteins that are involved in processes such as mRNA transport, fatty acid beta oxidation, electron transport chain, response to oxidative stress and chromatin DNA binding (Table A4.7j; Table A4.8n). Five proteins with innate immune system involvement are found in this cluster; these include 2 peroxiredoxin proteins and a Glutathione S transferase which exhibit antioxidant function, an insulin-like growth factor-binding protein complex acid labile subunit (a SLIT protein), and a FUN protein LOC100647796 that has lipid binding activity and is implicated in pathogen recognition.

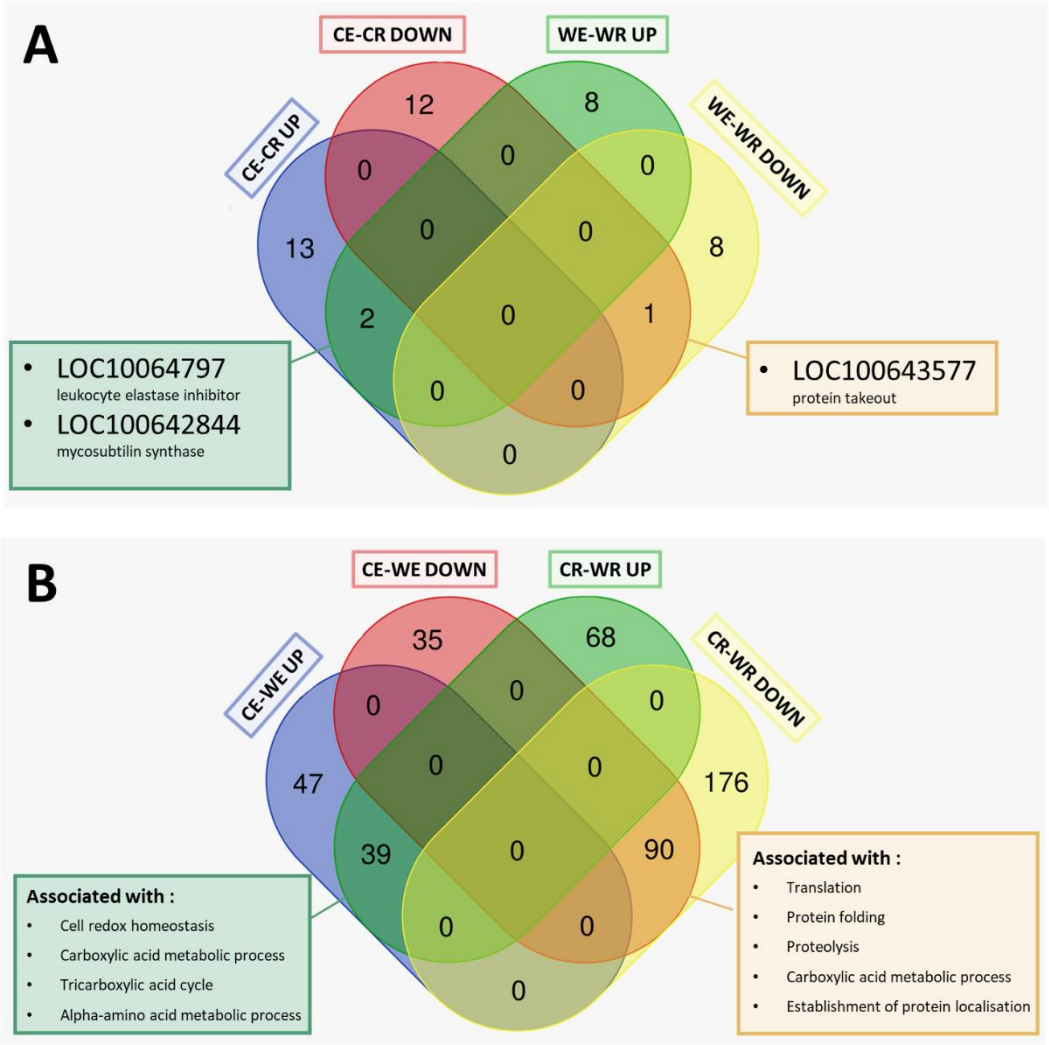
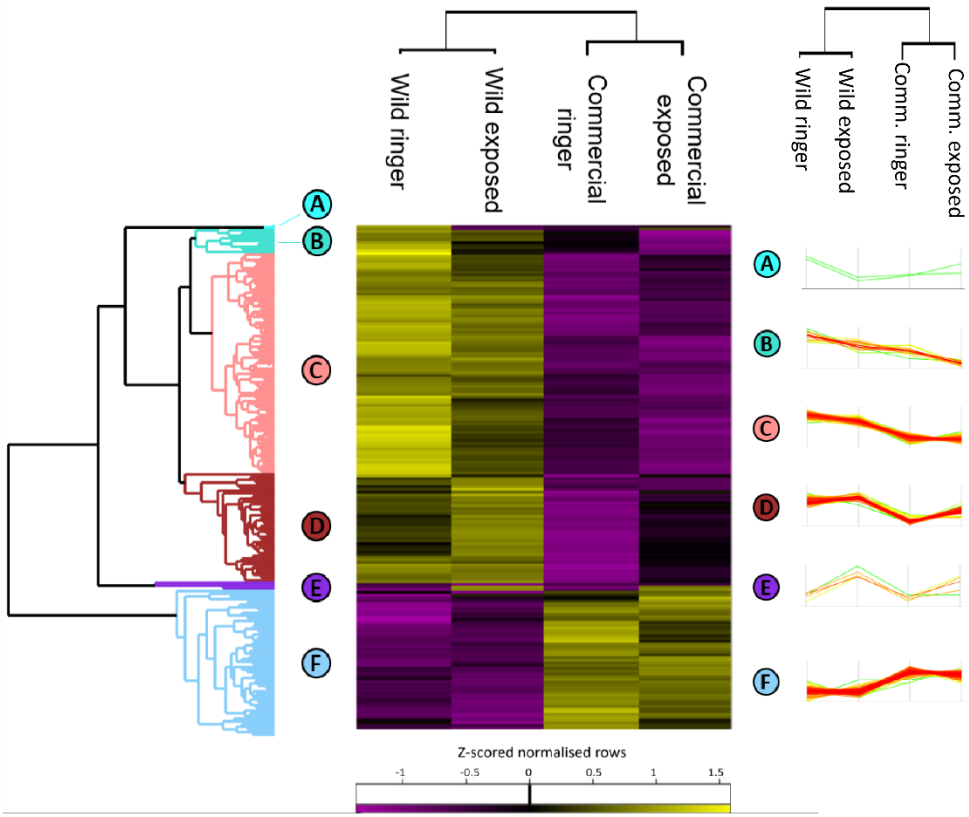


Figure 4.10 Venn diagrams showing number of overlapping proteins between (A) SSDA proteins in commercial vs wild comparisons and (B) SSDA proteins in *E. coli* infected vs ringer control comparisons. The labelled groups share the amount of proteins indicated where their shapes intersect. Shared proteins are shown for (A) *E. coli* infected and ringer control comparisons and (B) commercial and wild comparisons. Locus ID's and protein names are listed for each overlapping protein group in (A) and STRING gene ontology terms are listed for the overlapping proteins in (B).



Cluster	GO term
A	(BP) Endosome organization
B	(BP) Proteasome-mediated ubiquitin-dependent protein catabolic process, protein processing, asymmetric cell division., regulation of hemocyte differentiation
C	(BP) proteasome-mediated ubiquitin-dependent, circadian rhythm, Toll signaling pathway, cytoplasmic translation, fatty acid oxidation, protein targeting to ER, glucose metabolic process
D	(BP) negative regulation of neuron death, regulation of lipid storage, endoplasmic reticulum to Golgi vesicle-mediated transport, ribonucleoprotein complex subunit organization, positive regulation of innate immune response
E	(BP) melanin metabolic process
F	(BP) oxidative phosphorylation, cellular amino acid catabolic process, response to oxidative stress, fatty acid beta-oxidation, tricarboxylic acid cycle, positive regulation of cytoskeleton organization, larval lymph gland hemopoiesis, chaperone-mediated protein folding

Figure 4.11 Hierarchical clustering of SSDA proteins in the fat bodies of wild and commercial *B. terrestris* bumblebee infected with E. coli and control samples. Hierarchical clustering of the z-scored normalised mean intensity values for 338 SSDA proteins of wild and commercial brains response to clothianidin exposure. Analysis determined six clusters labelled and coloured accordingly. Alongside these clusters are treatment expression profiles and a table showing TopGO gene ontology terms associated with each cluster.

(e) Proteomic response of wild B. terrestris fat body to E. coli infection

Student's t-test found only 19 SSDA proteins, with 10 proteins increased in abundance in response to *E. coli* infection in wild worker fat body samples when compared to wild ringer controls, and 9 proteins decreased in abundance in the same (Figure 4.12A; Table A4.5a). All SSDA proteins in this comparison are shown in Table 4.5.

Two immune related proteins were found to be increased in abundance in response to *E. coli* infection in wild *B. terrestris* fat bodies. These were an anti-microbial peptide (defensin precursor) and a serine protease inhibitor (leukocyte elastase inhibitor). Also increased in abundance were two proteins involved in cuticle formation (glycine-rich cell wall structural protein and mycosubtilin synthase), and two proteins relating to actin binding and the cytoskeleton (actin-related protein 2 and F-actin-capping protein). The remaining proteins increased in abundance in the wild *E. coli* fat bodies were involved in calcium binding, heme-binding and nucleotide biosynthesis. TopGO found enrichment in this increased in abundance group in 'aromatic compound biosynthetic process' (Table A4.8a) and STRING analysis found enrichment in 'regulation of secondary metabolic process' (Table A4.7a).

Two proteins that were decreased in abundance in response to *E. coli* infection in wild bumblebees were linked to spliceosome complex and RNA binding (U6 snRNA-associated Sm-like protein LSm6 and splicing factor 3B). Two additional proteins were involved in protein processing, targeting and folding (mitochondrial intermediate peptidase and prefoldin). The remaining proteins decreased in abundance in response to *E. coli* infection in wild bees are associated with several different processes (Table 4.5), ranging from circadian rhythms (protein takeout) to transcription (DNA-directed RNA polymerase II).

Neither proteins increased nor decreased in abundance in this comparison were associated with any specific KEGG pathway. Blast KOALA results do not show much distinction in the general processes increased in abundance in wild *E. coli*-infected bees verses those in wild control samples given the small number of proteins in each group, although the ringer fat bodies have a higher proportion of proteins involved in genetic information processing while E. coli samples have the presence of proteins involved in environmental information processing and organismal systems (Figure 4.13 A - B).

Table 4.5 Top 10 SSDA proteins in *E. coli* and Ringer treated *B. terrestris* fat body proteome comparisons. The fold change, accession number and name of the top 10 SSDA proteins increased and decreased in abundance in *E. coli* treated and Ringer control wild and commercial fat bodies, ranked from highest intensity to lowest. Relative fold change reflects the mean protein LFQ intensity difference between exposed and control fat body proteomes. Fold changes highlighted in orange represent proteins increased in abundance in *B. terrestris* fat bodies in *E. coli* treated bees and those in blue represent proteins increased in abundance in the ringer control fat bodies.

	Wild *E. coli* – Wild ringer			**Commercial *E. coli* – Commercial ringer**		
	Fold change	Accession no.	Protein name	Fold change	Accession no.	Protein name
↑	4.64	XP_012165421.1	Mycosubtilin synthase	5.69	XP_012165421.1	Mycosubtilin synthase
	4.18	XP_003399040.1	Calcineurin	3.34	XP_003399319.1	Palmitoyl-protein thioesterase
	3.84	XP_003397327.1	Leukocyte elastase inhibitor	3.24	XP_003397327.1	Leukocyte elastase inhibitor
	2.58	XP_012176389.1	Actin-related protein	3.08	XP_003402120.1	H/ACA ribonucleoprotein complex
	2.53	NP_001267838.1	Defensin precursor	2.22	XP_020724355.1	Uncharacterized protein LOC100649724
	2.3	XP_012175644.1	Uncharacterized protein LOC105667096	1.56	XP_003400735.1	Congested-like trachea protein
	1.51	XP_003402123.1	Cytochrome b5	1.32	XP_003393042.1	Uncharacterized protein LOC100645359
	1.48	XP_020721219.1	Glycine-rich cell wall structural protein	1.25	XP_003401193.1	60S ribosomal protein
	1.21	XP_012164469.1	F-actin-capping protein	1.24	XP_003395599.1	Threo-3-hydroxyaspartate ammonia-lyase
	1.2	XP_020722313.1	Uridine 5-monophosphate synthase	1.23	XP_003398167.2	Hypoxia up-regulated protein
↓	-3.01	XP_003397291.1	Protein takeout	-3.88	XP_003394087.2	Hsp90 co-chaperone Cdc37
	-2.42	XP_012167997.1	DNA-directed RNA polymerase II	-1.67	XP_012165287.1	Uncharacterized protein LOC100648321
	-2.05	XP_012176317.1	U6 snrna-associated Sm protein	-1.55	XP_003397695.1	Peroxiredoxin-6
	-1.98	XP_003396719.1	Prefoldin	-1.46	XP_020720722.1	Glutamate--cysteine ligase catalytic
	-1.31	XP_003400665.1	Vacuolar protein sorting-associated protein	-1.44	XP_012163601.1	Glutathione S-transferase
	-1.31	XP_020721776.1	Splicing factor 3B subunit 4	-1.27	XP_012172650.1	Poly(rc)-binding protein
	-1.22	XP_003397437.1	Mitochondrial intermediate peptidase	-1.26	XP_012166085.1	Ubiquitin carboxyl-terminal hydrolase
	-1.11	XP_003401313.1	Actin-related protein 1	-1.21	XP_012163407.1	Ubiquitin-conjugating enzyme
	-1.09	XP_012170950.1	GMP synthase	-1.21	XP_012173007.1	Phospholipase A-2-activating protein
				-1.17	XP_012165957.1	Cat eye syndrome critical region protein 5

(f) Proteomic response of commercial B. terrestris fat body to E. coli infection

Student's t-tests found 15 SSDA increased in abundance proteins in response to *E. coli* infection in commercial bees, and 13 SSDA decreased in abundance proteins in response to the same (Figure 4.12B; Table A4.5b). The significantly increased in abundance proteins were found by a STRING analysis (Table A4.7b) to be enriched in functions relating to the endoplasmic reticulum (e.g., FUN protein LOC100649724, protein transport protein Sec24C, protein disulfide-isomerase and endoplasmin), cytoplasmic vesicle (protein transport protein Sec24C, protein transport protein Sec31A and FUN protein LOC100649724) and regulation of secondary metabolic process (mycosubtilin synthase; Table 4.5). The serpin, leukocyte elastase inhibitor, is also significantly increased in abundance in commercial *E. coli* exposed bees alongside proteins associated with the ribosome (H/ACA ribonucleoprotein complex protein and 60S ribosomal protein L26). A TopGO analysis found similar terms enriched (e.g., 'endoplasmic reticulum organisation', 'COPII-coated ER to Golgi transport vesicle') but in addition found enrichment in 'melanin metabolic process' (Table A4.8c).

Significantly decreased in abundance proteins in commercial fat bodies in response to *E. coli* infection include those relating to proteolysis (protein krasavietz, phospholipase A-2-activating protein and ubiquitin-conjugating enzyme E2-17 kDa) and proteosome formation (26S proteasome non-ATPase regulatory protein). Two immune-related proteins are also decreased in abundance in response to *E. coli* infection in commercial bees, these being a peroxiredoxin and glutathione S-transferase, both involved in antioxidant activities. While STRING found no general enrichment among decreased in abundance proteins, TopGO found enrichment in these genes among a number of terms e.g., 'cellular oxidant detoxification' and 'peroxidase activity' (Table A4.8d). Another key process linked to decreased in abundance proteins appears to include thermosensory behaviour (poly(rC)-binding protein 4).

Blast KOALA found that in commercial *E. coli* samples, there were a higher proportion of SSDA proteins involved in genetic information processing while in control samples, there was a higher proportion of proteins linked with biosynthesis of other secondary metabolites and environmental information processing (Figure 4.13 C - D).

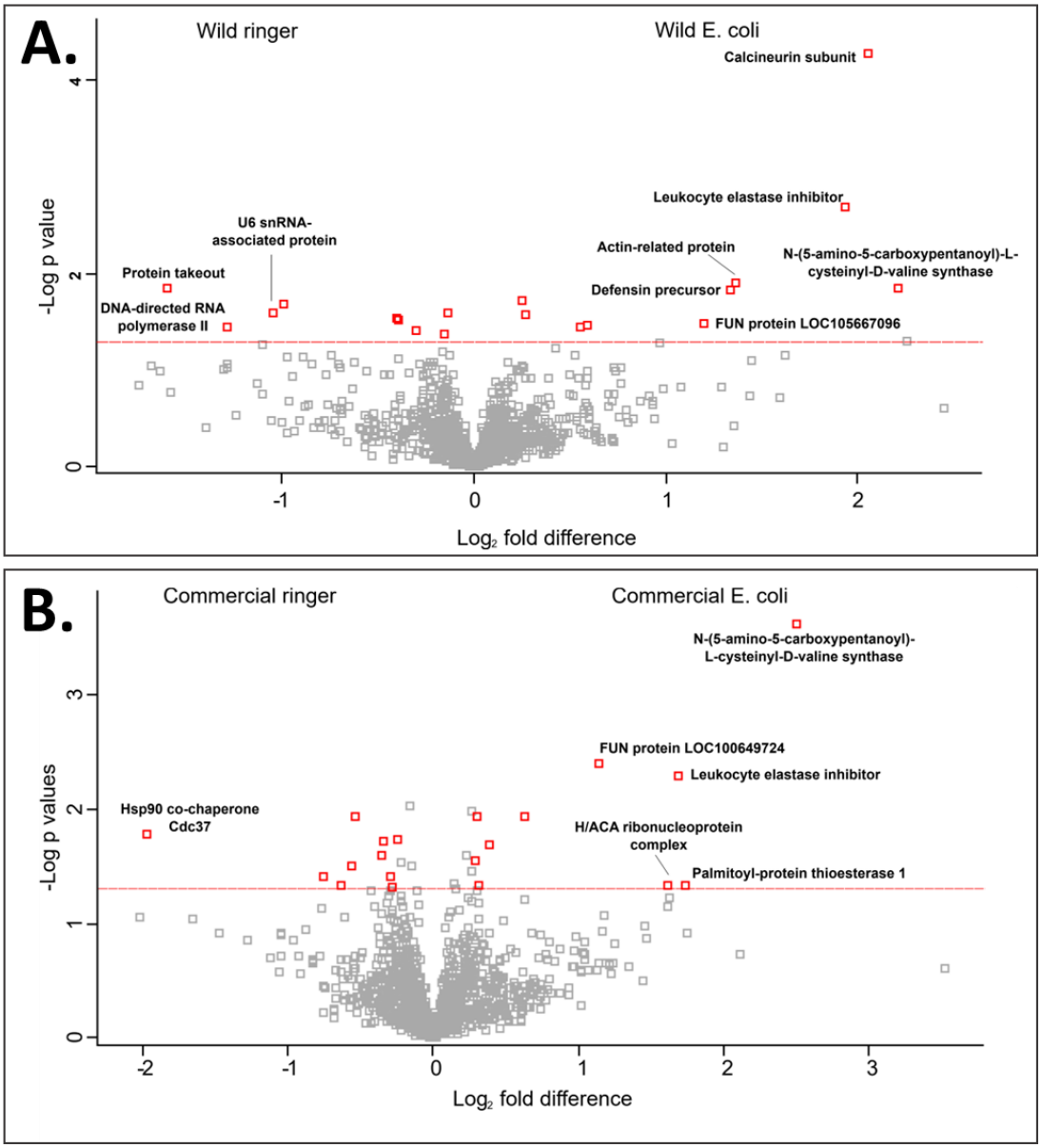


Figure 4.12 Differential abundance of proteins in (A) wild *E. coli* infected fat bodies compared with wild ringer control fat bodies and in (B) commercial *E. coli* infected fat bodies compared with commercial ringer control fat bodies. The $-\log_{10} p$ value and fold change ($\log_2$ mean LFQ intensity difference) are represented on the y- and x-axis respectively. All proteins above the red dashed line, indicating the cut-off point for significance ($p < 0.05$), are statistically significant differentially abundance proteins. Datapoints highlighted with red squares represent the top 10 most differentially abundant proteins in each group. Only those proteins with a relative fold change greater than 2 were annotated.

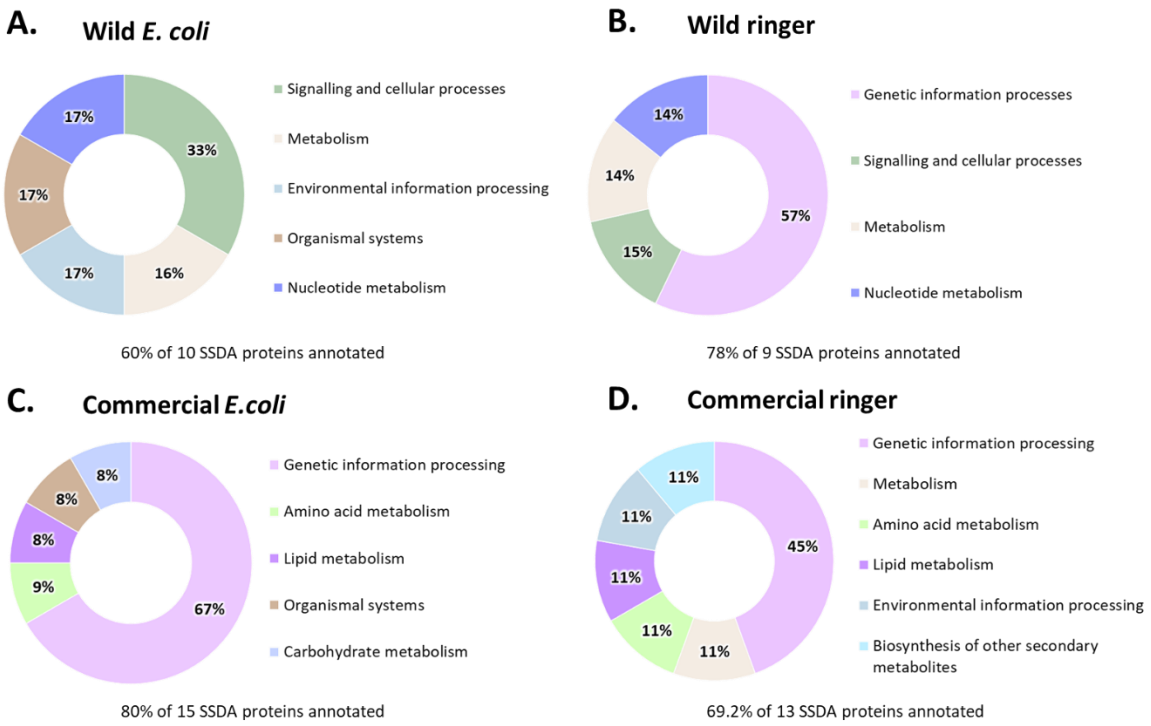


Figure 4.13 KEGG pathways enriched in all SSDA proteins increased in abundance within each group in the *E. coli* infected and ringer control comparisons. Relative proportions (%) of proteins annotated with various KEGG pathway categories in SSDA proteins increased in abundance (with % of annotated SSDA proteins) in (A) wild *E. coli* infected and (B) wild ringer control fat bodies in the WE – WR comparison and (C) commercial *E. coli* infected and (D) commercial ringer control fat bodies in the CE – CR comparison.

(g) Proteomic differences between wild and commercial B. terrestris fat bodies infected with E. coli

There was a total of 211 SSDA proteins when the commercial *E. coli* protein intensities were compared to wild *E. coli* protein intensities in a Student's t-test (Table A4.5c). Of these, 86 proteins were increased in abundance in the commercial fat bodies, and 125 were increased in abundance in the wild fat bodies (Figure 4.14 A).

TopGO analysis found several GO terms that were enriched among significantly increased in abundance proteins in commercial *E. coli* infected fat bodies in comparison to wild infected samples. These included: 'mRNA splicing, via spliceosome', 'mitotic cell cycle', 'determination of adult lifespan', 'fatty acid beta-oxidation', 'spliceosomal complex' and 'chromatin DNA binding' (Table A4.5e). STRING analysis found enrichment in similar terms e.g., 'mRNA metabolic process', 'RNA transport', 'chromatin' and 'antioxidant activity' (Figure 4.16; Table A4.7c).

The top ten most differentially abundant proteins increased in abundance in commercial infected fat bodies (Table 4.6) have functions relating to mRNA processing (polyadenylate-binding protein and serrate RNA effector molecule), antioxidant activity (peroxiredoxin) and nuclear transport (nuclear pore complex protein Nup50). Many more significantly increased in abundance proteins outside the top ten proteins also show involvement in mRNA processing, nucleocytoplasmic transport and amino acid metabolism. Blast KOALA found enrichment among proteins increased in abundance in *E. coli* infected commercial fat bodies in several pathways including fatty acid degradation, spliceosome, and nucleocytoplasmic transport (Figures A4.1 – A4.3).

Among the top ten proteins increased in abundance in wild *B. terrestris* fat body samples infected with *E. coli* (Table 4.6) were proteins involved in peroxisome functioning and detoxification (alpha-methylacyl-CoA racemase, D-aspartate oxidase, and probable serine hydrolase), immune effector processes (apidaicin) and intermembrane lipid transfer (alpha-tocopherol transfer protein). Among all SSDA proteins increased in abundance in *E. coli* infected wild fat bodies, were 26 proteins involved in proteolysis, 17 associated with carboxylic acid metabolic process and 22 involved in translation. These processes were reflected in STRING and TopGO enriched terms e.g., 'fatty acid oxidation', 'proteasome complex', 'peroxisome', 'cytoplasmic translation' and 'endoplasmic reticulum' (Table A4.7d, Table A4.8f). Other GO terms highlighted were 'circadian rhythm' (15 proteins) and 'toll signalling' (14 proteins), however the SSDA proteins in these two categories almost completely overlap. Blast KOALA found enrichment in several KEGG pathways, such as ribosome, protein processing in the endoplasmic reticulum, and proteasome (Figures A4.4 - A4.6).

When both groups are compared using Blast KOALA annotations, the commercial *E. coli* fat bodies show a higher proportion of genes associated with carbohydrate, amino acid and nucleotide metabolism whereas the wild *E. coli* group shows a much larger proportion of genes involved in genetic information processing (Figure 4.15 A - B).

(h) Proteomic comparison of wild and commercial control fat body samples

When commercial and wild ringer controls were compared with a Student's t-test, a total of 373 SSDA proteins were identified, with 107 proteins increased in abundance in the commercial control samples and 266 in the wild controls **(**Figure 4.14 B).

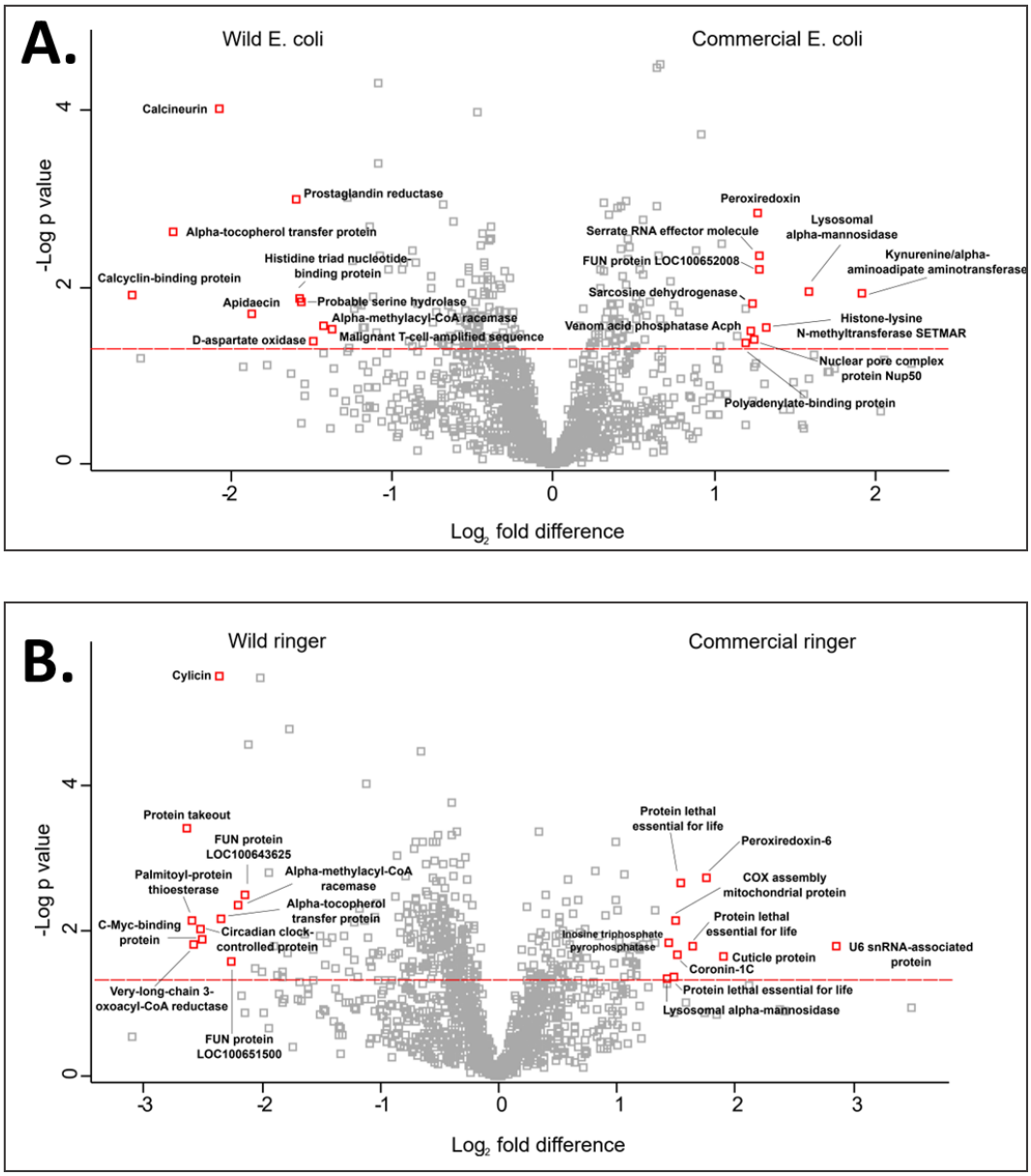


Figure 4.14 Differential abundance of proteins in (A) commercial *E. coli* infected fat bodies compared with wild *E. coli* infected fat bodies and in (B) commercial ringer control fat bodies compared with wild ringer control fat bodies. The -log10 *p* value and fold change (log2 mean LFQ intensity difference) are represented on the y- and x-axis respectively. All proteins above the red dashed line, indicating the cut-off point for significance ($p < 0.05$), are statistically significant differentially abundance proteins. Datapoints highlighted with red squares represent the top 10 most differentially abundant proteins in each group. Only those proteins with a relative fold change greater than 2 were annotated.

In the top ten proteins increased in abundance in commercial ringer control fat bodies, there were three protein lethal(2) essential for life proteins in the top ten most significantly differential proteins (Table 4.6). Other proteins in the top ten were linked to defence response to fungus (coronin-1C), lysozyme (lysosomal alpha-mannosidase), peroxidase functioning (peroxiredoxin) and the cuticle (cuticle protein 16.5). Overall, SSDA proteins increased in abundance in this group compared to wild controls showed involvement in cell development (17), carboxylic acid metabolic process (19) and cellular respiration (10). STRING enrichment analysis found this group to be associated with terms such as 'mitochondrion', 'cytoskeleton organisation', 'ATP metabolic pathway', 'TCA cycle' and 'myosin complex' (Table A4.7e). TopGO found similar terms including 'muscle structure development', 'mitochondrial respiratory chain complex assembly' and 'actin cytoskeleton' as well

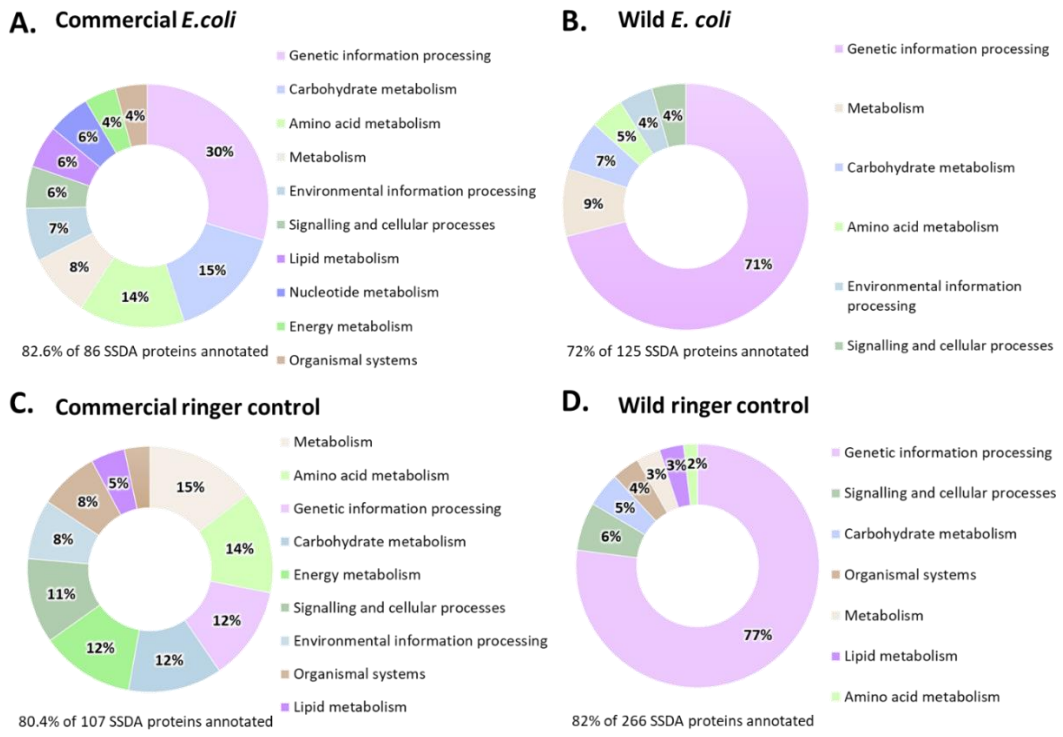


Figure 4.15 KEGG pathways enriched in all SSDA proteins increased in abundance within each group in the commercial – wild comparisons. Relative proportions (%) of proteins annotated with various KEGG pathway categories in SSDA proteins increased in abundance (with % of annotated SSDA proteins) in (A) commercial *E. coli* infected and (B) wild *E. coli* infected fat bodies in the CE – WE comparison and (C) commercial ringer control and (D) wild ringer control fat bodies in the CR – WR comparison.

as terms such as ‘peroxidase activity’ and ‘determination of adult lifespan’ (Table A4.8g). KEGG pathways that showed enrichment through Blast KOALA analysis included the TCA cycle, oxidative phosphorylation, and fatty acid degradation (Figures A4.7 – A4.9).

Key processes represented in SSDA proteins increased in abundance in wild controls when compared to commercial control fat bodies were peptide metabolic processes (83 proteins), translation (80 proteins), proteolysis (42 proteins), carboxylic acid metabolic process (24 proteins) and protein folding (16). These processes were reflected in both STRING and TopGO enrichment analyses, with terms such as ‘proteosome complex’, ‘peroxisome’, ‘translation’, ‘endoplasmic reticulum’ and ‘fatty acid metabolic process’ included in their outputs (Figure 4.17; Table A4.7f; Table A4.8i). Proteins in the top ten significantly differential proteins (Table 4.6) were specifically also involved in fatty acid metabolism (very-long-chain 3-oxoacyl-CoA reductase and palmitoyl-protein thioesterase), detoxification (alpha-methylacyl-CoA racemase), membrane transport (alpha-tocopherol transfer protein) and circadian rhythms (circadian clock-controlled protein and protein takeout).

TopGO also found enrichment in terms associated with ‘circadian rhythm’ (25 proteins) and ‘Toll signalling pathway’ (23 proteins), however all proteins with the ‘Toll signalling’ GO term also were annotated with the ‘circadian rhythm’ term. Blast KOALA found enrichment in among all SSDA proteins increased in abundance in wild control fat bodies in pathways such as ribosome, protein processing in the endoplasmic reticulum and proteosome (Figure A4.10-A4.12). Overall, Blast KOALA found proteins significantly increased in abundance in commercial control samples to be more involved in carbohydrate, amino acid and energy metabolic processes while wild fat bodies had a higher proportion of genes involved in genetic information processing (Figure 4.15).

Table 4.6 Top 10 SSDA proteins in wild and commercial *B. terrestris* fat body proteome comparisons. The fold change, accession number and name of the top 10 SSDA proteins increased and decreased in abundance in wild and commercial fat bodies, ranked from highest intensity to lowest. Relative fold change reflects the mean protein LFQ intensity difference between exposed and control fat body proteomes. Fold changes highlighted in orange represent proteins increased in abundance in *B. terrestris* fat bodies commercial fat bodies and those in blue represent proteins increased in abundance in wild fat bodies.

	Commercial *E. coli* – Wild *E. coli*			**Commercial ringer – Wild ringer**		
	Fold change	Accession no.	Protein name	Fold change	Accession no.	Protein name
↑	3.75	XP_003393516.1	Kynurenine/alpha-aminoadipate aminotransferase	7.28	XP_003401541.1	U6 snrna-associated protein
	2.99	XP_012176736.1	Lysosomal alpha-mannosidase	3.74	XP_003394953.1	Cuticle protein
	2.49	XP_012173827.2	Histone-lysine N-methyltransferase SETMAR	3.41	XP_003397695.1	Peroxiredoxin
	2.42	XP_012167732.1	Uncharacterized protein LOC100652008	3.12	XP_012170000.1	Protein lethal essential for life
	2.42	XP_012169739.1	Serrate RNA effector molecule	2.91	XP_003393098.1	Protein lethal essential for life
	2.4	XP_003397695.1	Peroxiredoxin	2.85	XP_012173067.1	Coronin-1C
	2.36	XP_012175066.1	Nuclear pore complex protein Nup50	2.82	XP_012175898.1	COX assembly mitochondrial protein
	2.35	XP_020724225.1	Sarcosine dehydrogenase	2.81	XP_012175695.2	Protein lethal essential for life
	2.33	XP_012170162.1	Venom acid phosphatase Acph	2.73	XP_003400266.1	Inosine triphosphate pyrophosphatase
	2.29	XP_020719246.1	Polyadenylate-binding protein	2.7	XP_012176736.1	Lysosomal alpha-mannosidase
↓	-6.11	XP_003394355.1	Calcyclin-binding protein	-6.21	XP_003397291.1	Protein takeout
	-5.12	XP_012168727.1	Alpha-tocopherol transfer protein-like	-6.01	XP_003399319.1	Palmitoyl-protein thioesterase
	-4.2	XP_003399040.1	Calcineurin	-5.96	XP_003402957.1	Very-long-chain 3-oxoacyl-coa reductase
	-3.65	XP_012175465.1	Apidaecin	-5.72	XP_003392987.1	C-Myc-binding protein
	-3.02	XP_020721705.1	Prostaglandin reductase	-5.64	XP_012167652.1	Circadian clock-controlled protein
	-2.97	XP_003397417.1	Histidine triad nucleotide-binding protein	-5.11	XP_012163313.1	Cylicin-1
	-2.94	XP_003394244.1	Probable serine hydrolase	-5.05	XP_012168727.1	Alpha-tocopherol transfer protein
	-2.8	XP_003402118.1	D-aspartate oxidase	-4.79	XP_003400408.1	Uncharacterized protein LOC100651500
	-2.67	XP_003394455.1	Alpha-methylacyl-coa racemase	-4.59	XP_003394455.1	Alpha-methylacyl-coa racemase
	-2.59	XP_003393639.1	Malignant T-cell-amplified sequence	-4.38	XP_020721756.1	Uncharacterized protein LOC100643625

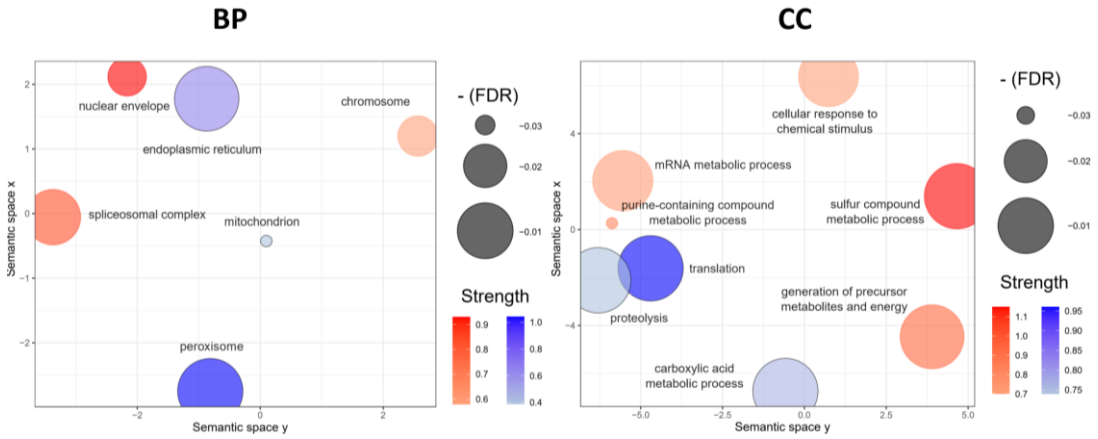


Figure 4.16 Revigo plots showing STRING GO terms associated with increased and decreased in abundance SSDA proteins in the *E. coli* infected commercial and wild *B. terrestris* fat bodies. GO terms associated with SSDA proteins increased in abundance (represented by red circles) and decreased in abundance (blue circles) in *E. coli* infected commercial fat bodies when compared with *E. coli* infected wild fat bodies. Size of circles represent the false discovery rate (FDR) multiplied by -1. The intensity of the colours reflects the strength of the enrichment effect is (Log10(observed / expected). Separate plots have been made for each GO term category: (A) Biological processes and (B) Cellular components.

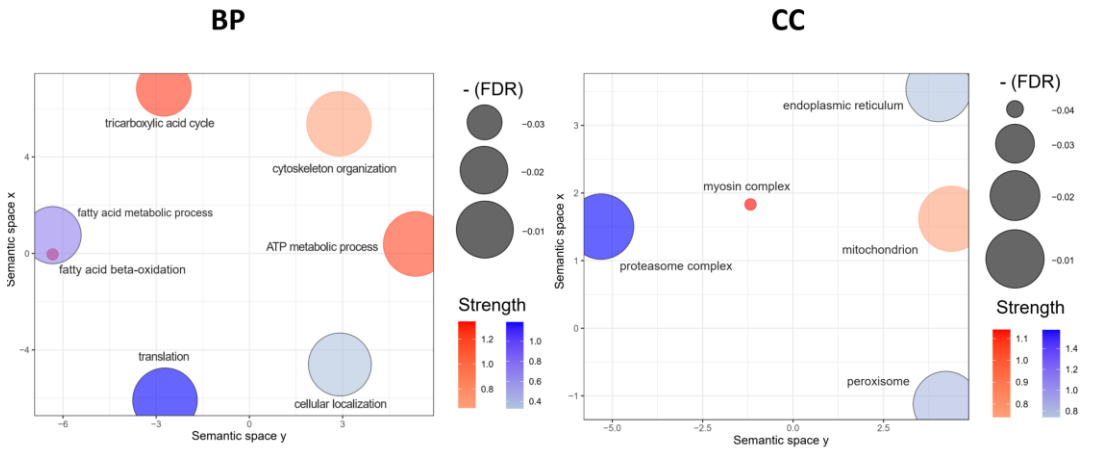


Figure 4.17 Revigo plots showing STRING GO terms associated with increased and decreased in abundance SSDA proteins in the commercial and wild ringer control fat bodies. GO terms associated with SSDA proteins increased in abundance (represented by red circles) and decreased in abundance (blue circles) in commercial control fat bodies when compared with wild control fat bodies. Size of circles represent the false discovery rate (FDR) multiplied by -1. The intensity of the colours reflects the strength of the enrichment effect is (Log10(observed / expected). Separate plots have been made for each GO term category: (A) Biological processes and (B) Cellular components.

4.4 Discussion

Understanding how wild and commercial bumblebees differ on a proteomic level will enable the identification of cellular and physiological traits associated with wild populations and those that are undergoing domestication. This will not only provide data on the evolutionary processes of domestication but will also potentially inform risk assessments of the possible impacts that commercial colony use and escape could have on wild pollinator populations e.g., hybridisation and the loss of advantageous alleles through introgression. This is essential if we are to continue to protect wild pollinators already facing declines (Goulson *et al.*, 2008). Furthermore, as much of bumblebee research is carried out on commercial colonies, it is important to investigate whether commercial colonies are representative of wild colonies in how they operate physiologically.

To investigate the distinctions between wild and commercial *B. terrestris* bumblebees, we carried out a proteomic analysis on two key bumblebee organs: the brain and fat body. We also explored the proteomic responses of these organs to two known stressors of bumblebee health: insecticide exposure and infection. Although many insecticides do not directly cause mortality in bumblebees, they can cause sublethal impacts on bee fitness e.g., foraging efficiency (Morandin *et al.*, 2005), colony growth (Mommaerts *et al.*, 2006), homing ability (Matsumoto, 2013) and immunity (Czerwinski and Sadd, 2017). We would expect that wild bumblebee populations are much more likely to be under selection to better handle insecticide exposure than commercial bumblebees and so may have developed physiological adaptations to detoxify or minimise the damage caused by such exposure. The increase of pathogen spread is also a serious threat to pollinator health (Colla *et al.*, 2006; Goulson *et al.*, 2008; Graystock *et al.*, 2013; Murray *et al.*, 2013). Wild bumblebees face infection and parasitism from a wide range of taxa. Commercial colonies are also known to carry pathogens such as *Crithidia bombi, Nosema bombi* and *Apicystis bombi* and can pass these to wild populations of bumblebee through their continued exportation (Colla *et al.*, 2006; Murray *et al.*, 2013). Wild bumblebees are likely exposed to a greater diversity of pathogen and parasite taxa and/or strains than commercial bumblebees are. We therefore expect that wild bumblebee immune systems have evolved under a different, possibly more complex set of selective pressures than the

immune systems of recent generations of commercial bumblebees. Indeed, we see in Chapter 3 that wild Irish *B. t. audax* appear to be experiencing a variety of selection pressures acting on their canonical immune genes. This could create a distinction in innate immune responses to a pathogen to which they are naïve (*E. coli*). In this study, we found that wild and commercial *B. terrestris* bumblebee workers have major proteomic distinctions in their brains and fat bodies, both endogenously and when they respond to neonicotinoid exposure and bacterial infection.

4.4.1 Shared and contrasting responses of wild and commercial B. terrestris bumblebees to 10ppb acute clothianidin exposure

Both wild and commercial bumblebee workers exhibited proteomic changes in their brains in response to 10ppb acute clothianidin exposure, while there were no observable impacts on neither wild nor commercial bumblebee behaviour, mortality or general fitness. We note that neither the different treatment groups or the wild or commercial status of the sampled bees could clearly separate samples in the PCA, which demonstrates the high levels of variability across samples in all groups. Many factors could potentially influence this observed variance across samples despite the workers being age controlled and treatment conditions being identical; for example, differences in physical size, designated task within their origin colony, success level or stage of origin colony life cycle and general health of origin colony.

However, in spite of the variability in samples, there were some commonalities in the responses of wild and commercial workers to clothianidin exposure. For example, both wild and commercial brains exposed to clothianidin showed a reduction in nuclear proteins (e.g., nucleolins), some antioxidant proteins (thioredoxin and glutaredoxin) and copper chaperones. Honeybee queens show a similar decrease in thioredoxin in response to both imidacloprid and coumaphos (Chaimanee *et al.*, 2016). However, in this study, honeybee workers had increased levels of this protein in response to the same treatments, implying that honeybee workers are more resilient to pesticide exposure than their queens. While wild and commercial *B. terrestris* may have specialised methods of detoxifying pesticides, as we will discuss later, the reduction in these particular proteins could indicate some disruption in metabolic and detoxification pathways as a result of neonicotinoid exposure.

Similarly, changes in copper chaperone levels may have impacts on copper availability and transport, which could impact bumblebee resilience as several antioxidant enzymes require copper to function e.g., superoxide dismutase (Husak, 2015). Also of note was the decreased amount in both wild and commercial clothianidin-exposed brains of the protein ubiquitin-40S ribosomal protein S27a which is involved in regulation of synapse numbers and memory formation. These may be molecular signatures that link to previous research findings on the negative impacts of insecticide exposure on learning and memory (Williamson and Wright, 2013; Stanley *et al.*, 2015; Piiroinen and Goulson, 2016; Tan *et al.*, 2017; Siviter *et al.*, 2018; Smith *et al.*, 2020; Siviter and Muth, 2022; but see Piiroinen *et al.*, 2016; Muth and Leonard, 2019). Also increased in both wild and commercial bumblebee brains exposed to clothianidin was chromobox protein, a component of heterochromatin. Chromobox protein homolog 5 has been implicated in response to DNA damage in humans (Gilmore *et al.*, 2016) and so an increase of abundance of this protein may indicate DNA-level stress due to neonicotinoid ingestion.

However, there were numerous distinctions also in the responses of wild and commercial bumblebee worker brains to clothianidin exposure. First, there is a larger change in commercial bumblebee brains in response to exposure when compared to the wild response, as indicated by volcano plots (Figure 4.4). This may an increased sensitivity to clothianidin exposure in commercial bees that they need to mount a larger response to combat this stressor. Wild workers, which are more likely to be under selection in the wild to cope with insecticidal threats, and so a smaller log-fold of protein changes could indicate they have a more specialised and effective response to counteract this exposure.

Choline ethanolamine kinase was increased in abundance in wild exposed *B. terrestris* workers but down in commercial exposed workers (but, as will be discussed later, was higher in commercial *B. terrestris* control brains than wild *B. terrestris* control brains). Choline ethanolamine kinase has a major role in phosphtidylcholine synthesis, an essential structural component of cell membranes of neuronal and glia cells (Adibhatla and Hatcher, 2007) and has been shown to enhance neuronal differentiation (Montaner *et al.*, 2018). The human ortholog of this gene are associated with intellectual disability and muscular dystrophy (Mitsuhashi and Nishino, 2013; Gramates *et al.*, 2022). The contrasting increase in abundance in wild exposed bees and decreased amount in

commercial exposed bees indicates, in this instance, an opposite response of these bees to clothianidin exposure. Impacts of these changes on fitness, behaviour and cognitive abilities should be investigated in the future to see if either response impacts resilience to neonicotinoid exposure.

In wild *B. terrestris*, proteins relating to processes such as gene expression, membrane synthesis and transport were increased in abundance in response to clothianidin exposure. Increases in protein levels associated with transcription, mRNA processing and splicing suggests that wild bees increase their capacity to regulate gene expression with likely consequences on the suite of genes being expressed and the levels to which they are expressed. Wild bees also had increased levels of phenoloxidase in response to clothianidin exposure. Interestingly, phenoloxidase levels have been shown to be lowered in the haemolymph of commercially-reared *B. impatiens* workers in response to 7ppb of imidacloprid (Czerwinski and Sadd, 2017). Gregorc *et al.* (2012) also found that prophenoloxidase-activating enzyme transcripts were elevated in *A. mellifera* larval responses to certain fungicides and insecticides. This suggests phenoloxidase may play a role in the brain's proteomic response to pesticide exposure in bumblebees or alternatively, could also be an indicator of stress.

Clothianidin exposure significantly increased levels of proteins involved in phosophotidylcholine biosynthesis such as choline ethanolamine kinase, choline transporter and 1-acyl-sn-glycerol-3-phosphate acyltransferase being increased in exposed wild *B. terrestris*. Phospholipids are key in membrane structure but also contribute to key cellular signalling processes that regulate a wide variety of cell processes (Vance and Vance, 2002). High-affinity choline transporter is a cell-membrane transporter involved in the movement of choline into acetylcholine-synthesising neurons. Choline ethanolamine kinase is also key in biosynthesis of phospholipids and is responsible for the biosynthesis of phosphocholine (Vance and Vance, 2002). It has been found previously that sublethal doses of clothianidin increase the levels of phospholipids such as phosphatidylcholine in the brains of honeybees, linking such changes in lipid composition to impacts on self-grooming behaviour (Morfin *et al.*, 2022). In contrast to our results, high affinity choline transporter decreased expression in response to thiacloprid in honeybee brains (Gregorc *et al.*, 2012). We hypothesise that in wild bees, clothianidin exposure may impact or alter the synthesis and, possibly, the functioning of these important

membrane components. Two proteins (mycosubtilin synthase and cysteine sulfinic acid decarboxylase), similar to the *Drosophila* cuticular pigmentation proteins Ebony and Black, were also increased in abundance in the wild bumblebee response to clothianidin. Both these proteins have been shown in *Drosophila melanogaster* to interact in the biogenic amine pathways (Gramates *et al.*, 2022). Mutants of these proteins interfere with pigmentation, vision and circadian rhythms of locomotor behaviour (Heisenberg, 1971; Wright, 1987; Newby and Jackson, 1991). This response is interesting as it has been found that neonicotinoids disrupt sleep and the circadian rhythms in *A. mellifera* (Tackenberg *et al.*, 2020).

Further proteins with elevated abundances in response to clothianidin exposure in wild *B. terrestris* brains were involved in translation and protein transport. There were also three eukaryotic translation factors decreased in abundance as well as a ribonucleotide protein in wild bees in response to clothianidin exposure, implying that there may have been a reduction in the capacity for or levels of protein synthesis in response to this insecticide in wild bees. The decrease of a eukaryotic translation factor has also been seen in *A. mellifera* nurse brains in response to a variety of pesticides (Zaluski *et al.*, 2020). We also see decreased abundances in two nucleolin proteins, found in the nucleolus, which are involved in cell proliferation, ribosomal biogenesis and apoptosis (Chen *et al.*, 1991; Ginisty *et al.*, 1998; Ginisty *et al.*, 1999). The decreased abundance of a protein similar to *A. mellifera* protein quiver may indicate changes in synaptic transmission and the regulation of sleep in wild bees as a result of neonicotinoid exposure (MacDougall *et al.*, 2020).

Processes that were represented in the commercial bumblebees' response to clothianidin exposure were detoxification, synapse organisation, signalling pathways and membrane transport. In commercial exposed bees, there was an increase abundance of detoxification proteins such as cytochrome P450 monooxygenase proteins, but also a down-regulation in proteins such as catalase, superoxide dismutase, glutathione-S-transferase and thioredoxin. Cytochrome P450 monooxygenase proteins are essential in the regulation of hormones, fatty acids and steroids as well as the metabolism of drugs and pesticides (Scott, 1999). They have also been linked with pesticide resistance (Yang *et al.*, 2018; Zhang *et al.*, 2022). This suggests that commercial bumblebees upregulate certain detoxification enzymes to combat pesticide exposure, while other detoxification proteins may be decreased. This

may be a method to optimise the detoxification response or perhaps the reduction in certain antioxidant proteins indicates stress in response to pesticide exposure. The reduction in certain antioxidant proteins in clothianidin-exposed commercial workers bees may consequently increase oxidative stress and reduce the resilience of these commercial bees to insecticide exposure. The decrease in abundance of these likely very useful proteins may be a result of relaxed selection, or the reduction of natural selection, on commercial lines whose fitness and reproduction in captive settings no longer depends on their ability to weather these agrochemicals in wild ecosystems (Mignon-Grasteau *et al.*, 2005).

Clothianidin in commercial workers caused increases in proteins associated with neuronal development (for instance, in mushroom bodies), scaffolding and neurotransmission (Nitta and Sugie, 2017), suggesting that neonicotinoid exposure has caused changes in neuronal structure and functioning. This may have downstream impacts on key traits that contribute to fitness such as learning and memory as has been observed in behavioural studies of insecticide impacts on bees (e.g., Williamson and Wright, 2013; Stanley *et al.*, 2015; Siviter and Muth, 2022) or may provide some restructuring that can compensate for neonicotinoid-induced impacts.

Clothianidin exposure increased the levels of several membrane transport proteins, including two that transport cationic or proton-coupled amino acids. The transport of amino acids to and from cells aids amino acid homeostasis and downstream processes such as translation, nerve transmission and regulation of cell growth (Castagna *et al.*, 1997). Other transporters such as sodium/hydrogen exchanger play important roles in cell cycle regulation, salt tolerance and regulation of intracellular pH (Ma and Haddad, 1997; Brett *et al.*, 2005). Levels of this exchanger were also shown to be increased in tobacco leafworm in response to fluralaner insecticide exposure (Jia *et al.*, 2020). The increased amount of these proteins may increase the commercial worker bees' capacity for salt and ion homeostasis, so that important processes, such as translation and the cell cycle, can be maintained in the face of pesticide exposure. Proteins associated with transcription and mRNA splicing were also increased in response to clothianidin exposure in commercial bees. Of these, C-Myc-binding protein may regulate the Myc transcription factor, which appears to contribute to cell growth and proliferation (Furrer *et al.*, 2010). A highly conserved pre-mRNA-processing-splicing factor that is highly involved in the spliceosome (Stanković *et al.*, 2020) was increased too in

response to clothianidin exposure, as well as a vacuolar sorting-associated protein possibly associated with transcriptional coregulator activity. Similarly, the increased levels of key regulators of gene expression such as splicing factors may help to sustain these important processes in a stressful cellular environment or may lead to alternative splicing that changes protein structure, function or localisation (Stamm *et al.*, 2005).

Several proteins related to neurodegeneration were also decreased in abundance after clothianidin exposure in commercial bees. For example, prosaposin is a precursor of sphingolipid activator proteins (saposins) which are essential in metabolism of sphingolipids and glycosphingolipids, important membrane components of eukaryotic cells (Ito, 2007). When lost in *Drosophila*, it is associated with neurodegeneration and reduced life expectancy (Sellin *et al.*, 2017). Sirtuins similarly are involved in processes relating to neurogenerative diseases such as protein aggregation and stress responses (Donmez, 2012). The *Drosophila* homolog of heat shock protein 75 kDa, Trap1, is a mitochondrial chaperone protein that regulates processes such as redox homeostasis, oxidative stress-induced cell death and apoptosis (Ramos Rego *et al.*, 2021). Loss of Trap1 causes an increased sensitivity to neurodegeneration as a result of oxidative stress and mitochondrial disfunction (Costa *et al.*, 2013). Target of rapamycin Lst8 was also decreased in abundance. Mutant Lst8 in *Drosophila* causes defects in cell growth, as it consists of a core component in TOR complexes that are involved in the key TOR pathway, essential regulators of lifespan and cellular growth (Katewa and Kapahi, 2011). The reduction in these proteins in commercial bumblebee worker brain proteomes indicates that exposure to clothianidin increases the likelihood of neurodegeneration in these bees.

Furthermore, levels of several proteins involved with protein biosynthesis, folding and degradation are decreased in response to clothianidin exposure in commercial brains. These include ribosomal proteins which both act as structural constituents of ribosomes, and a prefoldin, a protein that enables binding to unfolded proteins. An E3 ubiquitin-protein ligase, an E2 ubiquitin conjugating enzyme and two peptidases are lowered in abundance, suggesting processes relating to protein ubiquitination and degradation are being impacted by exposure to this neonicotinoid.

4.4.2 Distinctions between the brain proteomes of wild and commercial bumblebees

There were several consistent differences between wild and commercial bumblebee brains regardless of treatment. Consistently increased in abundance in commercial *B. terrestris* brain proteomes were proteins potentially involved in biogenic amine pathways, detoxification, and cytoskeletal organisation. Wild bumblebee brains had increased levels of proteins associated with mitochondrial transmembrane transport, electron transport chain, regulation of actin cytoskeleton and nuclear export.

The previously mentioned enzyme mycosubtilin synthase, similar to Ebony in *Drosophila* and therefore possibly implicated in pigmentation processes and photoreceptor activity (Newby and Jackson, 1991), was consistently increased in commercial bumblebees when compared to wild. This difference is intriguing as Ebony in *Drosophila* has roles in behavioural rhythmicity. In *Drosophila*, the *ebony* gene is expressed cyclically in circadian rhythm and is thought to play a role in glial cell control of dopaminergic functions (Suh, 2008). As commercial bumblebees may be under relaxed selection in their captive rearing environments and may have less exposure to the regular cyclic cues provided by natural environments (e.g., temperature shifts associated with day-night cycle), it is possible they require increased levels of this protein to maintain their circadian rhythms. Alternatively, as both the wild and commercial bumblebees used in this experiment were reared in a lab-setting in constant darkness, is possible wild *B. terrestris* removed from external, natural cues had an impacted circadian rhythm compared to commercial bumblebees which had been captively reared for many generations. Furthermore, the protein cysteine sulfinic acid decarboxylase similar to *Drosophila* protein Black, discussed earlier, was significantly higher in abundance in wild exposed bees compared with commercials. If these proteins act similarly in bumblebees as they do in *Drosophila*, it is possible there are fundamental differences in how pigmentation and circadian rhythms operate and are regulated in wild and commercial bumblebee brains.

Interestingly, commercial bumblebees also had consistently higher levels of certain antioxidant and detoxification proteins than wild workers, including a glutathione-S-transferase. Glutathione-S-transferase (GST) is associated with resistance to almost all major classes of chemical insecticides (Pavlidi *et al.*, 2018; Shi *et al.*, 2018); glutathione, the protein on which GST acts, also plays roles in metabolism of nutrients and regulation of cellular events (Wu *et al.*, 2004). In addition, when commercial

control brains were compared with wild control samples, commercial bumblebee brains contained higher levels of proteins such as thioredoxin, glutaredoxin and copper transport protein ATOX1 also shown to have antioxidant roles (Arnér and Holmgren, 2000; Holmgren, 2000; Hatori and Lutsenko, 2016). This suggests that commercial bumblebees may have an increased capacity for pesticide detoxification than wild bees. However, as mentioned earlier, when exposed to clothianidin these bees downregulate several detoxification enzymes, including a second glutathione-S-transferase. Furthermore, several proteins increased in wild exposed bees when compared to commercial exposed brains indicate differences in mechanisms of detoxification between both groups. For example, glutamate—cysteine ligase regulatory subunit, which is significantly higher in exposed wild workers than commercial workers, is a protein involved in glutathione biosynthesis. This pathway and the *Drosophila* homolog of this protein, GCS light chain, has been shown to be key in the defence against arsenic (Muñiz Ortiz *et al.*, 2009). The same protein in *Drosophila* has also been implicated in long term memory (Akalal *et al.*, 2011). Wild exposed bees also have an alcohol dehydrogenase (ADH), which has oxidoreductase activity, higher in abundance compared with commercial exposed. Future behavioural and mortality studies comparing wild and commercial bumblebees will help decipher the true impact these proteomic differences have on the overall fitness and insecticide resistance in these bees.

Control commercial bumblebee bee brains had higher amounts of phenoloxidase compared with control wild bees, however we saw an increase in phenoloxidase expression in wild workers in response to clothianidin exposure. This suggests the phenoloxidase system is more responsive in wild workers to insecticide exposure which may be an adaptation in wild bees that increases resistance to insecticides. Liu *et al.* (2009) found that butane-fipronil resistant diamondback moths (*P. xylostella*) had higher phenoloxidase activity than susceptible moths, possibly because increased levels of phenoloxidase can decrease cuticle penetration as well as increase immune ability (Wilson *et al.*, 2001). The higher endogenous levels of phenoloxidase in commercial bumblebee brains may have benefits for general fitness and immunity (Pauwels *et al.*, 2011).

Commercial bumblebees also had consistently higher levels of glycerol-3-phosphate phosphatase than in wild bees. In mammals, this protein hydrolyses glycerol-3-

phosphate into glycerol and, so, regulates levels of glycerol-3-phosphate (Mugabo *et al.*, 2016; Possik *et al.*, 2022). Glycerol-3-phosphate is an intermediate in many metabolic pathways e.g., glucose, lipid and energy metabolism (The UniProt Consortium, 2021). In addition, the product of this enzyme, glycerol, is used by organisms as an adaptation to stress e.g., freezing (Raymond, 1992; Storey and Storey, 2012) and hyperosmotic environments (Hohmann, 2002; He *et al.*, 2009). Although the impact of increased abundance of this enzyme in bumblebees is unknown, it's possible it may underly differences in the regulation of metabolic pathways, and potentially in adaptive responses, between commercial and wild *B. terrestris* bumblebees.

Many proteins increased generally in commercial bumblebees were involved in mitotic cytokinesis such as dynactin and myosin regulatory light chain (Matsumura *et al.*, 2001; Delcros *et al.*, 2006). Furthermore, filamin organises the F-actin cytoskeleton in many scenarios e.g., neuronal growth cones (Zheng *et al.*, 2011). Several tubulin proteins, important in several cellular processes such as mitosis, cellular migration and differentiation (Breuss *et al.*, 2017) were higher in abundance in control commercial bumblebees compared with wild. These distinctions suggest that wild and commercial bumblebees may have differences in the organisation of cytoskeletons in neural cells with possible impacts on how cell cycles are carried out and how neuronal connections are made. Adenylyl cyclase-associated protein 1, similar to *Drosophila* capulet, had higher levels in wild exposed bees than commercial bees. Capulet negatively regulates the assembly of actin filaments, and has been shown to be involved in processes such as axon guidance in photoreceptor cells (Marrone *et al.*, 2011). Wild control bees had increased levels of disco-interacting protein, also implicated in axon guidance, further suggesting that differences may exist between wild and commercial bumblebees in cytoskeletal regulation and organisation of neurons when they are exposed to insecticide and possibly, endogenously.

Proteins associated with the mitochondrial electron transport chain were also higher in abundance generally in wild bee brains compared with commercial bee brains, although control commercial bees had higher levels of cytochrome c oxidase copper chaperone than wild bees, a key protein in oxidative phosphorylation and generation of ATP in mitochondria (Stiburek and Zeman, 2010). These distinctions in protein levels suggest that there could be differences between wild and commercial

bumblebees in the processes that produces ATP and reactive oxygen species required for cellular signalling processes (Nolfi-Donegan *et al.*, 2020). Wild control bumblebee brains had comparatively higher levels of proteins associated with mitochondrial protein import also, suggesting wild workers may have a higher capacity than commercial workers to import products into the mitochondrial matrix. It has been suggested that the components involved in mitochondrial protein import are key regulators in response to physiological stress and reflect the energetic state of the mitochondria (Nargund *et al.*, 2012; Harbauer *et al.*, 2014). For example, in diabetic mice, proteins involved in mitochondrial importation processes had increased abundance in response to rising glucose levels, possibly to increase transport of proteins involved in oxidative phosphorylation and make this process more efficient (Wada and Kanwar, 1998). Further research into the implications of this difference between wild and commercial bumblebees is needed to elucidate the impacts on fitness and whether the increased amount of mitochondrial protein importers is an indicator of stress in control wild bees.

In addition, commercial exposed bees also had significantly higher levels of proteins associated with vesicles and vesicle traffic than found in wild exposed bees. One such protein was intersection 1, a highly conserved protein that plays a role in regulation of synaptic vesicles during neurotransmission (Pechstein *et al.*, 2010). Other vesicle related proteins included here were coatomer subunit gamma, which forms part of COPI vesicles that transport cargo to and from the Golgi apparatus and endoplasmic reticulum, and synaptojanin-1, involved in the uncoating of endocytic vesicles (The UniProt Consortium, 2021; Gramates *et al.*, 2022). This may indicate that commercial bees have a higher capacity for vesicle transport than wild bees when exposed to clothianidin. Exposed commercial bumblebees also had significantly higher intensities than exposed wild bumblebees of the ragulator complex protein LAMTOR4, a protein that is involved in the TOR pathway associated with cell growth, suggesting potential differences between wild and commercial bumblebees in how cell growth is regulated during this exposure.

When the proteomes of wild and commercial clothianidin exposed brains were compared, commercial bumblebees had several processes represented by proteins increased in abundance. These processes included DNA and RNA binding, detoxification proteins and cytokinesis (Weinhold *et al.*, 1990; Matsumura *et al.*,

2001; Delcros *et al.*, 2006; Furrer *et al.*, 2010; Zheng *et al.*, 2011; Baltz *et al.*, 2012). Control commercial *B. terrestris* brains had higher levels of DNA-directed RNA polymerase II than wild control samples, suggesting that commercials could have altered regulation of processes governing transcription and gene expression compared with wild *B. terrestris* bees.

In comparison with commercial bumblebees exposed to clothianidin, wild exposed bumblebees had significantly higher levels of proteins associated with translation and ribosome biogenesis, including translation initiation factor, ribosomal proteins and a signal-recognition particle which targets secretory proteins to the rough endoplasmic reticulum (The UniProt Consortium, 2021; Gramates *et al.*, 2022). This suggests an upregulation of translational processes or an increase in the capacity of translation in wild exposed bumblebees compared with commercial exposed bees, perhaps a method in wild bumblebees to counteract insecticide-induced stress. Proteins relating to translation were also increased in wild control samples compared with commercial control bees, suggesting that there are consistent distinctions in this process between these two groups. In addition, control wild bees also had higher expression of two proteins involved in spliceosome activities than seen in control commercial bees, which could result in alternative splicing activities of mRNA, suggesting that processes relating to gene expression differ in wild and commercial bumblebees even before they are exposed to an insecticide.

The significant elevation of phospholipid biosynthesis related proteins in clothianidin exposed wild bees such as high-affinity choline transporter, 1-acyl-sn-glycerol-3-phosphate acyltransferase, phosphatidylinositide phosphatase SAC2 and ethanolamine kinase suggest that there may be differences between how these important cell membrane components in the brain are responding to the clothianidin exposure in wild and commercial bees. Phospholipids and their biosynthesis are implicated in many human neurodegenerative diseases (Schwarz *et al.*, 2008; Dickson, 2019). In this way, it's possible these protein distinctions could indicate differences between wild and commercial bumblebees in how phospholipids are regulated and synthesised, which may have downstream impacts on the central nervous system and even behaviour (Morfin *et al.*, 2022). Increased levels of proteins associated with neurotransmission, twinfilin and phosphatidylinositide phosphatase SAC2 in wild bumblebees also implies that there may be differences in the regulation of

neurotransmission and signal transduction between wild and commercial bumblebees when exposed to clothianidin.

4.4.3 Shared proteomic responses to E. coli infection in wild and commercial fat bodies

When *E. coli* infected fat body samples were compared with fat bodies from bees injected with Ringer controls, it was found that very few proteins changed significantly in response to infection with the Gram-negative bacteria. This was supported by the PCA which displayed high sample variability and no clear distinction between the *E. coli* infection and control groups. This implies that wounding in these bees elicited a similar response to infection with *E. coli.* This may be due to the worker bees in this experiment pre-empting an infection upon wounding. *B. terrestris* has been shown to do this in another study by upregulating AMPs such as abaecin and defensin in response to sterile wounding (Erler *et al.*, 2011). In developing *Drosophila* larvae, sterile wounding was enough to elicit the development of lamellocytes (cells in the haemolymph which carry out encapsulation; Márkus *et al.* 2005).

Five proteins were found to increase in abundance in both wild and commercial bumblebees in response to *E. coli* infection. Leukocyte elastase inhibitor is a serine protease inhibitor (also known as a serpin). This protein is essential in the regulation of innate immune response and cellular homeostasis (Choi *et al.*, 2019). The increase in abundance of this protein likely indicates the need to protect cells from proteases secreted into the haemolymph that trigger signalling cascades of the innate immune response to combat infection (Torriglia *et al.*, 2017).

Another protein increased in response to *E. coli* infection in both groups was a catalytic subunit of serine/threonine-protein phosphatase 6. In *Drosophila*, protein phosphatase 6 (PP6) is a negative regulator of the c-Jun amino-terminal kinase (JNK) and AMP kinase (AMPK) signalling (Gramates *et al.*, 2022). The JNK pathway is essential for the infection-induced alteration of gene expression that facilitates the production of antimicrobial peptides (AMPS) and epithelial shedding (Kallio *et al.*, 2005; Zhai *et al.*, 2018) but may play a role in regulating apoptosis (De Smaele *et al.*, 2001). Also, AMPK, when activated, has been shown to be essential for viral infection (McArdle *et al.*, 2012). It may be that upregulating negative regulators of these pathways has a

protective role against invaders and stress-induced apoptosis. This is further supported by Chi *et al.* (2018), which found that another protein phosphatase, PPV, helps to regulate JNK activated apoptosis during wing development in *Drosophila*. Calcineurin, a calcium binding protein, has been shown to support the insect innate immune response in *Drosophila* larva by promoting a nitric oxide (NO) signal that initiates the production of AMPs (Dijkers and O'Farrell, 2007).

Cytochrome b5, a hemeprotein that is involved in the transfer of electrons in processes such as desaturation of fatty acids and reactions catalysed by cytochrome P450 (Porter, 2002). In *Anopheles gambiae* midguts, cytochrome P450 proteins have been shown to become differentially expressed in response to an *E. coli* and *S. aureus* challenges (Dong *et al.*, 2009). Furthermore, cytochrome P450s were also differentially expressed in response to *Plasmodium* parasite infection in *A. gambiae*, suggesting a likely role of cytochrome P450s and proteins that facilitate their activity in defence against infection (Dong *et al.*, 2006). Infectious agents and host defence responses may produce toxins such as reactive oxygen species which require host detoxification to protect cells and function (Mazet and Boucias, 1996; Dubovskiy *et al.*, 2008).

Mycosubtilin synthase was also increased among wild and commercial *B. terrestris* brains infected with *E. coli*. Interestingly, this was a protein that was upregulated in wild worker response to clothianidin exposure and was differentially expressed in wild and commercial worker brains (discussed earlier). This protein, similar to *Drosophila* Ebony, is likely involved in pigmentation, development of the visual system and circadian rhythm of locomotory behaviour (Newby and Jackson, 1991; Suh, 2008). This protein and its functioning in bumblebees would be an intriguing avenue to explore in future research to understand its role in both immunity and resistance to insecticides.

Both wild and commercial bumblebees had decreased levels of protein takeout in response to *E. coli* infection. This protein belongs is predicted to have juvenile hormone (JH) binding activity which may, like other juvenile hormone binding proteins, may facilitate the distribution of this hormone to target cells and tissues and may implicate it in developmental and reproductive processes (Kim *et al.*, 2017). When protein takeout is mutated in *Drosophila*, disruption is seen in male reproductive behaviour (Dauwalder *et al.*, 2002), starvation responses and circadian

rhythms (Sarov-Blat *et al.*, 2000). In honeybees, JH is involved in caste differentiation and division of labour amongst workers (Robinson, 1987; Schulz *et al.*, 2002). In *B. terrestris*, JH is involved in reproduction and acts in tissues such as fat body and ovaries (Amsalem *et al.*, 2014; Shpigler *et al.*, 2014), and has also been shown to have impacts on aggressive and dominant behaviours (Pandey *et al.*, 2020). Knowing this, a reduction of protein takeout may impact reproductive activities and other processes regulated by juvenile hormone.

4.4.4 Differential proteomic responses of wild and commercial fat bodies to E. coli infection

While the PCA did not show a clear distinction between *E. coli* and Ringer control treatments, it very clearly separated wild and commercial fat body groups. In terms of differences in the response to *E. coli* infection, wild workers had a small set of other proteins that showed differential expression in response to *E. coli* infection. Several of these were involved in actin filament regulation, suggesting changes to cytoskeletal organisation in response to Gram-negative bacteria infection. Actin-regulating proteins have changed in expression in response to pathogen infection in *Sarcophaga bullata* (Masova *et al.*, 2010) and *Drosophila melanogaster* (Vierstraete *et al.*, 2004). Changes in cytoskeletal component regulation of fat body cells may facilitate the exocytosis of molecules associated humoral defence such as antimicrobial peptides (Shandala and Brooks, 2012), but may also alter cell to cell communication within the fat body itself (Ugrankar *et al.*, 2022). Antimicrobial peptides (AMPs) are key effectors in the immune response of insects to many pathogens. Defensin, one such insect AMP, was increased in abundance in the wild response to *E. coli* infection. This protein has been observed to be active against Gram-positive bacteria, *E. coli* and certain fungi (Lowenberger *et al.*, 1995; Rees *et al.*, 1997; Lee *et al.*, 2004). Defensins are highly potent to certain bacteria as it is thought they can create channels within bacterial cytoplasmic membranes (Cociancich *et al.*, 1993).

Nucleic acids, RNA and DNA, are made up of nucleotides, and the production and metabolism of nucleotides by hosts is required by pathogens such as viruses to support their own growth and replication (Wang *et al.*, 2016). Uridine monophosphate synthetase is involved in pyrimidine biosynthesis, and so an increase in the levels of this protein in wild fat bodies in response to *E. coli* infection may upregulate the

production of these nitrogenous bases to the benefit of either the wild *B. terrestris* hosts or the invader. Bacteria may have co-evolved to stimulate hosts to upregulate nucleotide biosynthesis to support their own growth, or hosts upregulate these processes to maintain important cellular processes during infection. *B. bombysepticus* infection in silkworms is associated with a similar upregulation of nucleotide metabolism (Huang *et al.*, 2009). Among the few proteins with decreased abundances in wild *B. terrestris* in response to infection with *E. coli* were proteins involved in splicing, transcription and folding. Cells in the wild *B. terrestris* fat body may alter gene expression, mRNA transcripts and post-translational modifications of proteins to counteract pathogen infection.

Commercial bumblebees had a similarly reduced proteomic response in their fat body to *E. coli* infection. The commercial fat body cells seemed to respond to *E. coli* infection by encouraging protein biosynthesis, decreasing protein degradation, and increasing the folding and/or transport of proteins in the endoplasmic reticulum. Interestingly, certain detoxification proteins, such as peroxiredoxin and glutathione S-transferase, were decreased in abundance in response to *E. coli* infection in commercial bumblebees, suggesting that the infected fat body has a reduced capacity for antioxidant and detoxification processes. Peroxiredoxins can also regulate the immune response of *Drosophila*, through inhibition or activation of key signalling molecules (Radyuk *et al.*, 2010). A decrease in the abundance of these proteins may, therefore, facilitate the signalling pathways that result in the production of effector molecules.

Several of the proteins increased in commercial bumblebees infected with *E. coli* were secretory proteins involved in the formation of the coat protein complex II (Bonifacino and Glick, 2004). Coat-protein complex II (COPII) facilitates the selective vesicular transport of proteins to the Golgi apparatus (Duden, 2003). A LAMP protein was also increased in abundance, involved in protein localisation but also more generally, LAMP proteins have roles in autophagy and lipid homeostasis (Chaudhry *et al.*, 2022). The endoplasmic reticulum is essential in cellular homeostasis through processing and folding of certain proteins involved in secretory pathways or those destined for the membrane. When the protein processing requirements become too much for the ER, it can trigger stress in the ER and the accumulation of unfolded proteins (Celli and Tsolis, 2015). The response to this stress, the unfolded-protein response (UPR) is an

adaptation organisms employ to survive this stress. For example, the UPR has shown, to initiate the immune deficiency (IMD) pathway in insects (Sidak-Loftis *et al.*, 2022). Characteristics of the UPR include increasing the capacity of ER protein folding, which may explain the increase in abundance of secretory proteins. In addition, a heat shock protein, endoplasmin, was also increased in abundance. This protein is a member of the HSP90 family, that has ATPase activity and is involved in the chaperoning of proteins within the endoplasmic reticulum to facilitate correct folding and secretion of, for example, insulin-like-growth factor (IGF). The IGF pathways is both pro-growth and anti-apoptosis, and is also involved in the response to endoplasmic reticulum stress (Novosyadlyy *et al.*, 2008). Usually the UDR is also accompanied by a reduction in mRNA translation (Walter and Ron, 2011), however, in this case commercial bumblebee fat bodies exhibit an increase in certain translation-related proteins such as ribosomal proteins in response to *E. coli* infection. Additionally, the ubiquitin dependent protein catabolic pathway appears decreased, suggesting proteins biosynthesis is increased while, in parallel, protein degradation is decreased. It is possible the commercial bumblebees are only beginning to exhibit signatures of the UDR at this timepoint post-infection. Future research could address this query by characterising both wild and bumblebee responses to infection over a longer timeframe and examine the rapidity of certain immune responses.

4.4.5 Consistent proteomic differences between the fat bodies of wild and commercial B. terrestris workers

Many of the proteomic distinctions between wild and commercial fat bodies were consistent whether exposed to *E. coli* infection or treated with Ringer solution, indicating that there are strong endogenous differences between the fat body protein composition of wild and commercial bumblebees. Consistently increased in abundance in commercials when compared with wilds were proteins representing several key processes: cellular redox homeostasis, fatty acid oxidation and the TCA cycle. Commercial bumblebees had consistently higher levels of three peroxiredoxins and a superoxide dismutase. This implies that, at least in terms of the reactions these proteins participate in, commercial bumblebee fat bodies may have a higher capacity for detoxification of reactive oxygen species that wild bumblebee fat bodies.

Enrichment in mitochondrion-related cellular components and the increase in abundance of proteins involved in fatty acid metabolism, the TCA cycle and one linked to the electron transport chain suggests that the processes within mitochondria to generate ATP are increased in commercial fat bodies compared with wild. Fatty-acid beta-oxidation supplies acetyl-CoA to the TCA cycle, and the TCA cycle can consequently supply the electron transport chain with reducing agents, $FADH_2$ and NADH (Alberts *et al.,* 2015). Interestingly, the wild fat bodies had other proteins linked to the TCA cycle and fatty acid oxidation increased in abundance when compared to commercial fat bodies in both treatments, suggesting that wild and commercial bumblebees regulate these pathways differently. Interestingly, wild bumblebees had fatty-acid oxidation associated proteins that were enriched for the cellular component peroxisome. Peroxisomal beta-oxidation of fatty acids mainly acts on substrates such as very long chain fatty acids and branched fatty acids, while mitochondrial fatty-acid oxidation tends to act on long chain fatty acids; Of the latter, the intended output is usually energy production, while in peroxisomes the breakdown of fatty acids contributes to specific fatty acid biosynthesis (Demarquoy and Le Borgne, 2015). It is possible that wild fat body cells have an increased capacity for biosynthetic pathways driven by peroxisomal fatty acid metabolism than commercial bumblebees. Similarly, commercial bumblebee fat body cells may have an increased ability to produce energy from mitochondrial based fatty acid oxidation than do wild bees. Interestingly, studies into domesticated species such as Atlantic salmon (*Salmo salmar*) found that domesticated varieties have altered gene expression compared with the wild variety, showing increased transcripts of genes associated with glucose and lipid metabolism alongside faster growth (Jin *et al.*, 2020). It is possible that we are seeing something similar with wild and commercial bumblebees which may have consequences on their fitness, nutrition, growth and health. Further research into the metabolic differences of domesticated and wild population of *B. terrestris* as well as the potential consequences of this will shed light not only on how these populations differ, but also on the impacts of evolutionary shifts in central metabolic pathways on the health and fitness of bumblebees.

Many proteins involved in proteolysis were consistently increased in abundance in wild bumblebees compared with commercial bumblebees regardless of treatment. Proteolysis is the process where proteins are broken down by proteases into their

amino acids components (Alberts *et al.*, 2015). Large protein complexes in the cytosol and nucleus called proteasomes use ATP to bind ubiquitin-tagged proteins, unfold and degrade proteins into short chains by funnelling them into a central core lined with proteases (Alberts *et al.*, 2015). In addition to its role in metabolising proteinaceous food sources, proteolysis is a very important regulatory system within the cell (Wolf and Menssen, 2018). For example, cell cycle progression is driven by proteolysis (King *et al.*, 1996). Proteolysis is essential also to degrade proteins that are misfolded or damaged. Importantly, the circadian rhythm is regulated by proteolysis. Several key clock proteins in *Drosophila* have been shown to be regulated through degradation by proteasome-mediated proteolysis (Gallego and Virshup, 2007). Transcripts associated with proteolysis were also shown to be expressed in a bimodal pattern over a period of 24hrs in Antarctic krill (*Euphausia superba),* which exhibit daily, cyclical behavioural patterns e.g., migration up and down the water column. The large amount of proteolysis associated proteins increased levels of abundance in wild bumblebee fat bodies compared with commercials suggests that wild bees have an increased ability to maintain cellular and protein cycles compared with the captive-bred variety (De Pittà *et al.*, 2013). Commercial bumblebees may not need to retain adaptations that regulate circadian rhythms to continue to survive and reproduce in commercial facilities. If these traits do not contribute to fitness, the processes underlying them may therefore be downregulated.

Wild *B. terrestris* worker fat bodies seem to have a strong signature of upregulation in translational processes, with many proteins including translation initiation factors, elongation factors and ribosomal proteins expressed at significantly higher intensities than those in commercial fat bodies of both treatments. This may indicate a higher level of protein production in wild fat body cells. Furthermore, wild fat bodies show increased levels of proteins associated with protein folding also, suggesting processes relating to post-translational protein modification are increasing in capacity, perhaps to deal with an increased level of protein biosynthesis compared with commercial bumblebees. Interestingly, it has been shown that *Glyphodes pyloalis* increases its expression of proteins involved in protein translation in response to heat stress, perhaps due to an increase in damage and misfolding of proteins (Liu *et al.*, 2017). Larva of *Temnothorax* upregulate genes associated with translation also as protein biosynthesis is an important process during larval growth and development (Gstöttl *et*

al., 2020). These studies imply the importance of upregulation protein biosynthesis in cells where there is a high demand for proteins to fulfil important processes or to counteract a decrease in protein quantity or quality. With potentially higher levels of proteolysis in wild *B. terrestris* fat bodies, possibly due to circadian rhythms maintained through selection in wild populations, perhaps there is a requirement of wild fat body cells to increase protein production to maintain an acceptable level of protein turnover for the upkeep of cellular processes.

Wild *B. terrestris* ringer control samples, compared with commercial control samples, had further increases of many of the consistently distinctive expressional patterns described above, including translation, protein folding and proteolysis further supporting the hypothesis that these processes operate or are regulated differently in wild and commercial *B. terrestris* bumblebees. Similarly, commercial controls showed further increase of expression of proteins involved in the electron transport chain and ATP synthesis when compared to wild control samples. Interesting, commercial control samples also had increased abundance levels of proteins involved in glutamine family metabolism. Glutamine metabolism is key in fundamental cellular processes such as the TCA cycle (Altman *et al.*, 2016) and so this potential upregulation compared with wild control samples may be linked to the increase of ATP synthesis associated proteins. Overall, this implies that commercial *B. terrestris* may generally have higher levels of ATP production than wild *B. terrestris* workers and, therefore, the many cellular processes that depend on ATP may operate more efficiently. This may be a response to injury and could impact the speed of wound healing in wild and commercial bumblebees in both ringer and *E. coli* treatments, but further research is needed to confirm this. Furthermore, differences in energy production, protein biosynthesis and subsequent regulation between wild and commercial bumblebees may underly general differences in behaviour such as reproduction (see Chapter 5).

When exposed to *E. coli* infection, one of the key commercial *B. terrestris* fat body cell processes represented by proteins increased in abundance in comparison to wild *B. terrestris* was nucleocytoplasmic transport. Nucleocytoplasmic transport refers to the transport of macromolecules between the nucleus and cytoplasm. Nuclear pore complexes form channels within the nuclear envelope that allows the movement of proteins and RNA molecules to and from the nucleus (Mirsalehi *et al.*, 2021). Commercial fat bodies had an increased abundance of four nuclear pore proteins

suggesting an upregulation of import and/or export of macromolecules from the nucleus of fat body cells (Terry *et al.*, 2007). A ran GTPase-activating protein and regulator of chromosome condensation (RCC1) were also both increased in commercial infected *B. terrestris* fat bodies compared with infected wild fat bodies. Both of these interact with Ran GTPase, an enzyme that regulates nuclear transport, the assembly of mitotic spindles and the cell cycle (Bischoff and Ponstingl, 1991; Seki *et al.*, 1996; Matunis *et al.*, 1998). The potential upregulation of these processes suggest that infected commercial bumblebee fat bodies have, when compared to wild *B. terrestris*, an altered regulation of nuclear transport and, potentially, cell replication processes.

Proteins linked to spliceosome and RNA splicing were also increased in abundance in commercial bumblebees infected with *E. coli* when compared to wild infected bumblebees. An increase in these proteins could mean that the fat body cells have an increased capacity for mRNA splicing compared with wild bumblebee fat body, perhaps causing distinction in splice variants of certain genes between these groups. The alterative of RNA splicing may also impact fitness of bumblebee workers dealing with a pathogenic infection. Splicing of various genes involved in the immune response could produce proteins better suited to defence against particular types of pathogens and illicit a stronger and more effective immune response. For example, the gene for Down syndrome cell adhesion molecule (Dscam) has thousands of splice variants and depending on the type of infection, different groups of splice variants can be expressed to combat infection (Dong *et al.*, 2006; Brites *et al.*, 2008). Domestication has been shown to impact alternative splicing in domesticated crop species (Ranwez *et al.*, 2017; Smith *et al.*, 2018; Yu *et al.*, 2020). This suggests domestication may have driven these possible distinctions in alternative splicing processing between wild and commercial *B. terrestris*. Future research into the impacts of an upregulated spliceosome processes on the immune response of bumblebees would provide essential information on the resilience of wild and commercial bumblebee to infection.

Among the proteins expressed at significantly higher intensities in wild, *E. coli* infected *B. terrestris* fat bodies than commercial were a peroxidase and an antimicrobial peptide (AMP), apidaecin. Peroxidases exhibit antioxidant activity and may confer some protection to wild bees against their own immune responses such as

melanisation (Nappi and Christensen, 2005). Wild bees may be more frequently exposed to parasites and subsequent melanisation responses than commercial bumblebees may increase selective pressures on antioxidant systems that protect cells from oxidative stress.

AMPs are generally known as the key effectors of the insect innate immune system (Li *et al.*, 2012). Apidaecin was first isolated from *A. mellifera* and is one of the major humoral components secreted into the haemolymph of honeybees when undergoing bacterial infection and are highly effective against the cell propagation of *E. coli* (Casteels *et al.*, 1989). It is also worth mentioning that the microbiome of insects can impact the expression of antimicrobial peptides. For example, in honeybees infected with *Nosema ceranae*, it was found that the presence of gut microbiota was associated with an increase in expression of apidaecin and other AMPs (Wu *et al.*, 2020). The response of wild bumblebees to express more apidaecin than commercial bumblebees in their fat bodies may indicate a specialised feature of immune response in wild bees that is adaptively selected for in the wild to effectively combat Gram-negative bacteria. Commercial bumblebees, in contract may not be exposed to many Gram-negative bacterial pathogens in captive rearing conditions, so perhaps there is relaxed selection acting on these systems. It is often seen that domesticated species lose anti-predator or anti-parasite traits due to predation or parasitism not imposing strong selective pressures on the genes linked to these characteristics in captivity or domesticated environments (Mikonranta *et al.*, 2012).

An enrichment was found in cytoplasmic stress granule among proteins with increased abundances in wild exposed *B. terrestris* fat bodies, compared with commercial. Environmental stressors such as temperature changes, oxidative stress and viral infections can cause eukaryotic cells to form stress granules (SGs; Anderson and Kedersha, 2006; Protter and Parker, 2016). The formation of stress granules is a method to mitigate damage resulting from these stressors and to support cell survival (Mahboubi and Stochaj, 2017). These non-membranous granules contain non-translating messenger ribonucleoproteins (mRNPs) and polyadenylated mRNAs and are shown to aggregate in cells in stressful conditions (Anderson and Kedersha, 2009). Stress granules can dynamically sequester these mRNPs to regulate protein biosynthesis (Ivanov *et al.*, 2019). This indicates that wild fat body cells could be experiencing stress from the bacterial infection and are forming these granules as a

method to counteract this stress by regulating translational processes. Again, this may be an adaptative response created through selective pressures in the wild population but would need further functional studies to confirm if this response is indeed happening.

Wild fat bodies also had higher expression of COP9 signalosome (CSN) associated proteins involved in protein deneddylation. This process involves the removal of a NEDD8 type ubiquitin like protein from another protein, usually Cullin proteins (Huntley *et al.,* 2015; Kim *et al.,* 2017). CSN regulates the ubiquitination activity of Cullin-Ring E3 ubiquitin ligases (Stratmann and Gusmaroli, 2012). CSN proteins have shown, through this activity, involvement in many cellular processes such as gene expression, lipid metabolism and anti-oxidant activity (Oron *et al.*, 2007; Nahlik *et al.*, 2010; Licursi *et al.*, 2014).

4.4.6 Differences between wild and commercial ringer treated fat body proteomes

Commercial control bees had significantly higher intensities of proteins associated with motor-related functions and cytoskeletal organisation. These included myosins, tropomyosins and troponins. Myosins, troponins and tropomyosins are typically associated with muscles, and so this may suggest that there was a higher proportion of co-extracted muscle alongside commercial fat bodies. However, some of these proteins are shown to respond to wounding and infection. For example, myosin, actin and tropomyosin were found to be increased in the fall armyworm moth (*Spodoptera frugiperda*) fat body cells post-injection with a virus (Provost *et al.*, 2011). Fat body cells are motile and, when the insect is wounded, fat body cells can migrate towards the site of wounding to assist with the sealing of the wound, local release of effector molecules and can work alongside macrophages to removed debris (Franz *et al.*, 2018). Myosin proteins help facilitate cellular contraction that helps fat body cells migrate to wound sites (Casano and Sixt, 2018). In mice, tropomyosin has been shown to be involved in cell migrations associated with wound healing (Lees *et al.*, 2013)

Also among the most significantly differential proteins that were higher in commercial control samples than wild controls were three protein lethal(2)essential for life proteins. These proteins belong to the heat-shock 20 protein family (HSP20; Gramates *et al.* 2022) and, like other heat shock proteins, are induced by stress and are involved

in chaperoning and protecting proteins. HSP20 proteins are important for heat tolerance in species such as *Bemisia tabaci* (Lü and Wan, 2011). HSP20 proteins increased in expression in *Drosophila* fly haemolymph in response to *E. coli* infection, suggesting immune stress can also increase expression of these processes. Some plant species exhibit upregulation of HSP20 proteins after wounding e.g., Swindell *et al.* (2007). Conversely, wounding in *Apis mellifera* was shown to decrease expression of heat shock proteins, but HSP20 proteins were not included in this analysis (McKinstry *et al.*, 2017). It is possible in this situation, HSP20 proteins are at higher concentrations in commercial bumblebees than wild bumblebees after being injected with Ringer solution to counteract the stress of wounding and may confer tolerance to commercial bees to this stress. Future studies should compare the proteomes of wild and commercial bumblebees in naïve and injury treatments to appropriately investigate the differential responses to wounding in these groups of bees.

4.4.7 Conclusion

B. terrestris workers of wild and commercial lineages reared in the same conditions have distinctive brain and fat body proteomes and these organs have differential proteomic responses to neonicotinoid exposure and pathogen infection, respectively. This could be an indication that commercial bumblebees have experienced physiological changes as a result of changed evolutionary pressures associated with the domestication process and their molecular responses to threats, while showing some commonalities, are likely not reflective of how wild bumblebees would respond in a natural setting. It follows that caution must be taken when extrapolating conclusions based on research using commercial bumblebees to a wild context. Furthermore, these proteomic distinctions in commercial bumblebees could impact how they would survive in the wild. These distinctions could be beneficial or disadvantageous. In either scenario, introductions of commercial bumblebees to a wild scenario could disrupt the ecological balance of wild populations e.g., through the addition of poorly adapted alleles to the gene pool or by outcompeting (or out-surviving) native pollinator populations.

Further research is required to fully characterise the consequences of the differential responses and proteomes of wild and commercial bumblebees on fitness and the

relative resilience against pesticide and pathogen exposure. Future investigations into the potential divergences between wild and domesticated groups of bumblebees should explore:

- circadian behaviours and their regulation at the expressional level,
- survival and/or recovery when exposed to higher doses of pesticide or more pathogenic infections,
- immune response to other types of representative microbial elicitors (e.g., fungi or Gram-positive bacteria, pathogenic and non-pathogenic strains) that may induce a general immune response (as shown by Larkin 2018),
- immune response to bumblebee specific pathogens and specific and locally found strains of those pathogens,
- temporal dynamics of the immune response,
- immune responses of different castes,
- distinctions between naïve and wounding responses,
- and whether these similar and contrasting responses are the same when colonies are place or established in the field.

Chapter 5

Colony growth dynamics and gut microbiome communities of lab-reared wild and commercial *B. t. audax* bumblebees

5.1 Introduction

Domestication can change the genetics, physiology and behaviour of an organism, and its interaction with other organisms including microbial symbionts, through three major genetic processes: genetic drift, inbreeding and artificial selection (Ollivier, 2002, as cited in Mignon-Grasteau *et al.*, 2005). These processes can create 'domestication syndromes' i.e., a set of traits found in domesticated populations which are not found in their wild counterparts. The formation of domestication syndromes can happen through conscious artificial selection for traits that are beneficial to humans. However, they can also happen through indirect and unintentional means. In particular, domestication has often included intentional genetic selection for increased production (Luiting, 1990). Many domesticated species are selected to grow and reproduce faster. For example, domestic chickens (*Gallus domesticus*) have undergone strong selection to have an increased rate of birth, where one hen can lay 300 eggs in a year (Bell *et al.*, 2001). Where wild populations often experience seasonal reproductive periods, domestication often causes changes to the timing of reproduction, and domestication syndromes in mammals frequently include shorter oestrous cycles (Lord *et al.*, 2013). Elements of honeybee lifecycles have also been altered through domestication, in particular certain strains of honeybee show a disinclination to swarm (Lecocq, 2018).

Understanding behavioural and symbiotic distinctions between wild and domesticated populations that have the potential to mix is important for effective risk assessment and risk mitigation that ensures conservation of native populations. One such example of this is in the case of bumblebees. *Bombus terrestris*, have a relatively recent domestication history, with commercial mass-rearing of colonies beginning just over 30 years ago (Velthuis and Van Doorn, 2006). Bumblebee colonies were sought by growers as bumblebees can buzz-pollinate crops such as tomatoes, saving growers from labour intensive and costly mechanical and/or hand pollination of plants (Van Ravestijn and Van der Sande, 1990). Commercial bumblebee rearing involves completion of the entire lifecycle of captive bumblebees in controlled conditions and, in addition, may employ methods to skip some stages such as diapause, which allows the production and availability of bumblebees year-round (Röseler, 1985; Van Heemert *et al.*, 1990; Velthuis and Van Doorn, 2006). These colonies are then exported for pollination services, often to countries beyond those in which they are

reared. Their use as pollination providers often leads to escape into the wild (e.g., Matsumura *et al.*, 2004) where they are known to establish populations and pose risks to wild pollinators, including pathogen spread (Graystock *et al.*, 2013), competition for nest and food resources (Ings *et al.*, 2006) and hybridisation with wild conspecifics (Cejas *et al.*, 2020).

Our findings in Chapter 2 indicate that commercial *B. t. audax* stocks likely originated from British *B. t. audax* populations. However, since then they may have been subjected to domestication-associated evolutionary pressures such as genetic drift and selection (conscious or otherwise). Although domestication of bumblebees is recent, domestication traits can occur in just a few generations, as seen in certain fish species (Blouin *et al.*, 2021). We anticipate that certain traits may be selected for by bumblebee rearing facilities that improve the quality, resilience, and lifespan of bumblebee colony products for the market. There have already been reports of selective breeding in captive bumblebee populations (Beekman *et al.*, 1999). The production of a high number of worker bees would also be an attractive trait to growers using these pollination products for their crops, as workers are the main foraging and pollinating force of the colony. The gene targets of these potential selective processes may impact behaviours that encourage colony initiation and growth in artificial settings. These traits will likely be distinctive from wild populations subjected to selective pressures shaped by wild environments.

There are some studies that could indicate the presence of domestication syndromes in commercial bumblebees. Commercial colonies have been previously shown to have higher nectar foraging rates than wild bumblebees in natural settings (Ings *et al.*, 2006). Furthermore, the same study found than commercial colonies had higher reproductive success i.e. produced more gynes than wild colonies did (Ings *et al.*, 2006). However, this study focussed on commercial bumblebees derived from subspecies *B. t. terrestris* and *B. t. dalmatinus*, compared with wild *B. t. audax*, and so may indicate subspecies distinctions rather than domestic traits. Other than this, research into domestication syndromes in bumblebees is lacking (Lecocq, 2018). In addition, certain traits that improve fitness in the wild may not be adaptive in a captive setting e.g., anti-predatory behaviours (Carrete and Tella, 2015), and so may be lost. This is seen in salmonid fish; when they are raised in a hatchery, they grow faster (Blouin *et al.*, 2021) but have poorer fitness when compared to wild counterparts when

both reproduce in a wild environment (Berntson *et al.*, 2011; Ford *et al.*, 2016). The loss of wild-adapted traits is worrying when considering commercial bumblebee colony use and frequent escape into the wild within areas of natural *B. terrestris* distribution, as they could hybridise with local, wild populations and dilute their adaptive traits, potentially triggering or exacerbating a decline in a population already facing stressors such as low resource availability and pathogen spread (Goulson *et al.*, 2008).

Domestication and captivity can also impact key fitness determinants of an organism, such as the symbiotic microbiome communities they host. Although heavily influenced by lifestyle and environmental inputs (Spor *et al.*, 2011), microbiomes can also be influenced by genetics and host genotype (Goodrich *et al.*, 2014; Bonder *et al.*, 2016). A study comparing the fecal microbiomes of wild, undomesticated Przewalki's horse (*Equus ferus przewalskii*) populations with domestic horses (*E. f. caballus*) found that the Przewalki's horse populations hosted a distinctive and more diverse fecal bacterial community than the domesticated population (Metcalf *et al.*, 2017). Similarly, overall alpha diversity of gut bacterial communities found in wild mice is higher than lab-reared mice (Bowerman *et al.*, 2021). Bee guts, like most animals, contain a community of microbes that influence the fitness of their host through impacts on digestion, nutrition and immunity (Saraiva *et al.*, 2015; Engel *et al.*, 2016; Mockler *et al.*, 2018; Rothman *et al.*, 2019; Daisley *et al.*, 2020). To our knowledge, only one study has been performed comparing wild and commercially reared *B. terrestris* bumblebee microbiomes. This study found that commercially-reared bumblebee workers, when restricted from foraging, have distinctive gut microbiomes compared with wild-caught bumblebee workers (Newbold *et al.*, 2015).

In this chapter, we compare wild and commercial colonies established and reared in an identical, lab-controlled setting. First, we aim to assess wild and commercial colonies for distinctions in colony establishment and growth in this lab setting. Successful colony establishment and rate of colony growth are key aspects of the bumblebee lifecycle essential for colony fitness as the success of these stages determine the colony's ability to forage for food, care for brood, regulate colony conditions and ultimately produce sexual individuals (Alford, 1975; Goulson, 2010a). Detailed observations and measurements of colony growth are a fundamental, yet time-consuming, method to assess bumblebee colony behaviour. For this reason, this

study's focus was limited to the colony establishment and initial growth phase. Furthermore, we examine differences between the gut microbiome communities of wild and commercial workers reared in identical conditions as changes to an organism's microbiome during the domestication process (due to environmental inputs or selective processes) may underly behavioural changes.

The findings of this study will shed light into how wild and commercial populations of *B. terrestris* differ in their colony development and microbial communities. Understanding the potential impacts of domestication on bumblebees and characterising the traits of commercial bumblebee varieties is essential in how we assess the risk of their use by growers in countries containing wild populations of *B. terrestris* and other pollinators. This is key if we are to support sustainable practices in crop and food production as well as continue protecting of wild and locally-adapted pollinator species.

5.2 Methods

To compare colony initiation and growth between wild and commercial lineages of *B. t. audax*, colonies were reared from wild-caught queens, and lab-mated commercial queens that underwent an artificial diapause (Table A5.1). Usually, the term 'gyne' is used to describe the female reproductive caste of colonies (destined to become queens) until she has founded a colony (and becomes a 'foundress') and rears workers (when she becomes a 'queen'). For simplicity, we use the term 'gyne' to refer to pre-diapause individuals, and 'queen' to describe post-diapause individuals undergoing colony initiation and rearing procedures, whether they produce workers or not.

5.2.1 Mating of commercial queens and artificial diapause

Four Koppert colonies were sourced from UniChem Ltd. (Co. Meath, Ireland) and randomly labelled 1 – 4. These colonies were fed pollen *ad libitum* (Biobest, Agralan Ltd.) and kept in the dark at approximately 24 +/- 1°C. For one month, 8th December 2020 - 6th January 2021, these colonies were monitored for the production of virgin gynes and males. These reproductive individuals were removed from their colonies

and inserted into plastic containers lined with sand and given 30% (w/v) sucrose solution and pollen (Biobest, Agralan Ltd.).

Gynes from one colony were selected to mate with males from another colony to ensure cross-colony mating. Matched gynes and males were released into a flight cage (either of 40cm x 60 cm x 40cm or 60cm x 90cm x 65cm dimension) together in batches at room temperature in daylight to encourage mating. Once a gyne and male were engaged in mating, the pair were removed and placed into a fresh plastic container where the colony origins and date of mating were recorded. In total, 52 gynes successfully mated with a male from a different colony. Mated gynes were allowed to rest in these labelled containers with access to sucrose and pollen for at least 24 hours and no longer than a week, except in the case of mated gynes KOPQ.51 and KOPQ.52, which mated and were put into diapause on the same day (11/01/21). At this stage, each mated gyne was placed into a 50ml falcon tube with air-holes drilled into the cap and sand (approximately 5ml) in the bottom. The falcon tubes holding an individual gyne each were wrapped with tinfoil (except the lid) to keep light out, placed upright in a falcon tube rack and stored in a cold room (2.5 +/- 1 °C, 850% =/-5% R.H.) All gynes were stored in these artificial diapause conditions between 80-90 days (Table 5.1).

5.2.2 Wild queen collections

A total of 59 wild queens were caught from five sites across Maynooth and Leixlip, Co. Kildare between the 25/02/21 and 24/03/21 (Table 5.2). While winter active gynes were seen during the month prior to collections, all queens caught after the 25/02/21, were considered to have gone through a winter diapause as at this point, as many of the individuals were carrying mites, a common trait of post-diapause queens. Queens were caught, usually while foraging, using a net and transferred to a collection tube. They were then transferred to colony initiation boxes (Figure 5.1) within the next two hours.

5.2.3 Colony initiation

The mated commercial queens were taken out of the cold room at a time where each had undergone between 80 - 90 days in this artificial diapause state. Wild *B. terrestris*

queens caught February – March 2020 were brought back to the lab. Both commercial and wild queens were treated in the same way from this point on. Sucrose consumption of colony boxes was measured once a week for the 15-week duration of monitoring. Each queen was placed into a small colony rearing box (Figure 5.1) with an upright pollen feeder, pollen bread (made with pollen and 50% sucrose) and an underneath sucrose feeder containing 50% (w/v) sucrose. Each box with a single queen was placed into a dark room at 29 +/- 1 °C and 50% +/- 10% R.H. They were given at least 48hrs to acclimatise to these conditions.

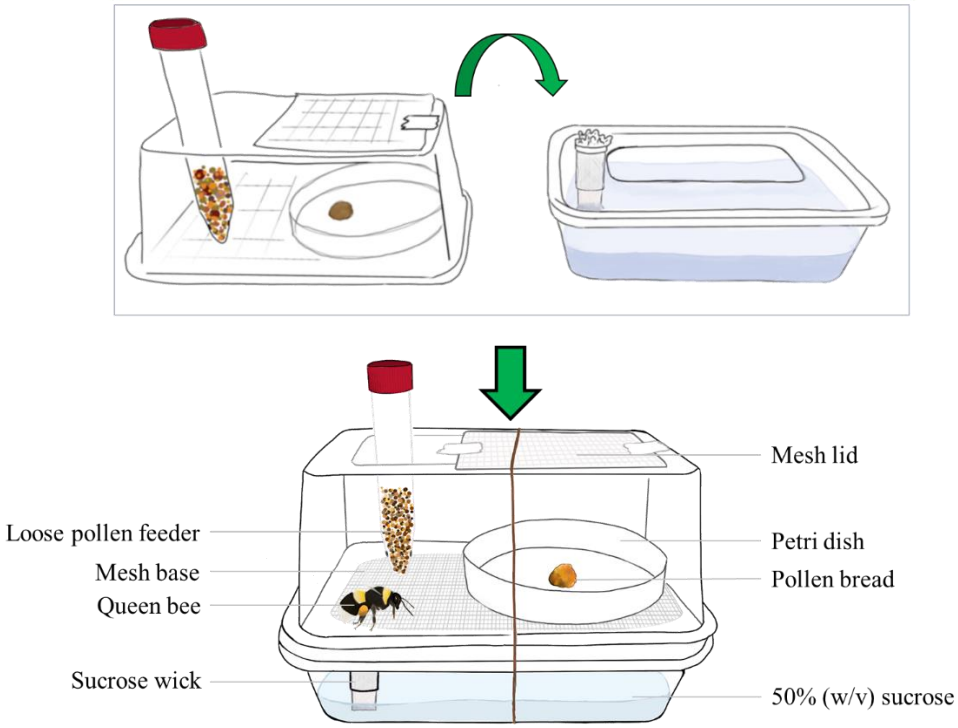


Figure 5.1 Set-up of the colony initiation box. The colony initiation box was comprised of a lower 50% (w/v) sucrose feeder box and an upper starter colony housing box. The upper colony initiation box had a mesh base, which allowed access to the sucrose contained underneath via a wick. There was also a square cut out of the top of this box to allow for observation and interaction with the colony, covered with a mesh lid. The upper box also had a vertical 15 ml tube with holes drilled in the bottom which functioned as a loose pollen feeder. A petri dish was placed on the mesh base at the opposite end of the upper box to the pollen feeder. On this petri dish, we placed a ball of pollen bread. The upper box was placed on top of the sucrose feeder box, and the two boxes were secured together with an elastic band. All wild-caught and commercial queens were each inserted into an individual colony initiation box and were later induced to lay eggs by the addition of two worker bees.

To initiate egg laying, two commercial *B. terrestris* workers were knocked out using CO_2 gas and their wings were cut to identify them as supplemented workers. If supplemented workers died prior to the queen's own workers emerging, these were replaced. Queens were monitored daily at this point. Pollen, pollen bread and sucrose were topped up as required. If wax moth larva began to appear, pollen in pollen feeders was removed and replaced with fresh pollen.

5.2.4 Colony growth and worker measurements

Queens were monitored daily. Notes were taken daily on appearance of egg batches, number of brood cells (larval and pupal cells) and if any new workers emerged. If a new worker had emerged in the previous 24 hours, the worker was removed from the colony, anaesthetised using CO_2 gas and given a unique identifier in the form of a numbered tag (Thorne; ABELO). The intertegular distance and thorax width of every new worker was also measured using a calliper. If a worker died and its tag was still attached, the code of the deceased worker was recorded. Once colonies reached 8-10 workers, brood, workers and queen were rehoused to a large colony rearing box (recycled Biobest colony box: 16cm x 22cm x 11cm; recycled Koppert colony box: 22cm x 22cm x 13cm; plastic container 15cm x 23cm x13cm) with supply of 30% (w/v) sucrose. Sucrose, pollen bread and pollen continued to be given as required. Colony growth continued to be monitored daily. Once a colony had reached 20+ workers, it was moved to temperature of 23-24°C as the colony, at this size, was considered better able to thermoregulate.

5.2.5 Data analysis of colony rearing data

All statistical tests were performed using R (4.2.1) on RStudio (v. 2022.12.0).

(a) Colony success and growth rates of wild and commercial colonies.

We calculated the number of workers produced by (worker-producing) colonies in the 60 days following the eclosion of their first worker. We defined colony initiation success as the production of at least one worker by a queen-right colony and examined differences between wild and commercial colony initiation success rates. Colony foundation success has been defined as the production of at least 10 workers (Gösterit

and Gurel, 2016). We assessed colony foundation success in wild and commercial groups with respect to this definition, but also analysed increasing levels of colony foundation success i.e., whether a colony produced at least 20, 30, 40, 50 and 60 workers. A colony with at least 50 workers is considered to be suitable to sell by commercial rearing companies (Gösterit and Gurel, 2016). Chi-squared tests were performed to analyse whether increasingly stringent definitions of colony foundation success differed between wild and commercial *B. terrestris* colonies whose queens had survived the first month after being introduced to the lab rearing environment. In both tests, Yates' continuity correction was included to account for low values in the data set.

To assess differences in growth rate between wild and commercial bumblebee colonies, worker-producing colonies that were observed for at least 50 days after the eclosion of their first worker and produced 10 or more workers were used to calculate mean growth rates (i.e. workers produced per day) for each colony in both the wild and commercial colony groups. Normality of the data was assessed using a Shapiro-Wilk test. The data were subsequently $\log_{10}$ transformed. A Welch's two sample t-test was performed to statistically test for differences in growth rates between wild and commercial colonies.

(b) Differences in mean worker sizes in wild and commercial colonies

Mean values for worker thorax width and intertegular (IT) distance were calculated for each colony in both commercial and wild groups. A Shapiro-Wilk test was used to confirm normality of the resulting data and a Welch's Two Sample t-test was carried out to test for difference between the wild and commercial mean thorax and IT measurements.

(c) Developmental times of the first worker produced by wild and commercial colonies

Developmental times for the first adult worker of each colony were estimated, in days, for two time periods:

- The combined length of the egg and larval stage, measured from the approximate date eggs were first laid until the first pupal cell appeared,
- And the length of the pupal stage, measured from the first appearance of a pupal cell to the first worker emergence.

A generalised linear model analysis using a Poisson regression was performed on number of days with colony type as an interaction to assess for differences in (1) combined egg and larval developmental periods, (2) pupal developmental periods and (3) total developmental periods of the first worker in each colony.

(d) Sucrose consumption rates in wild and commercial worker-producing colonies

Sucrose consumption was analysed for small colonies still in the initiation pods supplied with 50% (w/v) sucrose and bigger colonies that had been rehoused to large boxes supplied with 30% (w/v) sucrose. Mean sucrose consumption per week and mean numbers of workers in the colony in the same week were repeatedly measured for each colony. For all data collected from colonies in smaller (with 50% (w/v) sucrose) and bigger (with 30% (w/v) sucrose) colony boxes, tests were conducted for linearity, homoskedasticity, normality and multicollinearity to meet the assumptions of a general linear model analysis. A general linear model analysis was carried out on each group including colony type as an interaction to test whether wild or commercial colonies had different sucrose consumption rates per number of workers. Correlation was also tested using Pearson's correlation formula.

5.2.6 Microbiome sample collection

From reared wild and commercial colonies discussed in 5.2.1, workers aged between 10 – 15 days old were removed from their colonies with a forceps, immobilised using CO_2 gas and inserted into individual, labelled 1.5 ml tubes that contained a small piece of tissue paper to keep sample dry. Samples were then stored in a -70°C freezer until dissections could take place.

5.2.7 Digestive tract dissection

After freezing, each bee was removed from its 1.5 ml tube and the abdomen was cut to remove it from the rest of the body. The abdomen was placed with its ventral surface facing up on a wax plate. Using a dissection microscope, the abdomen was pinned at the top left and, with the abdomen stretched exposing the pleural membranes, in the muscle of the aculeus. With sterile scissors, an incision was made laterally along the top of the first sternum, between the fifth and sixth sternum and along the right-hand side of all sterna from posterior to anterior of the abdomen. The ventral portion of the abdomen was opened out using sterile tweezers and pinned to the wax plate. Ovaries and excess tissue were removed from the abdomen and the entire digestive tract was then removed and placed into a new, labelled 1.5ml tube containing 200µl Qiagen buffer ATL and frozen at -20°C freezer until DNA extractions could take place.

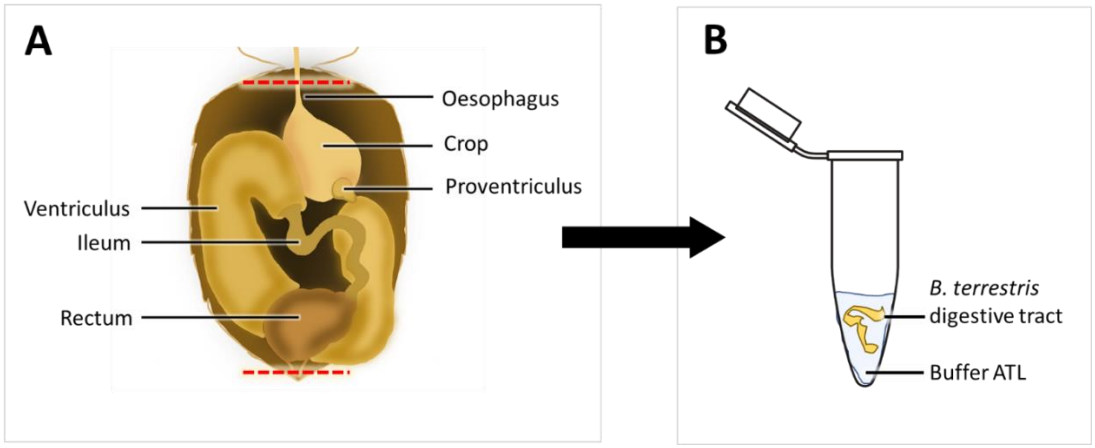


Figure 5.3 The dissection of the *B. terrestris* gut (A) The bumblebee gut within the abdomen as observed during ventral-view dissection. For each bee, the entire digestive tract between the dashed red lines was dissected out and was placed into (B) a 1.5ml tube containing 200µl of Qiagen Buffer ATL prior to DNA extraction.

5.2.8 DNA extraction

A QIAGEN DNeasy DNA extraction kit was used to extract microbial DNA from the guts of bumblebees. DNA extraction was performed on dissected guts stored in 200µl of Qiagen buffer ATL in 1.5 ml tubes (Figure 5.2). Approximately 1ml of lysozyme solution (10mg/ml) was prepared in advance of DNA extraction.

Each of the dissected guts stored in 200µl of Qiagen buffer ATL (furthermore referred to as samples) were removed from the -20°C freezer and were allowed to defrost at room temperature. Once defrosted, each sample was homogenised using a motorised grinder and a clean, autoclaved pestle. While leaving each pestle in its respective sample tube, 50µl of freshly prepared lysozyme (10mg/ml) was pipetted into each sample. Each sample was then mixed by hand with its respective pestle while ensuring all gut tissue was in solution. The pestles were then removed from each sample and the lids of each sample tube closed. Sample tubes were inserted into a pre-heated incubator at 37°C for 30 mins and were vortexed at every 5 mins during this incubation.

Once incubation was completed, approximately 300mg of acid-washed glass beads were added to each sample tube. 200µl of buffer AL was then added to each sample tube and tubes were vortexed. Each sample tube containing the glass beads and buffer AL were inserted into a bead beater and this was run at 30Hz for 5 mins. Once samples were removed from the bead beater, 20µl of Proteinase K was added to each. Samples were inserted into a preheated incubator at 56°C for 1 hour and were vortexed at the beginning and halfway through this incubation. Once incubation had completed, 200µl of chilled RNA pure Ethanol was added to each sample. Each sample tube was vortexed and glass beads were then left to settle at the bottom of each sample tube. From each sample, the supernatant was pipetted up, while avoiding the glass beads, and placed into a fresh, labelled Qiagen DNEasy Spin Column sitting in a fresh Qiagen collection tube. When all samples were loaded onto a labelled column, all columns were spun on a microcentrifuge at 8000rpm for 1 min. The supernatant and collection tube were discarded from each column and the column was placed into a new collection tube. 500µl of Qiagen Buffer AW1 was added to each column and all columns were spun again at 8000rpm for 1 min. Supernatant and column were discarded again, columns were placed in fresh collection tubes and to each column 500µl of Qiagen Buffer AW2 was added. All columns were spun for a further 3 min at 14000rpm. Supernatant and collection tubes were discarded, and each column was then inserted into a fresh, labelled 1.5ml.

Onto each column, 150µl of Qiagen buffer AE was added and columns were then allowed to incubate at room temperature for 1 min. All columns sitting in their

respective 1.5 ml tubes were then spun at 8000rpm for 1 min. This step was repeated with columns in fresh, 1.5ml tubes and 100µl of Buffer AE for increased yield.

5.2.9 PCRs and gel electrophoresis

To confirm that both bacterial and fungal DNA was extracted by DNA extraction protocol, a PCR on both 16S and ITS was carried out and run on 1% agarose gel at 130V for 30 minutes.

For the 16S PCR amplification, 515F (5'-GTGCCAGCMGCCGCGGTAA-3'; Sigma Genosys) and 806R (5'-GGACTACHVHHHTWTCTAAT-3'; Sigma Genosys) primers were used to confirm presence of bacterial DNA in the sample. In each 25µl reaction tube, 14.75µl of PCR H_2O (distilled water twice filtered using a 50ml Filtropur S 0.2µm sterile syringe filter head (Sarstedt)), 5µl of 5x Green GoTaq Buffer (Promega), 2.5µl of $MgCl_2$ (25mM; Promega), 0.5µl of dNTPs (10mM; Promega), 0.5µl of both forward and reverse primers (both 10mM) and 0.25µl of GoTaq (5U/µl; Promega) were combined with 1µl of extracted DNA from a bumblebee gut. 16S PCR reaction tubes were run on a Thermocycler as follows:

- Initial denaturation at 94°C for 5 minutes;
- 35 x cycles of:
    - 94°C for 1 minute,
    - 56°C for 1 minute,
    - 72°C for 1 minute;
- Final extension at 72°C for 10 minutes.

For the ITS PCR amplification, ITS 1F (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Sigma Genosys) and ITS 1R (5'- GCTGCGTTCTTCATCGATGC-3; Sigma Genosys) primers were used to confirm presence of fungal DNA in the sample. In each 25µl reaction tube, 12.05µl of PCR H_2O, 5µl of 5x Green GoTaq Buffer, 3µl of $MgCl_2$ (25mM), 0.5µl of dNTPs (10mM), 1.5µl of DMSO, 1µl of both forward and reverse primers (both 10mM) and 0.2µl of GoTaq (5U/µl) were combined with 1µl of sample DNA. ITS PCR reaction tubes were run on a Thermocycler as follows:

- Initial denaturation at 95°C for 3 minutes;

- 40 x cycles of:
    - 94°C for 30 seconds,
    - 50°C for 30 seconds,
    - 72°C for 30 seconds;
- Final extension at 72°C for 30 minutes.

These PCRs were carried out for all extracted gut DNA extraction.

5.2.10 Library preparation, sequencing and data analysis

18 samples of DNA extracted from digestive tracts of 9 individual wild *B. terrestris audax* and 9 commercial *B. terrestris* workers were sent to Novogene for amplicon-based metagenomic sequencing and data analysis. All samples underwent both 16S (v4) and ITS (ITS1-5F) amplicon-based sequencing to target bacterial and fungal organisms present in the gut microbiome. Novogene performed PCR amplification and generated libraries for each sample. Samples were sequenced on a NovaSeq 6000 using a pair-ended approach (250bp). Pair-ended were merged by FLASH (v1.2.7; Each sample met the requirement of having over 75% of reads with a Q score of at least 30 and generating 30,000 tags of raw data. Filtering was performed using Qiime (v1.7.0; Caporaso *et al.,* 2010) on raw reads and chimera sequences were and removed using the SLVA138 database (http://www.arb-silva.de/) with the UCHIME algorithm (Edgar *et al.,* 2011). On remaining data, sequences with 97% similarity were clustered and organised into operational taxonomic units (OTUs) by Uparse (v7.0.1090; Edgar, 2013). Representative sequences for each OTU were screened by QIIME using Mothur method against the SSUrRNA database from SILVA138. Phylogenetic relationships were determined between OTU groups using MUSCLE (v3.8.31; Edgar, 2004). Standard sequence numbers were used to normalise OTU abundance information for each sample. All the following analyses were carried out on this normalised data.

QIIME was used to calculate several indices of alpha diversity (including observed-species and Shannon indices) and beta diversity (weighted and unweighted Unifrac). A Principal Component Analysis (PCA) were performed and visualised on R (v2.15.3) using FactomineR and ggplot2 packages. Wilcoxon two rank sum tests were performed to test for significance between diversity estimates between wild and

commercial bumblebee gut microbiomes in both bacterial and fungal communities. Anosim, MRPP and t-test analyses were also performed on R.

5.3 Results

5.3.1 Bumblebee queen survival rates

Of the 52 commercial *B. terrestris* bumblebee queens mated and put through an artificial diapause, 13 queens (25%) were dead upon the end of their diapause period. Both wild-caught and commercial bumblebee queen groups had several individuals die in the first month of being in the initiation boxes in the lab. Of the 55 wild-caught queens set up in initiation pods, 13 queens died in the first month of initiation (23.6%), with 11 of these dying in the first five days post lab introduction. Among the 39 commercial bumblebee queens, seven queens died in the first month post lab introduction (18%). All of these queens died within the first two weeks.

5.3.2 Colony initiation and foundation success rates

We examined the differences in colony initiation success (where a queen had laid at least one worker producing egg) between wild and commercial queens. We also compared colony foundation success rates at increasing levels of success (i.e., how many produced ≥10, ≥20, ≥30, ≥40, ≥50 and ≥60 workers by day 60 post first worker eclosion). Wild colonies and commercial colonies had an initiation success rate of 72.1% and 71.9%, respectively, with dead queens excluded (Table A5.2). Commercial queens showed higher colony foundation success rates at each defined level of success (Figure 5.3). Indeed, the difference between the proportion of successful commercial and wild colonies increased as the definition of success became more stringent, indicating that commercial queens are more successful at producing colonies of higher numbers of workers than wild queens (Chi-squared; $x^2 = 4.34$, df = 1, p-value = 0.037; Figure 5.3).

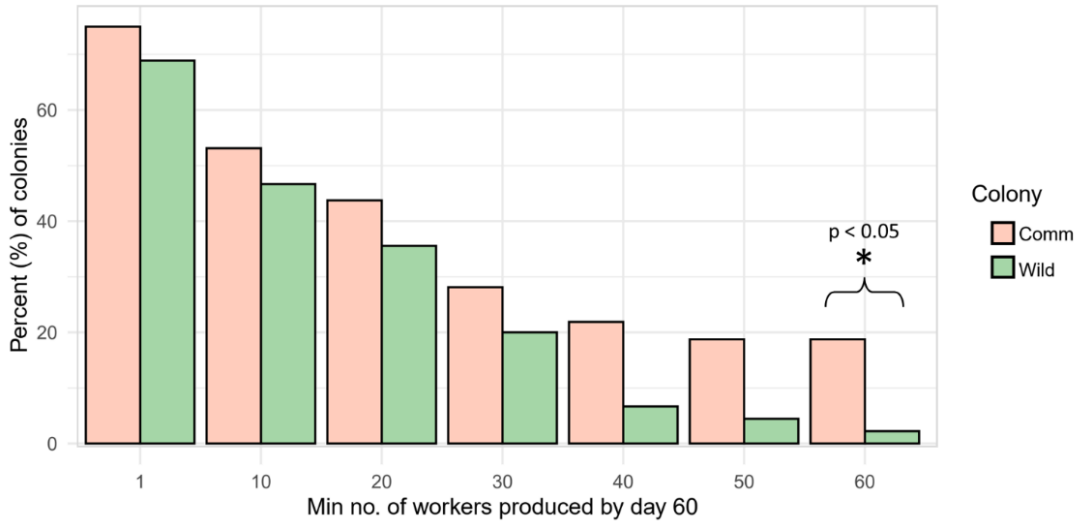


Figure 5.3 Success rates of wild and commercial colonies at various success levels of worker production. Percentage of wild (green) and commercial (orange) colonies that were successful at producing a given number of workers by day 60 after the eclosion of the first worker. Significant differences between wild and commercial colony success rates were determined by chi-squared tests and are indicated with *p* values and asterisks.

5.3.3 Wild and commercial colony growth rates

To determine if relatively successful, worker-producing wild and commercial colonies had differing growth rates, we assessed mean cumulative worker production and mean growth rate of colonies producing 10 or more workers which were observed for 50 days post first worker eclosion. The mean cumulative production of workers per day of colonies in the commercial group was consistently higher than colonies in the wild group after day 3 until the end of the observation period (Figure 5.4 A; Table A5.3). By day 50 post first worker eclosion, wild colonies had a mean worker total of 22.41 (s= 12.18), while commercial colonies had a mean worker total of 36.94 (s = 32.69). Log_{10} mean worker production rates between the wild and commercial groups were significantly different (t = 2.4311, df = 18.36, p-value = 0.0255), with successful commercial colonies having significantly higher colony growth rates (μ = 0.94 workers/day, s = 0.6) than wild colonies (μ = 0.49 workers/day, s = 0.21; Figure 5.4 B). Mean commercial colony worker production rates also exhibited a higher level of variance than wild colonies.

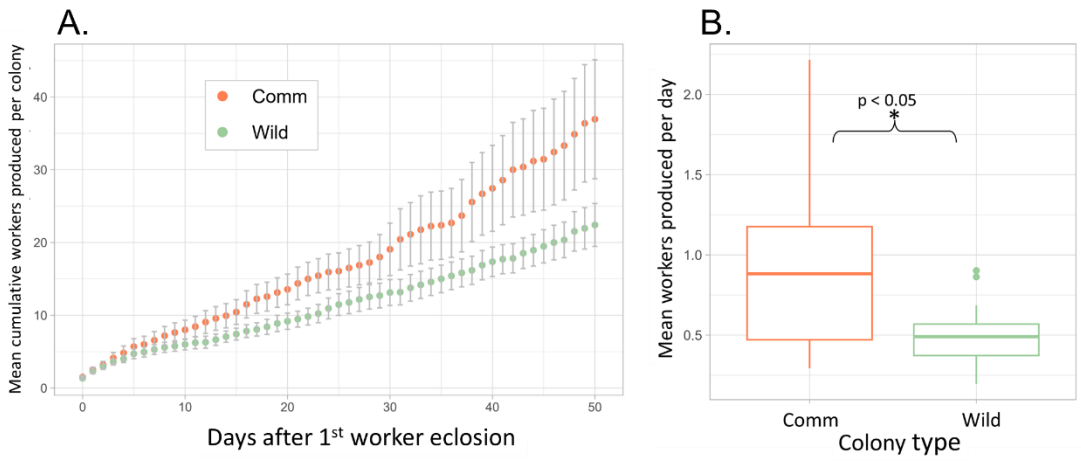


Figure 5.4 Wild and commercial colony growth rates. (A) Mean cumulative total workers per colony in the wild (green) and commercial (orange) group with standard error bars across 50 days post first worker eclosion in each colony. Day 0 is defined as the day on which the first worker eclosed. (B) Boxplot of wild (green) and commercial (orange) mean growth rates (workers produced per day) per colony after 50 days post first worker eclosion showing a significant difference as determined by Welch's t-test (t = 2.4311, df = 18.36, *p*-value = 0.0255).

5.3.4 Length of adult worker development in wild and commercial colonies

To investigate factors behind the increased growth rate of commercial colonies compared to wild colonies, we compared the length of first worker developmental periods between wild and commercial colonies. Overall, we found no significant differences between the developmental periods of the first worker in the wild and commercial groups (Slope (SE) = -0.034 (0.053), $p = 0.477$; Figure 5.5 C; Table A5.4). We also found no significant difference between the developmental periods of the combined egg and larval stage (Slope (SE) = -0.012 (0.066), $p = 0.854$) and the pupal stage (Slope (SE) = -0.082 (0.089), $p = 0.35$; Figure 5.5 A, B). The mean total length in days for the first worker to develop from egg to adult was 27.37 days (s = 4.48). For the commercial group, the mean total developmental period was 26.78 days (s = 4.01) and for the wild group, it was 27.81 days (s = 4.81). Mean pupal developmental length in wild and commercial colonies was 10.29 days (s = 1.57) and 9.48 days (s = 0.95) respectively. In both colony groups, the mean combined egg and larva developmental period had a higher standard deviation than the pupal developmental periods (wild colonies: $\bar{x}$ = 17.52 days, s = 4.23; commercial colonies: $\bar{x}$ = 17.3 days, s = 3.75).

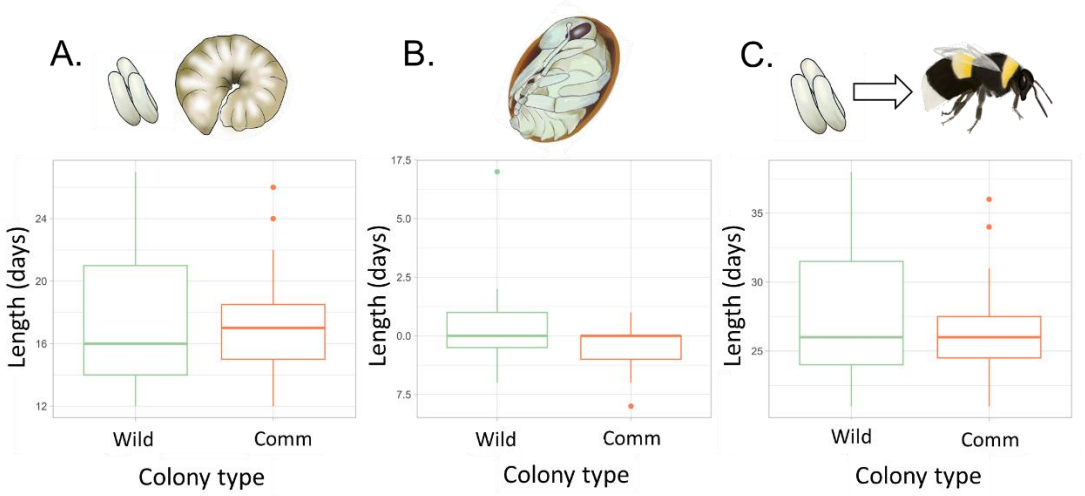


Figure 5.5 First worker developmental times in wild and commercial colonies. Developmental length (in days) of (A) combined egg and larval stage, (B) pupal stage and (C) all stages for the first adult worker in worker-producing wild and commercial colonies. Median values are represented by thick horizontal lines, box represents the interquartile range and whiskers mark the min and max values in each group. Individual circles are outliers in each data set.

5.3.5 Worker sizes

In total, there were 1,957 workers measured (Table A5.5) and used to assess differences of size between wild (n = 899 workers) and commercial colonies (n = 1,058 workers). When wild and commercial worker data were pooled, thorax widths had a range of 2.03 – 5.27 mm, and IT distance within a range of 1.8 – 4.71 mm. We observed that worker sizes within each colony in both groups showed similar levels of variance, with most colonies producing a mixture of relatively small and big workers (Figure A5.1 A - B). Workers in wild and commercial colonies had a very similar distribution of thorax width and IT distances, with higher similar median values, interquartile ranges and minimum maximum values (Figure 5.6 A, C).

To see whether commercial and wild colonies produced workers of differing sizes, we compared the mean worker thorax widths and IT distances per colony wild and commercial (Figure 5.6 B, D). A t-test performed on wild and commercial mean worker thorax widths per colony found no significant difference between these groups (t = 0.65908, df = 48.894, p = 0.5129). Results of t-test on wild and commercial mean worker IT distances per colony also found no significant difference (t = 0.42974, df = 48.61, p = 0.6693).

5.3.6 Sucrose consumption

To compare sucrose consumption per worker amount, from wild and commercial colonies, we analysed weekly sucrose consumption (g) against the mean total of workers in the colony that week for colonies in initiation pods (given 50% w/v sucrose) and in large colony boxes (given 30% w/v sucrose). The mean weekly sucrose consumption values and corresponding mean weekly worker total were found for each colony and by plotting these values on a scatter plot, we found that there was no significant positive correlation between weekly mean sucrose consumption and weekly mean worker total in colonies in the initiation pods ($r = 0.26$, $t = 1.73$, $df = 43$, p-value = 0.091; Figure 5.7A; Table A5.6). In contrast, when the same analysis was repeated for the bigger colonies that had been rehoused in the large colony boxes and given 30% (w/v) sucrose, a significant correlation was found ($r = 0.89$, $t = 10.59$, $df = 28$, p-value = 2.7×10^{-11}; Figure 5.8A).

A general linear model was used to test for difference in mean sucrose consumption per worker total in wild and commercials and found no difference between rates of sucrose consumption in either the initiation pod analysis (Slope (SE) = -0.3747 (0.4667), $p = 0.4267$; Figure 5.7B) nor the large colony boxes (Slope (SE) = 0.7033 (0.5323), p= 0.198; Figure 5.8B).

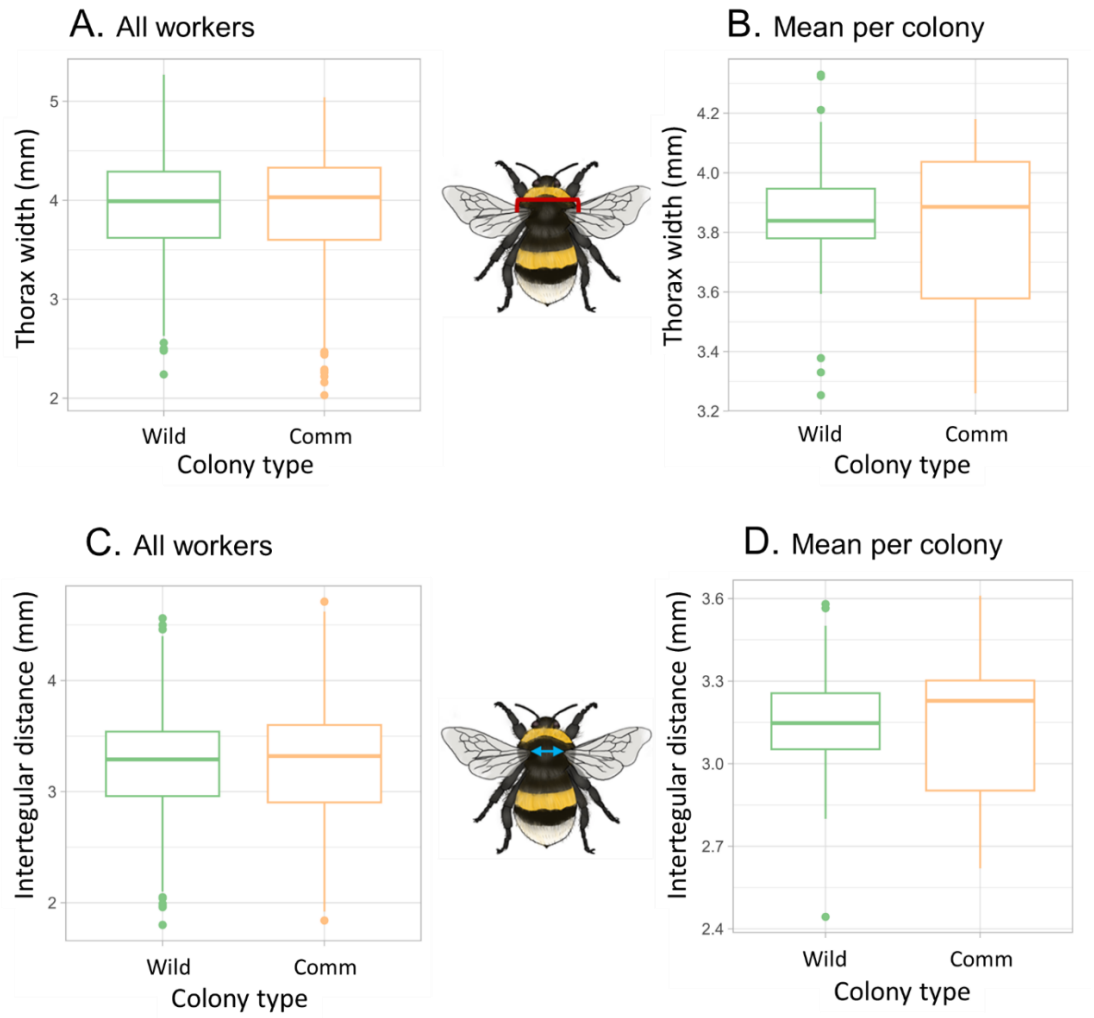


Figure 5.6 Wild and commercial worker thorax widths and intertegular distances (mm). Boxplots displaying (A) thorax widths data for all workers in wild (green) and commercial (orange) groups, (B) mean thorax widths distance per colony data in wild and commercial (orange) groups, (C) intertegular distance data for all workers in wild and commercial groups and (D) mean intertegular distance per colony data in wild and commercial groups. Median values are represented by thick horizontal lines, each box represents the interquartile range and whiskers mark the min and max values in each group. Individual circles are outliers in each data set.

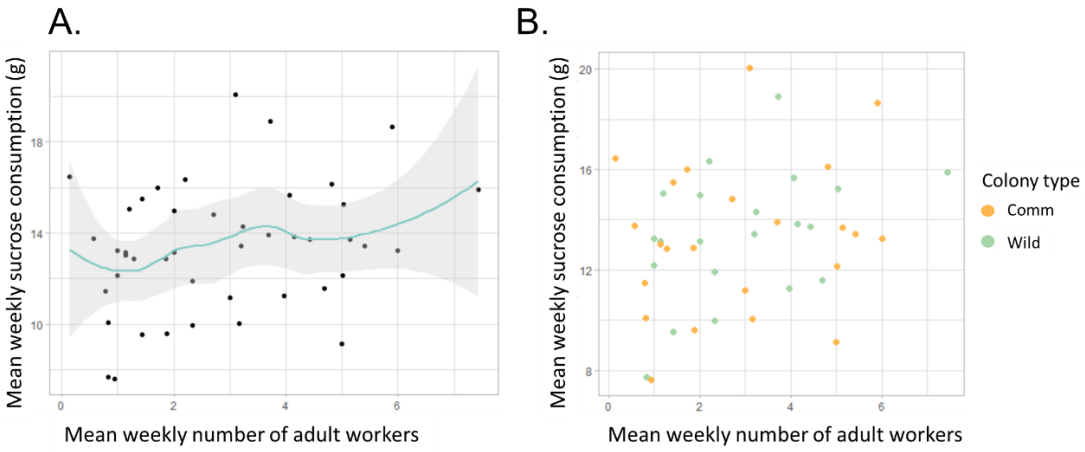


Figure 5.7 Relationship between mean weekly sucrose consumption and mean weekly worker total in wild and commercial colonies in initiation pods. (A) Mean weekly sucrose consumption and mean weekly worker with line to visualise correlation. This correlation is insignificant (Slope (SE) = -0.3747 (0.4667), p = 0.4267). (B) Mean weekly sucrose consumption and mean weekly worker of wild (green) and commercial (in orange) colonies.

Figure 5.8 Relationship between mean weekly sucrose consumption and mean weekly worker total in wild and commercial colonies in large colony boxes. (A) Mean weekly sucrose consumption and mean weekly worker with line to visualise correlation. This positive correlation is significant (r = 0.89, t = 10.59, df = 28, p-value = 2.7 x 10^{-11}), as is indicated by the asterisk and p-value. (B) Mean weekly sucrose consumption and mean weekly worker of wild (green) and commercial (in orange) colonies.

5.3.7 16S microbiome sequencing results

A total of 1,813,091 effective tags that could be used for downstream analyses were filtered from the total 1,965,589 raw tags. These effective tags correspond to sequences containing the 16S rRNA V4 amplicon region found in bacterial species. Of these effective tags, 906,640 were from the commercial group, and 906,451 from the wild group. There were 2,070 OTUs shared between wild and commercial groups, with 1211 unique OTUs in the wild group and 1043 unique tags in the commercial group (Table A5.7a).

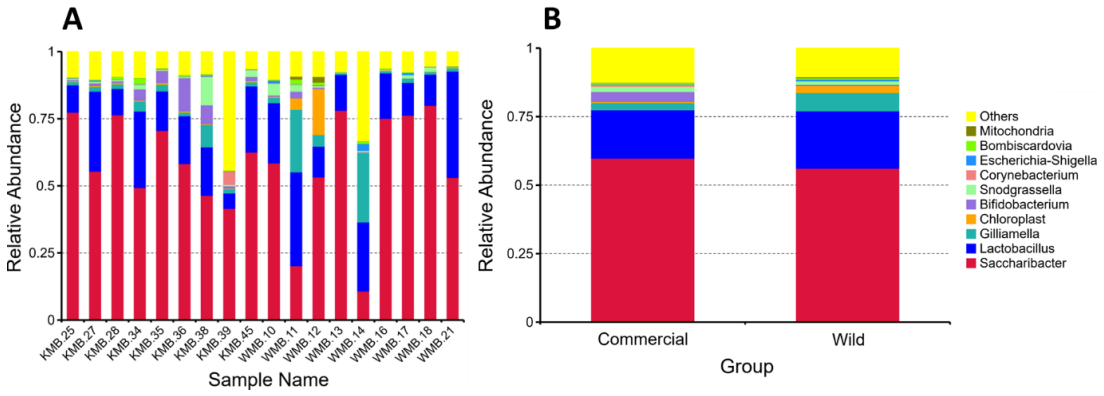


Figure 5.9 Relative abundance of bacterial genera in commercial and wild bumblebee gut microbiomes. (A) Relative abundance of bacterial general in individual samples and (B) mean relative abundance of bacterial general in commercial and wild bumblebee groups. Genera are represented by colours as shown by legend.

We observed subtle differences between the less represented genera among the wild and commercial group, such as an overall larger proportion of Bifidobacterium in the commercial group, and a larger relative abundance of *Gilliamellia* in the wild group (Figure 5.9 B). These slight differences observed between the groups can be explained by a few individual samples within each group (Figure 5.9 A; Table A5.7b). Two wild samples (WMB11 and WMB14) had large Gilliamella abundances (>20% relative abundance in each sample compared to <10% in all other samples) and low *Saccharibacter* (<25% relative abundance in each sample compared to >40% in all other samples). Four commercial samples (KMB34, KMB35, KMB36, KMB38) had larger individual relative abundances (>4%) of Bifidobacterium than calculated other individuals (<3%). However, the overall relative abundances were largely similar between the two groups, with both having over 50% relative abundance of

Saccharibacter and 15-20% *Lactobacillus* in the group comparison. A significant difference was found in the total of observed species between wild and commercial bumblebees (Wilcoxon rank sum: $p < 0.05$; Figure 5.10 A; Table A5.7c). No significant difference was found between the alpha diversity (Shannon index) between groups (Wilcoxon rank sum: $p = 0.49$; Figure 5.10 B; Table A5.7c).

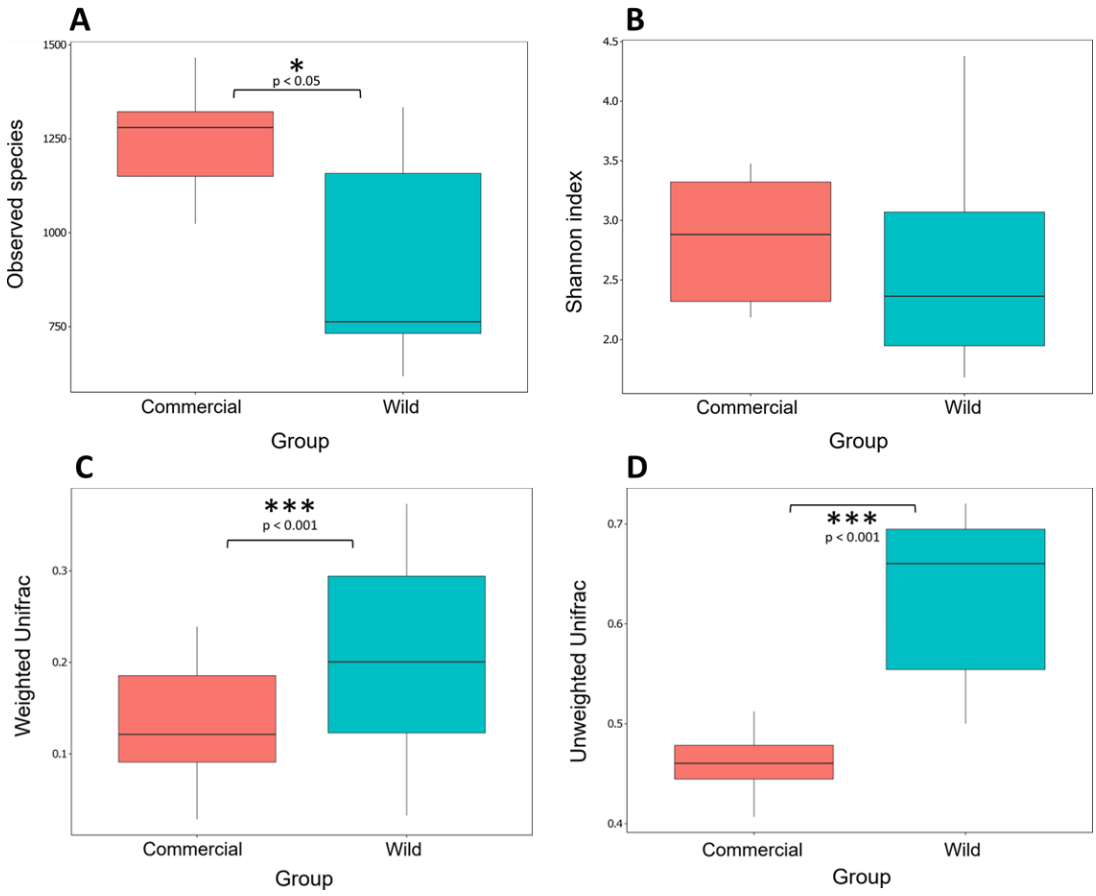


Figure 5.10 Alpha and beta diversity indices in the bacterial gut microbiomes of wild and commercial bumblebees. Alpha diversity indices (A) observed species and (B) Shannon index for bacterial communities wild and commercial bumblebee gut microbiome. Beta diversity estimates (C) weighted and (D) unweighted Unifrac distances for bacterial communities in wild and commercial gut microbiomes.

Significant differences were found between wild and commercial groups in both unweighted (Wilcoxon sum rank test: $p < 0.001$) and weighted (Wilcoxon sum rank test: $p < 0.001$) Unifrac distances (Figure 5.10 C, D). However, variation among groups was not significantly larger than the variation within groups, although a positive R value and a low p-value (Anosim: R = 0.083, $p = 0.071$; Figure A5.4 Table A5.7d) indicate a trend that is worth further investigation. There were also no

statistically significant differences in the structure of the microbial communities among groups (MRPP: A= 0.035, p = 0.57; Table A5.7d). t-tests revealed that there were statistical differences (p < 0.05) between wild and commercial bees in the abundances of certain genera (Table A5.7e).

Wild workers had significantly higher levels of microbes of the *Pseudomonas* and *Thiobacillus* genera and within the Muribaculaceae family, while commercial workers had a higher abundance of *Bifidobacterium*. Several species were significantly different in abundance between wild and commercial groups including *Bifidobacterium actinocolliforme* (Table 5.1; Table A5.7f).

5.3.8 ITS microbiome sequencing results

Raw tags matching the ITS gene amplicon region (ITS1-5F) numbered 1,030,052. Once filtered and chimeras removed, 721,960 effective tags remained. These remaining tags included 312,060 tags corresponding to the commercial group, and 409,900 to the wild group. Of the OTUs, 156 were shared between wild and commercial groups. Wild and commercial microbiomes contained 203 and 198 unique OTUs respectively (TableA5.8a). Commercial bumblebees as a group had a larger relative abundance of microorganisms of the genus Wickerhamomyces but a lower relative abundance of Candida species than wild bumblebees (Figure 5.11 B). There was a visibly high level of variance across individual samples in both groups (Figure 5.11 A; Table A5.8b). Wild samples WMB11 and WMB12 show a high abundance (>75%) of 'Other' species not included in the overall top 10 genera.

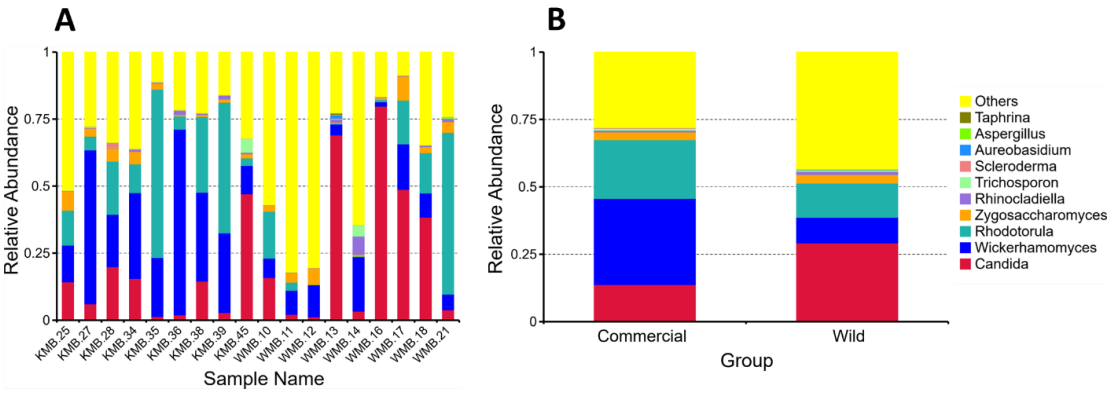


Figure 5.11 Relative abundance of fungal genera in commercial and wild bumblebee gut microbiomes. (A) Relative abundance of fungal general in individual samples and (B) mean relative abundance of fungal general in commercial and wild bumblebee groups. Genera are represented by colours as shown by legend.

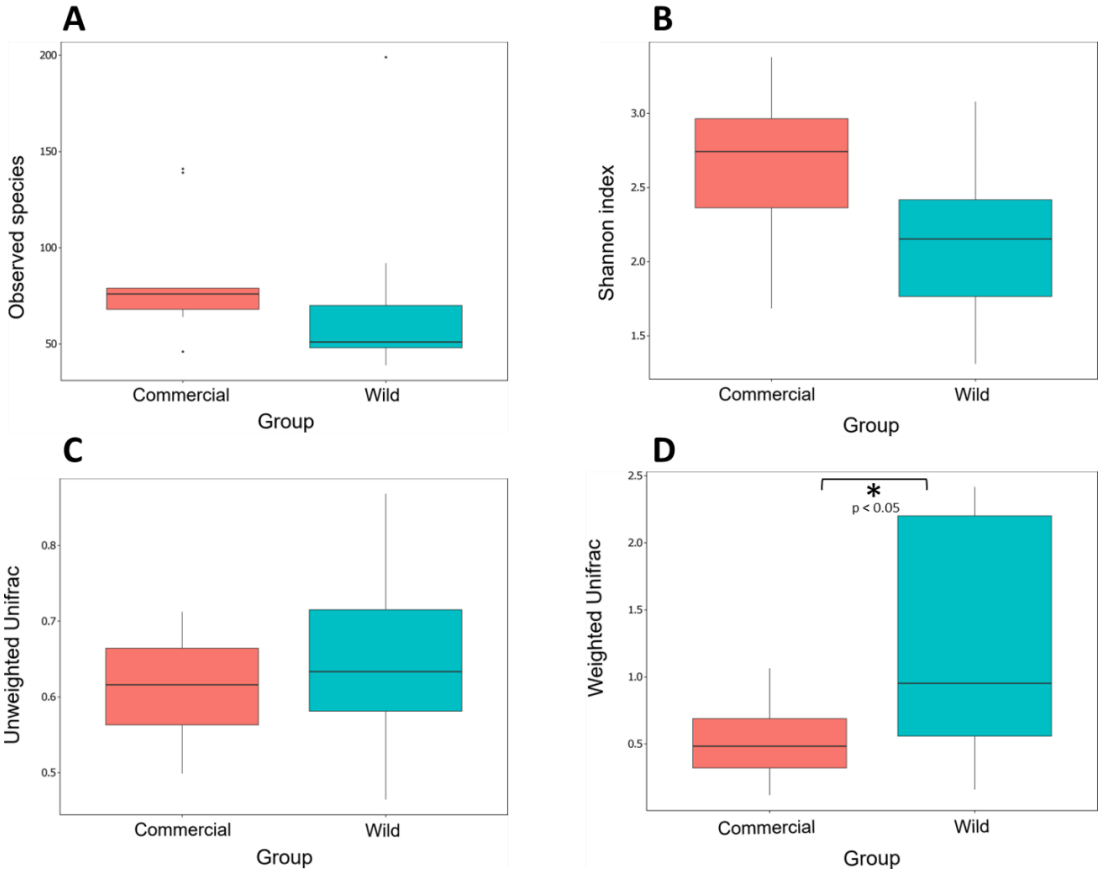


Figure 5.12 Alpha and beta diversity indices in the fungal gut microbiomes of wild and commercial bumblebees. Alpha diversity indices (A) observed species and (B) Shannon index for fungal communities wild and commercial bumblebee gut microbiome. Beta diversity estimates (C) weighted and (D) unweighted Unifrac distances for fungal communities in wild and commercial gut microbiomes.

Table 5.1 Differentially abundant bacterial species between wild and commercial gut microbiomes. Top 5 most differentially abundant species that are significantly different between wild and commercial bumblebee worker gut microbiomes alongside relative abundance difference values, mean relative abundances in wild and commercial microbiomes with standard deviation and p-value indicating the significance of the difference between wild and commercial gut microbiomes. Orange cells show the top 5 most differential species that are significantly higher in the commercial, and green cells show the top 5 most differential species that are significantly higher in the wild group.

		Commercial group		Wild group		
Bacterial species	**Difference in relative abundance**	**Mean relative abundance**	**Standard deviation**	**Mean relative abundance**	**Standard deviation**	**P-value**
Bifidobacterium actinocoloniiforme	0.0062	0.0067	0.0079	0.00051	0.00072	0.048
Azoarcus sp	0.00031	0.00055	0.00019	0.00024	0.00021	0.005
Planoglabratella opercularis	0.00022	0.00025	0.00018	2.73E-05	4.18E-05	0.0057
Pseudoxanthomonas mexicana	0.00017	0.0003	0.00013	0.00013	0.00011	0.01
Lacibacterium aquatile	0.00013	0.00019	9.06E-05	5.95E-05	5.79E-05	0.0023
Lactobacillus johnsonii	0.00085	3.47E-05	2.04E-05	0.00088	0.001	0.038
Ralstonia pickettii	0.00056	0.00039	0.00012	0.00095	0.00037	0.0015
Pseudomonas tolaasii	0.00035	5.08E-05	3.11E-05	0.00040	0.00035	0.017
Lactobacillus murinus	0.00033	3.22E-05	3.41E-05	0.00036	0.00039	0.035
Bacterium CYCU-0215	0.00028	0	0	0.00028	0.00031	0.029

Table 5.2 Differentially abundance fungal species between wild and commercial bumblebee gut microbiomes. All differentially abundant fungal species between wild and commercial bumblebee gut microbiomes, difference value, mean relative abundances in wild and commercial microbiomes with standard deviation and p-value.

		Commercial		Wild		
Fungal species	**Difference in relative abundance**	**Mean relative abundance**	**Standard deviation**	**Mean relative abundance**	**Standard deviation**	***p*-value**
Wickerhamomyces anomalus	0.22	0.32	0.2	0.095	0.06	0.009
Penicillium commune	0.00056	0.00095	0.00063	0.00038	0.00046	0.048
Malassezia restricta	3.83E-05	4.47E-05	4.79E-05	6.39E-06	1.92E-05	0.049
Clavulinopsis corniculata	5.11E-05	5.11E-05	4.49E-05	0	0	0.0092

5.4 Discussion

In this study, we found that colonies founded by commercial *B. terrestris audax* queens had higher colony foundation success rates and produced workers at faster rates than colonies founded by wild *B. t. audax* queens under captive rearing conditions. All colonies followed the exact same rearing procedures in terms of temperatures, humidity, provision of pollen and sucrose except for commercial queens undergoing an artificial diapause, and wild queens experiencing a natural one. The increased rate of worker production and colony growth in commercial colonies could not be explained by differences in individual worker developmental times, differences in input into worker development (as inferred from worker sizes) or by differences in sucrose consumption between wild and commercial stocks. In terms of digestive tract microbial communities, there were some distinctions in both bacterial and fungal populations, however further research is required into how certain microbe communities impact factors influence colony success both under captive rearing and natural conditions. We identified clear differences in the colony foundation success between wild-caught, Irish and commercial lines of *B. terrestris* bumblebees, which indicates a potential domestication syndrome within commercial stocks of bumblebees. This demonstrates the likely benefit of using commercial bumblebee colonies for pollination services as they may produce higher numbers of workers, the pollinating force of the colony, when compared to wild bumblebee colonies of the same species. From an ecological perspective, this also suggests a competitive advantage for commercial colonies over wild populations as the extent of success in colony foundation may, in part, determine reproductive success of the colony later in the season. For example, bigger colonies may have an increased ability to buffer worker losses (Müller and Schmid-Hempel, 1992), defend against parasites such as cuckoo bumblebees (Fisher, 1984; Müller and Schmid-Hempel, 1992; Malfi, *et al.* 2019), cope with neonicotinoid exposure (Crall *et al.*, 2019) and better exploit foraging resources as they become available over the season (Weinberg and Plowright, 2006). Therefore, we urge caution be taken in the use of commercial bumblebee colonies to avoid escape and/or mixing with wild populations of bumblebees.

5.4.1 Distinctions and similarities in growth dynamics and behaviour of wild and commercial B. terrestris audax colonies

There are several studies that support our findings of higher success rates and higher production of workers in commercial colonies than in wild. We assume that, given the time period of these studies, the commercial *B. terrestris* used in some of these previous studies was a variety of the *dalmatinus* subspecies, as this was preferred by commercial companies as it produces large colonies and has high foundation success rates in captivity (Velthuis and Van Doorn, 2006). Even so, there is previous evidence for commercial colonies differing from wild ones: a study comparing developmental characteristics in wild-caught *B. terrestris dalmatinus* queens and commercial *B. terrestris* queens found that commercial colonies produced significantly more workers than wild colonies (Gösterit and Gürel, 2005). Additionally, wild colonies in this study didn't produce any gynes while commercial colonies produced an average of 60.8 ± 12.7 gynes (Gösterit and Gürel, 2005). A similar study, published 11 years later, found that commercial genotypes of *B. terrestris* produced significantly more gynes and workers than wild *B. t. dalmatinus* colonies reared under the same conditions, including a similar diapause (Gösterit and Baskar, 2016). This study also found that commercial *B. t. dalmatinus* queens took less time than the wild variety to begin laying, laid more egg cells in the first brood and that the maternal genotype was likely more predictive of colony success (Gösterit and Baskar, 2016). Similarly, Ings *et al.* (2006) compared colony performance of commercial *B. terrestris* and wild *B. t. audax* colonies in the field in England, and found commercial colonies had higher reproductive success rates (no. of colonies producing gynes) and higher levels of success (no. of gynes produced). However, no work has previously been conducted on wild *B. t. audax* from Ireland in comparison to commercial colonies.

Increased colony growth rate could be caused by a decrease in the length of time it takes for adult worker bees to develop. However, pupal developmental length of the first worker was highly consistent across wild and commercial colonies. Pupal development is influenced by temperature (Tian and Hines, 2018), so consistent developmental lengths in this stage suggest colonies had similar incubation levels. The combined egg and larval developmental period was more variable. Alford (1971) states that bumblebee eggs usually hatch in the order they are laid and that all eggs tend to hatch over a period of two to three days, but that sometimes eggs laid in more

peripheral positions hatch last, suggesting that temperature may regulate the timing of egg hatching. Larval growth rate can also be influenced by diet and frequency of feeding (Kato *et al.*, 2022). Differences in incubation and larval feeding may have caused this variability in developmental length between all colonies. As developmental times are highly similar across wild and commercial groups, this does not appear to be a factor behind the increased growth rate of commercial colonies.

We also investigated whether commercial colonies consume more sucrose solution per worker present in colony than wild colonies. Carbohydrate intake can influence many aspects of bumblebee health and fitness. For example, phenoloxidase activity, haemolymph proteins and lysozyme concentrations are all influenced by carbohydrate intake (Cotter *et al.*, 2011). Unsurprisingly, we found a direct correlation between sucrose consumption and the size of colony in terms of worker numbers once colonies were larger and transferred to the colony foundation boxes supplied with 30% sucrose. The lack of a correlation between sucrose consumption and worker numbers in the initiation pods was likely due to variability in the amount of brood in these colonies. We found that the consumption rates were very similar between wild and commercial bumblebee colonies in colony foundation boxes. Therefore, in terms of sucrose solution, all colonies had similar nutritional intake that, when colonies were a larger size, was proportional to worker numbers.

Pollen consumption can be difficult to measure accurately in *B. terrestris* colonies. Instead, we examined worker size to determine if there were different nutritional inputs into developing brood of wild and commercial colonies. It is generally accepted that worker size is determined by its nutritional intake in its larval stage (Schmid-Hempel and Schmid-Hempel, 1998; Goulson, 2010a). This is supported by the common observation that workers produced later in the colony lifecycle are larger than previous broods, likely due to increased brood care by sisters and increased feedings (Hobbs, 1965; Knee and Medler, 1965; Shpigler *et al.*, 2013). *In-vitro* brood rearing has also shown a clear link between frequency of feeding and larval size (Kato *et al.*, 2022). Furthermore, colonies can exhibit trade-offs between worker size and worker quantity e.g Malfi *et al.* (2019) observed that when small bumblebee colonies were supplemented with additional resources, they produced smaller numbers of large workers.

We found that there was no difference in worker sizes between wild and commercial colonies. All worker-producing colonies had similar variability in the sizes of workers too, a common feature of bumblebee colonies which is thought to determine division of labour within the colony (alloethism; Goulson *et al.*, 2002). This could also mean that size-dependent abilities such as worker eyesight, tolerance of temperatures and flight (Goulson *et al.*, 2002; Spaethe and Weidenmüller, 2002) were similar between wild and commercial colonies, although further research would be required to investigate the differences of physical and mental capacities of wild and commercial bumblebees. However, these colonies were reared in a captive setting with identical diets. Ings *et al.* (2006) found that commercial *B. terrestris* colonies (likely ssp. *dalmatinus*) produced larger foragers than wild colonies and foraged more efficiently. Further research should explore whether similarities in worker size and resource consumption between commercial and wild colonies of *B. t. audax* continue to be observed in natural settings with full access to natural resources. This will provide insight into differences between wild and commercial *B. terrestris* foraging efficiency and diet preferences and the impact of these potential differences on worker production and size. With no significant differences observed between wild and commercial colonies in the nutritional input into workers nor developmental times, a likely answer to how commercial colonies grew faster than wild colonies in this experiment seems to be that commercial queens tend to lay more viable, fertilised eggs than wild queens or a higher proportion of brood survives to adulthood.

5.4.2 Distinctions in gut microbial communities of lab-reared wild and commercial B. t. audax workers

We examined the bacterial and fungal microbial communities from the digestive tracts of worker bees from wild and commercial, lab-reared colonies to identify potential symbiont differences that could explain differences observed in colony performance.

Overall, wild and commercial worker bacterial symbiont communities were very similar. Bumblebees, like other eusocial corbiculate bees, have a core set of gut bacteria including *Snodgrassella*, *Gilliamella*, *Lactobacillus Firm-4* and *Firm-5* and *Bifidobacterium* (Kwong *et al.*, 2017). Usually, these core bacteria are the major components of *Bombus* spp. digestive tract microbiomes (Bosmans *et al.*, 2018; Hammer *et al.*, 2021; Cullen *et al.*, 2023). Interestingly, in both wild and commercial

groups the dominant bacterial genus was the non-core *Saccharibacter* (Acetobacteraceae family), which contributed to over half the average relative abundance in both groups. *Saccharibacter*-type bacteria are a type of acetic acid bacteria that are symbiotic with insects (Crotti *et al.,* 2010; Chouaia *et al.,* 2014) The only described species of the genus *Saccharibacter* is *S. floricola,* which is a Gram-negative bacteria found in pollen (Jojima *et al.,* 2004). Furthermore, several closely-related species genomes labelled as *Parasaccharibacter apium* (associated with nurse honeybee crops; Corby-Harris *et al.* 2014) or *Saccharibacter sp.* have been found to be the same species as *Bombella apis* (Smith *et al.,* 2021). Species of the Acetobacteraceae in the guts of wild *Bombus* spp. have shown positive associations with *Crithidia* infection (Cariveau *et al.,* 2014).

It is possible rearing conditions and food provisions may have resulted in the gut microbiomes of colonies being dominated by non-core bacteria. Acetobacteraceae species are often linked to insects that depend on sugar-based diets (Crotti *et al.,* 2009). All colonies in this experiment were fed sucrose-only sugar solutions and honeybee-collected pollen, however many bumblebees used in microbiome research are provided with commercial sugar solutions such as Biogluc ® (Meeus *et al.,* 2013; Newbold *et al.,* 2015; Billiet *et al.,* 2017) which can include a variety of sugars (Billiet *et al.,* 2016). One study shows that the sugar solution (either Biogluc®, sucrose-rich and fructose-rich solutions) provided to workers did not alter the gut microbiomes of workers, although the workers older than four days used in this experiment had been kept in colonies fed on Biogluc ® solution prior to the experimental treatments (Billiet *et al.,* 2016). We suggest investigation into the effects of different kinds of sugar solution provided to colonies from foundation on the resulting symbiotic gut microbiomes of colony individuals. It is possible the nutritionally simple, sucrose-only solution provided to colonies in this experiment meant that bumblebees required a higher amount of a sugar-utilising species, such as *Saccharibacter* (Kindaichi *et al.,* 2016), in their guts to derive sufficient nutrition from the provided resources.

In spite of overall similarities in bacterial gut microbiomes between wild and commercial groups, there were some distinctions between groups and a high degree of variability across samples of both groups, especially the wild workers. Variability across worker microbiomes reflects the variability seen in Chapter 4 in both brain and fat-body samples. As mentioned then, this may be influenced by variability in the

origin colony's overall health and growth rate, the worker's role within colony, worker size and potentially parasite loads in colonies, which we did not examine. Commercial microbiomes included significantly more species than the wild. In addition, community structure was also significantly different between the wild and commercial groups based on both weighted and unweighted Unifrac distance values, with the wild group having more phylogenetically dissimilar communities in each measurement. We found that commercial bumblebee workers were found to have significantly more *Bifidobacterium* in their gut microbiomes than wild workers, in particular the species *Bifidobacterium actinocoloniiforme*. Interestingly, high *Bifidobacterium* levels in the gut microbiome are associated with ovipositing queens (Wang *et al.,* 2019). Furthermore, in humans, *Bifidobacterium* is present at higher levels in late pregnancy than in people giving birth pre-term (Dahl *et al.,* 2017). *Bifidobacterium* produces lactic acid which can suppress the growth of harmful bacteria such as *E. coli* and reduce gut inflammation (Fukuda *et al.,* 2011). Bumblebee workers inherit much of their gut microbial communities from their maternal queen (Su *et al.,* 2021) and so it is possible that commercial queens may also had higher levels of *Bifidobacterium* in their gut microbiomes and this may have resulted in an increased fecundity. Future research should investigate associations between queen microbiome, particularly the relationship of *Bifidobacterium* levels, and colony success.

Wild workers had a significantly higher abundance of species in the *Pseudomonas* genus. One species, identified as *Pseudomonas tolaasi*, was also found to be significantly higher in wild than in commercial microbiomes although we expect, as the relative abundance of the genus was higher than that found for this species alone, that other *Pseudomonas* species present in wild samples were not identified to species-level. *P. tolaasi* is a pathogen of mushrooms (Cutri *et al.,* 1984). In general, Pseudomonas species are a soil-dwelling species (Sah and Singh, 2016) but have been found in both nectar and pollen samples and have been shown to survive and spread in *A. mellifera* hives (Pattemore *et al.*, 2014). Furthermore, *Pseudomonas* often is a component of the *Bombus* microbiome (Wang *et al.,* 2019; Li *et al.,* 2021). Some *Pseudomonas* species are linked to detoxification (Ceja-Navarro *et al.,* 2015), digestion (Briones-Roblero *et al.,* 2017) and possibly nitrogen balance in insects (Morales-Jiménez *et al.,* 2013).

Two species of *Lactobacillus* (*L. johnsonii* and *L. murinus*) were also significantly higher in abundance in wild bumblebee microbiomes than in commercial. The *Lactobacillus* genus is often a core group of *Bombus* microbiomes (Killer *et al.*, 2014). *L. murinus* is a symbiotic bacterial species associated with rodents (Hemme *et al.*, 1980), while *L. johnsonii* has been found in the guts of honeybees and is associated with colony fitness (Audisio *et al.*, 2011). Wild workers also had higher abundances of the genera Muribaculaceae and Thiobacillus. Muribaculaceae have been found in the gut microbiomes of the fall armyworm (*Spodoptera frugiperda;* Lv *et al.*, 2021), but are more commonly associated with mammalian gut microbial communities, such as mice (Ormerod *et al.*, 2016; Schmidt *et al.*, 2019). Thiobacillus species are widespread in many types of environment, but are commonly associated with soil (Akhtar *et al.*, 2012). In this experiment wild bumblebee queens were caught post diapause, which usually occurs in loose soil, and so perhaps accumulated small amounts of soil bacteria in their guts which may have been passed to their daughters. Furthermore, queens used in this experiment will likely have originated from wild colonies of *B. terrestris*, which are usually based underground and often in disused small mammal burrows (Alford, 2009; Goulson, 2010a).

In terms of their fungal gut microbiome, there was a high degree of variability across all samples as was seen in the bacterial gut microbiome analysis. Wild and commercial bumblebees also exhibited large distinctions in overall community composition. When comparing Figure 5.9 and 5.11, it appears that, while wild and commercial bumblebee workers had subtle distinctions in the relative abundance of bacterial gut microbes, the distinctions between groups are much greater when examining the fungal microbiome. Wild bumblebee guts had a more phylogenetically dissimilar fungal microbiome than commercial bumblebee guts. Commercial bumblebee gut microbiomes had an overall higher alpha diversity than wild bumblebees, although the difference was insignificant. Wild bumblebee guts had a fungal microbiome largely composed of *Candida* spp. while the biggest fungal group in the commercial worker microbiome group was the yeast *Wickerhamomyces.* While no significant differences were found between wild and commercial *Candida* sp., likely due to the high degree of variability across samples in the relative abundance of fungal microbes, commercial bumblebees had overall lower relative abundances of these species compared with wild. Bee-associated yeasts were present in both wild and commercial bumblebees including *C.*

bombi and *C. apicola* (Kurtzman *et al.,* 2011; Pozo *et al.,* 2018; Praet *et al.,* 2018; Pozo *et al.,* 2020; Cullen *et al.,* 2023).

Commercial workers had significantly higher abundances of the species *Wickerhamomyces anomalus* than wild workers. *W. anomalus* is commonly associated with bee digestive tracts (Gilliam *et al.*, 1974; Yun *et al.*, 2018; Bosmans *et al.,* 2018; Tauber *et al.,* 2019). It has also been found to be associated with other insects from several orders including Diptera, Hemiptera and Coleoptera. In particular, it has been found in the guts and reproductive organs of *Anopheles stephensi* mosquitos (Ricci *et al.,* 2011). Interestingly, some strains of *W. anomalus* produce antimicrobial compounds which may protect its host from pathogen threat (Ricci *et al.,* 2011; Cappelli *et al.,* 2014) and so this fungal species has been suggested as a potential avenue toward controlling vector-borne diseases such as malaria (Cappelli *et al.*, 2021). The non-social lizard beetle, *Doubledaya bucculenta*, passes *W. anomalus* to offspring via the mother's ovipositor and subsequent larvae are nutritionally dependent on this symbiont (Toki *et al.*, 2012; Toki *et al.*, 2013). *W. anomalus* may provide protective and nutritional benefits to developing brood, adult workers and the queen in commercial colonies which could explain the increased success and growth rate of these colonies compared to the wild. Commercial bumblebees also had significantly higher amounts of the species *Malassezia restricta* in their fungal gut microbiome than wild bumblebees. This species has also been found to be a core component of the fungal gut microbiomes of two species of stingless bees (Liu *et al*., 2021). Interestingly, *M. restricta* is more well known for its presence in human gut microbiomes and links to gut diseases such as colorectal cancer and Crohn's disease (Coker *et al.,* 2019; Limon *et al.,* 2019). This species is also known as a commensal fungus found on human skin (Cho, 2022). Why commercial bumblebees in this experiment had higher amounts of this species in their fungal microbiome compared with wild remains unknown to us, but perhaps their closer association with humans in captive bumblebee rearing facilities increases the likelihood of transmission of classically human-associated fungal commensals to bumblebees.

5.4.3 Rearing environments could prime commercial B. terrestris queens to produce successful colonies

Overall, the findings of this experiment suggest two potential reasons behind the increased foundation and growth success of commercial colonies. Environmental conditions associated with commercial rearing facilities may prime commercial bumblebee queens to be more successful in founding colonies that grow at faster rates than colonies reared from wild-caught, Irish *B. t. audax* queens.

For example, artificial diapause may give freshly imported commercial colonies a competitive advantage over wild bumblebee queens and colonies active during the same season. This may explain differences in success among colonies reared in this experiment from wild-caught queens, who underwent a natural diapause, and commercial queens, who were put through an artificial diapause that was likely shorter than the average natural diapause. Varying lengths of diapause can impact queen survival and colony characteristics, with shorter diapause periods usually being more favourable (Beekman *et al.*, 1998; Beekman and Van Stratum, 2000; Gösterit and Gurel, 2009). While commercial queen diapause survival rates were similar to other experiments including bumblebees undergoing a similar diapause regime (Gösterit and Gurel, 2009) we cannot say if natural diapause conditions of the wild-caught queen had similar or different rates of survival. Queens of commercial colonies are likely subjected to less risky diapause conditions whereas wild bumblebee queens will have been exposed to the full complement of risks associated with diapause in the wild e.g., increased parasitism (Colgan *et al.,* 2019, 2020). We assume that benefits from artificial diapause conditions are restricted to only the subsequent generation of bees. Further research should explore the relative success of commercial and wild colonies where queens have undergone a similar artificial or similar natural diapause.

Although we did not explore the issue of pathogen prevalence among wild and commercial colonies, we acknowledge that many queens, both wild and commercial, were likely infected with pathogens (Colla *et al.,* 2006; Murray *et al.,* 2013; Mockler *et al.,* 2018) and this could have impacted reproduction rates (e.g., Yourth and Schmid-Hempel, 2006; Otti and Schmid-Hempel, 2007). Future research should be dedicated to understanding whether different genotypes of parasites and pathogens exist between wild and commercial populations and if these have similar impacts on reproduction across wild and commercial bumblebees.

Distinctions in gut microbial communities, as described above, could influence bumblebee queen fecundity for example, through provision of nutritional and immune benefits. When nematodes (*Caenorhabditis elegans*) host particular *E. coli* strains capable of arsenic biotransformation, they experience a decrease in fecundity, likely due to increased toxicity (Zhou *et al.*, 2020). Supplementation of certain bacterial species to *B. terrestris* colonies had the effect of increasing egg laying rates and higher brood size (Pozo *et al.*, 2021). Certain microbiome communities of the ladybird, *Henosepilachna vigintioctopunctata*, were shown to correlate with an increased development of ovaries and testes (Li *et al.*, 2021). Microbes in developing insect larva may also influence their survival by providing nutritional benefits to the larva (Zhang *et al.*, 2022) or by playing key roles in development (Paludo *et al.*, 2018). Distinctions between wild and commercial worker microbiomes likely originate from the vertical transmission of distinctive gut microbial communities from their queens (Koch *et al.*, 2022). Commercial bumblebees queens may have inherited a microbiome which has been shaped by many years of controlled breeding and rearing in artificial settings, as has been shown for other domesticated species (Metcalf *et al.*, 2017; Bowerman *et al.*, 2021). In addition, wild bumblebee queens were likely exposed to more diverse or rarer microbes by developing, foraging and undergoing diapause in natural settings (Newbold *et al.*, 2015). Future research should examine how bumblebee symbionts can influence bumblebee colony initiation and foundation success. Further characterisation of the gut microbiomes of freshly imported commercial colonies and commercial-descended, feral colonies will also help identify how much of the commercial bumblebee microbiome is shaped by their environment during captive rearing and if, once feral, bumblebees of commercial lineage gain a microbiome similar to wild conspecifics, as suggested by Newbold *et al.* (2015). If this is the case, it should also be investigated whether feral bumblebees with microbiomes typical of wild conspecifics exhibit the same increased success in colony establishment and growth.

5.4.4 Selection of commercial B. terrestris may have altered colony growth characteristics

Commercial colonies may have a specific genotype that benefits colony growth and success. In Chapter 2, we observed differences in genotype between wild, *Irish B. t.*

audax and both British and commercial *B. t. audax* populations. It is possible that artificial selection in commercial bumblebee populations has driven these genomic distinctions and created genetically determined domestic traits in commercial bumblebees that results in improved colony success compared with wild populations. It is also possible that commercial bumblebee stocks originate from British populations of *B. t. audax*, as is suggested by our findings in Chapter 2. To our knowledge, there has been no comparative studies of physiology, behaviour, or fitness between the wild populations of *B. t. audax* in Ireland and Britain. Any genotypically determined distinctions between wild Irish and commercial *B. t. audax* could simply reflect the already present genomic distinctions between these two wild populations. However, there is evidence that domestication has altered colony growth characteristics and reproductive success in other bumblebee populations (Gösterit and Baskar, 2016). Furthermore, as domestication-induced changes can happen very rapidly in captive populations (Islam *et al.*, 2020), and we have no knowledge whether commercial companies supplement their captive stocks with wild samples, it is quite possible commercial *B. t. audax* colonies may have acquired a domestication syndrome that increases reproductive success. It remains to be seen how the possible domestic trait of increased reproduction in commercial bee colonies impacts other physiological processes in these bee populations. e.g., through an energetic trade-off in favour of reproduction. For example, a study on Japanese quails which had been artificially selected to produce larger eggs found that they had a higher resting metabolic rate than quails not selected for this trait (Pick *et al.*, 2016). Although this study didn't find a corresponding increase in oxidative damage, the authors do not rule out the possibility of a trade-off in other functions such as immunity or brain mass (Knowles *et al.*, 2009; Kotrschal *et al.*, 2013; Ebneter *et al.*, 2016). Distinctions in microbiome communities between wild and commercial workers may also be explained by the differing genotypes of wild and commercial bumblebees. There is evidence to support that host genotype can affect symbiotic microbe colonisation and interactions in plants (Smith and Goodman, 1999), aphids (Chong and Moran, 2016), rodents (Spor *et al.*, 2011), humans (Bolnick *et al.*, 2014). Sauers and Sadd (2019) investigated gut colonisation in wild and commercial *B. impatiens* colonies and also used strains of *Snodgrassella alvi* cultured from each found that both host genotype and microbial symbiont genotype can influence microbial colonisation success. The findings of this study suggest that the specificity of the host-symbiont relationship may

be influenced by fine-scale changes within the genomes of either the host or the symbiont and suggests that co-evolution may occur between host and symbiont (Poisot *et al.*, 2011; Sauers and Sadd, 2019). Interestingly, genes involved in immune function appear to be key determinants for microbial diversity in the gut (Spor *et al.*, 2011). Future research should examine the degree of importance of genotype in wild and commercial (both newly imported and feral) bumblebees in determining symbiotic, microbial communities.

5.4.5 Conclusion

In this experiment, we have observed traits in commercial bumblebees, possibly linked to the domestication process, that are indicative of a competitive risk posed towards wild bumblebees and pollinators by importation of managed bumblebees. Firstly, a higher level of workers produced by commercial colonies and the trend of increasingly more colonies being imported into Ireland every year (Appendix A1.1) could mean wild pollinators will have more competition for floral resources (Goulson, 2010). The level of success a colony experiences is in its early life can also determine the reproductive success of the colony. For example, Pelletier and McNeil (2003) found that colonies with higher nectar and pollen supplies from early colony life produced more workers and had higher production of reproductive individuals. With higher numbers of workers, a colony has a higher foraging capacity, which can ensure food availability and support increased production of new males and gynes. This is particularly important in the case of gynes, as bigger sized gynes are more likely to survive diapause (Beekman *et al.*, 1998). As commercial bumblebees in Ireland are considered a 'native' subspecies, they are often used in open fields or semi-open crop polytunnels (Appendix A1.1). Therefore, there is a great risk that reproductive individuals could escape into the wild. Increased production and survival of gynes from commercial colonies may cause competition with wild bumblebee species for diapause sites, and subsequently nesting sites (Goulson, 2010). Furthermore, if either males or gynes escape, there is a chance of hybridisation with wild *B. t. audax* populations which could cause the loss of wild-adapted, advantageous alleles or, if domestic traits spread into wild populations, could alter the reproductive behaviours of wild *B. t. audax* populations. Any change to the behaviour or fitness of wild *B. terrestris* populations could have major ecological repercussions across many

ecosystems due to the common-status and generalist behaviour of this species (Dafni *et al.*, 2010; Falk, 2019). In addition, high numbers of workers produced by commercial colonies increases the likelihood of mixing between commercial and wild populations. This could increase the spread of pathogens to wild pollinator communities which is a well-documented and widely-acknowledged risk associated with imported bumblebee use (Colla *et al.*, 2006; Arbetman *et al.*, 2013; Graystock *et al.*, 2013; Murray *et al.*, 2013).

Our findings strongly suggest the need for stringent regulations and management regarding the use of commercial bumblebees. We highly recommend the use of queen excluders to be used on all imported bumblebee colonies and that colonies be destroyed at the end of their use on a crop to limit the escape of reproductive individuals and limit the mixing of wild and commercial pollinator populations. This study also highlights the potential that even recently domesticated populations of bumblebee can exhibit traits that differ from wild populations, which is an area that requires further investigation to confirm the existence of domestication syndromes in bumblebees and understand the consequences of these changes on the risks posed to wild pollinators.

Chapter 6

General discussion

6.1 Revisiting the thesis aims

Bees are highly valuable pollinators in both ecological and agricultural settings around the world. Almost 90% of the ~352,000 angiosperm plant species depend on animal-mediated pollination to successfully reproduce (Ollerton *et al.,* 2011). The contribution of bees and other pollinators are essential for the healthy functioning of ecosystems and the provision of associated ecosystem services as well as for human health and the economy (Daily, 2003; Potts *et al.,* 2016). The pollination services provided by pollinators are valued at €15 billion per year across Europe (Leonhardt *et al.,* 2013). However, in spite of their importance, many populations of bees are experiencing declines across Europe and North America (Kosior *et al.,* 2007; Colla and Packer, 2008; Nieto, 2014; Mazed *et al.,* 2022). Several stressors are considered to play a role in the decline of bee populations, including habitat loss, climate change, use of pesticides and the spread of pathogens (Colla *et al.,* 2006; Goulson *et al.,* 2008; Szabo *et al.,* 2012; Czerwinski and Sadd, 2017; Giannini *et al.,* 2017). Research into pollinator biology and understanding how responses on genetic, physiological, and behavioural levels contribute to fitness is more important than ever if we are to prevent further declines of these essential organisms.

Bombus terrestris, the earth bumblebee, is a bumblebee species ubiquitous across Europe but exhibits much variation within its distribution as exemplified by its classification into nine subspecies (Rasmont *et al.,* 2008). *B. terrestris* is one of the main bumblebee species studied in the literature and has been used for characterisations of bumblebee organs and physiological systems (Riddell *et al.,* 2011; Colgan *et al.,* 2011; Barribeau and Schmid-Hempel, 2013; Larkin, 2018; Colgan *et al.,* 2019) as well as for investigations into bumblebee responses to stressors (Schmid-Hempel and Schmid-Hempel, 1998; Imhoof and Schmid-Hempel, 1999; Bortolotti *et al.,* 2001; Mallon *et al.,* 2003; Tyler *et al.,* 2006; Yourth *et al.,* 2008; Botías *et al.,* 2021) and general bumblebee behaviour, health and ecology (Beekman and Van Stratum, 2000; Cnaani *et al.,* 2002; Goulson *et al.,* 2002; Kraus *et al.,* 2009; Gösterit, 2016; Billiet *et al.,* 2017). Additionally, this species' amenability to being reared under captive conditions (Velthuis and Van Doorn, 2006) alongside its tendency to grow large colonies (Alford, 1975; Goulson, 2010a), has led to *B. terrestris* becoming the preferred bumblebee species for the commercial production of colonies (Velthuis and

Van Doorn, 2006). However, the use of imported commercial colonies of bumblebees not only poses threats to wild conspecific populations, but it may also threaten other wild species of pollinator. Commercial bumblebees often carry parasites such as *Crithidia* and *Nosema bombi* (Colla *et al.,* 2006; Murray *et al.,* 2013) which, when colonies are used in close proximity to wild pollinators, are thought to spread to many different pollinating species in a process called pathogen spillover (Szabo *et al.,* 2012; Graystock *et al.,* 2013). This has been shown by several studies where parasite loads are higher in wild bumblebees the closer they are found to sites using commercial colonies (Colla *et al.,* 2006, Murray *et al.,* 2013). Commercial bumblebees may also be able to compete with wild pollinators for nesting or floral resources (Ings *et al.,* 2006; Goulson, 2010b). Finally, in situations where commercial bumblebees are used within the natural distribution of the species, there is a risk of hybridisation with locally adapted, distinctive subspecies (Seabra *et al.,* 2019; Cejas *et al.,* 2020).

My thesis aimed to explore and characterise distinctions between native Irish *B. t. audax* bumblebees and other wild and commercial populations of *B. t. audax*. Investigating how selection is acting in these populations and evaluating the possible impacts of differential selective forces in these populations is essential to fully understanding and mitigating the potential risks that face them, for example, through pathogen spread or hybridisation with non-native populations.

Human activities such as rapid increase in movement of goods and transport, has increased the frequency of previously isolated populations coming into contact with one another. Invasive species and hybridisation have had serious impacts on many ecosystems and indigenous populations (Huxel, 1999; Meeus *et al.,* 2011; Arbetman *et al.,* 2013). Considering the widespread use of commercial *B. terrestris* colonies, and given the many instances of escape into locations with distinctive subspecies (Seabra *et al.,* 2019; Cejas *et al.,* 2020) or outside its natural distribution (Matsumura *et al.,* 2004; Torretta *et al.,* 2006; Arbetman *et al.,* 2013), there is a clear need to characterise potentially distinctive or vulnerable wild populations of *B. terrestris* before they are lost through hybridisation. While several subspecies of *B. terrestris* have been described, there has been only one other study to our knowledge (Moreira *et al.,* 2015) that has carried out a genetic evaluation of the shared British and Irish subspecies *B. t. audax*. To protect distinctive populations of valuable pollinators, they must be identified first through population genetic and genomic approaches. We used whole

genome sequencing to perform a population genomics study on the populations of *B. terrestris* in Ireland and Britain as this carries many advantages over the commonly used population genetics approaches. For example, it is possible to use a much larger pool of neutral loci which increases the accuracy of tests of relatedness, admixture and inbreeding (Allendorf *et al.,* 2010). The use of whole genomes also allows investigation into selective processes that act across significant stretches of DNA and result in patterns indicative of positive, purifying or balancing selection (Ellegren and Sheldon, 2008). This can provide insight into functionally important genes, as well as neutral evolving loci (Allendorf *et al.,* 2010). We explored differences between Irish and British populations of *B. t. audax* in overall genetic distinctness in addition to genetic variants that are undergoing differential selection between populations. We also examined differential signatures of selection between wild and commercial populations of *B. t. audax* to potentially identify genes that have changed due to the domestication process in captive bred bumblebees.

In Chapter 3, we perform a study also using population genomics to uncover the types of selective pressures acting within the canonical immune gene set of the Irish population of *B. t. audax*. Insects have been subjected to rapid changes in their landscapes as a consequence of the intensification of agriculture and urbanisation (Raven and Wagner, 2021). These changes to their landscapes as well as an associated influx of stressors such as agrochemical use, fragmented habitats, loss of available resources, pathogen spread and climate change have caused dramatic declines in insect numbers and biodiversity (FitzPatrick *et al.,* 2007; Jha, 2015; Hallmann *et al.,* 2017; Leather, 2017; Vogel, 2017; Janzen and Hallwachs, 2019; Sánchez-Bayo and Wyckhuys, 2019; Gunstone *et al.,* 2021; Nessel *et al.,* 2022). However, some insect populations may be able to adapt to the stressors they face in their local environments. For example, evidence has been found in Elaterid beetles (*Limonius* spp.) for adaptation to agricultural fields with specific pesticide regimes (Andrews *et al.,* 2020). In the case of bumblebees, one of the proposed drivers of decline is the spread of pathogens to wild pollinator communities through the worldwide trade in commercial bumblebees (Goka *et al.,* 2001; Colla *et al.,* 2006; Graystock *et al.,* 2013; Murray *et al.,* 2013).

Pathogens exert strong selection pressure on genes involved in immunity (Roux *et al.* 2014; Shultz and Sackton 2019). Given the potential link between pathogen spillover

and pollinator decline (Colla *et al.*, 2006; Goulson, 2010b; Wagner, 2020), understanding how selection is shaping immune genes within a wild bumblebee population can provide information on how resilient a population is to pathogenic threat while also identifying specific genes targeted by selection which may be key in immunity. To examine types of selection and the categories of immune genes they were acting on (e.g., recognition, signalling, regulatory or effector), we identified genomic regions of significantly high or low genetic diversity as well as regions exhibiting high levels of extended haplotype homozygosity. Furthermore, we searched for SNPs with high impacts, e.g., those causing early stop codons that may impact gene product formation and function, within immune genes that could be indicative of redundancy.

My thesis also aimed to explore phenotypic differences between wild, Irish *B. t. audax* and commercial *B. t. audax.* This was of interest as domestication of insects such as *Bombyx mori* and of many mammalian and fish species has resulted in conspicuous domestication syndromes (Jensen, 2006; Lecocq, 2018). Domestication syndromes are the set of genetic characters and phenotypic traits that a captive population acquires throughout the domestication process, and can appear in as little as one generation of captive rearing (Islam *et al.*, 2020). The potential for domestication-related changes to genes and traits of bumblebees is quite possible, as their lifecycles can be controlled fully by humans in captivity and may be completely isolated from wild gene pools (Lecocq, 2018). The acquisition of domestic traits in commercial bumblebees could heighten the perceived risk associated with hybridisation of wild conspecifics with escaped males and gynes from commercial *B. terrestris* colonies. For example, traits associated with domestication can often include a loss of fitness-related genes (Carrete and Tella, 2015) which, if introduced to wild populations, could impact their survival and fitness. This may exacerbate the stressors already facing wild populations of pollinators such as pesticide use and climate change (Goulson *et al.*, 2008).

Using label-free quantitative mass spectrometry, my research also explored proteomic differences between Irish and commercial *B. t. audax* in two key organs; the fat body and the brain. The fat body of insects plays key roles in energy metabolism, protein and lipid storage as well as the production of key haemolymph proteins, including those involved in development, lipid transport and immunity (Arrese and Soulages, 2010; Azeez *et al.*, 2014; Klowden, 2013). The insect brain is one of the main

components of the central nervous system and is responsible for a variety of processes essential to life such as detecting and processing stimuli, coordinating locomotion and storing memories (Davis, 1993; Fahrbach, 2006; Nation, 2008; Matsui *et al.,* 2009; Klowden, 2013). In addition, my research investigated differences and similarities between wild and commercial *B. t. audax* responses to two key threats: insecticide exposure and bacterial infection. These stressors are, of course, very timely to explore as they have been linked to wild pollinator declines (FitzPatrick *et al.,* 2007; Kosior *et al.,* 2007; Goulson *et al.,* 2008; Graystock *et al.,* 2013). Understanding differences between the responses and resilience of wild and commercial *B. terrestris* might help to predict potentially harmful consequences of hybridisation as well as provide insight into how selection, both in natural and captive settings, can influence adaptive traits.

My research also aimed to explore early-stage colony establishment and growth dynamics of wild and commercial bumblebees reared under the same conditions. It is widely acknowledged that when commercial companies began domesticating bumblebees, there were preferences for species and subspecies of bumblebees which produced larger workers and grew larger colonies, such as *B. t. dalmatinus* (Ings *et al.,* 2006; Velthuis and Van Doorn, 2006; Rasmont *et al.,* 2008). It is possible that domesticated populations of bumblebees have also been selected for traits that result in an improved product, such as increased growth rate of workers, female based sex ratios and docility (Evans, 2017). In fact, there is evidence captive bred *B. t. dalmatinus* show an increased growth and success rate compared with wild conspecifics that are reared in the same conditions (Gösterit and Baskar, 2016). Domestication can also cause changes to symbiotic communities associated with the captive host populations (Metcalf *et al.,* 2017; Bowerman *et al.,* 2021; Glazko *et al.,* 2021). We aimed to identify potential domestication-driven distinctions between the growth dynamics and microbiomes of commercially reared and wild-caught *B. t. audax* colonies. Such information would greatly inform risk assessments on the competitive ability and potential consequences of hybridisation associated with imported *B. terrestris* colony use in Ireland.

6.2 Summary of key thesis findings

The first results chapter in this thesis, Chapter 2, focusses on our population genomics study of Irish and British populations of the currently described *B. t. audax* subspecies (Rasmont *et al.,* 2008). We found evidence that this subspecies is composed of two discrete populations: a genetically distinctive Irish population and a British population that is closer in relatedness to mainland European subspecies such as *B. t. terrestris* and *B. t. dalmatinus*. The identification of a distinctive Irish *B. terrestris* population in Ireland mirrors the existence of many other distinctive species found on the island (Sleeman, 2014). The apparently common occurrence of distinctive biodiversity evolving in Ireland suggests that the wildlife here is sufficiently isolated from mixing with other gene pools. Islands are known for higher levels of endemism but also higher extinction risks due to smaller land masses and for this reason, they are commonly prioritised by conservation efforts (Estoup *et al.,* 1996; Whittaker and Fernández-Palacios, 2007; Kier *et al.,* 2009). We also anticipate that the identification of this genetic resource in Ireland will allow for an increased level of caution and protection from the threats associated with commercial bumblebees.

In addition, we find evidence of admixture in the Irish population, possibly a result of hybridisation with currently or previously imported varieties of commercial *B. terrestris.* Previously in Ireland and the UK, a genotype considered to be a blend of *B. t. terrestris* and *B. t. dalmatinus* was imported from commercial facilities for supplemental crop pollination services (Ings *et al.,* 2006; Rasmont *et al.* 2008; Moreira *et al.* 2015). Due to growing concerns that imported colonies could cause genetic homogenisation through hybridisation with the native *B. t. audax* subspecies and competition with all pollinating species (Ings *et al.,* 2006; Teagasc, 2012; Moreira *et al.,* 2015) and the introduction of regulations which controlled import of bumblebee subspecies (Natural England, 2018), commercial companies now sell captive-reared *B. t. audax* as a substitute product (Biobest, 2011). However, to our knowledge, it is not clear where commercial bumblebee facilities sourced their initial stock of *B. t. audax* and how often captive stocks are supplemented with wild genotypes, although this is thought to be limited (Lecocq, 2018). According to our findings, commercial *B. t. audax* from at least one of the suppliers is more similar in genetic makeup to British populations. Considering the low sample sizes for our commercial representatives, we

can only offer a tentative hypothesis that the British population was the original source of *B. t. audax* bees for commercial use, however this group also clustered with mainland European subspecies which, as mentioned before, used to be the main subspecies used in commercial *B. terrestris* bumblebee rearing (Ings *et al.,* 2006; Rasmont *et al.,* 2008). Commercial *B. t. audax* bumblebees sourced from the second commercial supplier were more distantly related to the British population than the first supplier, suggestive that different companies offering what is considered the same subspecies of bumblebee may, in fact, be offering two distinctive genotypes. Our study highlights the need for a thorough genetic evaluation of commercially bred subspecies of *B. terrestris* to assess the suitability of each captive variety for use in importing countries, particularly where colonies are being used without barriers to escape into natural landscapes. More specifically, our study shows the clear distinction between Irish and commercially provided *B. t. audax* and emphasises the likelihood of potential hybridisation of wild and imported individuals.

We also found evidence that both Irish and British *B. t. audax* populations are undergoing distinctive selective processes suggesting these populations may be adapting to the conditions and changes occurring within their environments. Interestingly, it was found that selection appears to be acting on genes associated with fundamental biological processes such as development and the nervous system. This may be a result of two different populations adapting to similar selective pressures in different ways, e.g., exposure to pesticides in agriculture, or may be an indicator that each population has location-specific pressures which are driving location-specific adaptive responses. Ireland and Britain likely have differences in land-use activities and changes to natural landscapes that could have caused differential selection in these populations. For example, the UK has a higher percentage of arable land, while Ireland agricultural land use is predominantly grassland (Eurostat, 2023). Differences in gene flow may also contribute to distinctive signatures of selection; from our results, the British population likely has more connectivity with mainland European populations, as was also found by Moreira *et al.* (2015), and so may also benefit from the introduction of beneficial alleles that evolve in those populations (Tigano and Friesen, 2016).

Our findings outlined in Chapter 2 provide context and foundation to subsequent chapters. In terms of Chapter 3, the findings from the population comparison of Irish

and British *B. t. audax* support the existence of a genetically distinctive and possibly locally adapted Irish *B. terrestris* population which shows distinguishing selection pressures from other populations. These results add justification for a deeper investigation into the dynamic processes of selection acting on an important physiological system within a genetically distinctive population. In Chapter 3, we explored the types of selection acting on canonical immune genes within the wild, Irish population of *B. t. audax* and found that functionally different components of the canonical immune genome were under differential selective forces. For example, we found signatures of positive selection in many genes encoding for scavenger receptor proteins, involved in recognition of pathogen associated molecular patterns, and possible signatures of purifying selection on genes encoding for participants in key signalling pathways of the innate immune system. We also find putative loss of function alleles in genes of CLIP serine proteases and one Serpin which could indicate relaxed evolution on genes encoding non-essential or redundant components of the immune system. Different types of selection acting on canonical immune genes reflect the functional diversity of gene products acting in innate immune pathways of Irish-caught *B. t. audax*. Similar insights into the diversity of selective pressures acting on immune genes have been shown in other insects (e.g., Tan *et al.,* 2021). Our research points towards potentially important gene products that are adapting to cope with immune challenges in their environment and those that potentially have essential roles to bumblebee survival and are therefore under constrained evolution to ensure these functions are conserved. Our findings of certain canonical immune genes showing signatures of recent selection highlights the potential adaptive capacity of this population of Irish *B. t. audax* to an increase of pathogenic stressors in their environment.

This study demonstrates the potential for genomics to investigate ecological questions and paves the way for future research to connect genotypes to functionally important proteins in ecologically important species and to understand how wild populations are adapting to their environments and changes therein (Allendorf *et al.,* 2010). However, whole genome sequencing is just one of many approaches available that can provide useful insight into signatures of selection acting on ecologically important traits. Another method often utilised to investigate genomic signatures of selection include RADseq (Restriction site-associated DNA sequencing) which involves examining

reduced genomes, allowing large numbers of polymorphic markers to be generated at a significantly reduced cost compared to whole genome sequencing (Baird *et al.*, 2008). This method is particularly useful for non-model organisms as it does not require any taxon-specific genomic resources (Andrews *et al.*, 2016), but is limited in how many adaptive loci it can detect (Lowry *et al.*, 2017). RADseq has been used recently to identify signatures of selection acting in *B. pascuorum* and *B. lapidarius* populations in both agricultural and natural settings by Hart *et al.* (2022). This study was able to identify several loci that may be involved in adaptation to agricultural stressors in genes encoding for neurodevelopment and detoxification. Further investigations into both general signatures of selection working on a population as well as focussed analyses, such as ours, on selection shaping important and functionally diverse physiological systems will help identify key loci and gene products contributing to resilience against many anthropogenic threats facing wild insects. Additionally, by assessing regions of the genome experiencing purifying, balancing and relaxed selection, we can gain insight into the dynamic evolutionary forces that constrain and allow adaptation in certain gene products over others. Studies such as these may also help to differentiate populations that are vulnerable to certain threats, e.g., infection, and those that show evidence for adaptation. This may help to focus conservation efforts on populations struggling to overcome the stressors in their environments.

Evidence from previous chapters of selective processes acting on immune genes within wild Irish populations (Chapter 3) alongside evidence of differential selection between Irish and commercial populations of *B. t. audax* on the nervous systems and processes involved in energy production and storage (e.g., the TCA cycle and lipid homeostasis; Chapter 2) could indicate that these biological processes may operate differently between wild and commercial populations due to selective pressures specific to their environments. Additionally, functional changes in these processes driven by changes on the genetic level could impact resilience to prominent stressors of pollinator populations such as increased pathogen spread and pesticide exposure (Palmer and Oldroyd, 2003; Enayati *et al.*, 2005; Bass *et al.*, 2015; Kim *et al.*, 2015). In the proteomic study outlined in Chapter 4, we found that workers from colonies reared from wild and commercial *B. t. audax* queens showed significant distinctions

in their brain and fat body proteomes, both in response to stressors (in this case, acute clothianidin exposure and bacterial infection) and endogenously.

When the brain proteomes of wild and commercial workers were compared, several consistent differences were found. These included an increased abundance of proteins associated with detoxification, cytoskeletal organisation, and biogenic amine pathways in commercial workers, while proteins associated with energy production were generally increased in abundance in wild bees. Responses to acute clothianidin exposure elicited some consistent responses from wild and commercial workers; for example, there was a common reduction in the abundance of proteins involved in nuclear transport, antioxidant activity and copper-chaperoning. Wild exposed bees also had decreased abundances of proteins involved in epithelial cell morphogenesis, oxidoreductase activities and regulation of gene expression which were not observed in other treatment groups. Differences in the proteomes of wild and commercial worker brains, particularly when exposed to an insecticide, may point towards differences in processes and traits that have been shaped by selection pressures of ancestral wild or commercial bumblebees. In particular, the differential abundances of proteins such as *D. melanogaster* homolog *black* and *ebony*, linked to circadian rhythms and biogenesis of pigments (Newby and Jackson, 1991; Phillips *et al.*, 2005), between wild and commercial workers were interesting for a number of reasons. Cysteine sulfinic acid decarboxylase, similar to *D. melanogaster* black, showed strong evidence for positive selection in the Irish population of *B. t. audax* when compared to commercial representatives in Chapter 2. *D. melanogaster* homolog *ebony* was also implicated in the immune response to *E. coli* in both wild and commercial bumblebees examined in Chapter 4. One would expect wild lineages of *B. t. audax* to be under strong selection for maintenance of circadian rhythms as day – night cycles help determine when worker bees in colonies can undertake activities such as foraging and for how long these activities can go on for, although not all individuals may show circadian behaviour (Yerushalmi *et al.*, 2006; Ingram *et al.*, 2009; Shemesh *et al.*, 2010). As captive bred bumblebees do not have to forage for food, perhaps dependence on circadian rhythm is decreased and, therefore, selection does not act on associated genes in the same way as wild populations. This could explain why we observed differences in selection and expression of associated genes between workers from colonies founded by wild and commercial *B. t. audax* queens. Functional

investigations into the roles of these proteins in bumblebees may help identify key biological processes that are differentiated in wild and domesticated bumblebees.

The differential responses to clothianidin exposure between wild and commercial bumblebee workers may also indicate differences in the resilience of wild and commercial bumblebees to cope with agrochemical exposure. Similar to selection pressures acting on the circadian rhythm genes of wild populations, one would expect that wild populations may be more exposed to stressors associated with agricultural practices, such as use of insecticides (Andrews *et al.,* 2020; Hart *et al.,* 2022). Differences in proteomic responses to this threat in wild and commercial bumblebee workers may result in differential resistance to these stressors. Interestingly, we saw commercial bumblebees having increased levels of proteins linked to detoxification, including a cytochrome P450 monooxygenase, and synapse organisation in response to clothianidin while wild exposed workers instead had increased abundances of proteins linked to gene expression and membrane synthesis. Further research is needed into the consequences of these expressional differences to understand if these correlate with benefits to survival or pesticide resistance in wild or commercial populations.

When examining differences in wild and commercial fat body proteomic responses to *E. coli* infection, there were very few differentially expressed proteins in response to *E. coli* infection in both wild and commercial workers. This may be due to the control treatment of Ringer solution injection eliciting a highly similar response to that elicited by *E. coli* infection treatment in both wild and commercial bees. However, there were a small number of proteins showing similar responses in wild and commercial bumblebee fat bodies to *E. coli* infection. For instance, a serpin, calcineurin and a protein similar to *D. melanogaster* Ebony were shown to be increased in abundance in both infected wild and commercial worker fat bodies, indicating the role of these proteins in conserved responses to gram-negative bacterial infection. Both wild and commercial groups exhibited a decreased abundance in protein takeout, which like Ebony, plays a role in circadian rhythms and developmental processes (Sarov-Blat *et al.,* 2000). In terms of differential responses to *E. coli* infection, wild bumblebee workers increased abundance of an antimicrobial peptide alongside proteins involved in actin filament regulation, while commercial bumblebees had increased abundances of proteins relating to protein biosynthesis and transport and decreased protein abundances associated with protein degradation.

The overwhelming finding from this experiment was that wild and commercial fat body proteomes had major distinctions regardless of treatment. Similar to our comparison of brain proteomes, we found consistent differences in abundances of proteins associated to detoxification, fatty acid metabolism and the TCA cycle and translation in wild and commercial bumblebee fat bodies. Interestingly, in Chapter 2, we found signatures of selection in the genomes of commercial bumblebees acting on genes associated with the TCA cycle and lipid homeostasis, indicating that the differences in fat body proteomes could be rooted in genotypic differences in these bees. To investigate whether proteomic differences confer any benefits to the resilience or health in wild or commercial bumblebees, future research should explore if and how insecticide exposure and pathogen infection impact fitness-related behaviours, such as navigation and foraging, and survival in these bumblebees. This will provide insight into the potential risk of outbreeding depression and introduction of maladaptive alleles into wild *B. terrestris* populations through hybridisation with commercial bumblebees.

Worker bees used in proteomics experiments were derived from lab-grown colonies (discussed in more detail in Chapter 5). Colonies were reared under identical conditions to investigate if genotype could influence colony rearing behaviours and the proteomic profiles of resulting worker bees. However, our rearing regime may have generated proteomic profiles specific to the rearing conditions which may not be observed in the real world, and so future research should investigate proteomic differences between wild-established bumblebees and commercial bumblebees reared in commercial facilities being used on crops.

Mass spectrometry-based proteomics is becoming a highly useful tool to address ecological questions (Baer and Millar, 2016). Our study identifies differences in the proteomes of two key organs in populations showing distinctive selective signatures (Chapter 2), which may reflect the potential functional impacts of genetic distinctions. Other studies have also used proteomics to investigate adaptations in certain populations to stressors or changes in their environments (Dalziel and Schulte, 2012; Papakostas *et al.,* 2012; Ebner *et al.,* 2019; Maher *et al.,* 2019). Proteomics is also being used to investigate the impacts of domestication and associated selective processes on the proteome (Rokas, 2009; Douxfils *et al.,* 2011; Hu *et al.,* 2013; Borziak *et al.,* 2016). It also provides the opportunity to test responses to the potential

drivers of selection themselves likely affecting many wild insect populations, such as pathogenic threat and exposure to agrochemicals (Colla *et al.*, 2006; Graystock *et al.*, 2013; Raven and Wagner, 2021). Identifying underlying processes and specific proteins involved in resistance and response to the stressors which insects face will be key in future research and conservation efforts aiming to reverse the declines of ecologically and economically vital pollinators. Furthermore, investigations into the proteomic profiles of domesticated populations may help identify markers associated with certain desired traits and help guide selective breeding practices (Bendixen *et al.*, 2011; Ghatak *et al.*, 2017).

We evaluated potential differences in behaviour and colony growth dynamics between colonies reared from *B. t. audax* queens sourced from the wild and from commercial colonies and found that commercial colonies were significantly more successful in establishing colonies that reached higher numbers of workers. Colonies reared from commercial queens also produced workers at a faster rate than wild colonies. Interestingly, there were no differences in the levels of sucrose consumed or in the sizes of workers produced between wild and commercial colonies. This suggests that nutritional inputs into developing workers were similar. Additionally, developmental periods were of very similar length between workers from wild and commercial colonies. It is possible that commercial queens were laying more eggs compared to the wild queens or that a higher proportion of eggs were making it to adulthood in commercial colonies than wild. In Chapter 2, we saw enrichment in genes under selection in commercial colonies for oocyte fate determination, TCA cycle and lipid homeostasis. In Chapter 4, we also find evidence of proteomic distinctions in the brains and fat bodies of workers. Domestication processes may have selected commercial bumblebee populations for higher output in terms of queen reproduction and caused unintentional changes to processes related to this such as egg development, energy use and storage. Proteomic distinctions in wild and commercial worker fat bodies outlined in Chapter 4 may reflect these functional differences between wild and commercial populations, which may in turn influence colony output. The fat body is important in coordination of insect growth and reproduction (Arrese *et al.*, 2010). Future research investigating differences in the proteomes of wild and commercial *B. terrestris* queen fat bodies may help identify differentially expressed proteins that could explain functional changes in reproductive output. However, environmental

conditions may also have affected reproductive outputs. Most notably, we were limited to catching wild queens once they emerged from natural diapause conditions in Spring while commercial queens underwent an artificial diapause. This may have given commercial queens an advantage in setting up more successful colonies (Beekman and Van Stratum, 2000; Gösterit and Gurel, 2009).

We also investigated distinctions in the gut microbiomes of wild and commercial worker bees, which may likely represent the microbial communities in their queens which are passed to offspring by vertical transmission (Su *et al.,* 2021). We found several distinctions in wild and commercial worker gut microbiomes which could reflect the different environments queens were sourced from (Newbold *et al.,* 2015) or, alternatively, could reflect differential genotypes as found in Chapter 2 (Spor *et al.,* 2011; Bolnick *et al.,* 2014; Chong and Moran, 2016). Distinctions in gut microbiota could also explain differences in reproductive outputs seen between wild and commercial colonies; for example, commercial workers had higher representation of *Bifidobacterium* species, which have been associated with oviposition (Wang *et al.,* 2019).

Our study shows that queens of a commercial lineage may be primed, through genetics or environment, to produce faster-growing and more successful colonies. This is supported by previous studies examining the colony behaviours of commercial colonies (Ings *et al.,* 2006; Gösterit and Baskar, 2016; Gösterit, 2021) and suggests that even commercial colonies that are taxonomically classified as subspecies found in the wild may exhibit very different behaviours. Future research is needed to tease apart the relative influence of genetics and environment on the phenotypic distinctions observed in commercial bumblebees when compared to wild *B. t. audax*. However, we expect the findings of Chapter 5 to have implications on the perceived risk associated with the use of imported bumblebee colonies.

We believe the research reported in this thesis highlights the utility of behavioural and ecological investigations when used alongside molecular approaches such as genomics and proteomics. Data on the intraspecific variation of behaviours and traits alongside molecular data may pave the way towards understanding the genetic and cellular processes that determine behaviours (Jensen, 2006; Rittschof and Hughes, 2018). Consequently, information on the differences in basic biology between

distinctive bumblebee populations may also help optimise computational models that are being developed to predict bumblebee behaviour and trends in response to certain conditions (e.g., Becher *et al.,* 2018).

6.3 Implications of research findings and future hypotheses

All organisms, whether extant or extinct, have been shaped by evolutionary processes. Evolution has created vast levels of biodiversity, or in the words of Darwin (1859) "endless forms most beautiful and wonderful" which are involved in interconnected ecosystems and provide mankind with countless resources and services (Turner *et al.,* 2007; Mace *et al.,* 2012). Bees are one such example of an evolved system which provides both humans and ecosystems alike with the essential service of pollination (Aizen *et al.,* 2009; Ollerton *et al.,* 2011). However, the world for these insects has undergone rapid changes over the last two centuries, driven by the intensification of agriculture, rising CO_2 levels which are contributing to climate change and habitat destruction (Houghton, 1994; Curtis *et al.,* 2018; Stokstad, 2018; WMO, 2018; Raven and Wagner, 2021). Bee populations are exhibiting declines (FitzPatrick *et al.* 2007; Kosior *et al.,* 2007; Colla and Packer, 2008; Goulson *et al.,* 2008; Meeus *et al.,* 2011) likely as a result of these factors and so, in order to conserve vital species and prevent further declines, research is necessary to understand the dynamics of important pollinator species, the threats they face and their responses to them.

In this thesis, I focussed on the bumblebee species *B. terrestris* which is one of the most well-studied bumblebee species and has been a key model in understanding behaviour, physiology, and genetic aspects of bumblebees. While there is a native population of *B. terrestris* in Ireland, commercial colonies of *B. terrestris* are imported by growers to supply crops with pollination services (Velthuis and Van Doorn, 2006; Murray *et al.,* 2013). The risks associated with the use of commercial bumblebees, such as parasite spread, hybridisation and competition (Ings *et al.,* 2005, 2006; Colla *et al.,* 2006; Graystock *et al.,* 2013; Murray *et al.,* 2013; Cejas *et al.,* 2020), demonstrate a need for further research to understand the characteristics and dynamics of wild and commercial bumblebee pollinators. The urgency to protect wild pollinators and reverse their declines is further evident by the EU's recent proposal that EU

member states must "reverse the decline of pollinator populations by 2030 and achieve thereafter an increasing trend of pollinator populations" (COM/2022/304).

At the heart of this thesis is the question of evolution - how does it act on wild and captive populations of *B. terrestris* and what are its consequences? Population genomics provided insight into distinctions between populations of *B. terrestris* in Britain and Ireland as well as deepened our understanding of how these populations are adapting and differentiating (Chapter 2 and Chapter 3). Furthermore, these studies provided preliminary insight into the genetic relationships between wild and commercial *B. terrestris* populations, by highlighting specific genes that are distinctive between them and also by finding evidence of hybridisation within the Irish *B. terrestris* population. Functional studies in this thesis found distinctions in phenotypes between wild and commercial bumblebees, such as proteomic profiles of key organs which play vital roles in bumblebee survival and fitness (Chapter 4), as well as colony growth behaviour and gut microbiota (Chapter 5), which may be a result of genetic distinctions between these populations.

The findings of this thesis Provide support for the hypothesis that commercial *B. t. audax* colonies exhibit distinctions from wild, Irish *B. t. audax* on genetic, proteomic and behavioural levels. There are several possible reasons behind this. Firstly, commercial *B. t. audax* may have been sourced from genetically dissimilar British *B. t. audax* populations and, so, may exhibit traits characteristic of this population. Furthermore, commercial, and wild populations of *B. t. audax* likely experience different selection pressures. Commercial bumblebees are likely bred in a closed system and so captive bred *B. t. audax* may have experienced elevated levels of genetic drift alongside selective pressures associated with artificial selection. Wild bumblebees in Ireland likely experience selective pressures specific to their natural environment and stressors therein, such as pathogenic threats, and we find evidence for this in Chapter 3. My thesis' findings underline the clear need for careful and appropriate management of commercial bumblebee colonies in Ireland, as outlined in Appendix A1.1, as well as the need for further investigation into bumblebee domestication. There is no doubt that domestication can cause significant alteration to the genetics and characteristics of captive populations (Jensen, 2006; Lecocq, 2018). By understanding the links between genotype and phenotype, commercial facilities can apply this knowledge to breeding practices to maintain stocks with beneficial traits

(Rothammer *et al.*, 2013) and importing countries can be informed on the conservation risks of certain commercial bumblebee genotypes.

I list below areas of future research which will help elucidate the causes and consequences of bumblebee domestication as well as further understanding of wild bumblebee fitness and adaptations to stressors within their environments:

- I hypothesise that the evidence supporting a distinctive Irish *B. terrestris* population may be predictive of other distinctive Irish pollinator populations. Future research should evaluate other pollinator species in Ireland on a genetic level as they may exhibit distinctive local adaptations that may be important to characterise and offer them protection as genetic resources;
- Genomic comparisons of Irish and British populations revealed distinctions in processes associated with the nervous system and development. Future research should investigate whether phenotypic distinctions linked to these processes exist between these populations and, in particular, if any phenotypic distinctions influence resilience to certain threats e.g., pesticide exposure. In addition, functional studies on the gene products of highlighted genes from both Chapter 2 and Chapter 3 findings should also be performed to identify potentially important roles of these products and if they change structurally or functionally in certain genotypes;
- Further genomic characterisations of the various subspecies of *B. terrestris* supplied by commercial companies and genomic comparisons of wild and commercial *B. terrestris* populations would provide information on selective forces acting within captive populations and potential traits under selection as a result of domestication;
- Similarly, there is a need to understand further phenotypic distinctions between wild and commercial bumblebees to fully assess the risks of competition and hybridisation. For example, behavioural studies on foraging and pollination efficiency would provide essential information on how necessary commercial bumblebees actually are for pollination services as well as allowing us to investigate potential competition with wild pollinators for floral resources;
- Evidence for differential growth dynamics of wild and commercial bumblebee colonies in particular warrants further investigation. For example, proteomic comparisons of wild and commercial *B. t. audax* queens may provide

information on whether commercial queens have particular processes upregulated in comparison to wild queens that facilitate an increased reproductive rate. An interesting focus would be a further characterisation of differences between wild and domesticated *B. terrestris* queen fat bodies to investigate whether there is a relationship between altered reproductive behaviours and changes in pathways impacting nutrition and energy storage. Furthermore, it should also be investigated whether certain microbial communities confer reproductive benefits in bumblebees.

To conclude, my thesis findings provide evidence for genetic distinctions and signatures of selection within the Irish population of *B. terrestris* and reveal potential genetic and phenotypic distinctions between wild and commercial bumblebees. I believe the combination of molecular and ecological approaches are providing new insights into pollinator health and adaptation and will deepen our understanding of the risks faced by pollinator populations, how these risks may be mitigated and, on a broader level, how important pollinators such as bees, have been and are being, evolved.

Chapter 7

Bibliography

7.0 Bibliography

Aaronson, D.S. and Horvath, C.M. (2002) ‘A road map for those who don’t know JAK-STAT’, Science, 296(5573), 1653–1655.

Abondio, P., Cilli, E., and Luiselli, D. (2022) ‘Inferring Signatures of Positive Selection in Whole-Genome Sequencing Data: An Overview of Haplotype-Based Methods’, Genes, 13(5), 926.

Adibhatla, R.M. and Hatcher, J.F. (2007) ‘Role of lipids in brain injury and diseases’, Future Lipidology, 2(4), 403–422.

Agaisse, H. and Perrimon, N. (2004) ‘The roles of JAK/STAT signaling in *Drosophila* immune responses’, Immunological Reviews, 198(1), 72–82.

Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., and Klein, A.M. (2009) ‘How much does agriculture depend on pollinators? Lessons from long-term trends in crop production’, Annals of Botany, 103(9), 1579–1588.

Akalal, D.-B.G., Yu, D., and Davis, R.L. (2011) ‘The long-term memory trace formed in the *Drosophila* α/β mushroom body neurons is abolished in long-term memory mutants’, Journal of Neuroscience, 31(15), 5643–5647.

Akhtar, M.S., Babel, S., Yadav, B., Yadav, R.S., and Panwar, J. (2012) ‘Potentiality of Thiobacillus in Agricultural System’, Advanced Science, Engineering and Medicine, 4, 77–80.

Aktar, W., Sengupta, D., and Chowdhury, A. (2009) ‘Impact of pesticides use in agriculture: their benefits and hazards’, Interdisciplinary Toxicology, 2(1), 1–12.

Albert, J. and Lingle, C. (1993) ‘Activation of nicotinic acetylcholine receptors on cultured *Drosophila* and other insect neurones.’, The Journal of Physiology, 463(1), 605–630.

Alberts, B., Bray, D., Hopkin, K., Johnson, A.D., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2015) Essential Cell Biology, Garland Science: New York, USA.

Alexa, A. and Rahnenführer, J. (2009) ‘Gene set enrichment analysis with topGO’, Bioconductor Improv, 27, 1–26.

Alexander, D.H., Novembre, J., and Lange, K. (2009) ‘Fast model-based estimation of ancestry in unrelated individuals’, Genome Research, 19(9), 1655–1664.

Alford, D. (1971) ‘Egg laying by bumble bee queens at the beginning of colony development’, Bee World, 52(1), 11–18.

Alford, D.V. (1969) ‘A Study of the Hibernation of Bumblebees (Hymenoptera: Bombidae) in Southern England’, The Journal of Animal Ecology, 38(1), 149.

Alford, D.V. (1975) Bumblebees, Davis-Poynter: London, UK.

Alford, D.V. (2009) The Life of the Bumblebee, Northern Bee Books.

Alger, S. A., Burnham, P. A., Boncristiani, H. F., & Brody, A. K. (2019) ‘RNA virus spillover from managed honeybees (*Apis mellifera*) to wild bumblebees (*Bombus spp.*)’, PloS One, 14(6), e0217822.

Alghamdi, A., Dalton, L., Phillis, A., Rosato, E., and Mallon, E.B. (2008) ‘Immune response impairs learning in free-flying bumble-bees’, Biology Letters, 4(5), 479–481.

Ali Mohammadie Kojour, M., Han, Y.S., and Jo, Y.H. (2020) ‘An overview of insect innate immunity’, Entomological Research, 50(6), 282–291.

Allendorf, F.W., Hohenlohe, P.A., and Luikart, G. (2010) ‘Genomics and the future of conservation genetics’, Nature Reviews Genetics, 11(10), 697–709.

Allendorf, F.W., Leary, R.F., Spruell, P., and Wenburg, J.K. (2001) ‘The problems with hybrids: setting conservation guidelines’, Trends in Ecology & Evolution, 16(11), 613–622.

Allmon, W.D. (1992) ‘A causal analysis of stages in allopatric speciation’, Oxford Surveys in Evolutionary Biology, 8, 219–219.

Altman, B.J., Stine, Z.E., and Dang, C.V. (2016) ‘From Krebs to clinic: glutamine metabolism to cancer therapy’, Nature Reviews Cancer, 16(10), 619–634.

Amor, M.D., Norman, M.D., Cameron, H.E., and Strugnell, J.M. (2014) ‘Allopatric speciation within a cryptic species complex of Australasian octopuses’, PLoS One, 9(6), e98982.

Amsalem, E., Teal, P., Grozinger, C.M., and Hefetz, A. (2014) ‘Precocene-I inhibits juvenile hormone biosynthesis, ovarian activation, aggression and alters sterility

signal production in bumble bee (*Bombus terrestris*) workers', Journal of Experimental Biology, 217(17), 3178–3185.

Amsalem, E., Twele, R., Francke, W., and Hefetz, A. (2009) 'Reproductive competition in the bumble-bee *Bombus terrestris*: Do workers advertise sterility?', Proceedings of the Royal Society B: Biological Sciences, 276(1660), 1295–1304.

An, C., Zhang, M., Chu, Y., and Zhao, Z. (2013) 'Serine protease MP2 activates prophenoloxidase in the melanization immune response of *Drosophila melanogaster*', PLoS One, 8(11), e79533.

Anderson, P. and Kedersha, N. (2006) 'RNA granules', Journal of Cell Biology, 172(6), 803–808.

Anderson, P. and Kedersha, N. (2009) 'Stress granules', Current Biology, 19(10), R397–R398.

Andrews, K.R., Gerritsen, A., Rashed, A., Crowder, D.W., Rondon, S.I., van Herk, W.G., Vernon, R., Wanner, K.W., Wilson, C.M., and New, D.D. (2020) 'Wireworm (Coleoptera: Elateridae) genomic analysis reveals putative cryptic species, population structure, and adaptation to pest control', Communications Biology, 3(1), 489.

Andrews, K.R., Good, J.M., Miller, M.R., Luikart, G., and Hohenlohe, P.A. (2016) 'Harnessing the power of RADseq for ecological and evolutionary genomics', Nature Reviews Genetics, 17(2), 81–92.

Andrews, S. (2010) 'FastQC: a quality control tool for high throughput sequence data', available: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [accessed 22 Aug 2022].

Arbetman, M.P., Gleiser, G., Morales, C.L., Williams, P., and Aizen, M.A. (2017) 'Global decline of bumblebees is phylogenetically structured and inversely related to species range size and pathogen incidence', Proceedings of the Royal Society B: Biological Sciences, 284(1859), 20170204.

Arbetman, M.P., Meeus, I., Morales, C.L., Aizen, M.A., and Smagghe, G. (2013) 'Alien parasite hitchhikes to Patagonia on invasive bumblebee', Biological Invasions, 15(3), 489–494.

Arnér, E.S. and Holmgren, A. (2000) ‘Physiological functions of thioredoxin and thioredoxin reductase’, European Journal of Biochemistry, 267(20), 6102–6109.

Arrese, E.L. and Soulages, J.L. (2010) ‘Insect fat body: energy, metabolism, and regulation’, Annual Review of Entomology, 55, 207.

Athenstaedt, K. and Daum, G. (2006) ‘The life cycle of neutral lipids: synthesis, storage and degradation’, Cellular and Molecular Life Sciences CMLS, 63(12), 1355–1369.

Audisio, M.C., Torres, M.J., Sabaté, D.C., Ibarguren, C., and Apella, M.C. (2011) ‘Properties of different lactic acid bacteria isolated from *Apis mellifera* L. bee-gut’, Microbiological Research, 166(1), 1–13.

Azeez, O.I., Meintjes, R., and Chamunorwa, J.P. (2014) ‘Fat body, fat pad and adipose tissues in invertebrates and vertebrates: the nexus’, Lipids in Health and Disease, 13(1), 1–13.

Baer, B. and Millar, A. (2016) ‘Proteomics in evolutionary ecology’, Journal of Proteomics, 135, 4–11.

Baer, B. and Schmid-Hempel, P. (2006) ‘Phenotypic variation in male and worker encapsulation response in the bumblebee *Bombus terrestris*’, Ecological Entomology, 31(6), 591–596.

Baird, N.A., Etter, P.D., Atwood, T.S., Currey, M.C., Shiver, A.L., Lewis, Z.A., Selker, E.U., Cresko, W.A., and Johnson, E.A. (2008) ‘Rapid SNP discovery and genetic mapping using sequenced RAD markers’, PloS one, 3(10), e3376.

Bakker, J.P. and Berendse, F. (1999) ‘Constraints in the restoration of ecological diversity in grassland and heathland communities’, Trends in Ecology & Evolution, 14(2), 63–68.

Balloux, F. and van Dorp, L. (2017) ‘Q&A: What are pathogens, and what have they done to and for us?’, BMC Biology, 15(1), 1–6.

Baltz, A.G., Munschauer, M., Schwanhäusser, B., Vasile, A., Murakawa, Y., Schueler, M., Youngs, N., Penfold-Brown, D., Drew, K., and Milek, M. (2012) ‘The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts’, Molecular Cell, 46(5), 674–690.

Banks, J.E., Banks, H.T., Myers, N., Laubmeier, A.N., and Bommarco, R. (2020) 'Lethal and sublethal effects of toxicants on bumble bee populations: a modelling approach', Ecotoxicology, 29, 237-245.

Bantz, A., Camon, J., Froger, J.-A., Goven, D., and Raymond, V. (2018) 'Exposure to sublethal doses of insecticide and their effects on insects at cellular and physiological levels', Current Opinion in Insect Science, 30, 73–78.

Barbara, G.S., Zube, C., Rybak, J., Gauthier, M., and Grünewald, B. (2005) 'Acetylcholine, GABA and glutamate induce ionic currents in cultured antennal lobe neurons of the honeybee, *Apis mellifera*', Journal of Comparative Physiology A, 191, 823–836.

Barilani, M., Bernard-Laurent, A., Mucci, N., Tabarroni, C., Kark, S., Perez Garrido, J.A., and Randi, E. (2007) 'Hybridisation with introduced chukars (*Alectoris chukar*) threatens the gene pool integrity of native rock (*A. graeca*) and red-legged (*A. rufa*) partridge populations', Biological Conservation, 137(1), 57–69.

Baron, G.L., Raine, N.E., and Brown, M.J. (2017) 'General and species-specific impacts of a neonicotinoid insecticide on the ovary development and feeding of wild bumblebee queens', Proceedings of the Royal Society B: Biological Sciences, 284(1854), 20170123.

Barreiro, L.B. and Quintana-Murci, L. (2010) 'From evolutionary genetics to human immunology: how selection shapes host defence genes', Nature Reviews Genetics, 11(1), 17–30.

Barribeau, S.M. and Schmid-Hempel, P. (2013) 'Qualitatively different immune response of the bumblebee host, *Bombus terrestris*, to infection by different genotypes of the trypanosome gut parasite, *Crithidia bombi*', Infection, Genetics and Evolution, 20, 249–256.

Barribeau, S.M., Sadd, B.M., du Plessis, L., Brown, M.J., Buechel, S.D., Cappelle, K., Carolan, J.C., Christiaens, O., Colgan, T.J., and Erler, S. (2015) 'A depauperate immune repertoire precedes evolution of sociality in bees', Genome Biology, 16(1), 1–21.

Bass, C., Denholm, I., Williamson, M.S., and Nauen, R. (2015) 'The global status of insect resistance to neonicotinoid insecticides', Insecticide and Acaricide Modes of Action and their Role in Resistance and its Management, 121, 78–87.

Batra, S.W. (1966) 'Nests and social behavior of halictine bees of India (Hymenoptera: Halictidae)', The Indian Journal of Entomology, 28, 375.

Becher, M.A., Twiston-Davies, G., Penny, T.D., Goulson, D., Rotheray, E.L., and Osborne, J.L. (2018) 'Bumble-BEEHAVE: A systems model for exploring multifactorial causes of bumblebee decline at individual, colony, population and community level', Journal of Applied Ecology, 55(6), 2790–2801.

Beekman, M. and Van Stratum, P. (2000) 'Does the diapause experience of bumblebee queens *Bombus terrestris* affect colony characteristics?', Ecological Entomology, 25(1), 1–6.

Beekman, M., Van Stratum, P., and Lingeman, R. (1998) 'Diapause survival and post-diapause performance in bumblebee queens (*Bombus terrestris*)', Entomologia Experimentalis et Applicata, 89(3), 207–214.

Beekman, M., Van Stratum, P., and Lingeman, R. (2000) 'Artificial rearing of bumble bees (*Bombus terrestris*) selects against heavy queens', Journal of Apicultural Research, 39(1–2), 61–65.

Beekman, M., Van Stratum, P., and Veerman, A. (1999) 'Selection for non-diapause in the bumblebee *Bombus terrestris*, with notes on the effect of inbreeding', Entomologia Experimentalis et Applicata, 93(1), 69–75.

Beenakkers, A.T., Van der Horst, D., and Van Marrewijk, W. (1984) 'Insect flight muscle metabolism', Insect Biochemistry, 14(3), 243–260.

Begon, M. and Townsend, C.R. (2020) Ecology: From Individuals to Ecosystems, John Wiley & Sons: West Sussex, UK.

Bell, D.D., Weaver, W.D., and North, M.O. (2001) Commercial Chicken Meat and Egg Production, Springer Science & Business Media.

Bendixen, E., Danielsen, M., Hollung, K., Gianazza, E., and Miller, I. (2011) 'Farm animal proteomics—a review', Journal of Proteomics, 74(3), 282–293.

Berendse, F., Elberse, W.T., and Geerts, R. (1992) ‘Competition and nitrogen loss from plants in grassland ecosystems’, Ecology, 73(1), 46–53.

Berntson, E.A., Carmichael, R.W., Flesher, M.W., Ward, E.J., and Moran, P. (2011) ‘Diminished Reproductive Success of Steelhead from a Hatchery Supplementation Program (Little Sheep Creek, Imnaha Basin, Oregon)’, Transactions of the American Fisheries Society, 140(3), 685–698.

Beye, M., Gattermeier, I., Hasselmann, M., Gempe, T., Schioett, M., Baines, J.F., Schlipalius, D., Mougel, F., Emore, C., and Rueppell, O. (2006) ‘Exceptionally high levels of recombination across the honey bee genome’, Genome Research, 16(11), 1339–1344.

Bilio, M. (2007) ‘Controlled reproduction and domestication in aquaculture’, Aquaculture Europe, 32(1), 5–14.

Billiet, A., Meeus, I., Van Nieuwerburgh, F., Deforce, D., Wäckers, F., and Smagghe, G. (2016) ‘Impact of sugar syrup and pollen diet on the bacterial diversity in the gut of indoor-reared bumblebees (*Bombus terrestris*)’, Apidologie, 47(4), 548–560.

Billiet, A., Meeus, I., Van Nieuwerburgh, F., Deforce, D., Wäckers, F., and Smagghe, G. (2017) ‘Colony contact contributes to the diversity of gut bacteria in bumblebees (*Bombus terrestris*)’, Insect Science, 24(2), 270–277.

Biobest (2011) ‘Success of Biobest’s Native UK Bumblebee Recently Proven by Science’, Biobest, available: https://www.biobestgroup.com/en/news/success-of-biobest%C2%92s-native-uk-bumblebee-recently-proven-by-science [accessed 27 Feb 2023].

Bischoff, F.R. and Ponstingl, H. (1991) ‘Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1’, Nature, 354(6348), 80–82.

Blouin, M.S., Wrey, M.C., Bollmann, S.R., Skaar, J.C., Twibell, R.G., and Fuentes, C. (2021) ‘Offspring of first-generation hatchery steelhead trout (*Oncorhynchus mykiss*) grow faster in the hatchery than offspring of wild fish, but survive worse in the wild: Possible mechanisms for inadvertent domestication and fitness loss in hatchery salmon’, PLoS One, 16(12), e0257407.

Bolnick, D.I., Snowberg, L.K., Caporaso, J.G., Lauber, C., Knight, R., and Stutz, W.E. (2014) 'Major Histocompatibility Complex class II b polymorphism influences gut microbiota composition and diversity', Molecular Ecology, 23(19), 4831–4845.

Boncristiani, H., Li, J., Evans, J.D., Pettis, J., and Chen, Y. (2011) 'Scientific note on PCR inhibitors in the compound eyes of honey bees, *Apis mellifera*', Apidologie, 42(4), 457–460.

Bonder, M.J., Kurilshikov, A., Tigchelaar, E.F., Mujagic, Z., Imhann, F., Vila, A.V., Deelen, P., Vatanen, T., Schirmer, M., Smeekens, S.P., Zhernakova, D.V., Jankipersadsing, S.A., Jaeger, M., Oosting, M., Cenit, M.C., Masclee, A.A.M., Swertz, M.A., Li, Y., Kumar, V., Joosten, L., Harmsen, H., Weersma, R.K., Franke, L., Hofker, M.H., Xavier, R.J., Jonkers, D., Netea, M.G., Wijmenga, C., Fu, J., and Zhernakova, A. (2016) 'The effect of host genetics on the gut microbiome', Nature Genetics, 48(11), 1407–1412.

Bonifacino, J.S. and Glick, B.S. (2004) 'The mechanisms of vesicle budding and fusion', Cell, 116(2), 153–166.

Bortolotti, L., Grazioso, E., Porrini, C., and Sbrenna, G. (2001) 'Effect of pesticides on the bumblebee *Bombus terrestris* L. in the laboratory', IOBC WPRS Bulletin 23(3), 31.

Borziak, K., Álvarez-Fernández, A., L Karr, T., Pizzari, T., and Dorus, S. (2016) 'The Seminal fluid proteome of the polyandrous Red junglefowl offers insights into the molecular basis of fertility, reproductive ageing and domestication', Scientific Reports, 6(1), 1–15.

Bosmans, L., Pozo, M.I., Verreth, C., Crauwels, S., Wilberts, L., Sobhy, I.S., Wäckers, F., Jacquemyn, H., and Lievens, B. (2018) 'Habitat-specific variation in gut microbial communities and pathogen prevalence in bumblebee queens (*Bombus terrestris*)', PLOS ONE, 13(10), e0204612.

Botías, C., Jones, J.C., Pamminger, T., Bartomeus, I., Hughes, W.O., and Goulson, D. (2021) 'Multiple stressors interact to impair the performance of bumblebee *Bombus terrestris* colonies', Journal of Animal Ecology, 90(2), 415–431.

Bourke, A.F. (1994) ‘Worker matricide in social bees and wasps’, Journal of Theoretical Biology, 167(3), 283–292.

Bowerman, K.L., Knowles, S.C.L., Bradley, J.E., Baltrūnaitė, L., Lynch, M.D.J., Jones, K.M., and Hugenholtz, P. (2021) ‘Effects of laboratory domestication on the rodent gut microbiome’, ISME Communications, 1(1), 49.

Brett, C.L., Donowitz, M., and Rao, R. (2005) ‘Evolutionary origins of eukaryotic sodium/proton exchangers’, American Journal of Physiology-Cell Physiology, 288(2), C223–C239.

Breuss, M.W., Leca, I., Gstrein, T., Hansen, A.H., and Keays, D.A. (2017) ‘Tubulins and brain development – The origins of functional specification’, Molecular and Cellular Neuroscience, 84, 58–67.

Brian, A.D. (1952) ‘Division of Labour and Foraging in *Bombus agrorum* Fabricius’, The Journal of Animal Ecology, 21(2), 223.

Briones-Roblero, C.I., Rodríguez-Díaz, R., Santiago-Cruz, J.A., Zúñiga, G., and Rivera-Orduña, F.N. (2017) ‘Degradation capacities of bacteria and yeasts isolated from the gut of *Dendroctonus rhizophagus* (Curculionidae: Scolytinae)’, Folia Microbiologica, 62(1), 1–9.

Brites, D., McTaggart, S., Morris, K., Anderson, J., Thomas, K., Colson, I., Fabbro, T., Little, T.J., Ebert, D., and Du Pasquier, L. (2008) ‘The Dscam homologue of the crustacean *Daphnia* is diversified by alternative splicing like in insects’, Molecular biology and evolution, 25(7), 1429–1439.

Brown, M.J., Schmid-Hempel, R., and Schmid-Hempel, P. (2003) ‘Strong context-dependent virulence in a host–parasite system: reconciling genetic evidence with theory’, Journal of Animal Ecology, 72(6), 994–1002.

Burbrink, F. T., Crother, B. I., Murray, C. M., Smith, B. T., Ruane, S., Myers, E. A., & Pyron, R. A. (2022) ‘Empirical and philosophical problems with the subspecies rank’, Ecology and Evolution, 12(7), e9069.

Buttermore, R. (1997) ‘Observations of successful *Bombus terrestris* (L.)(Hymenoptera: Apidae) colonies in southern Tasmania’, Australian Journal of Entomology, 36(3), 251–254.

Cameron, S.A., Hines, H.M., and Williams, P.H. (2007) 'A comprehensive phylogeny of the bumble bees (*Bombus*)', Biological Journal of the Linnean Society, 91(1), 161–188.

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F., and Griswold, T.L. (2011) 'Patterns of widespread decline in North American bumble bees', Proceedings of the National Academy of Sciences, 108(2), 662–667.

Campos, J. and Hartenstein, V. (1985) 'The embryonic development of *Drosophila melanogaster*', Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormone, Springer-Verlag, Berlin, Germany, 218–225.

Canale, C.I. and Henry, P.-Y. (2010) 'Adaptive phenotypic plasticity and resilience of vertebrates to increasing climatic unpredictability', Climate Research, 43(1–2), 135–147.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010) 'QIIME allows analysis of high-throughput community sequencing data', Nature Methods, 7(5), 335–336.

Cappelli, A., Favia, G., and Ricci, I. (2021) '*Wickerhamomyces anomalus* in Mosquitoes: A Promising Yeast-Based Tool for the "Symbiotic Control" of Mosquito-Borne Diseases', Frontiers in Microbiology, 11, 621605.

Cappelli, A., Ulissi, U., Valzano, M., Damiani, C., Epis, S., Gabrielli, M.G., Conti, S., Polonelli, L., Bandi, C., and Favia, G. (2014) 'A *Wickerhamomyces anomalus* killer strain in the malaria vector *Anopheles stephensi*', PloS One, 9(5), e95988.

Cariveau, D.P., Elijah Powell, J., Koch, H., Winfree, R., and Moran, N.A. (2014) 'Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*)', The ISME Journal, 8(12), 2369–2379.

Carlsson, J., Cross, T.F., McGinnity, P., Prodöhl, P.A., and McDevitt, A.D. (2014) 'The use of genetics to infer the origins of the terrestrial and freshwater elements of the Irish fauna', Irish Naturalists' Journal, 33, 8–27.

Carrete, M. and Tella, J.L. (2015) ‘Rapid loss of antipredatory behaviour in captive-bred birds is linked to current avian invasions’, Scientific Reports, 5(1), 1–8.

Cartereau, A., Pineau, X., Lebreton, J., Mathé-Allainmat, M., Taillebois, E., and Thany, S.H. (2022) ‘Impairments in learning and memory performances associated with nicotinic receptor expression in the honeybee *Apis mellifera* after exposure to a sublethal dose of sulfoxaflor’, Plos One, 17(8), e0272514.

Casano, A.M. and Sixt, M. (2018) ‘A Fat Lot of Good for Wound Healing’, Developmental Cell, 44(4), 405–406.

Castagna, M., Shayakul, C., Trotti, D., Sacchi, V.F., Harvey, W.R., and Hediger, M.A. (1997) ‘Molecular characteristics of mammalian and insect amino acid transporters: implications for amino acid homeostasis.’, The Journal of Experimental Biology, 200(2), 269–286.

Casteels, P., Ampe, C., Jacobs, F., Vaeck, M., and Tempst, P. (1989) ‘Apidaecins: antibacterial peptides from honeybees.’, The EMBO Journal, 8(8), 2387–2391.

Castelli, L., Branchiccela, B., Romero, H., Zunino, P., & Antúnez, K. (2022) ‘Seasonal dynamics of the honey bee gut microbiota in colonies under subtropical climate: Seasonal dynamics of honey bee gut microbiota’, Microbial Ecology, 83(2), 492-500.

Castillo, J. C., Reynolds, S. E., & Eleftherianos, I. (2011) ‘Insect immune responses to nematode parasites’, Trends in Parasitology, 27(12), 537-547.

Ceja-Navarro, J.A., Vega, F.E., Karaoz, U., Hao, Z., Jenkins, S., Lim, H.C., Kosina, P., Infante, F., Northen, T.R., and Brodie, E.L. (2015) ‘Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee’, Nature Communications, 6(1), 1–9.

Cejas, D., López-López, A., Muñoz, I., Ornosa, C., and De la Rúa, P. (2020) ‘Unveiling introgression in bumblebee (*Bombus terrestris*) populations through mitogenome-based markers’, Animal Genetics, 51(1), 70–77.

Celli, J. and Tsolis, R.M. (2015) ‘Bacteria, the endoplasmic reticulum and the unfolded protein response: friends or foes?’, Nature Reviews Microbiology, 13(2), 71–82.

Chaimanee, V., Evans, J.D., Chen, Y., Jackson, C., and Pettis, J.S. (2016) 'Sperm viability and gene expression in honey bee queens (*Apis mellifera*) following exposure to the neonicotinoid insecticide imidacloprid and the organophosphate acaricide coumaphos', Journal of Insect Physiology, 89, 1–8.

Chand, S., Chand, S., and Raula, B. (2022) 'Usage of Silkworm Materials in Various Ground of Science and Research', Journal of Natural Fibers, 1–14.

Chanda, S. and Xu-Friedman, M.A. (2010) 'Neuromodulation by GABA converts a relay into a coincidence detector', Journal of Neurophysiology, 104(4), 2063–2074.

Chapman, J.R., Hill, T., and Unckless, R.L. (2019) 'Balancing selection drives the maintenance of genetic variation in *Drosophila* antimicrobial peptides', Genome Biology and Evolution, 11(9), 2691–2701.

Charles, H.M. and Killian, K.A. (2015) 'Response of the insect immune system to three different immune challenges', Journal of Insect Physiology, 81, 97–108.

Charroux, B. and Royet, J. (2010) '*Drosophila* immune response: From systemic antimicrobial peptide production in fat body cells to local defense in the intestinal tract', Fly, 4(1), 40–47.

Chase, M.R., Raina, K., Bruno, J., and Sugumaran, M. (2000) 'Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*', Insect Biochemistry and Molecular Biology, 30(10), 953–967.

Chaudhry, N., Sica, M., Surabhi, S., Hernandez, D.S., Mesquita, A., Selimovic, A., Riaz, A., Lescat, L., Bai, H., and Macintosh, G.C. (2022) 'Lamp1 mediates lipid transport, but is dispensable for autophagy in *Drosophila*', Autophagy, 18(10), 1–16.

Chen, C.-M., Chiang, S.-Y., and Yeh, N.-H. (1991) 'Increased stability of nucleolin in proliferating cells by inhibition of its self-cleaving activity.', Journal of Biological Chemistry, 266(12), 7754–7758.

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018) 'fastp: an ultra-fast all-in-one FASTQ preprocessor', Bioinformatics, 34(17), i884–i890.

Chi, C., Wang, L., Lan, W., Zhao, L., and Su, Y. (2018) 'PpV, acting via the JNK pathway, represses apoptosis during normal development of *Drosophila* wing', Apoptosis, 23(9), 554–562.

Chinnici, J. P. (1971) ‘Modification of recombination frequency in Drosophila. I. Selection for increased and decreased crossing over’, Genetics, 69(1), 71.

Cho, O., Matsumoto, Y., Yamada, T., & Sugita, T. (2022) ‘Establishment of a gene recombination method for a major human skin commensal fungus, *Malassezia restricta*, using *Agrobacterium tumefaciens*-mediated gene transfer system’, Medical Mycology, 60(11), myac077.

Choi, I.K. and Hyun, S. (2012) ‘Conserved microRNA miR-8 in fat body regulates innate immune homeostasis in *Drosophila*’, Developmental & Comparative Immunology, 37(1), 50–54.

Choi, Y.J., Kim, S., Choi, Y., Nielsen, T.B., Yan, J., Lu, A., Ruan, J., Lee, H.-R., Wu, H., and Spellberg, B. (2019) ‘SERPINB1-mediated checkpoint of inflammatory caspase activation’, Nature Immunology, 20(3), 276–287.

Chong, R.A. and Moran, N.A. (2016) ‘Intraspecific genetic variation in hosts affects regulation of obligate heritable symbionts’, Proceedings of the National Academy of Sciences, 113(46), 13114–13119.

Chouaia, B., Gaiarsa, S., Crotti, E., Comandatore, F., Degli Esposti, M., Ricci, I., Alma, A., Favia, G., Bandi, C., and Daffonchio, D. (2014) ‘Acetic acid bacteria genomes reveal functional traits for adaptation to life in insect guts’, Genome Biology and Evolution, 6(4), 912–920.

Chouaia, B., Rossi, P., Epis, S., Mosca, M., Ricci, I., Damiani, C., Ulissi, U., Crotti, E., Daffonchio, D., and Bandi, C. (2012) ‘Delayed larval development in *Anopheles mosquitoes* deprived of Asaiabacterial symbionts’, BMC Microbiology, 12(1), 1–8.

Cingolani, P., Platts, A., Wang, L.L., Coon, M., Nguyen, T., Wang, L., Land, S.J., Lu, X., and Ruden, D.M. (2012) ‘A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3’, Fly, 6(2), 80–92.

Clayton, J.B., Gomez, A., Amato, K., Knights, D., Travis, D.A., Blekhman, R., Knight, R., Leigh, S., Stumpf, R., and Wolf, T. (2018) ‘The gut microbiome of nonhuman primates: lessons in ecology and evolution’, American Journal of Primatology, 80(6), e22867.

Cnaani, J., Schmid-Hempel, R., and Schmidt, J.O. (2002) 'Colony development, larval development and worker reproduction in *Bombus impatiens* Cresson', Insectes Sociaux, 49(2), 164–170.

Cociancich, S., Ghazi, A., Hetru, C., Hoffmann, J., and Letellier, L. (1993) 'Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus*', Journal of Biological Chemistry, 268(26), 19239–19245.

Coker, O. O., Nakatsu, G., Dai, R. Z., Wu, W. K. K., Wong, S. H., Ng, S. C., Chan, F.K.L., Sung, J.J.Y., and Yu, J. (2019) 'Enteric fungal microbiota dysbiosis and ecological alterations in colorectal cancer', Gut, 68(4), 654-662.

Colgan, T.J., Arce, A.N., Gill, R.J., Ramos Rodrigues, A., Kanteh, A., Duncan, E.J., Li, L., Chittka, L., and Wurm, Y. (2022) 'Genomic Signatures of Recent Adaptation in a Wild Bumblebee', Molecular Biology and Evolution, 39(2), msab366.

Colgan, T.J., Carolan, J.C., Bridgett, S.J., Sumner, S., Blaxter, M.L., and Brown, M.J. (2011) 'Polyphenism in social insects: insights from a transcriptome-wide analysis of gene expression in the life stages of the key pollinator, *Bombus terrestris*', BMC Genomics, 12(1), 1–20.

Colgan, T.J., Carolan, J.C., Sumner, S., Blaxter, M.L., and Brown, M.J. (2020) 'Infection by the castrating parasitic nematode *Sphaerularia bombi* changes gene expression in *Bombus terrestris* bumblebee queens', Insect Molecular Biology, 29(2), 170–182.

Colgan, T.J., Finlay, S., Brown, M.J., and Carolan, J.C. (2019) 'Mating precedes selective immune priming which is maintained throughout bumblebee queen diapause', BMC Genomics, 20(1), 1–18.

Colla, S.R. and Packer, L. (2008) 'Evidence for decline in eastern North American bumblebees (Hymenoptera: Apidae), with special focus on *Bombus affinis* Cresson', Biodiversity and Conservation, 17(6), 1379–1391.

Colla, S.R., Otterstatter, M.C., Gegear, R.J., and Thomson, J.D. (2006) 'Plight of the bumble bee: pathogen spillover from commercial to wild populations', Biological Conservation, 129(4), 461–467.

Collett, T.S., Graham, P., Harris, R.A., and Hempel-de-Ibarra, N. (2006) 'Navigational memories in ants and bees: memory retrieval when selecting and following routes', Advances in the Study of Behavior, 36, 123–172.

Collins, D. (2014) 'The role of small RNAs in caste determination and differentiation in the bumble bee, *Bombus terrestris*', doctoral dissertation, University of East Anglia.

Collins, D.H., Mohorianu, I., Beckers, M., Moulton, V., Dalmay, T., and Bourke, A.F. (2017) 'MicroRNAs associated with caste determination and differentiation in a primitively eusocial insect', Scientific Reports, 7(1), 1–9.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., and Léopold, P. (2003) 'A nutrient sensor mechanism controls *Drosophila* growth', Cell, 114(6), 739–749.

Cook, J.M. and Crozier, R.H. (1995) 'Sex determination and population biology in the Hymenoptera', Trends in Ecology & Evolution, 10(7), 281–286.

Cooper, D. and Eleftherianos, I. (2017) 'Memory and specificity in the insect immune system: current perspectives and future challenges', Frontiers in Immunology, 8, 539.

Corbet, S.A., Fussell, M., Ake, R., Fraser, A., Gunson, C., Savage, A., and Smith, K. (1993) 'Temperature and the pollinating activity of social bees', Ecological entomology, 18(1), 17–30.

Corby-Harris, V., Snyder, L.A., Schwan, M.R., Maes, P., McFrederick, Q.S., and Anderson, K.E. (2014) 'Origin and effect of Alpha 2.2 Acetobacteraceae in honey bee larvae and description of *Parasaccharibacter apium* gen. nov., sp. nov', Applied and Environmental Microbiology, 80(24), 7460–7472.

Cordes, N., Huang, W.-F., Strange, J.P., Cameron, S.A., Griswold, T.L., Lozier, J.D., and Solter, L.F. (2012) 'Interspecific geographic distribution and variation of the pathogens *Nosema bombi* and *Crithidia species* in United States bumble bee populations', Journal of Invertebrate Pathology.

Costa, A., Loh, S., and Martins, L.M. (2013) '*Drosophila* Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease', Cell Death & Disease, 4(1), e467–e467.

Costa, C.P., Leza, M., Duennes, M.A., Fisher, K., Vollaro, A., Hur, M., Kirkwood, J.S., and Woodard, S.H. (2022) 'Pollen diet mediates how pesticide exposure impacts brain gene expression in nest-founding bumble bee queens', Science of The Total Environment, 833, 155216.

Cotter, S.C., Simpson, S.J., Raubenheimer, D., and Wilson, K. (2011) 'Macronutrient balance mediates trade-offs between immune function and life history traits', Functional Ecology, 25(1), 186–198.

Cox, J. and Mann, M. (2007) 'Is Proteomics the New Genomics?', Cell, 130(3), 395–398.

Crall, J.D., De Bivort, B.L., Dey, B., and Ford Versypt, A.N. (2019) 'Social buffering of pesticides in bumblebees: agent-based modeling of the effects of colony size and neonicotinoid exposure on behavior within nests', Frontiers in Ecology and Evolution, 7, 51.

Cremer, S. (2019) 'Social immunity in insects', Current Biology, 29(11), R458–R463.

Cremer, S., Armitage, S. A., & Schmid-Hempel, P. (2007) 'Social immunity', Current Biology, 17(16), R693-R702.

Crespel, A., Rime, H., Fraboulet, E., Bobe, J., and Fauvel, C. (2008) 'Egg quality in domesticated and wild seabass (*Dicentrarchus labrax*): a proteomic analysis', Cybium, Revue Internationale d'Ichtyologie, 32(2).

Crotti Elena, Rizzi Aurora, Chouaia Bessem, Ricci Irene, Favia Guido, Alma Alberto, Sacchi Luciano, Bourtzis Kostas, Mandrioli Mauro, Cherif Ameur, Bandi Claudio, and Daffonchio Daniele (2010) 'Acetic Acid Bacteria, Newly Emerging Symbionts of Insects', Applied and Environmental Microbiology, 76(21), 6963–6970.

Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A., Ricci, I., Negri, I., Scuppa, P., Rossi, P., and Ballarini, P. (2009) 'Asaia, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders', Environmental microbiology, 11(12), 3252–3264.

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, 161074.

Curtis, P.G., Slay, C.M., Harris, N.L., Tyukavina, A., and Hansen, M.C. (2018) 'Classifying drivers of global forest loss', Science, 361(6407), 1108–1111.

Cutri, S.S., Macauley, B.J., and Roberts, W.P. (1984) 'Characteristics of pathogenic non-fluorescent (smooth) and non-pathogenic fluorescent (rough) forms of *Pseudomonas tolaasii* and *Pseudomonas gingeri*', Journal of Applied Bacteriology, 57(2), 291–298.

Czerwinski, M.A. and Sadd, B.M. (2017) 'Detrimental interactions of neonicotinoid pesticide exposure and bumblebee immunity', Journal of Experimental Zoology Part A: Ecological and Integrative Physiology, 327(5), 273–283.

DAFM, 'Survey of pollinator use by growers conducted in 2013' [unpublished], Department of Agriculture, Food and the Marine.

Dafni, A., Kevan, P., Gross, C.L., and Goka, K. (2010) '*Bombus terrestris*, pollinator, invasive and pest: An assessment of problems associated with its widespread introductions for commercial purposes', Applied Entomology and Zoology, 45(1), 101–113.

Dahl, C., Stanislawski, M., Iszatt, N., Mandal, S., Lozupone, C., Clemente, J.C., Knight, R., Stigum, H., and Eggesbø, M. (2017) 'Gut microbiome of mothers delivering prematurely shows reduced diversity and lower relative abundance of Bifidobacterium and Streptococcus', PLoS ONE, 12(10), e0184336.

Dai, Z.-J., Sun, W., and Zhang, Z. (2019) 'Comparative analysis of iTRAQ-based proteomes for cocoons between the domestic silkworm (*Bombyx mori*) and wild silkworm (*Bombyx mandarina*)', Journal of Proteomics, 192, 366–373.

Daily, G. (2003) 'What are ecosystem services', Global environmental challenges for the twenty-first century: Resources, consumption and sustainable solutions, 227–231.

Daisley, B.A., Chmiel, J.A., Pitek, A.P., Thompson, G.J., and Reid, G. (2020) 'Missing microbes in bees: how systematic depletion of key symbionts erodes immunity', Trends in Microbiology, 28(12), 1010–1021.

Dalziel, A.C. and Schulte, P.M. (2012) 'Ecological proteomics: finding molecular markers that matter', Molecular Ecology, 21, 3382-3384.

Damodaram, K.J.P., Kempraj, V., Aurade, R.M., Rajasekhar, S.B., Venkataramanappa, R.K., Nandagopal, B., and Verghese, A. (2014) 'Centuries of domestication has not impaired oviposition site-selection function in the silkmoth, *Bombyx mori*', Scientific Reports, 4(1), 1–6.

Danecek, P., Auton, A., Abecasis, G., Albers, C.A., Banks, E., DePristo, M.A., Handsaker, R.E., Lunter, G., Marth, G.T., and Sherry, S.T. (2011) 'The variant call format and VCFtools', Bioinformatics, 27(15), 2156–2158.

Darwin Tree of Life Project Consortium (2022) 'Sequence locally, think globally: The Darwin tree of life project', Proceedings of the National Academy of Sciences, 119(4), e2115642118.

Darwin, C. (1868) The Variation of Animals and Plants under Domestication, J. Murray: University of Lausanne.

Dauwalder, B., Tsujimoto, S., Moss, J., and Mattox, W. (2002) 'The *Drosophila* takeout gene is regulated by the somatic sex-determination pathway and affects male courtship behavior', Genes & Development, 16(22), 2879–2892.

Davis, E. S., Murray, T. E., FitzPatrick, Ú. N. A., Brown, M. J., & Paxton, R. J. (2010) Landscape effects on extremely fragmented populations of a rare solitary bee, *Colletes floralis*. Molecular Ecology, 19(22), 4922-4935.

Davis, R.L. (1993) 'Mushroom bodies and *Drosophila* learning', Neuron, 11(1), 1–14.

De Luca, P.A. and Vallejo-Marin, M. (2013) 'What's the 'buzz'about? The ecology and evolutionary significance of buzz-pollination', Current Opinion in Plant Biology, 16(4), 429–435.

De Pittà, C., Biscontin, A., Albiero, A., Sales, G., Millino, C., Mazzotta, G.M., Bertolucci, C., and Costa, R. (2013) 'The Antarctic Krill *Euphausia superba* Shows Diurnal Cycles of Transcription under Natural Conditions', PLoS ONE, 8(7), e68652.

De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D.U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001) 'Induction of gadd45β by NF-κB downregulates pro-apoptotic JNK signalling', Nature, 414(6861), 308–313.

Décory, M.S.M. (2019) ‘A Universal Definition of ‘Domestication’to Unleash Global Animal Welfare Progress’, Presented at the Derecho Animal. Forum of Animal Law Studies, 39–55.

Delcros, J.-G., Prigent, C., and Giet, R. (2006) ‘Dynactin targets Pavarotti-KLP to the central spindle during anaphase and facilitates cytokinesis in *Drosophila* S2 cells’, Journal of Cell Science, 119(21), 4431–4441.

Demarquoy, J. and Le Borgne, F. (2015) ‘Crosstalk between mitochondria and peroxisomes’, World Journal of Biological Chemistry, 6(4), 301.

DeNieu, M., Mounts, K., and Manier, M. (2019) ‘Two gut microbes are necessary and sufficient for normal cognition in *Drosophila melanogaster*’, bioRxiv, 593723.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G., and Pennacchio, F. (2013) ‘Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees’, Proceedings of the National Academy of Sciences, 110(46), 18466–18471.

Dickson, E.J. (2019) ‘Recent advances in understanding phosphoinositide signaling in the nervous system’, F1000Research, 8.

Dijkers, P.F. and O’Farrell, P.H. (2007) ‘*Drosophila* calcineurin promotes induction of innate immune responses’, Current Biology, 17(23), 2087–2093.

Directive, H.A.T. (1992) ‘Council Directive 92/65/EEC of 13 July 1992 laying down animal health requirements governing trade in and imports into the Community of animals, semen, ova and embryos not subject to animal health requirements laid down in specific Community rules referred to in Annex A (I) to Directive 90/425/EEC’, Official Journal L, 268(14/09), 0054–0072.

Dong, Y., Aguilar, R., Xi, Z., Warr, E., Mongin, E., and Dimopoulos, G. (2006) ‘*Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species’, PLoS Pathogens, 2(6), e52.

Dong, Y., Manfredini, F., and Dimopoulos, G. (2009) ‘Implication of the mosquito midgut microbiota in the defense against malaria parasites’, PLoS Pathogens, 5(5), e1000423.

Dong, Y., Taylor, H.E., and Dimopoulos, G. (2006) ‘AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system’, PLoS Biology, 4(7), e229.

Donmez, G. (2012) ‘The neurobiology of sirtuins and their role in neurodegeneration’, Trends in Pharmacological Sciences, 33(9), 494–501.

Doublet, V., Poeschl, Y., Gogol-Doering, A., Alaux, C., Annoscia, D., Aurori, C., Barribeau, S.M., Bedoya-Reina, O.C., Brown, M.J., and Bull, J.C. (2017) ‘Unity in defence: honeybee workers exhibit conserved molecular responses to diverse pathogens’, BMC Genomics, 18(1), 1–17.

Douglas, A. (1998) ‘Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*’, Annual Review of Entomology, 43(1), 17–37.

Douxfils, J., Mathieu, C., Mandiki, S.N., Milla, S., Henrotte, E., Wang, N., Vandecan, M., Dieu, M., Dauchot, N., and Pigneur, L.-M. (2011) ‘Physiological and proteomic evidences that domestication process differentially modulates the immune status of juvenile Eurasian perch (*Perca fluviatilis*) under chronic confinement stress’, Fish & Shellfish Immunology, 31(6), 1113–1121.

Downer, R. and Matthews, J. (1976) ‘Patterns of lipid distribution and utilisation in insects’, American Zoologist, 16(4), 733–745.

Dubovskiy, I., Martemyanov, V., Vorontsova, Y., Rantala, M., Gryzanova, E., and Glupov, V. (2008) ‘Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of *Galleria mellonella* L. larvae (Lepidoptera, Pyralidae)’, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 148(1), 1–5.

Duchateau, M.J. and Velthuis, H.H.W. (1988) ‘Development and Reproductive Strategies in *Bombus terrestris* Colonies’, Behaviour, 107(3–4), 186–207.

Duden, R. (2003) ‘ER-to-Golgi transport: Cop I and Cop II function’, Molecular Membrane Biology, 20(3), 197–207.

Dudley, N. and Alexander, S. (2017) ‘Agriculture and biodiversity: a review’, Biodiversity, 18(2–3), 45–49.

Early, A.M., Arguello, J.R., Cardoso-Moreira, M., Gottipati, S., Grenier, J.K., and Clark, A.G. (2017) 'Survey of global genetic diversity within the *Drosophila* immune system', Genetics, 205(1), 353–366.

Ebach, M. C., & Williams, D. M. (2009) 'How objective is a definition in the subspecies debate?', Nature, 457(7231), 785–785.

Ebner, J.N., Ritz, D., and von Fumetti, S. (2019) 'Comparative proteomics of stenotopic caddisfly Crunoecia irrorata identifies acclimation strategies to warming', Molecular Ecology, 28(19), 4453–4469.

Ebneter, C., Pick, J. L., & Tschirren, B. (2016) 'A trade-off between reproductive investment and maternal cerebellum size in a precocial bird', Biology Letters, 12(12), 20160659.

Edgar, R.C. (2004) 'MUSCLE: multiple sequence alignment with high accuracy and high throughput', Nucleic Acids Research, 32(5), 1792–1797.

Edgar, R.C. (2013) 'UPARSE: highly accurate OTU sequences from microbial amplicon reads', Nature Methods, 10(10), 996–998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011) 'UCHIME improves sensitivity and speed of chimera detection', Bioinformatics, 27(16), 2194–2200.

Edmands, S., & Timmerman, C. C. (2003) 'Modeling factors affecting the severity of outbreeding depression', Conservation Biology, 17(3), 883-892.

Eleftherianos, I., Boundy, S., Joyce, S. A., Aslam, S., Marshall, J. W., Cox, R. J., Simpson, T.J., Clarke, D.J., ffrench-Constant, R.H. and Reynolds, S. E. (2007) 'An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition', Proceedings of the National Academy of Sciences, 104(7), 2419-2424.

Eleftherianos, I., Marokhazi, J., Millichap, P. J., Hodgkinson, A. J., Sriboonlert, A., & Reynolds, S. E. (2006) 'Prior infection of *Manduca sexta* with non-pathogenic *Escherichia coli* elicits immunity to pathogenic *Photorhabdus luminescens*: roles of immune-related proteins shown by RNA interference', Insect Biochemistry and Molecular Biology, 36(6), 517-525.

Eleftherianos, I., Xu, M., Yadi, H., Ffrench-Constant, R. H., & Reynolds, S. E. (2009) 'Plasmatocyte-spreading peptide (PSP) plays a central role in insect cellular immune defenses against bacterial infection', Journal of Experimental Biology, 212(12), 1840-1848.

Ellegaard, K.M., Tamarit, D., Javelind, E., Olofsson, T.C., Andersson, S.G., and Vásquez, A. (2015) 'Extensive intra-phylotype diversity in *lactobacilli* and *bifidobacteria* from the honeybee gut', BMC genomics, 16(1), 1–22.

Ellegren, H. and Sheldon, B.C. (2008) 'Genetic basis of fitness differences in natural populations', Nature, 452(7184), 169–175.

Ellstrand, N.C. and Schierenbeck, K.A. (2000) 'Hybridization as a stimulus for the evolution of invasiveness in plants?', Proceedings of the National Academy of Sciences, 97(13), 7043–7050.

Ellstrand, N.C., Heredia, S.M., Leak-Garcia, J.A., Heraty, J.M., Burger, J.C., Yao, L., Nohzadeh-Malakshah, S., and Ridley, C.E. (2010) 'Crops gone wild: evolution of weeds and invasives from domesticated ancestors', Evolutionary Applications, 3(5-6), 494–504.

Enayati, A.A., Ranson, H., and Hemingway, J. (2005) 'Insect glutathione transferases and insecticide resistance', Insect Molecular Biology, 14(1), 3–8.

Engel, P. and Moran, N.A. (2013) 'The gut microbiota of insects – diversity in structure and function', FEMS Microbiology Reviews, 37(5), 699–735.

Engel, P., Kwong, W.K., McFrederick, Q., Anderson, K.E., Barribeau, S.M., Chandler, J.A., Cornman, R.S., Dainat, J., De Miranda, J.R., and Doublet, V. (2016) 'The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions', MBio, 7(2), e02164-15.

Engel, P., Martinson, V.G., and Moran, N.A. (2012) 'Functional diversity within the simple gut microbiota of the honey bee', Proceedings of the National Academy of Sciences, 109(27), 11002–11007.

Engel, P., Stepanauskas, R., and Moran, N.A. (2014) 'Hidden diversity in honey bee gut symbionts detected by single-cell genomics', PLoS Genetics, 10(9), e1004596.

Erler, S., Popp, M., and Lattorff, H.M.G. (2011) 'Dynamics of Immune System Gene Expression upon Bacterial Challenge and Wounding in a Social Insect (*Bombus terrestris*)', PLoS ONE, 6(3), e18126.

Estoup, A., Scholl, A., Pouvreau, A., and Solignac, M. (1995) 'Monoandry and polyandry in bumble bees (Hymenoptera; Bombinae) as evidenced by highly variable microsatellites', Molecular Ecology 4(1), 89-94.

Estoup, A., Solignac, M., Cornuet, J., Goudet, J., and Scholl, A. (1996) 'Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe', Molecular Ecology, 5(1), 19–31.

European Union (2022) 'Proposal for a regulation of the European parliament and of the Council on nature restoration (COM/2022/304)', available: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=COM:2022:304:FIN [accessed 27 Feb 2023]

Eurostat (2023) Share of Main Land Types in Utilised Agricultural Area (UAA) by NUTS 2 Regions [online], Eurostat, available: https://ec.europa.eu/eurostat/cache/metadata/en/tai05_esmsip2.htm [accessed 14 Feb 2023].

Evans, E. (2017) 'From humble bee to greenhouse pollination workhorse: can we mitigate risks for bumble bees?', Bee World, 94(2), 34–41.

Evans, J., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J., Jiang, H., Kanost, M., Thompson, G., Zou, Z., and Hultmark, D. (2006) 'Immune pathways and defence mechanisms in honey bees *Apis mellifera*', Insect Molecular Biology, 15(5), 645–656.

Fahrbach, S.E. (2006) 'Structure of the mushroom bodies of the insect brain', Annual Reviews Entomology, 51, 209–232.

Falk, S. (2019) Field Guide to the Bees of Great Britain and Ireland, Bloomsbury Publishing.

Farris, S.M. and Sinakevitch, I. (2003) 'Development and evolution of the insect mushroom bodies: towards the understanding of conserved developmental mechanisms in a higher brain center', Arthropod Structure & Development, 32(1), 79–101.

Faust, G.G. and Hall, I.M. (2014) ‘SAMBLASTER: fast duplicate marking and structural variant read extraction’, Bioinformatics, 30(17), 2503–2505.

Feder, M. and Walser, J. (2005) ‘The biological limitations of transcriptomics in elucidating stress and stress responses’, Journal of Evolutionary Biology, 18(4), 901–910.

Fernandez-Fournier, P., Lewthwaite, J. M., & Mooers, A. Ø. (2021) ‘Do we need to identify adaptive genetic variation when prioritizing populations for conservation?’, Conservation Genetics, 22, 205-216.

Ferrandon, D., Imler, J.-L., and Hoffmann, J.A. (2004) ‘Sensing infection in *Drosophila*: Toll and beyond’, Presented at the Seminars in Immunology, Elsevier, 43–53.

Ferrer-Admetlla, A., Liang, M., Korneliussen, T., and Nielsen, R. (2014) ‘On detecting incomplete soft or hard selective sweeps using haplotype structure’, Molecular Biology and Evolution, 31(5), 1275–1291.

Ferrier, S., Ninan, K., Leadley, P., Alkemade, R., Acosta-Michlik, L., Akçakaya, H., and Kabubo-Mariara, J. (2016) ‘IPBES (2016): Summary for policymakers of the assessment report of the methodological assessment of scenarios and models of biodiversity and ecosystem services’.

Figueroa, L.L., Maccaro, J.J., Krichilsky, E., Yanega, D., and McFrederick, Q.S. (2021) ‘Why did the bee eat the chicken? Symbiont Gain, Loss, and Retention in the Vulture Bee Microbiome’, Mbio, 12(6), e02317-21.

Fijarczyk, A. and Babik, W. (2015) ‘Detecting balancing selection in genomes: limits and prospects’, Molecular Ecology, 24(14), 3529–3545.

Finnegan, L.A., Edwards, C.J., and Rochford, J.M. (2008) ‘Origin of, and conservation units in, the Irish red squirrel (*Sciurus vulgaris*) population’, Conservation Genetics, 9(5), 1099–1109.

Fisher, R.M. (1984) ‘Evolution and host specificity: a study of the invasion success of a specialized bumblebee social parasite’, Canadian Journal of Zoology, 62(8), 1641–1644.

FitzPatrick, Ú., Murray, T., Byrne, A.W., Paxton, R., and Brown, M. (2006) Regional Red List of Irish Bees, National Parks and Wildlife Service (Ireland) and Environment and Heritage Service (N. Ireland).

FitzPatrick, Ú., Murray, T.E., Paxton, R.J., Breen, J., Cotton, D., Santorum, V., and Brown, M.J. (2007) 'Rarity and decline in bumblebees–a test of causes and correlates in the Irish fauna', Biological conservation, 136(2), 185–194.

FitzPatrick, Ú., Stout, J., Bertrand, C., Bradley, K., Clabby, G., Keena, C., and Walsh, J. (2015) 'All-Ireland pollinator plan 2015–2020', National Biodiversity Data Centre: Waterford, Ireland.

Ford, M.J., Murdoch, A.R., Hughes, M.S., Seamons, T.R., and LaHood, E.S. (2016) 'Broodstock history strongly influences natural spawning success in hatchery steelhead (*Oncorhynchus mykiss*)', PLoS ONE, 11(10), e0164801.

Formenti, G., Theissinger, K., Fernandes, C., Bista, I., Bombarely, A., Bleidorn, C., Ciofi, C., Crottini, A., Godoy, J.A., Höglund, J., Malukiewicz, J., Mouton, A., *et al.* (2022) 'The era of reference genomes in conservation genomics', Trends in Ecology & Evolution, 37(3), 197–202, available: https://doi.org/10.1016/j.tree.2021.11.008.

Fowler, A.C., Eadie, J.M., and Engilis, A. (2009) 'Identification of endangered Hawaiian ducks (*Anas wyvilliana*), introduced North American mallards (*A. platyrhynchos*) and their hybrids using multilocus genotypes', Conservation Genetics, 10, 1747–1758.

Francisco, F.O., Santiago, L.R., Mizusawa, Y.M., Oldroyd, B.P., and Arias, M.C. (2016) 'Genetic structure of island and mainland populations of a Neotropical bumble bee species', Journal of insect conservation, 20(3), 383–394.

Franklin, M.T., Winston, M.L., and Morandin, L.A. (2004) 'Effects of clothianidin on *Bombus impatiens* (Hymenoptera: Apidae) colony health and foraging ability', Journal of Economic Entomology, 97(2), 369–373.

Franz, A., Wood, W., and Martin, P. (2018) 'Fat Body Cells Are Motile and Actively Migrate to Wounds to Drive Repair and Prevent Infection', Developmental Cell, 44(4), 460-470.e3.

Free, J. (1971) ‘Stimuli Eliciting Mating Behaviour of Bumblebee (*Bombus Pratorum* L.) Males’, Behaviour, 40(1–2), 55–61.

Free, J.B. and Butler, C.G. (1959) Bumblebees, Collins New Naturalist Library, Collins.

Freeman, M.R. and Doherty, J. (2006) ‘Glial cell biology in *Drosophila* and vertebrates’, Trends in Neurosciences, 29(2), 82–90.

Freitak, D., Wheat, C. W., Heckel, D. G., & Vogel, H. (2007) ‘Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*’, BMC Biology, 5, 1-13.

Frison, T.H. (1927) ‘The fertilization and hibernation of queen bumblebees under controlled conditions. (Bremidae: Hym.)’, Journal of Economic Entomology, 20(3), 522–527.

Fründ, J., Zieger, S.L., and Tscharntke, T. (2013) ‘Response diversity of wild bees to overwintering temperatures’, Oecologia, 173(4), 1639–1648.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J.M., Topping, D.L., and Suzuki, T. (2011) ‘*Bifidobacteria* can protect from enteropathogenic infection through production of acetate’, Nature, 469(7331), 543–547.

Furrer, M., Balbi, M., Albarca-Aguilera, M., Gallant, M., Herr, W., and Gallant, P. (2010) ‘*Drosophila* Myc interacts with host cell factor (dHCF) to activate transcription and control growth’, Journal of Biological Chemistry, 285(51), 39623–39636.

Fürst, M., McMahon, D.P., Osborne, J., Paxton, R., and Brown, M. (2014) ‘Disease associations between honeybees and bumblebees as a threat to wild pollinators’, Nature, 506(7488), 364–366.

Gadau, J., Gerloff, C.U., Krüger, N., Chan, H., Schmid-Hempel, P., Wille, A., and Page, R.E. (2001) ‘A linkage analysis of sex determination in *Bombus terrestris* (L.) (Hymenoptera: Apidae)’, Heredity, 87(2), 234–242.

Gallego, M. and Virshup, D.M. (2007) ‘Post-translational modifications regulate the ticking of the circadian clock’, Nature Reviews Molecular Cell Biology, 8(2), 139–148.

Garbis, S., Lubec, G., and Fountoulakis, M. (2005) ‘Limitations of current proteomics technologies’, Journal of Chromatography A, 1077(1), 1–18.

Garibaldi, L.A., Aizen, M.A., Klein, A.M., Cunningham, S.A., and Harder, L.D. (2011) ‘Global growth and stability of agricultural yield decrease with pollinator dependence’, Proceedings of the National Academy of Sciences, 108(14), 5909–5914.

Garrison, E. and Marth, G. (2012) ‘Haplotype-based variant detection from short-read sequencing’, arXiv preprint arXiv:1207.3907.

Gauthier, M. and Grünewald, B. (2012) ‘Neurotransmitter systems in the honey bee brain: functions in learning and memory’, Honeybee Neurobiology and Behavior: a tribute to Randolf Menzel, 155–169.

Gepts, P. (2010) ‘Crop domestication as a long-term selection experiment’, Plant Breeding Reviews, 24(2), 1–44.

Gérard, M., Vanderplanck, M., Wood, T., and Michez, D. (2020) ‘Global warming and plant–pollinator mismatches’, Emerging Topics in Life Sciences, 4(1), 77–86.

Ghatak, A., Chaturvedi, P., and Weckwerth, W. (2017) ‘Cereal crop proteomics: systemic analysis of crop drought stress responses towards marker-assisted selection breeding’, Frontiers in Plant Science, 8, 757.

Ghoneim, K. (2019) ‘Characterization of Qualitative and Quantitative Haemogram Parameters in Insects: Current Concepts and Future Prospects’, Egyptian Academic Journal of Biological Sciences (A. Entomology), 12(1), 9-63.

Giannini, T.C., Costa, W.F., Cordeiro, G.D., Imperatriz-Fonseca, V.L., Saraiva, A.M., Biesmeijer, J., and Garibaldi, L.A. (2017) ‘Projected climate change threatens pollinators and crop production in Brazil’, PLoS ONE, 12(8), e0182274.

Gillespie and, J.P., Kanost, M.R., and Trenczek, T. (1997) ‘Biological mediators of insect immunity’, Annual Review of Entomology, 42(1), 611–643.

Gillet, L.C., Leitner, A., and Aebersold, R. (2016) ‘Mass spectrometry applied to bottom-up proteomics: entering the high-throughput era for hypothesis testing’, Annual Review of Analytical Chemistry, 9, 449–472.

Gilliam, M., Wickerham, L., Morton, H.L., and Martin, R.D. (1974) 'Yeasts isolated from honey bees, *Apis mellifera*, fed 2, 4-D and antibiotics', Journal of Invertebrate Pathology, 24(3), 349–356.

Gilmore, J.M., Sardiu, M.E., Groppe, B.D., Thornton, J.L., Liu, X., Dayebgadoh, G., Banks, C.A., Slaughter, B.D., Unruh, J.R., Workman, J.L., Florens, L., and Washburn, M.P. (2016) 'WDR76 Co-Localizes with Heterochromatin Related Proteins and Rapidly Responds to DNA Damage', PLOS ONE, 11(6), e0155492.

Ginisty, H., Amalric, F., and Bouvet, P. (1998) 'Nucleolin functions in the first step of ribosomal RNA processing', The EMBO journal, 17(5), 1476–1486.

Ginisty, H., Sicard, H., Roger, B., and Bouvet, P. (1999) 'Structure and functions of nucleolin', Journal of Cell Science, 112(6), 761–772.

Giuliani, A., Bui, T. T., Helmy, M., & Selvarajoo, K. (2022) 'Identifying toggle genes from transcriptome-wide scatter: A new perspective for biological regulation', Genomics, 114(1), 215-228.

Glazko, V.I., Zybaylov, B.L., Kosovsky, Y.G., Glazko, G.V., and Glazko, T.T. (2021) 'Domestication and microbiome', The Holocene, 31(10), 1635–1645.

Gogoi, P., Baruah, P., and Nath, S.C. (1997) 'Antifungal Activity of the Essential Oil of *Litsea cubeba* Pers.', Journal of Essential Oil Research, 9(2), 213–215.

Goka, K., Okabe, K., Yoneda, M., and Niwa, S. (2001) 'Bumblebee commercialization will cause worldwide migration of parasitic mites', Molecular ecology, 10(8), 2095–2099.

Goldsmith, M.R., Shimada, T., and Abe, H. (2005) 'The genetics and genomics of the silkworm, *Bombyx mori*', Annual Review of Entomology, 50, 71.

Gon III, S.M. and Price, E.O. (1984) 'Invertebrate domestication: Behavioral considerations', Bioscience, 34(9), 575–579.

Goodrich, J.K., Waters, J.L., Poole, A.C., Sutter, J.L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J.T., Spector, T.D., Clark, A.G., and Ley, R.E. (2014) 'Human Genetics Shape the Gut Microbiome', Cell, 159(4), 789–799.

Gorman, M.J. and Paskewitz, S.M. (2001) ‘Serine proteases as mediators of mosquito immune responses’, Insect Biochemistry and Molecular Biology, 31(3), 257–262.

Gösterit, A. (2016) ‘Adverse effects of inbreeding on colony foundation success in bumblebees, *Bombus terrestris* (Hymenoptera: Apidae)’, Applied Entomology and Zoology, 51(4), 521–526.

Gösterit, A. and Baskar, V.C. (2016) ‘Impacts of commercialization on the developmental characteristics of native *Bombus terrestris* (L.) colonies’, Insectes Sociaux, 63(4), 609–614.

Gösterit, A. and Ekran (2021) ‘Growth Characteristics of Commercial Bumblebee Colonies in Open Field Conditions May Be Evidence for Their Invasion Potential’, Journal, 13(1), 5–8.

Gösterit, A. and Gürel, F. (2005) ‘Comparison of development patterns of imported and native *Bombus terrestris* L.(Hymenoptera: Apidae) colonies in the Mediterranean coastal region’, Turkish Journal of Veterinary & Animal Sciences, 29(2), 393–398.

Gösterit, A. and Gurel, F. (2009) ‘Effect of different diapause regimes on survival and colony development in the bumble bee, *Bombus terrestris*’, Journal of Apicultural Research, 48(4), 279–283.

Gösterit, A. and Gurel, F. (2016) ‘Male remating and its influences on queen colony foundation success in the bumblebee, *Bombus terrestris*’, Apidologie.

Goulson, D. (2003) ‘Effects of introduced bees on native ecosystems’, Annual Review of Ecology, Evolution, and Systematics, 34, 1–26.

Goulson, D. (2010a) Bumblebees: Behaviour, Ecology, and Conservation, Oxford University Press: Oxford, UK.

Goulson, D. (2010b) ‘Impacts of non-native bumblebees in Western Europe and North America’, Applied Entomology and Zoology, 45(1), 7–12.

Goulson, D. and Hanley, M.E. (2004) ‘Distribution and forage use of exotic bumblebees in South Island, New Zealand’, New Zealand Journal of Ecology, 225–232.

Goulson, D., Hughes, W., Derwent, L., and Stout, J. (2002) ‘Colony growth of the bumblebee, *Bombus terrestris*, in improved and conventional agricultural and suburban habitats’, Oecologia, 130(2), 267–273.

Goulson, D., Lye, G.C., and Darvill, B. (2008) ‘Decline and Conservation of Bumble Bees’, Annual Review of Entomology, 53(1), 191–208.

Goulson, D., Peat, J., Stout, J.C., Tucker, J., Darvill, B., Derwent, L.C., and Hughes, W.O.H. (2002) ‘Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency?’, Animal Behaviour, 64(1), 123–130.

Goulson, D., Stout, J.C., and Kells, A.R. (2002) ‘Do exotic bumblebees and honeybees compete with native flower-visiting insects in Tasmania?’, Journal of Insect Conservation, 6(3), 179–189.

Government of England (1981) Wildlife and Countryside Act 1981, available: https://www.legislation.gov.uk/ukpga/1981/69 [accessed 27 Feb 2023]

Government of Ireland (2011) S.I. No. 477/2011. The European Communities (Birds and Natural Habitats) Regulations 2011, Dublin: The Stationary Office.

Gramates, L.S., Agapite, J., Attrill, H., Calvi, B.R., Crosby, M.A., Dos Santos, G., Goodman, J.L., Goutte-Gattat, D., Jenkins, V.K., and Kaufman, T. (2022) ‘FlyBase: a guided tour of highlighted features’, Genetics, 220(4), iyac035.

Graystock, P., Meeus, I., Smagghe, G., Goulson, D., & Hughes, W. O. (2016) ‘The effects of single and mixed infections of *Apicystis bombi* and deformed wing virus in *Bombus terrestris*’, Parasitology, 143(3), 358-365.

Graystock, P., Yates, K., Evison, S.E., Darvill, B., Goulson, D., and Hughes, W.O. (2013) ‘The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies’, Journal of Applied Ecology, 50(5), 1207–1215.

Gregorc, A., Evans, J.D., Scharf, M., and Ellis, J.D. (2012) ‘Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and *Varroa* mites (*Varroa destructor*)’, Journal of Insect Physiology, 58(8), 1042–1049.

Gressel, J. (2005) Crop Ferality and Volunteerism, CRC Press.

Griffin, C.R., Shallenberger, R.J., Fefer, S.I., Sharitz, R., and Gibbons, J. (1989) 'Hawaii's endangered waterbirds: a resource management challenge', Presented at the Freshwater Wetlands and Wildlife. Department of Energy Symposium, 1165–1175.

Grimm, N.B., Faeth, S.H., Golubiewski, N.E., Redman, C.L., Wu, J., Bai, X., and Briggs, J.M. (2008) 'Global change and the ecology of cities', Science, 319(5864), 756–760.

Grixti, J.C., Wong, L.T., Cameron, S.A., and Favret, C. (2009) 'Decline of bumble bees (*Bombus*) in the North American Midwest', Biological Conservation, 142(1), 75–84.

Gstöttl, C., Stoldt, M., Jongepier, E., Bornberg-Bauer, E., Feldmeyer, B., Heinze, J., and Foitzik, S. (2020) 'Comparative analyses of caste, sex, and developmental stage-specific transcriptomes in two *Temnothorax* ants', Ecology and Evolution, 10(10), 4193–4203.

Guerra-Sanz, J.M. (2008) 'Crop pollination in greenhouses', Bee pollination in Agricultural Ecosystems, 27–47.

Gunstone, T., Cornelisse, T., Klein, K., Dubey, A., and Donley, N. (2021) 'Pesticides and soil invertebrates: A hazard assessment', Frontiers in Environmental Science, 9, 122.

Hahn, D.A. and Denlinger, D.L. (2007) 'Meeting the energetic demands of insect diapause: nutrient storage and utilization', Journal of Insect Physiology, 53(8), 760–773.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., and Hörren, T. (2017) 'More than 75 percent decline over 27 years in total flying insect biomass in protected areas', PLoS ONE, 12(10), e0185809.

Hamill, R.M., Doyle, D., and Duke, E.J. (2006) 'Spatial patterns of genetic diversity across European subspecies of the mountain hare, *Lepus timidus* L.', Heredity, 97(5), 355–365.

Hammer, T.J., Easton-Calabria, A., and Moran, N.A. (2022) 'Microbiome assembly and maintenance across the lifespan of bumble bee workers', bioRxiv.

Hammer, T.J., Le, E., Martin, A.N., and Moran, N.A. (2021) 'The gut microbiota of bumblebees', Insectes Sociaux, 68(4), 287–301.

Harbauer, A.B., Zahedi, R.P., Sickmann, A., Pfanner, N., and Meisinger, C. (2014) 'The Protein Import Machinery of Mitochondria—A Regulatory Hub in Metabolism, Stress, and Disease', Cell Metabolism, 19(3), 357–372.

Harpur, B.A. and Rehan, S.M. (2021) 'Connecting social polymorphism to single nucleotide polymorphism: population genomics of the small carpenter bee, *Ceratina australensis*', Biological Journal of the Linnean Society, 132(4), 945–954.

Harris, E. V., de Roode, J. C., & Gerardo, N. M. (2019) 'Diet–microbiome–disease: Investigating diet's influence on infectious disease resistance through alteration of the gut microbiome', PLoS pathogens, 15(10), e1007891.

Hart, A.F., Verbeeck, J., Ariza, D., Cejas, D., Ghisbain, G., Honchar, H., Radchenko, V.G., Straka, J., Ljubomirov, T., and Lecocq, T. (2022) 'Signals of adaptation to agricultural stress in the genomes of two European bumblebees', Frontiers in Genetics, 13, 2868.

Hart, S.R. and Gaskell, S.J. (2005) 'LC–tandem MS in proteome characterization', TrAC Trends in Analytical Chemistry, 24(7), 566–575.

Hartl, D.L. and Brown, S.W. (1970) 'The origin of male haploid genetic systems and their expected sex ratio', Theoretical Population Biology, 1(2), 165–190.

Hartl, D.L., Clark, A.G., and Clark, A.G. (1997) Principles of Population Genetics, Sinauer associates Sunderland.

Hassett, J., Browne, K.A., McCormack, G.P., Moore, E., Society, N.I.H.B., Soland, G., and Geary, M. (2018) 'A significant pure population of the dark European honey bee (*Apis mellifera mellifera*) remains in Ireland', Journal of Apicultural Research, 57(3), 337–350.

Hastings, P.J., Lupski, J.R., Rosenberg, S.M., and Ira, G. (2009) 'Mechanisms of change in gene copy number', Nature Reviews Genetics, 10(8), 551–564.

Hatori, Y. and Lutsenko, S. (2016) 'The role of copper chaperone Atox1 in coupling redox homeostasis to intracellular copper distribution', Antioxidants, 5(3), 25.

Hayden, B., Pulcini, D., Kelly-Quinn, M., O'Grady, M., Caffrey, J., McGrath, A., and Mariani, S. (2010) 'Hybridisation between two cyprinid fishes in a novel habitat: genetics, morphology and life-history traits', BMC Evolutionary Biology, 10(1), 1–11.

He, Y., Meng, X., Fan, Q., Sun, X., Xu, Z., and Song, R. (2009) 'Cloning and characterization of two novel chloroplastic glycerol-3-phosphate dehydrogenases from *Dunaliella viridis*', Plant Molecular Biology, 71(1), 193–205.

Hedrick, P., Lee, R., and Hurt, C. (2006) 'The endangered Sonoran topminnow: examination of species and ESUs using three mtDNA genes', Conservation Genetics, 7(4), 483–492.

Heinrich, B. (1972) 'Physiology of brood incubation in the bumblebee queen, *Bombus vosnesenskii*', Nature, 239, 223–225.

Heinrich, B. and Vogt, F.D. (1993) 'Abdominal temperature regulation by arctic bumblebees', Physiological Zoology, 66(2), 257–269.

Heisenberg, M. (1971) 'Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*', Journal of Experimental Biology, 55(1), 85–100.

Hemme, D., Raibaud, P., Ducluzeau, R., Galpin, J., Sicard, P., and Van Heijenoort, J. (1980) '"*Lactobacillus murinus*" n. sp., a new species of the autochtoneous dominant flora of the digestive tract of rat and mouse', Presented at the Annales de Microbiologie, 131(3), 297–308.

Heraghty, S.D., Sutton, J.M., Pimsler, M.L., Fierst, J.L., Strange, J.P., and Lozier, J.D. (2020) 'De novo genome assemblies for three North American bumble bee species: *Bombus bifarius, Bombus vancouverensis,* and *Bombus vosnesenskii*', G3: Genes, Genomes, Genetics, 10(8), 2585–2592.

Hines, H.M. (2008) 'Historical biogeography, divergence times, and diversification patterns of bumble bees (Hymenoptera: Apidae: *Bombus*)', Systematic Biology, 57(1), 58–75.

Hingston, A.B., Herrmann, W., and Jordan, G.J. (2006) ‘Reproductive success of a colony of the introduced bumblebee *Bombus terrestris* (L.) (Hymenoptera : Apidae) in a Tasmanian National Park’, Australian Journal of Entomology 45(2), 137-141.

Hjort, C.K., Paris, J.R., Olsson, P., Herbertsson, L., de Miranda, J.R., Dudaniec, R.Y., and Smith, H.G. (2022) ‘Genomic divergence and a lack of recent introgression between commercial and wild bumblebees (*Bombus terrestris*)’, Evolutionary Applications, 15(3), 365–382.

Hobbs, G.A. (1965) ‘Ecology of Species of *Bombus* Latr. (Hymenoptera: Apidae) in Southern Alberta. II. Subgenus *Bombias* Robt.’, Canadian Entomologist, 97(February), 120–128.

Hohmann, S. (2002) ‘Osmotic stress signaling and osmoadaptation in yeasts’, Microbiology and Molecular Biology Reviews, 66(2), 300–372.

Holmgren, A. (2000) ‘Antioxidant function of thioredoxin and glutaredoxin systems’, Antioxidants & Redox Signaling, 2(4), 811–820.

Hoshizaki, D.K. (1998) ‘Chapter 6 - Fat Body’, in The Insects: Structure and Function, Cambridge University Press: Cambridge, UK.

Hoshizaki, D.K., Blackburn, T., Price, C., Ghosh, M., Miles, K., Ragucci, M., and Sweis, R. (1994) ‘Embryonic fat-cell lineage in *Drosophila melanogaster*’, Development, 120(9), 2489–2499.

Houghton, R.A. (1994) ‘The Worldwide Extent of Land-Use Change’, BioScience, 44(5), 305–313.

Hovav, R., Udall, J.A., Chaudhary, B., Hovav, E., Flagel, L., Hu, G., and Wendel, J.F. (2008) ‘The evolution of spinnable cotton fiber entailed prolonged development and a novel metabolism’, PLoS Genetics, 4(2), e25.

Howard, D., Watkins, J., Clarke, R., Barnett, C., and Stark, G. (2003) ‘Estimating the extent and change in broad habitats in Great Britain’, Journal of Environmental Management, 67(3), 219–227.

Howard, S.R. (2021) ‘Wild non-eusocial bees learn a colour discrimination task in response to simulated predation events’, The Science of Nature, 108(4), 1–8.

Howe, K.L., Achuthan, P., Allen, J., Allen, J., Alvarez-Jarreta, J., Amode, M.R., Armean, I.M., Azov, A.G., Bennett, R., Bhai, J., Billis, K., Boddu, S., Charkhchi, M., Cummins, C., Da Rin Fioretto, L., Davidson, C., Dodiya, K., El Houdaigui, B., *et al.* (2021) 'Ensembl 2021', Nucleic Acids Research, 49(D1), D884–D891.

Hu, G., Koh, J., Yoo, M., Grupp, K., Chen, S., and Wendel, J.F. (2013) 'Proteomic profiling of developing cotton fibers from wild and domesticated *Gossypium barbadense*', New Phytologist, 200(2), 570–582.

Huang, L., Cheng, T., Xu, P., Cheng, D., Fang, T., and Xia, Q. (2009) 'A Genome-Wide Survey for Host Response of Silkworm, *Bombyx mori* during Pathogen *Bacillus bombyseptieus* Infection', PLoS ONE, 4(12), e8098.

Huang, Q., Chen, Y.P., Wang, R.W., Cheng, S., and Evans, J.D. (2016) 'Host-parasite interactions and purifying selection in a microsporidian parasite of honey bees', PLoS ONE, 11(2), e0147549.

Huntley, R.P., Sawford, T., Mutowo-Meullenet, P., Shypitsyna, A., Bonilla, C., Martin, M.J., and O'Donovan, C. (2015) 'The GOA database: gene ontology annotation updates for 2015', Nucleic Acids Research, 43(D1), D1057–D1063.

Husak, V. (2015) 'Copper and copper-containing pesticides: metabolism, toxicity and oxidative stress', Journal of Vasyl Stefanyk Precarpathian National University, 2(1), 39–51.

Huxel, G.R. (1999) 'Rapid displacement of native species by invasive species: effects of hybridization', Biological Conservation, 89(2), 143–152.

Hyman, S.E. (2005) 'Neurotransmitters', Current biology, 15(5), R154–R158.

Imhoof, B. and Schmid-Hempel, P. (1999) 'Colony success of the bumble bee, *Bombus terrestris*, in relation to infections by two protozoan parasites, *Crithidia bombi* and *Nosema bombi*', Insectes Sociaux, 46(3), 233–238.

Imler, J.-L. and Hoffmann, J.A. (2001) 'Toll receptors in innate immunity', Trends in cell biology, 11(7), 304–311.

Ingram, K.K., Krummey, S., and LeRoux, M. (2009) 'Expression patterns of a circadian clock gene are associated with age-related polyethism in harvester ants, Pogonomyrmex occidentalis', BMC ecology, 9, 1–9.

Ings, T., Ward, N., and Chittka, L. (2006) ‘Can commercially imported bumble bees out-compete their native conspecifics?’, Journal of Applied Ecology, 43(5), 940–948.

Ings, T.C., Raine, N.E., and Chittka, L. (2005) ‘Mating preference in the commercially imported bumblebee species *Bombus terrestris* in Britain (Hymenoptera: Apidae)’, Entomologia Generalis, 28(3), 233.

Ings, T.C., Raine, N.E., and Chittka, L. (2009) ‘A population comparison of the strength and persistence of innate colour preference and learning speed in the bumblebee *Bombus terrestris*’, Behavioral Ecology and Sociobiology, 63, 1207–1218.

Inoue, M.N., Yokoyama, J., and Washitani, I. (2008) ‘Displacement of Japanese native bumblebees by the recently introduced *Bombus terrestris* (L.)(Hymenoptera: Apidae)’, Journal of Insect Conservation, 12(2), 135–146.

International Pest Control (2017) ‘Biobest invented the buzziness’, International Pest Control, 59(5), 264.

Islam, S.S., Yates, M.C., and Fraser, D.J. (2020) ‘Single generation exposure to a captive diet: a primer for domestication selection in a salmonid fish?’, bioRxiv, 2020.01.24.919175.

Issaq, H.J. and Veenstra, T.D. (2008) ‘Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives’, Biotechniques, 44(5), 697–700.

Ito, M. (2007) ‘Degradation of Glycolipids’, Comprehensive Glycoscience, 3, 193–208.

Ivanov, P., Kedersha, N., and Anderson, P. (2019) ‘Stress granules and processing bodies in translational control’, Cold Spring Harbor Perspectives in Biology, 11(5), a032813.

Jackson, J.M., Pimsler, M.L., Oyen, K.J., Strange, J.P., Dillon, M.E., and Lozier, J.D. (2020) ‘Local adaptation across a complex bioclimatic landscape in two montane bumble bee species’, Molecular Ecology, 29(5), 920–939.

Janeway Jr, C. A., Travers, P., Walport, M., & Shlomchik, M. J. (2001) ‘Principles of innate and adaptive immunity’ In Immunobiology: The Immune System in Health and Disease, 5th edition. Garland Science: New York, USA.

Jang, S. and Kikuchi, Y. (2020) ‘Impact of the insect gut microbiota on ecology, evolution, and industry’, Current Opinion in Insect Science, 41, 33–39.

Janzen, D.H. and Hallwachs, W. (2019) ‘Perspective: Where might be many tropical insects?’, Biological Conservation, 233, 102–108.

Jauker, F., Bondarenko, B., Becker, H.C., and Steffan-Dewenter, I. (2012) ‘Pollination efficiency of wild bees and hoverflies provided to oilseed rape’, Agricultural and Forest Entomology, 14(1), 81–87.

Jensen, P. (2006) ‘Domestication—from behaviour to genes and back again’, Applied Animal Behaviour Science, 97(1), 3–15.

Jha, S. (2015) ‘Contemporary human-altered landscapes and oceanic barriers reduce bumble bee gene flow’, Molecular Ecology, 24(5), 993–1006.

Jia, Z.-Q., Liu, D., Peng, Y.-C., Han, Z.-J., Zhao, C.-Q., and Tang, T. (2020) ‘Identification of transcriptome and fluralaner responsive genes in the common cutworm *Spodoptera litura* Fabricius, based on RNA-seq’, BMC Genomics, 21(1), 120.

Jiggins, F. and Kim, K. (2007) ‘A screen for immunity genes evolving under positive selection in *Drosophila*’, Journal of Evolutionary Biology, 20(3), 965–970.

Jin, Y., Olsen, R.E., Harvey, T.N., Østensen, M.-A., Li, K., Santi, N., Vadstein, O., Bones, A.M., Vik, J.O., Sandve, S.R., and Olsen, Y. (2020) ‘Comparative transcriptomics reveals domestication-associated features of Atlantic salmon lipid metabolism’, Molecular Ecology, 29(10), 1860–1872.

Jin, Z. and Xie, T. (2007) ‘Dcr-1 maintains *Drosophila* ovarian stem cells’, Current Biology, 17(6), 539–544.

Johnson, K.N. (2015) ‘Bacteria and antiviral immunity in insects’, Current Opinion in Insect Science, 8, 97–103.

Jojima, Y., Mihara, Y., Suzuki, S., Yokozeki, K., Yamanaka, S., and Fudou, R. (2004) ‘*Saccharibacter floricola gen. nov.*, sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen’, International Journal of Systematic and Evolutionary Microbiology, 54(6), 2263–2267.

Jones, L. F., Lou, R. N., Murray, C. S., Robert, D., Bourne, C. M., Bouchard, C., Kučka, M., Chan Y.F., Carlon, D.B., Wiley, D.N., Therkildsen, N.O., and Baumann, H. (2023) 'Two distinct population clusters of northern sand lance (*Ammodytes dubius*) on the northwest Atlantic shelf revealed by whole genome sequencing', ICES Journal of Marine Science, 80(1), 122-132.

Joseph, S.B. and Kirkpatrick, M. (2004) 'Haploid selection in animals', Trends in Ecology & Evolution, 19(11), 592–597.

Kakumanu, M. L., Reeves, A. M., Anderson, T. D., Rodrigues, R. R., & Williams, M. A. (2016) 'Honey bee gut microbiome is altered by in-hive pesticide exposures', Frontiers in Microbiology, 7, 1255.

Kallio, J., Leinonen, A., Ulvila, J., Valanne, S., Ezekowitz, R.A., and Rämet, M. (2005) 'Functional analysis of immune response genes in *Drosophila* identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells', Microbes and Infection, 7(5–6), 811–819.

Kanehisa, M., Sato, Y., and Morishima, K. (2016) 'BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences', Journal of Molecular Biology, 428(4), 726–731.

Kaneko, T. and Silverman, N. (2005) 'Bacterial recognition and signalling by the *Drosophila* IMD pathway', Cellular Microbiology, 7(4), 461–469.

Kaneko, T., Yano, T., Aggarwal, K., Lim, J.-H., Ueda, K., Oshima, Y., Peach, C., Erturk-Hasdemir, D., Goldman, W.E., Oh, B.-H., Kurata, S., and Silverman, N. (2006) 'PGRP-LC and PGRP-LE have essential yet distinct functions in the *Drosophila* immune response to monomeric DAP-type peptidoglycan', Nature Immunology, 7(7), 715–723.

Kanost, M.R. and Gorman, M.J. (2008) 'Phenoloxidases in insect immunity', Insect Immunology, 1, 69–96.

Karr, T. (2008) 'Application of proteomics to ecology and population biology', Heredity, 100(2), 200–206.

Katewa, S.D. and Kapahi, P. (2011) ‘Role of TOR signaling in aging and related biological processes in *Drosophila melanogaster*’, Experimental Gerontology, 46(5), 382–390.

Kato, Y., Kikuta, S., Barribeau, S.M., and Inoue, M.N. (2022) ‘In vitro larval rearing method of eusocial bumblebee *Bombus terrestris* for toxicity test’, Scientific Reports, 12(1), 15783.

Kaufmann, C. and Brown, M.R. (2008) ‘Regulation of carbohydrate metabolism and flight performance by a hypertrehalosaemic hormone in the mosquito *Anopheles gambiae*’, Journal of insect physiology, 54(2), 367–377.

Kelemen, E.P. and Rehan, S.M. (2021) ‘Conservation insights from wild bee genetic studies: Geographic differences, susceptibility to inbreeding, and signs of local adaptation’, Evolutionary Applications, 14(6), 1485–1496.

Kent, C.F., Dey, A., Patel, H., Tsvetkov, N., Tiwari, T., MacPhail, V.J., Gobeil, Y., Harpur, B.A., Gurtowski, J., and Schatz, M.C. (2018) ‘Conservation genomics of the declining North American bumblebee *Bombus terricola* reveals inbreeding and selection on immune genes’, Frontiers in Genetics, 9, 316.

Khaitovich, P., Weiss, G., Lachmann, M., Hellmann, I., Enard, W., Muetzel, B., Wirkner, U., Ansorge, W., and Pääbo, S. (2004) ‘A neutral model of transcriptome evolution’, PLoS Biology, 2(5), e132.

Kier, G., Kreft, H., Lee, T.M., Jetz, W., Ibisch, P.L., Nowicki, C., Mutke, J., and Barthlott, W. (2009) ‘A global assessment of endemism and species richness across island and mainland regions’, Proceedings of the National Academy of Sciences, 106(23), 9322–9327.

Killer, J., Votavova, A., Valterova, I., Vlkova, E., Rada, V., and Hroncová, Z. (2014) ‘Lactobacillusbombi sp. nov., from the digestive tract of laboratory-reared bumblebee queens (*Bombus terrestris*)’, International journal of systematic and evolutionary microbiology, 64(8), 2611–2617.

Kim, I.H., Pham, V., Jablonka, W., Goodman, W.G., Ribeiro, J.M., and Andersen, J.F. (2017) ‘A mosquito hemolymph odorant-binding protein family member specifically binds juvenile hormone’, Journal of Biological Chemistry, 292(37), 15329–15339.

Kim, J.I., Kwon, M., Kim, G.-H., Kim, S.Y., and Lee, S.H. (2015) 'Two mutations in nAChR beta subunit is associated with imidacloprid resistance in the *Aphis gossypii*', Journal of Asia-Pacific Entomology, 18(2), 291–296.

Kim, K., Yoon, J., Yim, J., and Kim, H.-J. (2017) 'Deneddylase 1 regulates deneddylase activity of the Cop9 signalosome in *Drosophila melanogaster*', Insect Science, 24(1), 27–34.

Kindaichi, T., Yamaoka, S., Uehara, R., Ozaki, N., Ohashi, A., Albertsen, M., Nielsen, P.H., and Nielsen, J.L. (2016) 'Phylogenetic diversity and ecophysiology of Candidate phylum Saccharibacteria in activated sludge', FEMS Microbiology Ecology, 92(6), fiw078.

King, R.W., Deshaies, R.J., Peters, J.-M., and Kirschner, M.W. (1996) 'How Proteolysis Drives the Cell Cycle', Science, 274(5293), 1652–1659.

Kingsolver, M.B., Huang, Z., and Hardy, R.W. (2013) 'Insect antiviral innate immunity: pathways, effectors, and connections', Journal of Molecular Biology, 425(24), 4921–4936.

Kinsella, R.J., Kähäri, A., Haider, S., Zamora, J., Proctor, G., Spudich, G., Almeida-King, J., Staines, D., Derwent, P., Kerhornou, A., Kersey, P., and Flicek, P. (2011) 'Ensembl BioMarts: a hub for data retrieval across taxonomic space', Database, 2011, bar030.

Kleijn, D., Kohler, F., Báldi, A., Batáry, P., Concepción, E., Clough, Y., Díaz, M., Gabriel, D., Holzschuh, A., and Knop, E. (2009) 'On the relationship between farmland biodiversity and land-use intensity in Europe', Proceedings of the Royal Society B: Biological Sciences, 276(1658), 903–909.

Klein, A.-M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., and Tscharntke, T. (2007) 'Importance of pollinators in changing landscapes for world crops', Proceedings of the royal society B: biological sciences, 274(1608), 303–313.

Klein, S., Cabirol, A., Devaud, J.-M., Barron, A.B., and Lihoreau, M. (2017) 'Why bees are so vulnerable to environmental stressors', Trends in Ecology & Evolution, 32(4), 268–278.

Klowden, M.J. (2013) Physiological Systems in Insects, Academic press.

Knee, W.J. and Medler, J.T. (1965) 'The Seasonal Size Increase of Bumblebee Workers (Hymenoptera: *Bombus*)', The Canadian Entomologist, 97(11), 1149–1155.

Knowles, S. C., Nakagawa, S., & Sheldon, B. C. (2009) 'Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach', *Functional Ecology*, 405-415.

Koch, H. and Schmid-Hempel, P. (2011) 'Socially transmitted gut microbiota protect bumble bees against an intestinal parasite', Proceedings of the National Academy of Sciences, 108(48), 19288–19292.

Koch, H., Abrol, D.P., Li, J., and Schmid-Hempel, P. (2013) 'Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees', Molecular Ecology, 22(7), 2028–2044.

Koch, H., Welcome, V., Kendal-Smith, A., Thursfield, L., Farrell, I.W., Langat, M.K., Brown, M.J., and Stevenson, P.C. (2022) 'Host and gut microbiome modulate the antiparasitic activity of nectar metabolites in a bumblebee pollinator', Philosophical Transactions of the Royal Society B, 377(1853), 20210162.

Kong, A., Gudbjartsson, D. F., Sainz, J., Jonsdottir, G. M., Gudjonsson, S. A., Richardsson, B., Sigurdardottir, S., Barnard, J., Hallbeck, B., Masson, G. *et al.* (2002) 'A high-resolution recombination map of the human genome', Nature Genetics, 31(3), 241-247.

Koppert Biological Systems (2011) 'Native Bumblebee Available for Growers in UK and Ireland: Koppert Biological Systems', Available: https://webcache.googleusercontent.com/search?q=cache:iscVlvK9CY0J:https://bit.ly/2rcMVuK&cd=12&hl=en&ct=clnk&gl=ie [accessed 20 Feb 2019].

Kosior, A., Celary, W., Olejniczak, P., Fijał, J., Król, W., Solarz, W., and Płonka, P. (2007) 'The decline of the bumble bees and cuckoo bees (Hymenoptera: Apidae: Bombini) of Western and Central Europe', Oryx, 41(1), 79–88.

Kotrschal, A., Rogell, B., Bundsen, A., Svensson, B., Zajitschek, S., Brännström, I., Immler, S., Maklakov, A.A., and Kolm, N. (2013) 'Artificial selection on relative

brain size in the guppy reveals costs and benefits of evolving a larger brain', *Current Biology*, *23*(2), 168-171.

Kraus, F., Wolf, S., and Moritz, R. (2009) 'Male flight distance and population substructure in the bumblebee *Bombus terrestris*', Journal of Animal Ecology, 247–252.

Kraus, F.B., Szentgyörgyi, H., Rożej, E., Rhode, M., Moroń, D., Woyciechowski, M., and Moritz, R.F. (2011) 'Greenhouse bumblebees (*Bombus terrestris*) spread their genes into the wild', Conservation Genetics, 12(1), 187–192.

Krieger, M. (1997) 'The other side of scavenger receptors: pattern recognition for host defense.', Current Opinion in Lipidology, 8(5), 275–280.

Krishnan, M., Bharathiraja, C., Pandiarajan, J., Prasanna, V.A., Rajendhran, J., and Gunasekaran, P. (2014) 'Insect gut microbiome–An unexploited reserve for biotechnological application', Asian Pacific Journal of Tropical Biomedicine, 4, S16–S21.

Kruska, D.C.T. (2005) 'On the evolutionary significance of encephalization in some eutherian mammals: effects of adaptive radiation, domestication, and feralization.', Brain, Behavior and Evolution, 65(2), 73-108.

Kurtzman, C. P. (2011) 'The yeasts: a taxonomic study. 5th Edition', Elsevier.

Kwong, W.K., Engel, P., Koch, H., and Moran, N.A. (2014) 'Genomics and host specialization of honey bee and bumble bee gut symbionts', Proceedings of the National Academy of Sciences, 111(31), 11509–11514.

Kwong, W.K., Mancenido, A.L., and Moran, N.A. (2017) 'Immune system stimulation by the native gut microbiota of honey bees', Royal Society Open Science, 4(2), 170003.

Kwong, W.K., Medina, L.A., Koch, H., Sing, K.-W., Soh, E.J.Y., Ascher, J.S., Jaffé, R., and Moran, N.A. (2017) 'Dynamic microbiome evolution in social bees', Science Advances, 3(3), e1600513.

Lacy, R.C. (1997) 'Importance of Genetic Variation to the Viability of Mammalian Populations', Journal of Mammalogy, 78(2), 320–335.

Lämsä, J., Kuusela, E., Tuomi, J., Juntunen, S., and Watts, P.C. (2018) 'Low dose of neonicotinoid insecticide reduces foraging motivation of bumblebees', Proceedings of the Royal Society B: Biological Sciences, 285(1883), 20180506.

Langmead, B. and Salzberg, S.L. (2012) 'Fast gapped-read alignment with Bowtie 2', Nature Methods, 9(4), 357–359.

Larkin, D.E. (2018) 'A proteomic and cytological characterisation of the buff-tailed bumblebee (*Bombus terrestris*) fat body and haemolymph-An immune perspective' doctoral dissertation, Maynooth University.

Larson, G. and Fuller, D.Q. (2014) 'The evolution of animal domestication', Annual Review of Ecology, Evolution, and Systematics, 45(1), 115–136.

Lawrie, D.S., Messer, P.W., Hershberg, R., and Petrov, D.A. (2013) 'Strong purifying selection at synonymous sites in *D. melanogaster*', PLoS Genetics, 9(5), e1003527.

Laycock, I., Lenthall, K.M., Barratt, A.T., and Cresswell, J.E. (2012) 'Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*)', Ecotoxicology, 21, 1937–1945.

Layer, R.M., Chiang, C., Quinlan, A.R., and Hall, I.M. (2014) 'LUMPY: a probabilistic framework for structural variant discovery', Genome Biology, 15(6), 1–19.

Lázaro, A. and Traveset, A. (2006) 'Reproductive success of the endangered shrub *Buxus balearica* Lam.(Buxaceae): pollen limitation, and inbreeding and outbreeding depression', Plant Systematics and Evolution, 261, 117–128.

Lazzaro, B.P. (2005) 'Elevated polymorphism and divergence in the class C scavenger receptors of *Drosophila melanogaster* and D. simulans', Genetics, 169(4), 2023–2034.

Le Conte, Y., Meixner, M. D., Brandt, A., Carreck, N. L., Costa, C., Mondet, F., & Büchler, R. (2020) 'Geographical distribution and selection of European honey bees resistant to *Varroa destructor*', Insects, 11(12), 873.

Leadbeater, E. and Chittka, L. (2007) 'The dynamics of social learning in an insect model, the bumblebee (*Bombus terrestris*)', Behavioral Ecology and Sociobiology, 61(11), 1789–1796.

Leather, S.R. (2017) '"Ecological Armageddon"-more evidence for the drastic decline in insect numbers', Annals of Applied Biology, 172(1), 1–3.

Lecocq, T. (2018) 'Insects: the disregarded domestication histories' in Animal Domestication, (Ed.) Teletchea, F., InTechOpen (2018), 35-68.

Lecocq, T., Rasmont, P., Harpke, A., and Schweiger, O. (2016) 'Improving International Trade Regulation by Considering Intraspecific Variation for Invasion Risk Assessment of Commercially Traded Species: The *Bombus terrestris* Case', Conservation Letters, 9(4), 281–289.

Lee, J.B., Park, K.-E., Lee, S.A., Jang, S.H., Eo, H.J., Jang, H.A., Kim, C.-H., Ohbayashi, T., Matsuura, Y., Kikuchi, Y., Futahashi, R., Fukatsu, T., and Lee, B.L. (2017) 'Gut symbiotic bacteria stimulate insect growth and egg production by modulating hexamerin and vitellogenin gene expression', Developmental & Comparative Immunology, 69, 12–22.

Lee, Y., Yun, E., Jang, W., Kim, I., Lee, J., Park, S., Ryu, K., Seo, S., Kim, C., and Lee, I. (2004) 'Purification, cDNA cloning and expression of an insect defensin from the great wax moth, *Galleria mellonella*', Insect Molecular Biology, 13(1), 65–72.

Lees, J.G., Ching, Y.W., Adams, D.H., Bach, C.T.T., Samuel, M.S., Kee, A.J., Hardeman, E.C., Gunning, P., Cowin, A.J., and O'Neill, G.M. (2013) 'Tropomyosin Regulates Cell Migration during Skin Wound Healing', Journal of Investigative Dermatology, 133(5), 1330–1339.

Leger, L. and McFrederick, Q.S. (2020) 'The gut–brain–microbiome axis in bumble bees', Insects, 11(8), 517.

Leonhardt, S.D., Gallai, N., Garibaldi, L.A., Kuhlmann, M., and Klein, A.-M. (2013) 'Economic gain, stability of pollination and bee diversity decrease from southern to northern Europe', Basic and Applied Ecology, 14(6), 461–471.

Levin, D.A. (2002) 'Hybridization and extinction: in protecting rare species, conservationists should consider the dangers of interbreeding, which compound the more well-known threats to wildlife', American Scientist, 90(3), 254–261.

Levin, T.C. and Malik, H.S. (2017) ‘Rapidly evolving Toll-3/4 genes encode male-specific toll-like receptors in *Drosophila*’, Molecular Biology and Evolution, 34(9), 2307–2323.

Li, H. and Durbin, R. (2009) ‘Fast and accurate short read alignment with Burrows–Wheeler transform’, Bioinformatics, 25(14), 1754–1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. (2009) ‘The sequence alignment/map format and SAMtools’, Bioinformatics, 25(16), 2078–2079.

Li, H., Zhao, C., Yang, Y., Zhou, Z., Qi, J., and Li, C. (2021) ‘The Influence of Gut Microbiota on the Fecundity of *Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae)’, Journal of Insect Science, 21(4), 15.

Li, K., Wang, L., Zhang, Z., Guo, Y., Guo, J., Chen, Y., Zhuang, D., and Li, J. (2021) ‘Dynamic change of gut microbiota in the male bee of *Bombus terrestris* (Hymenoptera: Apidae)’, Journal of Agricultural Science, 13(9), 155 – 162.

Li, L., Solvi, C., Zhang, F., Qi, Z., Chittka, L., and Zhao, W. (2021) ‘Gut microbiome drives individual memory variation in bumblebees’, Nature Communications, 12(1), 1–10.

Li, S., Yu, X., and Feng, Q. (2019) ‘Fat body biology in the last decade’, Annual Review of Entomology, 64, 315–333.

Li, Y., Xiang, Q., Zhang, Q., Huang, Y., and Su, Z. (2012) ‘Overview on the recent study of antimicrobial peptides: origins, functions, relative mechanisms and application’, Peptides, 37(2), 207–215.

Licursi, V., Salvi, C., De Cesare, V., Rinaldi, T., Mattei, B., Fabbri, C., Serino, G., Bramasole, L., Zimbler, J.Z., and Pick, E. (2014) ‘The COP9 signalosome is involved in the regulation of lipid metabolism and of transition metals uptake in *Saccharomyces cerevisiae*’, The FEBS journal, 281(1), 175–190.

Liczner, A.R. and Colla, S.R. (2019) ‘A systematic review of the nesting and overwintering habitat of bumble bees globally’, Journal of Insect Conservation, 23(5–6), 787–801.

Ligoxygakis, P., Pelte, N., Hoffmann, J.A., and Reichhart, J.-M. (2002) 'Activation of *Drosophila* Toll during fungal infection by a blood serine protease', Science, 297(5578), 114–116.

Limon, J. J., Tang, J., Li, D., Wolf, A. J., Michelsen, K. S., Funari, V., Gargus, M., Nguyen, C., Sharma, P., Maymi, V.I., Iliev, I.D. *et al.* (2019) '*Malassezia* is associated with Crohn's disease and exacerbates colitis in mouse models', Cell Host & Microbe, 25(3), 377-388.

Lin, C.-Y., Chuang, C.-C., Hua, T.-E., Chen, C.-C., Dickson, B.J., Greenspan, R.J., and Chiang, A.-S. (2013) 'A comprehensive wiring diagram of the protocerebral bridge for visual information processing in the *Drosophila* brain', Cell reports, 3(5), 1739–1753.

Lin, G., Huang, Z., Wang, L., Chen, Z., Zhang, T., Gillman, L.N., and Zhao, F. (2019) 'Evolutionary Rates of Bumblebee Genomes Are Faster at Lower Elevations', Molecular Biology and Evolution, 36(6), 1215–1219.

Liu, H., Hall, M. A., Brettell, L. E., Halcroft, M., Wang, J., Nacko, S., Spooner-Hart, R., Cook, J.M., Riegler, M., and Singh, B. (2021) 'Gut microbial diversity in stingless bees is linked to host wing size and is influenced by geography', bioRxiv, 2021-07.

Liu, H., Jia, Y., Sun, X., Tian, D., Hurst, L.D., and Yang, S. (2017) 'Direct determination of the mutation rate in the bumblebee reveals evidence for weak recombination-associated mutation and an approximate rate constancy in insects', Molecular Biology and Evolution, 34(1), 119–130.

Liu, S., Niu, H., Xiao, T., Xue, C., Liu, Z., and Luo, W. (2009) 'Does phenoloxidase contributed to the resistance? Selection with butane-fipronil enhanced its activities from diamondback moths', The Open Biochemistry Journal, 2009(3), 9-13.

Liu, Y., Su, H., Li, R., Li, X., Xu, Y., Dai, X., Zhou, Y., and Wang, H. (2017) 'Comparative transcriptome analysis of *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) reveals novel insights into heat stress tolerance in insects', BMC Genomics, 18(1), 974.

Locatelli, F., Bundrock, G., and Müller, U. (2005) 'Focal and temporal release of glutamate in the mushroom bodies improves olfactory memory in *Apis mellifera*', Journal of Neuroscience, 25(50), 11614–11618.

Lockey, K.H. (1988) 'Lipids of the insect cuticle: origin, composition and function', Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 89(4), 595–645.

Lohr, J. N., & Haag, C. R. (2015) 'Genetic load, inbreeding depression, and hybrid vigor covary with population size: an empirical evaluation of theoretical predictions', Evolution, 69(12), 3109-3122.

Lord, K., Feinstein, M., Smith, B., and Coppinger, R. (2013) 'Variation in reproductive traits of members of the genus *Canis* with special attention to the domestic dog (*Canis familiaris*)', Behavioural Processes, 92, 131–142.

Lowenberger, C., Bulet, P., Charlet, M., Hetru, C., Hodgeman, B., Christensen, B.M., and Hoffmann, J.A. (1995) 'Insect immunity: Isolation of three novel inducible antibacterial defensins from the vector mosquito, *Aedes aegypti*', Insect Biochemistry and Molecular Biology, 25(7), 867–873.

Lowenstein, D.M., Matteson, K.C., and Minor, E.S. (2015) 'Diversity of wild bees supports pollination services in an urbanized landscape', Oecologia, 179(3), 811–821.

Lowry, D.B., Hoban, S., Kelley, J.L., Lotterhos, K.E., Reed, L.K., Antolin, M.F., and Storfer, A. (2017) 'Breaking RAD: an evaluation of the utility of restriction site-associated DNA sequencing for genome scans of adaptation', Molecular Ecology Resources, 17(2), 142–152.Lü, Z.-C. and Wan, F.-H. (2011) 'Using double-stranded RNA to explore the role of heat shock protein genes in heat tolerance in *Bemisia tabaci* (Gennadius)', Journal of Experimental Biology, 214(5), 764–769.

Lucia, M., Reynaldi, F. J., Sguazza, G. H., & Abrahamovich, A. H. (2014) 'First detection of deformed wing virus in *Xylocopa augusti* larvae (Hymenoptera: Apidae) in Argentina', Journal of Apicultural Research, 53(4), 466-468.

Luikart, G., Sherwin, W., Steele, B., and Allendorf, F. (1998) 'Usefulness of molecular markers for detecting population bottlenecks via monitoring genetic change', Molecular Ecology, 7(8), 963–974.

Luiting, P. (1990) ‘Genetic variation of energy partitioning in laying hens: causes of variation in residual feed consumption’, World’s Poultry Science Journal, 46(2), 133–152.

Lv, D., Liu, X., Dong, Y., Yan, Z., Zhang, X., Wang, P., Yuan, X., and Li, Y. (2021) ‘Comparison of Gut Bacterial Communities of Fall Armyworm (Spodoptera frugiperda) Reared on Different Host Plants’, International Journal of Molecular Sciences, 22(20).

Lye, G., Jennings, S.N., Osborne, J.L., and Goulson, D. (2011) ‘Impacts of the use of nonnative commercial bumble bees for pollinator supplementation in raspberry’, Journal of Economic Entomology, 104(1), 107–114.

Ma, E. and Haddad, G.G. (1997) ‘Expression and localization of Na+/H+ exchangers in rat central nervous system’, Neuroscience, 79(2), 591–603.

MacDougall, A., Volynkin, V., Saidi, R., Poggioli, D., Zellner, H., Hatton-Ellis, E., Joshi, V., O’Donovan, C., Orchard, S., and Auchincloss, A.H. (2020) ‘UniRule: a unified rule resource for automatic annotation in the UniProt Knowledgebase’, Bioinformatics, 36(17), 4643–4648.

Mace, G.M., Norris, K., and Fitter, A.H. (2012) ‘Biodiversity and ecosystem services: a multilayered relationship’, Trends in Ecology & Evolution, 27(1), 19–26.

MacInnis, G. and Forrest, J.R.K. (2019) ‘Pollination by wild bees yields larger strawberries than pollination by honey bees’, Journal of Applied Ecology, 56(4), 824–832.

Mack, P.D., Kapelnikov, A., Heifetz, Y., and Bender, M. (2006) ‘Mating-responsive genes in reproductive tissues of female *Drosophila melanogaster*’, Proceedings of the National Academy of Sciences, 103(27), 10358–10363.

Mahboubi, H. and Stochaj, U. (2017) ‘Cytoplasmic stress granules: Dynamic modulators of cell signaling and disease’, Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1863(4), 884–895.

Maher, T., Mirzaei, M., Pascovici, D., Wright, I.J., Haynes, P.A., and Gallagher, R.V. (2019) ‘Evidence from the proteome for local adaptation to extreme heat in a widespread tree species’, Functional Ecology, 33(3), 436–446.

Maia, K.P., Rasmussen, C., Olesen, J.M., and Guimarães Jr, P.R. (2019) 'Does the sociality of pollinators shape the organisation of pollination networks?', Oikos, 128(5), 741–752.

Mäki-Petäys, H. and Breen, J. (2007) 'Genetic vulnerability of a remnant ant population', Conservation Genetics, 8, 427–435.

Makki, R., Meister, M., Pennetier, D., Ubeda, J.-M., Braun, A., Daburon, V., Krzemień, J., Bourbon, H.-M., Zhou, R., and Vincent, A. (2010) 'A short receptor downregulates JAK/STAT signalling to control the *Drosophila* cellular immune response', PLoS biology, 8(8), e1000441.

Malfi, R.L., Crone, E., and Williams, N. (2019) 'Demographic benefits of early season resources for bumble bee (*B. vosnesenskii*) colonies', Oecologia, 191(2), 377–388.

Mallon, E.B., Brockmann, A., and Schmid-Hempel, P. (2003) 'Immune response inhibits associative learning in insects', Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(1532), 2471–2473.

Mallon, E.B., Loosli, R., And Schmid-Hempel, P. (2003) 'Specific versus nonspecific immune defense in The Bumblebee, *Bombus terrestris* L.', Evolution, 57(6), 1444–1447.

Manley, R., Boots, M., and Wilfert, L. (2015) 'Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors', Journal of Applied Ecology, 52(2), 331–340.

Márkus, R., Kurucz, É., Rus, F., and Andó, I. (2005) 'Sterile wounding is a minimal and sufficient trigger for a cellular immune response in *Drosophila melanogaster*', Immunology Letters, 101(1), 108–111.

Marrone, A.K., Kucherenko, M.M., Rishko, V.M., and Shcherbata, H.R. (2011) 'New dystrophin/dystroglycan interactors control neuron behavior in *Drosophila* eye', BMC neuroscience, 12(1), 1–14.

Marshall, T. and Spalton, J. (2000) 'Simultaneous inbreeding and outbreeding depression in reintroduced Arabian oryx', Presented at the Animal Conservation Forum, Cambridge University Press, 241–248.

Martins, G. and Ramalho-Ortigao, J. (2012) ‘Oenocytes in insects’, Invertebrate Survival Journal, 9(2), 139–152.

Martinson, V.G., Danforth, B.N., Minckley, R.L., Rueppell, O., Tingek, S., and Moran, N.A. (2011) ‘A simple and distinctive microbiota associated with honey bees and bumble bees’, Molecular Ecology, 20(3), 619–628.

Martinson, V.G., Moy, J., and Moran, N.A. (2012) ‘Establishment of characteristic gut bacteria during development of the honeybee worker’, Applied and Environmental Microbiology, 78(8), 2830–2840.

Masova, A., Sanda, M., Jiracek, J., and Selicharova, I. (2010) ‘Changes in the proteomes of the hemocytes and fat bodies of the flesh fly Sarcophaga bullata larvae after infection by Escherichia coli’, Proteome Science, 8(1), 1.

Matsui, T., Matsumoto, T., Ichihara, N., Sakai, T., Satake, H., Watari, Y., and Takeda, M. (2009) ‘The pars intercerebralis as a modulator of locomotor rhythms and feeding in the American cockroach, *Periplaneta americana*’, Physiology & Behavior, 96(4–5), 548–556.

Matsumoto, T. (2013) ‘Reduction in homing flights in the honey bee *Apis mellifera* after a sublethal dose of neonicotinoid insecticides’, Bulletin of Insectology, 66(1), 1–9.

Matsumura, C., Yokoyama, J., and Washitani, I. (2004) ‘Invasion status and potential ecological impacts of an invasive alien bumblebee, *Bombus terrestris* L.(Hymenoptera: Apidae) naturalized in Southern Hokkaido, Japan’, Global Environmental Research, 8(1), 51–66.

Matsumura, F., Totsukawa, G., Yamakita, Y., and Yamashiro, S. (2001) ‘Role of myosin light chain phosphorylation in the regulation of cytokinesis’, Cell Structure and Function, 26(6), 639–644.

Matunis, M.J., Wu, J., and Blobel, G. (1998) ‘SUMO-1 modification and its role in targeting the Ran GTPase-activating protein, RanGAP1, to the nuclear pore complex’, The Journal of Cell Biology, 140(3), 499–509.

Mavrouli, M.D., Tsakas, S., Theodorou, G.L., Lampropoulou, M., and Marmaras, V.J. (2005) ‘MAP kinases mediate phagocytosis and melanization via prophenoloxidase

activation in medfly hemocytes', Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1744(2), 145–156.

Mayr, E. (1982) 'Of what use are subspecies?', The Auk, 99(3), 593-595.

Mazed, M., Afroz, M., and Rahman, M. (2022) 'Global decline of insects: A review from agricultural perspective', Agricultural Reviews, 43(3), 334–340.

Mazet, I. and Boucias, D. (1996) 'Effects of the fungal pathogen, *Beauveria bassiana* on protein biosynthesis of infected *Spodoptera exigua* larvae', Journal of Insect Physiology, 42(2), 91–99.

Maziarz, M., Chung, C., Drucker, D.J., and Emili, A. (2005) 'Integrating Global Proteomic and Genomic Expression Profiles Generated from Islet α Cells: Opportunities and Challenges to Deriving Reliable Biological Inferences', Molecular & Cellular Proteomics, 4(4), 458–474.

McArdle, J., Moorman, N.J., and Munger, J. (2012) 'HCMV targets the metabolic stress response through activation of AMPK whose activity is important for viral replication', PLoS Pathogens, 8(1), e1002502.

McDevitt, A.D., Edwards, C.J., O'Toole, P., O'Sullivan, P., O'Reilly, C., and Carden, R.F. (2009) 'Genetic structure of, and hybridisation between, red (*Cervus elaphus*) and sika (*Cervus nippon*) deer in Ireland', Mammalian Biology, 74(4), 263–273.

McKinstry, M., Chung, C., Truong, H., Johnston, B.A., and Snow, J.W. (2017) 'The heat shock response and humoral immune response are mutually antagonistic in honey bees', Scientific Reports, 7(1), 1–14.

McLean, J.E., Seamons, T.R., Dauer, M.B., Bentzen, P., and Quinn, T.P. (2008) 'Variation in reproductive success and effective number of breeders in a hatchery population of steelhead trout (*Oncorhynchus mykiss*): examination by microsatellite-based parentage analysis', Conservation Genetics, 9(2), 295–304.

McMahon, D. P., Fürst, M. A., Caspar, J., Theodorou, P., Brown, M. J., & Paxton, R. J. (2015) 'A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees', Journal of Animal Ecology, 84(3), 615-624.

McMullan, J. (2018) 'Adaptation in honey bee (*Apis mellifera*) colonies exhibiting tolerance to *Varroa destructor* in Ireland', Bee World, 95(2), 39-43.

Meeus, I., Brown, M.J., De Graaf, D.C., and Smagghe, G. (2011) ‘Effects of invasive parasites on bumble bee declines’, Conservation Biology, 25(4), 662–671.

Meeus, I., Mommaerts, V., Billiet, A., Mosallanejad, H., Van de Wiele, T., Wäckers, F., and Smagghe, G. (2013) ‘Assessment of mutualism between *Bombus terrestris* and its microbiota by use of microcolonies’, Apidologie, 44(6), 708–719.

Memmott, J., Waser, N.M., and Price, M.V. (2004) ‘Tolerance of pollination networks to species extinctions’, Proceedings of the Royal Society of London. Series B: Biological Sciences, 271(1557), 2605–2611.

Meng, L., Huo, X., Feng, M., Fang, Y., Han, B., Hu, H., Wu, F., and Li, J. (2018) ‘Proteomics reveals the molecular underpinnings of stronger learning and memory in eastern compared to western bees’, Molecular & Cellular Proteomics, 17(2), 255–269.

Metcalf, J.L., Song, S.J., Morton, J.T., Weiss, S., Seguin-Orlando, A., Joly, F., Feh, C., Taberlet, P., Coissac, E., Amir, A., Willerslev, E., Knight, R., McKenzie, V., and Orlando, L. (2017) ‘Evaluating the impact of domestication and captivity on the horse gut microbiome’, Scientific Reports, 7(1), 15497.

Michener, C.D. (2000) The Bees of the World, JHU Press.

Michener, C.D. and Grimaldi, D.A. (1988) ‘The oldest fossil bee: Apoid history, evolutionary stasis, and antiquity of social behavior’, Proceedings of the National Academy of Sciences, 85(17), 6424–6426.

Mignon-Grasteau, S., Boissy, A., Bouix, J., Faure, J.-M., Fisher, A.D., Hinch, G.N., Jensen, P., Le Neindre, P., Mormede, P., and Prunet, P. (2005) ‘Genetics of adaptation and domestication in livestock’, Livestock Production Science, 93(1), 3–14.

Mikonranta, L., Friman, V.-P., and Laakso, J. (2012) ‘Life History Trade-Offs and Relaxed Selection Can Decrease Bacterial Virulence in Environmental Reservoirs’, PLoS ONE, 7(8), e43801.

Mills, M. C., & Mathieson, I. (2022) ‘The challenge of detecting recent natural selection in human populations’, Proceedings of the National Academy of Sciences, 119(15), e2203237119.

Mirsalehi, A., Markova, D.N., Eslamieh, M., and Betrán, E. (2021) 'Nuclear transport genes recurrently duplicate by means of RNA intermediates in *Drosophila* but not in other insects', BMC Genomics, 22(1), 876.

Mitsuhashi, S. and Nishino, I. (2013) 'Megaconial congenital muscular dystrophy due to loss-of-function mutations in choline kinase β', Current Opinion in Neurology, 26(5), 536–543.

Mockler, B.K., Kwong, W.K., Moran, N.A., and Koch, H. (2018) 'Microbiome structure influences infection by the parasite *Crithidia bombi* in bumble bees', Applied and Environmental Microbiology, 84(7), e02335-17.

Möllmann, J.S. and Colgan, T.J. (2021) 'Genomic architecture and sexually dimorphic expression underlying immunity in the red mason bee, *Osmia bicornis*', Insect Molecular Biology, 31(6), 686-700.

Mommaerts, V., Sterk, G., and Smagghe, G. (2006) 'Hazards and uptake of chitin synthesis inhibitors in bumblebees *Bombus terrestris*', Pest Management Science: formerly Pesticide Science, 62(8), 752–758.

Montalvo, A.M. and Ellstrand, N.C. (2001) 'Nonlocal transplantation and outbreeding depression in the subshrub *Lotus scoparius* (Fabaceae)', American Journal of Botany, 88(2), 258–269.

Montaner, A., da Silva Santana, T.T., Schroeder, T., Einicker-Lamas, M., Girardini, J., Costa, M.R., and Banchio, C. (2018) 'Specific Phospholipids Regulate the Acquisition of Neuronal and Astroglial Identities in Post-Mitotic Cells', Scientific Reports, 8(1), 460.

Mooney, H., Larigauderie, A., Cesario, M., Elmquist, T., Hoegh-Guldberg, O., Lavorel, S., Mace, G.M., Palmer, M., Scholes, R., and Yahara, T. (2009) 'Biodiversity, climate change, and ecosystem services', Current Opinion in Environmental Sustainability, 1(1), 46–54.

Morales-Jiménez, J., Vera-Ponce de León, A., García-Domínguez, A., Martínez-Romero, E., Zúñiga, G., and Hernández-Rodríguez, C. (2013) 'Nitrogen-Fixing and Uricolytic Bacteria Associated with the Gut of *Dendroctonus rhizophagus* and

Dendroctonus valens (Curculionidae: Scolytinae)', Microbial Ecology, 66(1), 200–210.

Morandin, L., Laverty, T., and Kevan, P. (2001) 'Effect of bumble bee (Hymenoptera: Apidae) pollination intensity on the quality of greenhouse tomatoes', Journal of Economic Entomology, 94(1), 172–179.

Morandin, L.A., Winston, M.L., Franklin, M.T., and Abbott, V.A. (2005) 'Lethal and sub-lethal effects of spinosad on bumble bees (*Bombus impatiens* Cresson)', Pest Management Science: formerly Pesticide Science, 61(7), 619–626.

Moreira, A.S., Horgan, F.G., Murray, T.E., and Kakouli-Duarte, T. (2015) 'Population genetic structure of *Bombus terrestris* in Europe: Isolation and genetic differentiation of Irish and British populations', Molecular Ecology, 24(13), 3257–3268.

Morelle, R. (2014) 'Bumblebees infected with honeybee diseases', BBC News, available at: https://www.bbc.com/news/science-environment-26242960 [accessed 17th May 2023]

Moretti, A., Mulé, G., Ritieni, A., Láday, M., Stubnya, V., Hornok, L., & Logrieco, A. (2008) 'Cryptic subspecies and beauvericin production by *Fusarium subglutinans* from Europe', International Journal of Food Microbiology, 127(3), 312-315.

Morfin, N., Fillier, T.A., Pham, T.H., Goodwin, P.H., Thomas, R.H., and Guzman-Novoa, E. (2022) 'First insights into the honey bee (*Apis mellifera*) brain lipidome and its neonicotinoid-induced alterations associated with reduced self-grooming behavior', Journal of Advanced Research, 37, 75–89.

Morlais, I., Ponçon, N., Simard, F., Cohuet, A., and Fontenille, D. (2004) 'Intraspecific nucleotide variation in *Anopheles gambiae*: new insights into the biology of malaria vectors.', American Journal of Tropical Medicine and Hygiene, 71(6), 795–802.

Mousseau, T., & Sikes, D. S. (2011) 'Almost but not quite a subspecies: a case of genetic but not morphological diagnosability in *Nicrophorus* (Coleoptera: Silphidae)', Biological Journal of the Linnean Society, 102(2), 311-333.

Mugabo, Y., Zhao, S., Seifried, A., Gezzar, S., Al-Mass, A., Zhang, D., Lamontagne, J., Attane, C., Poursharifi, P., and Iglesias, J. (2016) 'Identification of a mammalian

glycerol-3-phosphate phosphatase: Role in metabolism and signaling in pancreatic β-cells and hepatocytes', Proceedings of the National Academy of Sciences, 113(4), E430–E439.

Müller, C.B. and Schmid-Hempel, P. (1992) 'Correlates of reproductive success among field colonies of *Bombus lucorum*: the importance of growth and parasites', Ecological Entomology, 17(4), 343–353.

Muñiz Ortiz, J.G., Opoka, R., Kane, D., and Cartwright, I.L. (2009) 'Investigating arsenic susceptibility from a genetic perspective in *Drosophila* reveals a key role for glutathione synthetase', Toxicological Sciences, 107(2), 416–426.

Murray, T.E., Coffey, M.F., Kehoe, E., and Horgan, F.G. (2013) 'Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations', Biological Conservation, 159, 269–276.

Murray, T.E., Kehoe, E., and Horgan, F.G. (2008) 'Trends in Commercially Reared Bumblebee Importation for Pollination in Ireland', Presented at the Environ, available: https://www.teagasc.ie/media/website/crops/horticulture/fruit-production/Environ2008_Poster.pdf [accessed 11 Aug 2022].

Muth, F. and Leonard, A. (2019) 'A neonicotinoid pesticide impairs foraging, but not learning, in free-flying bumblebees', Scientific Reports, 9(1), 1–13.

Myllymäki, H., Valanne, S., and Rämet, M. (2014) 'The *Drosophila* IMD signaling pathway', The Journal of Immunology, 192(8), 3455–3462.

Nahlik, K., Dumkow, M., Bayram, Ö., Helmstaedt, K., Busch, S., Valerius, O., Gerke, J., Hoppert, M., Schwier, E., and Opitz, L. (2010) 'The COP9 signalosome mediates transcriptional and metabolic response to hormones, oxidative stress protection and cell wall rearrangement during fungal development', Molecular microbiology, 78(4), 964–979.

Nappi, A.J. and Christensen, B.M. (2005) 'Melanogenesis and associated cytotoxic reactions: Applications to insect innate immunity', Insect Biochemistry and Molecular Biology, 35(5), 443–459.

Nargund, A.M., Pellegrino, M.W., Fiorese, C.J., Baker, B.M., and Haynes, C.M. (2012) 'Mitochondrial Import Efficiency of ATFS-1 Regulates Mitochondrial UPR Activation', Science, 337(6094), 587–590.

Nation, J.L. (2008) Insect Physiology and Biochemistry, CRC press: Boca Raton, USA.

Natural England (2018) Bumblebees: Licence for Emergency Release of Non-Native Species for Pollination or Research (CL28) [online], available: https://www.gov.uk/government/publications/bumblebees-licence-to-release-them-for-pollination-and-research/licence-to-permit-the-release-of-non-native-sub-species-of-bumblebee-bombus-terrestris-in-commercial-glass-houses-for-research [accessed 4 Nov 2022].

Negroni, M.A., Segers, F.H., Vogelweith, F., and Foitzik, S. (2020) 'Immune challenge reduces gut microbial diversity and triggers fertility-dependent gene expression changes in a social insect', BMC Genomics, 21, 1–16.

Nessel, M.P., Konnovitch, T., Romero, G.Q., and González, A.L. (2022) 'Decline of insects and arachnids driven by nutrient enrichment: A meta-analysis', Ecology, e3897.

Nesvizhskii, A.I. and Aebersold, R. (2005) 'Interpretation of shotgun proteomic data', Molecular & Cellular Proteomics, 4(10), 1419–1440.

Newbold, L.K., Oliver, A.E., Cuthbertson, L., Walkington, S.E., Gweon, H.S., Heard, M.S., and van der Gast, C.J. (2015) 'Rearing and foraging affects bumblebee (*Bombus terrestris*) gut microbiota', Environmental Microbiology Reports, 7(4), 634–641.

Newby, L.M. and Jackson, F.R. (1991) '*Drosophila* Ebony Mutants Have Altered Circadian Activity Rhythms but Normal Eclosion Rhythms', Journal of Neurogenetics, 7(2–3), 85–101.

Nicholls, E. and Hempel de Ibarra, N. (2017) 'Assessment of pollen rewards by foraging bees', Functional Ecology, 31(1), 76–87.

Nielsen, E.E., Hansen, M.M., and Loeschcke, V. (1997) 'Analysis of microsatellite DNA from old scale samples of Atlantic salmon *Salmo salar*: a comparison of genetic composition over 60 years', Molecular Ecology, 6(5), 487–492.

Nielsen, R., Williamson, S., Kim, Y., Hubisz, M.J., Clark, A.G., and Bustamante, C. (2005) ‘Genomic scans for selective sweeps using SNP data’, Genome Research, 15(11), 1566–1575.

Nieto, A. (2014) ‘European red list of bees’, European Commission: Luxembourg.

Nikoh, N., Hosokawa, T., Moriyama, M., Oshima, K., Hattori, M., and Fukatsu, T. (2014) ‘Evolutionary origin of insect–Wolbachia nutritional mutualism’, Proceedings of the National Academy of Sciences, 111(28), 10257–10262.

Nilsen, K.-A., Ihle, K.E., Frederick, K., Fondrk, M.K., Smedal, B., Hartfelder, K., and Amdam, G.V. (2011) ‘Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology’, Journal of Experimental Biology, 214(9), 1488–1497.

Nish, S. and Medzhitov, R. (2011) ‘Host defense pathways: role of redundancy and compensation in infectious disease phenotypes’, Immunity, 34(5), 629–636.

Nitta, Y. and Sugie, A. (2017) ‘DISCO interacting protein 2 determines direction of axon projection under the regulation of c-Jun N-terminal kinase in the *Drosophila* mushroom body’, Biochemical and Biophysical Research Communications, 487(1), 116–121.

NNSS (2009) Information about GB Non-Native Species Risk Assessment (*Bombus terrestris*) [online], available: https://www.nonnativespecies.org/assets/Uploads/RA_Bombus_terrestris_sp_revised_2014_final_postsc-2.pdf [accessed 4 Nov 2022].

Nolfi-Donegan, D., Braganza, A., and Shiva, S. (2020) ‘Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement’, Redox Biology, 37, 101674.

Novosyadlyy, R., Kurshan, N., Lann, D., Vijayakumar, A., Yakar, S., and LeRoith, D. (2008) ‘Insulin-like growth factor-I protects cells from ER stress-induced apoptosis via enhancement of the adaptive capacity of endoplasmic reticulum’, Cell Death & Differentiation, 15(8), 1304–1317.

Nyholm, S.V. and Graf, J. (2012) ‘Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses’, Nature Reviews Microbiology, 10(12), 815–827.

O’Connor, T.P. (1997) ‘Working at relationships: another look at animal domestication’, Antiquity, 71(271), 149–156.

O’Donnell, S., Reichardt, M., and Foster, R. (2000) ‘Individual and colony factors in bumble bee division of labor (*Bombus bifarius nearcticus* Handl; Hymenoptera, Apidae)’, Insectes Sociaux, 47(2), 164–170.

O’Meara, D.B., Edwards, C.J., Sleeman, D.P., Cross, T.F., Statham, M.J., Mcdowell, J.R., Dillane, E., Coughlan, J.P., O’leary, D., O’reilly, C., Bradley, D.G., and Carlsson, J. (2012) ‘Genetic structure of Eurasian badgers *Meles meles* (Carnivora: Mustelidae) and the colonization history of Ireland’, Biological Journal of the Linnean Society, 106(4), 893–909.

Obbard, D.J., Jiggins, F.M., Halligan, D.L., and Little, T.J. (2006) ‘Natural selection drives extremely rapid evolution in antiviral RNAi genes’, Current Biology, 16(6), 580–585.

OECD (2017) ‘Test No. 247: Bumblebee, Acute Oral Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2, No. 247’. OECD Publishing: Paris, France.

Ollerton, J., Winfree, R., and Tarrant, S. (2011) ‘How many flowering plants are pollinated by animals?’, Oikos, 120(3), 321–326.

Ollivier, L. (2002) Éléments de génétique quantitative 2nd edn., INRA:Paris, France.

Ormerod, K.L., Wood, D.L.A., Lachner, N., Gellatly, S.L., Daly, J.N., Parsons, J.D., Dal’Molin, C.G.O., Palfreyman, R.W., Nielsen, L.K., Cooper, M.A., Morrison, M., Hansbro, P.M., and Hugenholtz, P. (2016) ‘Genomic characterization of the uncultured Bacteroidales family S24-7 inhabiting the guts of homeothermic animals’, Microbiome, 4(1), 36.

Oron, E., Tuller, T., Li, L., Rozovsky, N., Yekutieli, D., Rencus-Lazar, S., Segal, D., Chor, B., Edgar, B.A., and Chamovitz, D.A. (2007) ‘Genomic analysis of COP9 signalosome function in *Drosophila melanogaster* reveals a role in temporal regulation of gene expression’, Molecular Systems Biology, 3(1), 108.

Osborne, J.L., Loxdale, H.D., and Woiwod, I.P. (2002) 'Monitoring insect dispersal: methods and approaches', Presented at the Dispersal Ecology: the 42nd Symposium of the British Ecological Society held at the University of Reading, 2-5 April 2001.

Otterstatter, M.C. and Thomson, J.D. (2007) 'Contact networks and transmission of an intestinal pathogen in bumble bee (*Bombus impatiens*) colonies', Oecologia, 154(2), 411–421.

Otterstatter, M.C. and Thomson, J.D. (2008) 'Does pathogen spillover from commercially reared bumble bees threaten wild pollinators?', PLoS ONE, 3(7), e2771.

Otti, O. and Schmid-Hempel, P. (2007) '*Nosema bombi*: a pollinator parasite with detrimental fitness effects', Journal of Invertebrate Pathology, 96(2), 118–124.

Ouborg, N.J., Pertoldi, C., Loeschcke, V., Bijlsma, R.K., and Hedrick, P.W. (2010) 'Conservation genetics in transition to conservation genomics', Trends in Genetics, 26(4), 177–187.

Palmer, K.A. and Oldroyd, B.P. (2003) 'Evidence for intra-colonial genetic variance in resistance to American foulbrood of honey bees (*Apis mellifera*): further support for the parasite/pathogen hypothesis for the evolution of polyandry', Naturwissenschaften, 90, 265–268.

Palmer, W.H., Hadfield, J.D., and Obbard, D.J. (2018) 'RNA-interference pathways display high rates of adaptive protein evolution in multiple invertebrates', Genetics, 208(4), 1585–1599.

Paludo, C.R., Menezes, C., Silva-Junior, E.A., Vollet-Neto, A., Andrade-Dominguez, A., Pishchany, G., Khadempour, L., do Nascimento, F.S., Currie, C.R., and Kolter, R. (2018) 'Stingless bee larvae require fungal steroid to pupate', Scientific Reports, 8(1), 1–10.

Pan, X., Pike, A., Joshi, D., Bian, G., McFadden, M.J., Lu, P., Liang, X., Zhang, F., Raikhel, A.S., and Xi, Z. (2018) 'The bacterium Wolbachia exploits host innate immunity to establish a symbiotic relationship with the dengue vector mosquito *Aedes aegypti*', The ISME Journal, 12(1), 277–288.

Pandey, A., Motro, U., and Bloch, G. (2020) 'Juvenile hormone interacts with multiple factors to modulate aggression and dominance in groups of orphan bumble bee (*Bombus terrestris*) workers', Hormones and behavior, 117, 104602.

Papakostas, S., Vasemaegi, A., VÄHÄ, J., Himberg, M., Peil, L., and Primmer, C.R. (2012) 'A proteomics approach reveals divergent molecular responses to salinity in populations of European whitefish (*Coregonus lavaretus*)', Molecular Ecology, 21(14), 3516–3530.

Parker, R., Melathopoulos, A.P., White, R., Pernal, S.F., Guarna, M.M., and Foster, L.J. (2010) 'Ecological adaptation of diverse honey bee (*Apis mellifera*) populations', PLoS ONE, 5(6), e11096.

Parmesan, C. (2006) 'Ecological and evolutionary responses to recent climate change', Annual Review of Ecology, Evolution, and Systematics, 637–669.

Patel, V.J., Thalassinos, K., Slade, S.E., Connolly, J.B., Crombie, A., Murrell, J.C., and Scrivens, J.H. (2009) 'A comparison of labeling and label-free mass spectrometry-based proteomics approaches', Journal of Proteome Research, 8(7), 3752–3759.

Pattemore, D., Goodwin, R., McBrydie, H., Hoyte, S., and Vanneste, J. (2014) 'Evidence of the role of honey bees (*Apis mellifera*) as vectors of the bacterial plant pathogen *Pseudomonas syringae*', Australasian Plant Pathology, 43(5), 571–575.

Patten, M. A. and Unitt, P. (2002) 'Diagnosability versus mean differences of Sage Sparrow subspecies', The Auk, 119(1), 26-35.

Patten, M.A. (2009) ''Subspecies' and 'race' should not be used as synonyms', Nature, 457(7226), 147-147.

Patten, M.A. (2015) 'Subspecies and the philosophy of science', The Auk, 132(2), 481-485.Pauls, S.U., Nowak, C., Bálint, M., and Pfenninger, M. (2013) 'The impact of global climate change on genetic diversity within populations and species', Molecular Ecology, 22(4), 925–946.

Pauwels, K., De Meester, L., Decaestecker, E., and Stoks, R. (2011) 'Phenoloxidase but not lytic activity reflects resistance against *Pasteuria ramosa* in *Daphnia magna*', Biology Letters, 7(1), 156–159.

Pavlidi, N., Vontas, J., and Van Leeuwen, T. (2018) ‘The role of glutathione S-transferases (GSTs) in insecticide resistance in crop pests and disease vectors’, Current Opinion in Insect Science, 27, 97–102.

Paxton, R.J. (2005) ‘Male mating behaviour and mating systems of bees: an overview’, Apidologie, 36(2), 145–156.

Pazaroviçi, P. and Pener, M. (1978) ‘The relations of the pars intercerebralis, corpora allata, and juvenile hormone to oocyte development and oviposition in the African migratory locust’, General and Comparative Endocrinology, 35(4), 375–386.

Peat, J., Darvill, B., Ellis, J., and Goulson, D. (2005) ‘Effects of climate on intra- and interspecific size variation in bumble-bees’, Functional Ecology, 19(1), 145–151.

Peat, J., Tucker, J., and Goulson, D. (2005) ‘Does intraspecific size variation in bumblebees allow colonies to efficiently exploit different flowers?’, Ecological Entomology, 30(2), 176–181.

Pechstein, A., Shupliakov, O., and Haucke, V. (2010) ‘Intersectin 1: a versatile actor in the synaptic vesicle cycle’, Biochemical Society Transactions, 38(1), 181–186.

Pedreschi, D., Kelly-Quinn, M., Caffrey, J., O’Grady, M., and Mariani, S. (2014) ‘Genetic structure of pike (*Esox lucius*) reveals a complex and previously unrecognized colonization history of Ireland’, Journal of Biogeography, 41(3), 548–560.

Peer, K. and Taborsky, M. (2005) ‘Outbreeding depression, but no inbreeding depression in haplodiploid ambrosia beetles with regular sibling mating’, Evolution, 59(2), 317–323.

Pelletier, L. and McNeil, J.N. (2003) ‘The effect of food supplementation on reproductive success in bumblebee field colonies’, Oikos, 103(3), 688–694.

Petanidou, T., Kallimanis, A.S., Sgardelis, S.P., Mazaris, A.D., Pantis, J.D., and Waser, N.M. (2014) ‘Variable flowering phenology and pollinator use in a community suggest future phenological mismatch’, Acta Oecologica, 59, 104–111.

Pfeifer, B., Wittelsbürger, U., Ramos-Onsins, S.E., and Lercher, M.J. (2014) ‘PopGenome: an efficient Swiss army knife for population genomic analyses in R’, Molecular Biology and Evolution, 31(7), 1929–1936.

Phillips, A.M., Smart, R., Strauss, R., Brembs, B., and Kelly, L.E. (2005) 'The *Drosophila* black enigma: the molecular and behavioural characterization of the black1 mutant allele', Gene, 351, 131–142.

Pick, J. L., Hutter, P., Ebneter, C., Ziegler, A. K., Giordano, M., & Tschirren, B. (2016) 'Artificial selection reveals the energetic expense of producing larger eggs', *Frontiers in Zoology*, *13*(1), 1-10.

Piiroinen, S. and Goulson, D. (2016) 'Chronic neonicotinoid pesticide exposure and parasite stress differentially affects learning in honeybees and bumblebees', Proceedings of the Royal Society B: Biological Sciences, 283(1828), 20160246.

Piiroinen, S., Botías, C., Nicholls, E., and Goulson, D. (2016) 'No effect of low-level chronic neonicotinoid exposure on bumblebee learning and fecundity', PeerJ, 4, e1808.

Pizzorno, M.C., Field, K., Kobokovich, A.L., Martin, P.L., Gupta, R.A., Mammone, R., Rovnyak, D., and Capaldi, E.A. (2021) 'Transcriptomic responses of the honey bee brain to infection with deformed wing virus', Viruses, 13(2), 287.

Plowright, R. and Jay, S. (1966) 'Rearing bumble bee colonies in captivity', Journal of Apicultural Research, 5(3), 155–165.

Poisot, T., Bever, J.D., Nemri, A., Thrall, P.H., and Hochberg, M.E. (2011) 'A conceptual framework for the evolution of ecological specialisation', Ecology Letters, 14(9), 841–851.

Porter, T.D. (2002) 'The roles of cytochrome b5 in cytochrome P450 reactions', Journal of Biochemical and Molecular Toxicology, 16(6), 311–316.

Possik, E., Schmitt, C., Al-Mass, A., Bai, Y., Côté, L., Morin, J., Erb, H., Oppong, A., Kahloan, W., and Parker, J.A. (2022) 'Phosphoglycolate phosphatase homologs act as glycerol-3-phosphate phosphatase to control stress and healthspan in *C. elegans*', Nature Communications, 13(1), 1–17.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., and Kunin, W.E. (2010) 'Global pollinator declines: trends, impacts and drivers', Trends in Ecology & Evolution, 25(6), 345–353.

Potts, S.G., Imperatriz Fonseca, V., Ngo, H.T., Biesmeijer, J.C., Breeze, T.D., Dicks, L., Garibaldi, L.A., Hill, R., Settele, J., and Vanbergen, A.J. (2016) 'Summary for policymakers of the assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production', available: https://www.actu-environnement.com/media/pdf/news-26331-synthese-ipbes-decideurs-pollinisateurs.pdf [accessed 27 Feb 2023].

Potts, S.G., Ngo, H.T., Biesmeijer, J.C., Breeze, T.D., Dicks, L.V., Garibaldi, L.A., Hill, R., Settele, J., and Vanbergen, A. (2016) 'The assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production', IPBES: Germany.

Pozo, M. I., Bartlewicz, J., Van Oystaeyen, A., Benavente, A., Van Kemenade, G., Wäckers, F., & Jacquemyn, H. (2018) 'Surviving in the absence of flowers: do nectar yeasts rely on overwintering bumblebee queens to complete their annual life cycle?', FEMS Microbiology Ecology, 94(12), fiy196.

Pozo, M. I., Van Kemenade, G., Van Oystaeyen, A., Aledón-Catalá, T., Benavente, A., Van den Ende, W., Wackers, F., and Jacquemyn, H. (2020) 'The impact of yeast presence in nectar on bumble bee behavior and fitness', *Ecological Monographs, 90*(1), e01393.

Pozo, M.I., Mariën, T., van Kemenade, G., Wäckers, F., and Jacquemyn, H. (2021) 'Effects of pollen and nectar inoculation by yeasts, bacteria or both on bumblebee colony development', Oecologia, 195(3), 689–703.

Praet, J., Parmentier, A., Schmid-Hempel, R., Meeus, I., Smagghe, G., & Vandamme, P. (2018) 'Large-scale cultivation of the bumblebee gut microbiota reveals an underestimated bacterial species diversity capable of pathogen inhibition', Environmental Microbiology, 20(1), 214-227.

Protter, D.S.W. and Parker, R. (2016) 'Principles and Properties of Stress Granules', Trends in Cell Biology, 26(9), 668–679.

Provost, B., Jouan, V., Hilliou, F., Delobel, P., Bernardo, P., Ravallec, M., Cousserans, F., Wajnberg, E., Darboux, I., Fournier, P., Strand, M.R., and Volkoff, A.-N. (2011) 'Lepidopteran transcriptome analysis following infection by phylogenetically

unrelated polydnaviruses highlights differential and common responses', Insect Biochemistry and Molecular Biology, 41(8), 582–591.

Quinlan, A.R. (2014) 'BEDTools: the Swiss-army tool for genome feature analysis', Current Protocols in Bioinformatics, 47(1), 11–12.

R Core Team (2021) R: A Language and Environment for Statistical Computing [online], Vienna, Austria: R Foundation for Statistical Computing, available: https://www.R-project.org/.

Radyuk, S.N., Michalak, K., Klichko, V.I., Benes, J., and Orr, W.C. (2010) 'Peroxiredoxin 5 modulates immune response in *Drosophila*', Biochimica et Biophysica Acta (BBA) - General Subjects, 1800(11), 1153–1163.

Raghanti, M.A. (2019) 'Domesticated species: It takes one to know one', Proceedings of the National Academy of Sciences, 116(29), 14401–14403.

Raine, N.E. and Chittka, L. (2007) 'Pollen foraging: learning a complex motor skill by bumblebees (*Bombus terrestris*)', Naturwissenschaften, 94(6), 459–464.

Ramos Rego, I., Santos Cruz, B., Ambrósio, A.F., and Alves, C.H. (2021) 'TRAP1 in Oxidative Stress and Neurodegeneration', Antioxidants, 10(11), 1829.

Randi, E. (2008) 'Detecting hybridization between wild species and their domesticated relatives', Molecular Ecology, 17(1), 285–293.

Randi, E., Davoli, F., Pierpaoli, M., Pertoldi, C., Madsen, A.B., and Loeschcke, V. (2003) 'Genetic structure in otter (*Lutra lutra*) populations in Europe: implications for conservation', Animal Conservation forum, 6(2), 93–100.

Ranta, E. (1982) 'Species structure of North European bumblebee communities', Oikos, 8(2), 202–209.

Ranwez, V., Serra, A., Pot, D., and Chantret, N. (2017) 'Domestication reduces alternative splicing expression variations in sorghum', PLoS ONE, 12(9), e0183454.

Rasmont, P., Coppée, A., Michez, D., and De Meulemeester, T. (2008) 'An overview of the *Bombus terrestris* (L. 1758) subspecies (Hymenoptera: Apidae)', Presented at the Annales de la Société Entomologique de France, Taylor & Francis, 243–250.

Rasmont, P., Franzen, M., Lecocq, T., Harpke, A., Roberts, S.P., Biesmeijer, J.C., Castro, L., Cederberg, B., Dvorak, L., and FitzPatrick, U. (2015) Climatic Risk and Distribution Atlas of European Bumblebees, Pensoft Publishers.

Raven, P.H. and Wagner, D.L. (2021) 'Agricultural intensification and climate change are rapidly decreasing insect biodiversity', Proceedings of the National Academy of Sciences, 118(2), e2002548117.

Rawlings, J.S., Rosler, K.M., and Harrison, D.A. (2004) 'The JAK/STAT signaling pathway', Journal of Cell Science, 117(8), 1281–1283.

Raymann, K. and Moran, N.A. (2018) 'The role of the gut microbiome in health and disease of adult honey bee workers', Current Opinion in Insect Science, 26, 97–104.

Raymann, K., Shaffer, Z., & Moran, N. A. (2017). Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. PLoS biology, 15(3), e2001861.

Raymond, J.A. (1992) 'Glycerol is a colligative antifreeze in some northern fishes', Journal of Experimental Zoology, 262(3), 347–352.

Reed, D.H. and Frankham, R. (2003) 'Correlation between Fitness and Genetic Diversity', Conservation Biology, 17(1), 230–237.

Rees, J.A., Moniatte, M., and Bulet, P. (1997) 'Novel antibacterial peptides isolated from a European bumblebee, *Bombus pascuorum* (Hymenoptera, Apoidea)', Insect Biochemistry and Molecular Biology, 27(5), 413–422.

Reynolds, M.P., Pierre, C.S., Saad, A.S.I., Vargas, M., and Condon, A.G. (2007) 'Evaluating Potential Genetic Gains in Wheat Associated with Stress-Adaptive Trait Expression in Elite Genetic Resources under Drought and Heat Stress', Crop Science, 47(S3), S-172.

Rhymer, J.M. and Simberloff, D. (1996) 'Extinction by hybridization and introgression', Annual Review of Ecology and Systematics, 27(1), 83–109.

Ricci, I., Damiani, C., Scuppa, P., Mosca, M., Crotti, E., Rossi, P., Rizzi, A., Capone, A., Gonella, E., and Ballarini, P. (2011) 'The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*', Environmental microbiology, 13(4), 911–921.

Ricci, I., Mosca, M., Valzano, M., Damiani, C., Scuppa, P., Rossi, P., Crotti, E., Cappelli, A., Ulissi, U., and Capone, A. (2011) 'Different mosquito species host *Wickerhamomyces anomalus* (*Pichia anomala*): perspectives on vector-borne diseases symbiotic control', Antonie van Leeuwenhoek, 99(1), 43–50.

Richards, O.W. and Davies, R.G. (2013) Imms' General Textbook of Entomology: Volume 2: Classification and Biology, Springer Science & Business Media.

Ricketts, T.H., Regetz, J., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Bogdanski, A., Gemmill-Herren, B., Greenleaf, S.S., Klein, A.M., and Mayfield, M.M. (2008) 'Landscape effects on crop pollination services: are there general patterns?', Ecology Letters, 11(5), 499–515.

Riddell, C., Sumner, S., Adams, S., and Mallon, E. (2011) 'Pathways to immunity: temporal dynamics of the bumblebee (*Bombus terrestris*) immune response against a trypanosomal gut parasite', Insect Molecular Biology, 20(4), 529–540.

Riddell, C.E., Lobaton Garces, J.D., Adams, S., Barribeau, S.M., Twell, D., and Mallon, E.B. (2014) 'Differential gene expression and alternative splicing in insect immune specificity', BMC Genomics, 15(1), 1–15.

Riessberger-Gallé, U., Hernández López, J., Schuehly, W., Crockett, S., Krainer, S., and Crailsheim, K. (2015) 'Immune responses of honeybees and their fitness costs as compared to bumblebees', Apidologie, 46(2), 238–249.

Rittschof, C.C. and Hughes, K.A. (2018) 'Advancing behavioural genomics by considering timescale', Nature Communications, 9(1), 489.

Robinson, G.E. (1987) 'Regulation of honey bee age polyethism by juvenile hormone', Behavioral Ecology and Sociobiology, 20(5), 329–338.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011) 'Integrative genomics viewer', Nature Biotechnology, 29(1), 24–26.

Roffet-Salque, M., Regert, M., Evershed, R.P., Outram, A.K., Cramp, L.J., Decavallas, O., Dunne, J., Gerbault, P., Mileto, S., and Mirabaud, S. (2015) 'Widespread exploitation of the honeybee by early Neolithic farmers', Nature, 527(7577), 226–230.

Roh, K.-B., Kim, C.-H., Lee, H., Kwon, H.-M., Park, J.-W., Ryu, J.-H., Kurokawa, K., Ha, N.-C., Lee, W.-J., and Lemaitre, B. (2009) 'Proteolytic cascade for the activation of the insect toll pathway induced by the fungal cell wall component', Journal of Biological Chemistry, 284(29), 19474–19481.

Rokas, A. (2009) 'The effect of domestication on the fungal proteome', Trends in Genetics, 25(2), 60–63.

Röseler, P.-F. (1985) 'A technique for year-round rearing of *Bombus terrestris* (Apidae, Bombini) colonies in captivity', Apidologie, 16(2), 165–170.

Rosinger, H.S., Geraldes, A., Nurkowski, K.A., Battlay, P., Cousens, R.D., Rieseberg, L.H., and Hodgins, K.A. (2021) 'The tip of the iceberg: Genome wide marker analysis reveals hidden hybridization during invasion', Molecular Ecology, 30(3), 810–825.

Rothammer, S., Seichter, D., Förster, M., and Medugorac, I. (2013) 'A genome-wide scan for signatures of differential artificial selection in ten cattle breeds', BMC Genomics, 14(1), 908.

Rother, L., Kraft, N., Smith, D.B., el Jundi, B., Gill, R.J., and Pfeiffer, K. (2021) 'A micro-CT-based standard brain atlas of the bumblebee', Cell and Tissue Research, 386(1), 29–45.

Rothman, J.A., Leger, L., Graystock, P., Russell, K., and McFrederick, Q.S. (2019) 'The bumble bee microbiome increases survival of bees exposed to selenate toxicity', Environmental Microbiology, 21(9), 3417–3429.

Roux, J., Privman, E., Moretti, S., Daub, J.T., Robinson-Rechavi, M., and Keller, L. (2014) 'Patterns of positive selection in seven ant genomes', Molecular Biology and Evolution, 31(7), 1661–1685.

Rowe, G., Harris, D.J., and Beebee, T.J.C. (2006) 'Lusitania revisited: A phylogeographic analysis of the natterjack toad Bufo calamita across its entire biogeographical range', Molecular Phylogenetics and Evolution, 39(2), 335–346.

Roy, S., Saha, T.T., Zou, Z., and Raikhel, A.S. (2018) 'Regulatory pathways controlling female insect reproduction', Annual Review of Entomology, 63, 489–511.

Royet, J., Gupta, D., and Dziarski, R. (2011) ‘Peptidoglycan recognition proteins: modulators of the microbiome and inflammation’, Nature Reviews Immunology, 11(12), 837–851.

Rundlöf, M., Andersson, G.K.S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B.K., Pedersen, T.R., Yourstone, J., and Smith, H.G. (2015) ‘Seed coating with a neonicotinoid insecticide negatively affects wild bees’, Nature, 521(7550), 77–80.

Rutkowski, D., Litsey, E., Maalouf, I., and Vannette, R.L. (2022) ‘Bee-associated fungi mediate effects of fungicides on bumble bees’, Ecological Entomology, 47(3), 411–422.

Rutrecht, S.T. and Brown, M.J. (2008) ‘The life-history impact and implications of multiple parasites for bumble bee queens’, International Journal for Parasitology, 38(7), 799–808.

Rutschmann, S., Kilinc, A., and Ferrandon, D. (2002) ‘Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in *Drosophila*’, The Journal of Immunology, 168(4), 1542–1546.

Ruz, L. (2002) ‘Bee pollinators introduced to Chile: a review’, Pollinating bees, 155–167.

Sadd, B.M., Barribeau, S.M., Bloch, G., De Graaf, D.C., Dearden, P., Elsik, C.G., Gadau, J., Grimmelikhuijzen, C.J., Hasselmann, M., and Lozier, J.D. (2015) ‘The genomes of two key bumblebee species with primitive eusocial organization’, Genome Biology, 16(1), 1–32.

Sah, S. and Singh, R. (2016) ‘Phylogenetical coherence of Pseudomonas in unexplored soils of Himalayan region’, 3 Biotech, 6(2), 1–10.

Sahle, H., Enbiyale, G., Negash, A., and Neges, T. (2018) ‘Assessment of honey production system, constraints and opportunities in Ethiopia’, Pharmacy and Pharmacology International Journal, 6(1), 42–47.

Sala, O.E., Stuart Chapin, F., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L.F., Jackson, R.B., and Kinzig, A. (2000) ‘Global biodiversity scenarios for the year 2100’, Science, 287(5459), 1770–1774.

Sánchez-Bayo, F. and Wyckhuys, K.A. (2019) ‘Worldwide decline of the entomofauna: A review of its drivers’, Biological Conservation, 232, 8–27.

Saraiva, M.A., Zemolin, A.P.P., Franco, J.L., Boldo, J.T., Stefenon, V.M., Triplett, E.W., de Oliveira Camargo, F.A., and Roesch, L.F.W. (2015) ‘Relationship between honeybee nutrition and their microbial communities’, Antonie van Leeuwenhoek, 107(4), 921–933.

Sarov-Blat, L., So, W.V., Liu, L., and Rosbash, M. (2000) ‘The *Drosophila* takeout gene is a novel molecular link between circadian rhythms and feeding behavior’, Cell, 101(6), 647–656.

Sauers, L.A. and Sadd, B.M. (2019) ‘An interaction between host and microbe genotypes determines colonization success of a key bumble bee gut microbiota member’, Evolution, 73(11), 2333–2342.

Schilling, M. and Cruse, H. (2020) ‘Decentralized control of insect walking: A simple neural network explains a wide range of behavioral and neurophysiological results’, PLoS Computational Biology, 16(4), e1007804.

Schmid-Hempel, P. (1995) ‘Parasites and social insects’, Apidologie, 26(3), 255–271.

Schmid-Hempel, P. (1998) *Parasites in social insects.* Princeton University Press: New Jersey, USA.

Schmid-Hempel, R. and Schmid-Hempel, P. (1998) ‘Colony performance and immunocompetence of a social insect, *Bombus terrestris*, in poor and variable environments’, Functional Ecology, 12(1), 22–30.

Schmid-Hempel, R. and Schmid-Hempel, P. (2000) ‘Female mating frequencies in *Bombus* spp. from Central Europe’, Insectes Sociaux, 47, 36 – 41.

Schmid-Hempel, R., Eckhardt, M., Goulson, D., Heinzmann, D., Lange, C., Plischuk, S., Escudero, L.R., Salathé, R., Scriven, J.J., and Schmid-Hempel, P. (2014) ‘The invasion of southern South America by imported bumblebees and associated parasites’, Journal of Animal Ecology, 83(4), 823–837.

Schmidt, C.J., Abasht, B., and McCarthy, F.M. (2023) ‘Proteomic insight into human directed evolution of the domesticated chicken *Gallus gallus*.’, bioRxiv, 2023–02.

Schmidt, E., Mykytczuk, N., and Schulte-Hostedde, A.I. (2019) ‘Effects of the captive and wild environment on diversity of the gut microbiome of deer mice (*Peromyscus maniculatus*)’, The ISME Journal, 13(5), 1293–1305.

Schouten, C., Lloyd, D., Ansharyani, I., Salminah, M., Somerville, D., and Stimpson, K. (2020) ‘The role of honey hunting in supporting subsistence livelihoods in Sumbawa, Indonesia’, Geographical Research, 58(1), 64–76.

Schulz, D.J., Sullivan, J.P., and Robinson, G.E. (2002) ‘Juvenile hormone and octopamine in the regulation of division of labor in honey bee colonies’, Hormones and Behavior, 42(2), 222–231.

Schwarz, E., Prabakaran, S., Whitfield, P., Major, H., Leweke, F., Koethe, D., McKenna, P., and Bahn, S. (2008) ‘High throughput lipidomic profiling of schizophrenia and bipolar disorder brain tissue reveals alterations of free fatty acids, phosphatidylcholines, and ceramides’, Journal of Proteome Research, 7(10), 4266–4277.

Seabra, S.G., Silva, S.E., Nunes, V.L., Sousa, V.C., Martins, J., Marabuto, E., Rodrigues, A.S.B., Pina-Martins, F., Laurentino, T.G., Rebelo, M.T., Figueiredo, E., and Paulo, O.S. (2019) ‘Genomic signatures of introgression between commercial and native bumblebees, *Bombus terrestris*, in western Iberian Peninsula-Implications for conservation and trade regulation’, Evolutionary Applications 12(4), 679 - 691.

Seki, T., Hayashi, N., and Nishimoto, T. (1996) ‘RCC1 in the Ran pathway’, The Journal of Biochemistry, 120(2), 207–214.

Sellin, J., Schulze, H., Paradis, M., Gosejacob, D., Papan, C., Shevchenko, A., Psathaki, O.E., Paululat, A., Thielisch, M., and Sandhoff, K. (2017) ‘Characterization of *Drosophila* Saposin-related mutants as a model for lysosomal sphingolipid storage diseases’, Disease Models & Mechanisms, 10(6), 737–750.

Semmens, T. (1993) ‘*Bombus terrestris* (L.) (Hymenoptera : Apoidae) now established in Tasmania’, Journal of the Australian Entomological Society, 32(4), 346.

Shakeel, M., Xu, X., De Mandal, S., and Jin, F. (2019) ‘Role of serine protease inhibitors in insect-host-pathogen interactions’, Archives of Insect Biochemistry and Physiology, 102(3), e21556.

Shandala, T. and Brooks, D.A. (2012) ‘Innate immunity and exocytosis of antimicrobial peptides’, Communicative & Integrative Biology, 5(2), 214–216.

Shaw, D. D. (1972) ‘Genetic and environmental components of chiasma control: II. The response to selection in Schistocerca’, Chromosoma, 37(3), 297-308.

Shemesh, Y., Eban-Rothschild, A., Cohen, M., and Bloch, G. (2010) ‘Molecular dynamics and social regulation of context-dependent plasticity in the circadian clockwork of the honey bee’, Journal of Neuroscience, 30(37), 12517–12525.

Sherman, P.W., Lacey, E.A., Reeve, H.K., and Keller, L. (1995) ‘The eusociality continuum’, Behavioral Ecology, 6(1), 102–108.

Shi, T., Burton, S., Wang, Y., Xu, S., Zhang, W., and Yu, L. (2018) ‘Metabolomic analysis of honey bee, *Apis mellifera* L. response to thiacloprid’, Pesticide Biochemistry and Physiology, 152, 17–23.

Shiga, S. and Numata, H. (2000) ‘The role of neurosecretory neurons in the pars intercerebralis and pars lateralis in reproductive diapause of the blowfly, *Protophormia terraenovae*’, Naturwissenschaften, 87(3), 125–128.

Shpigler, H., Amsalem, E., Huang, Z.Y., Cohen, M., Siegel, A.J., Hefetz, A., and Bloch, G. (2014) ‘Gonadotropic and physiological functions of juvenile hormone in bumblebee (*Bombus terrestris*) workers’, PLoS ONE, 9(6), e100650.

Shpigler, H., Tamarkin, M., Gruber, Y., Poleg, M., Siegel, A.J., and Bloch, G. (2013) ‘Social influences on body size and developmental time in the bumblebee *Bombus terrestris*’, Behavioral Ecology and Sociobiology, 67(10), 1601–1612.

Shuai, K. and Liu, B. (2003) ‘Regulation of JAK–STAT signalling in the immune system’, Nature Reviews Immunology, 3(11), 900–911.

Shultz, A.J. and Sackton, T.B. (2019) ‘Immune genes are hotspots of shared positive selection across birds and mammals’, Elife, 8, e41815.

Shykoff, J.A. and Schmid-Hempel, P. (1991) ‘Parasites and the advantage of genetic variability within social insect colonies’, Proceedings of the Royal Society of London. Series B: Biological Sciences, 243(1306), 55–58.

Sidak-Loftis Lindsay C., Rosche Kristin L., Pence Natasha, Ujczo Jessica K., Hurtado Joanna, Fisk Elis A., Goodman Alan G., Noh Susan M., Peters John W., and Shaw Dana K. (2022) 'The Unfolded-Protein Response Triggers the Arthropod Immune Deficiency Pathway', mBio, 13(4), e00703-22.

Singhania, A. and Grueber, W.B. (2014) 'Development of the embryonic and larval peripheral nervous system of *Drosophila*', Wiley Interdisciplinary Reviews: Developmental Biology, 3(3), 193–210.

Siva-Jothy, M.T., Moret, Y., and Rolff, J. (2005) 'Insect immunity: an evolutionary ecology perspective', Advances in Insect Physiology, 32, 1–48.

Siviter, H. and Muth, F. (2022) 'Exposure to the novel insecticide flupyradifurone impairs bumblebee feeding motivation, learning, and memory retention', Environmental Pollution, 307, 119575.

Siviter, H., Koricheva, J., Brown, M.J., and Leadbeater, E. (2018) 'Quantifying the impact of pesticides on learning and memory in bees', Journal of Applied Ecology, 55(6), 2812–2821.

Skoulakis, E.M., Kalderon, D., and Davis, R.L. (1993) 'Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory', Neuron, 11(2), 197–208.

Sladen, F.W.L. (1912) The Humble-Bee Its Life-History and How to Domesticate It with Descriptions of All the British Species of Bombus and Psithyrus, MacMillan & Company Ltd.: London, UK.

Sleeman, D.P. (2014) 'Ireland, an unexpected focus for genetic biodiversity and the ecological implications', Irish Naturalists' Journal, 33, 3–7.

Smith, C.C., Srygley, R.B., Healy, F., Swaminath, K., and Mueller, U.G. (2017) 'Spatial structure of the mormon cricket gut microbiome and its predicted contribution to nutrition and immune function', Frontiers in Microbiology, 8, 801.

Smith, C.C.R., Tittes, S., Mendieta, J.P., Collier-zans, E., Rowe, H.C., Rieseberg, L.H., and Kane, N.C. (2018) 'Genetics of alternative splicing evolution during sunflower domestication', Proceedings of the National Academy of Sciences, 115(26), 6768–6773.

Smith, D.B., Arce, A.N., Ramos Rodrigues, A., Bischoff, P.H., Burris, D., Ahmed, F., and Gill, R.J. (2020) 'Insecticide exposure during brood or early-adult development reduces brain growth and impairs adult learning in bumblebees', Proceedings of the Royal Society B, 287(1922), 20192442.

Smith, E.A., Anderson, K.E., Corby-Harris, V., McFrederick, Q.S., Parish, A.J., Rice, D.W., and Newton, I.L.G. (2021) 'Reclassification of seven honey bee symbiont strains as *Bombella apis*', bioRxiv, 2020.05.06.081802.

Smith, K.P. and Goodman, R.M. (1999) 'Host variation for interactions with beneficial plant-associated microbes', Annual Review of Phytopathology, 37, 473.

Sommer, S. (2005) 'The importance of immune gene variability (MHC) in evolutionary ecology and conservation', Frontiers in Zoology, 2(1), 1–18.

Spaethe, J. and Weidenmüller, A. (2002) 'Size variation and foraging rate in bumblebees (*Bombus terrestris*)', Insectes Sociaux, 49(2), 142–146.

Spor, A., Koren, O., and Ley, R. (2011) 'Unravelling the effects of the environment and host genotype on the gut microbiome', Nature Reviews Microbiology, 9(4), 279–290.

Sreeman, S.M., Vijayaraghavareddy, P., Sreevathsa, R., Rajendrareddy, S., Arakesh, S., Bharti, P., Dharmappa, P., and Soolanayakanahally, R. (2018) 'Introgression of physiological traits for a comprehensive improvement of drought adaptation in crop plants', Frontiers in chemistry, 6, 92.

St. Leger, R.J. (1995) 'The role of cuticle-degrading proteases in fungal pathogenesis of insects', Canadian Journal of Botany, 73(S1), 1119–1125.

Stamm, S., Ben-Ari, S., Rafalska, I., Tang, Y., Zhang, Z., Toiber, D., Thanaraj, T.A., and Soreq, H. (2005) 'Function of alternative splicing', Gene, 344, 1–20.

Stanković, D., Claudius, A.-K., Schertel, T., Bresser, T., and Uhlirova, M. (2020) 'A *Drosophila* model to study retinitis pigmentosa pathology associated with mutations in the core splicing factor Prp8', Disease Models & Mechanisms, 13(6), dmm043174.

Stanley, D. (2006) 'Prostaglandins and other eicosanoids in insects: biological significance', Annual Reviews of Entomology, 51, 25–44.

Stanley, D.A. and Raine, N.E. (2016) ‘Chronic exposure to a neonicotinoid pesticide alters the interactions between bumblebees and wild plants’, Functional Ecology, 30(7), 1132–1139.

Stanley, D.A., Smith, K.E., and Raine, N.E. (2015) ‘Bumblebee learning and memory is impaired by chronic exposure to a neonicotinoid pesticide’, Scientific Reports, 5(1), 1–10.

Stapley, J., Feulner, P. G., Johnston, S. E., Santure, A. W., & Smadja, C. M. (2017) ‘Recombination: the good, the bad and the variable’, Philosophical Transactions of the Royal Society B: Biological Sciences, 372(1736), 20170279.

Steiner, H. (2004) ‘Peptidoglycan recognition proteins: on and off switches for innate immunity’, Immunological Reviews, 198(1), 83–96.

Stelzer, R.J., Chittka, L., Carlton, M., and Ings, T.C. (2010) ‘Winter Active Bumblebees (*Bombus terrestris*) Achieve High Foraging Rates in Urban Britain’, PLoS ONE, 5(3), p.e9559.

Stiburek, L. and Zeman, J. (2010) ‘Assembly factors and ATP-dependent proteases in cytochrome c oxidase biogenesis’, Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1797(6–7), 1149–1158.

Stokstad, E. (2018) ‘New global study reveals the ‘staggering’ loss of forests caused by industrial agriculture’, Science, 10.

Stolle, E., Wilfert, L., Schmid-Hempel, R., Schmid-Hempel, P., Kube, M., Reinhardt, R., and Moritz, R.F. (2011) ‘A second generation genetic map of the bumblebee *Bombus terrestris* (Linnaeus, 1758) reveals slow genome and chromosome evolution in the Apidae’, BMC Genomics, 12(1), 1–17.

Storey, K.B. and Storey, J.M. (2012) ‘Insect cold hardiness: metabolic, gene, and protein adaptation’, Canadian Journal of Zoology, 90(4), 456–475.

Stout, J. C., Kells, A. R., and Goulson, D. (2002) Pollination of the invasive exotic shrub *Lupinus arboreus* (Fabaceae) by introduced bees in Tasmania. Biological Conservation, 106(3), 425-434.

Stout, J.C. and Goulson, D. (2000) ‘Bumble bees in Tasmania: their distribution and potential impact on Australian flora and fauna’, Bee World, 81(2), 80-86.

Stout, J.C., Murphy, J.T., and Kavanagh, S. (2019) Assessing Market and Non-Market Values of Pollination Services in Ireland (Pollival), Environmental Protection Agency Research Report, Johnstown Castle, Co. Wexford, Ireland.

Strand, M.R. (2008) 'The insect cellular immune response', Insect Science, 15(1), 1–14.

Stratmann, J.W. and Gusmaroli, G. (2012) 'Many jobs for one good cop – The COP9 signalosome guards development and defense', Plant Science, 185–186, 50–64.

Su, Q., Wang, Q., Mu, X., Chen, H., Meng, Y., Zhang, X., Zheng, L., Hu, X., Zhai, Y., and Zheng, H. (2021a) 'Strain-level analysis reveals the vertical microbial transmission during the life cycle of bumblebee', Microbiome, 9(1), 1–14.

Suh, J. (2008) 'Characterization of ebony, a circadian output gene in *Drosophila melanogaster*', doctoral dissertation, Tufts University.

Sun, C., Huang, J., Wang, Y., Zhao, X., Su, L., Thomas, G.W.C., Zhao, M., Zhang, X., Jungreis, I., Kellis, M., Vicario, S., Sharakhov, I.V., Bondarenko, S.M., Hasselmann, M., Kim, C.N., Paten, B., Penso-Dolfin, L., Wang, L., Chang, Y., Gao, Q., *et al.* (2020) 'Genus-wide characterization of bumblebee genomes provides insights into their evolution and variation in ecological and behavioral traits', Molecular Biology and Evolution, 38(2), 486-501.

Supek, F., Bošnjak, M., Škunca, N., and Šmuc, T. (2011) 'REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms', PLoS ONE, 6(7), e21800.

Svärdson, G. (1970) 'Significance of introgression in coregonid evolution', Biology of Coregonid Fishes, 33–59.

Svensson, B., Lagerlöf, J., and Svensson, B.G. (2000) 'Habitat preferences of nest-seeking bumble bees (Hymenoptera: Apidae) in an agricultural landscape', Agriculture, Ecosystems & Environment, 77(3), 247–255.

Swindell, W.R., Huebner, M., and Weber, A.P. (2007) 'Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways', BMC Genomics, 8(1), 125.

Szabo, N.D., Colla, S.R., Wagner, D.L., Gall, L.F., and Kerr, J.T. (2012) 'Do pathogen spillover, pesticide use, or habitat loss explain recent North American bumblebee declines?', Conservation Letters, 5(3), 232–239.

Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N.T., Morris, J.H., and Bork, P. (2019) 'STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets', Nucleic Acids Research, 47(D1), D607–D613.

Szpiech, Z.A. (2021) 'selscan 2.0: scanning for sweeps in unphased data', bioRxiv.

Szpiech, Z.A., Novak, T.E., Bailey, N.P., and Stevison, L.S. (2021) 'Application of a novel haplotype-based scan for local adaptation to study high-altitude adaptation in rhesus macaques', Evolution Letters, 5(4), 408–421.

Tackenberg, M.C., Giannoni-Guzmán, M.A., Sanchez-Perez, E., Doll, C.A., Agosto-Rivera, J.L., Broadie, K., Moore, D., and McMahon, D.G. (2020) 'Neonicotinoids disrupt circadian rhythms and sleep in honey bees', Scientific Reports, 10(1), 17929.

Tadkowski, T.M. and McCann, F.V. (1980) 'Embryonic development of an insect myocardium', Experientia, 36(1), 105–106.

Tan, K. C., Ipcho, S. V., Trengove, R. D., Oliver, R. P., & Solomon, P. S. (2009) 'Assessing the impact of transcriptomics, proteomics and metabolomics on fungal phytopathology', Molecular Plant Pathology, 10(5), 703-715.

Tan, K., Wang, C., Dong, S., Li, X., and Nieh, J.C. (2017) 'The pesticide flupyradifurone impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults', Scientific Reports, 7(1), 1–9.

Tan, W., Talla, V., Mongue, A.J., de Roode, J.C., Gerardo, N.M., and Walters, J.R. (2021) 'Population genomics reveals variable patterns of immune gene evolution in monarch butterflies (*Danaus plexippus*)', Molecular Ecology, 30(18), 4381–4391.

Tarpy, D. R., Mattila, H. R., & Newton, I. L. (2015) 'Development of the honey bee gut microbiome throughout the queen-rearing process', Applied and Environmental Microbiology, 81(9), 3182-3191.

Tauber, J.P., Nguyen, V., Lopez, D., and Evans, J.D. (2019) ‘Effects of a Resident Yeast from the Honeybee Gut on Immunity, Microbiota, and Nosema Disease’, Insects, 10(9).

Teacher, A.G.F., Garner, T.W.J., and Nichols, R.A. (2009) ‘European phylogeography of the common frog (*Rana temporaria*): routes of postglacial colonization into the British Isles, and evidence for an Irish glacial refugium’, Heredity, 102(5), 490–496.

Teagasc (2012) ‘Managing bumblebee imports to maintain pollinator diversity’, available: https://www.teagasc.ie/media/website/publications/2010/Bumblebee-imports-5633.pdf [accessed 4 Nov 2022].

Teagasc, ‘Survey of commercial pollinator use by growers conducted in 2007 and 2009’ [unpublished], Teagasc.

Teletchea, F. and Fontaine, P. (2014) ‘Levels of domestication in fish: implications for the sustainable future of aquaculture’, Fish and fisheries, 15(2), 181–195.

Tellier, A., Moreno-Gámez, S., and Stephan, W. (2014) ‘Speed of adaptation and genomic footprints of host–parasite coevolution under arms race and trench warfare dynamics’, Evolution, 68(8), 2211–2224.

Tepedino, V.J. (1979) ‘The importance of bees and other insect pollinators in maintaining floral species composition’, Great Basin Naturalist Memoirs, 139–150.

Terry, L.J., Shows, E.B., and Wente, S.R. (2007) ‘Crossing the Nuclear Envelope: Hierarchical Regulation of Nucleocytoplasmic Transport’, Science, 318(5855), 1412–1416.

The UniProt Consortium (2021) ‘UniProt: the universal protein knowledgebase in 2021’, Nucleic acids research, 49(D1), D480–D489.

Thompson, H.M. and Hunt, L.V. (1999) ‘Extrapolating from honeybees to bumblebees in pesticide risk assessment’, Ecotoxicology, 8(3), 147–166.

Thomson, J.D. (2010) ‘Flowering phenology, fruiting success and progressive deterioration of pollination in an early-flowering geophyte’, Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1555), 3187–3199.

Thorpe, R.S., Surget-Groba, Y., and Johansson, H. (2010) 'Genetic tests for ecological and allopatric speciation in anoles on an island archipelago', PLoS Genetics, 6(4), e1000929.

Tian, L. and Hines, H.M. (2018) 'Morphological characterization and staging of bumble bee pupae', PeerJ, 6, e6089.

Tigano, A. and Friesen, V.L. (2016) 'Genomics of local adaptation with gene flow', Molecular Ecology, 25(10), 2144–2164.

Tilman, D. (1999) 'Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices', Proceedings of the national Academy of Sciences, 96(11), 5995–6000.

Tilman, D., Fargione, J., Wolff, B., D'antonio, C., Dobson, A., Howarth, R., Schindler, D., Schlesinger, W.H., Simberloff, D., and Swackhamer, D. (2001) 'Forecasting agriculturally driven global environmental change', Science, 292(5515), 281–284.

Tison, L., Duer, A., Púčiková, V., Greggers, U., and Menzel, R. (2020) 'Detrimental effects of clothianidin on foraging and dance communication in honey bees', PLoS ONE, 15(10), e0241134.

Toki, W., Takahashi, Y., and Togashi, K. (2013) 'Fungal Garden Making inside Bamboos by a Non-Social Fungus-Growing Beetle', PLoS ONE, 8(11), e79515.

Toki, W., Tanahashi, M., Togashi, K., and Fukatsu, T. (2012) 'Fungal farming in a non-social beetle', PLoS ONE, 7(7), e41893.

Tomizawa, M. (2013) 'Chemical biology of the nicotinic insecticide receptor', in Advances in Insect Physiology, Elsevier, 63–99.

Tomizawa, M. and Casida, J.E. (2001) 'Structure and diversity of insect nicotinic acetylcholine receptors', Pest Management Science, 57(10), 914–922.

Tomizawa, M. and Casida, J.E. (2005) 'Neonicotinoid insecticide toxicology: mechanisms of selective action', Annual Review of Pharmacology and Toxicology, 45, 247.

Torretta, J.P., Medan, D., and Abrahamovich, A.H. (2006) ‘First record of the invasive bumblebee *Bombus terrestris* (L.)(Hymenoptera, Apidae) in Argentina’, Transactions of the American Entomological Society, 132(3), 285–289.

Torriglia, A., Martin, E., and Jaadane, I. (2017) ‘The hidden side of SERPINB1/Leukocyte Elastase Inhibitor’, SI: Cartilage Biology and Pathology, 62, 178–186.

Trpis, M. and Hausermann, W. (1975) ‘Demonstration of differential domesticity of Aedes aegypti (L.)(Diptera, Culicidae) in Africa by mark-release-recapture’, Bulletin of Entomological Research, 65(2), 199–208.

Tsakas, S. and Marmaras, V. (2010) ‘Insect immunity and its signalling: an overview’, Invertebrate Survival Journal, 7(2), 228–238.

Turner, W.R., Brandon, K., Brooks, T.M., Costanza, R., Da Fonseca, G.A., and Portela, R. (2007) ‘Global conservation of biodiversity and ecosystem services’, BioScience, 57(10), 868–873.

Tyler, E.R., Adams, S., and Mallon, E.B. (2006) ‘An immune response in the bumblebee, *Bombus terrestris* leads to increased food consumption’, BMC Physiology, 6(1), 6.

Ugrankar, R., Tran, S., Bowerman, J., Kovalenko, A., Paul, B., and Henne, W.M. (2022) ‘The fat body cortical actin network regulates *Drosophila* inter-organ nutrient trafficking and adipocyte cell size’, bioRxiv.

Unckless, R.L. and Lazzaro, B.P. (2016) ‘The potential for adaptive maintenance of diversity in insect antimicrobial peptides’, Philosophical Transactions of the Royal Society B: Biological Sciences, 371(1695), 20150291.

Uneme, H. (2011) ‘Chemistry of clothianidin and related compounds’, Journal of Agricultural and Food Chemistry, 59(7), 2932–2937.

UniProt (2023) ‘UniProt: the Universal Protein knowledgebase in 2023’, Nucleic Acids Research, 51(D1), D523–D531.

Valanne, S., Wang, J.-H., and Rämet, M. (2011) ‘The *Drosophila* toll signaling pathway’, The Journal of Immunology, 186(2), 649–656.

Valcu, C.-M. and Kempenaers, B. (2015) ‘Proteomics in behavioral ecology’, Behavioral Ecology, 26(1), 1–15.

Van Alphen, J. J., & Fernhout, B. J. (2020) ‘Natural selection, selective breeding, and the evolution of resistance of honeybees (*Apis mellifera*) against *Varroa*’, Zoological Letters, 6(1), 1-20.

Van Arnam, E.B., Currie, C.R., and Clardy, J. (2018) ‘Defense contracts: molecular protection in insect-microbe symbioses’, Chemical Society Reviews, 47(5), 1638–1651.

Van den Eijnde, J., De Ruijter, A., and Van der Steen, J. (1990) ‘Method for rearing *Bombus terrestris* continuously and the production of bumblebee colonies for pollination purposes.’, Presented at the VI International Symposium on Pollination 288, 154–158.

van den Ouweland, J.M. and Kema, I.P. (2012) ‘The role of liquid chromatography–tandem mass spectrometry in the clinical laboratory’, Journal of Chromatography B, 883, 18–32.

Van Heemert, C., De Ruijter, A., Van den Eijnde, J., and Van der Steen, J. (1990) ‘Year-round production of bumble bee colonies for crop pollination’, Bee World, 71(2), 54–56.

Van Ravestijn, W. and Van der Sande, J. (1990) ‘Use of bumblebees for the pollination of glasshouse tomatoes’, Presented at the VI International Symposium on Pollination 288, 204–212.

Van Vaerenbergh, M., Debyser, G., Smagghe, G., Devreese, B., and de Graaf, D.C. (2015) ‘Unraveling the venom proteome of the bumblebee (*Bombus terrestris*) by integrating a combinatorial peptide ligand library approach with FT-ICR MS’, Toxicon, 102, 81–88.

Vance, D.E. and Vance, J.E. (2002) Biochemistry of Lipids, Lipoproteins and Membranes, Elsevier: Amsterdam, The Netherlands.

Vasimuddin, M., Misra, S., Li, H., and Aluru, S. (2019) ‘Efficient architecture-aware acceleration of BWA-MEM for multicore systems’, Presented at the 2019 IEEE

International Parallel and Distributed Processing Symposium (IPDPS), IEEE, 314–324.

Vatsiou, A.I., Bazin, E., and Gaggiotti, O.E. (2016) ‘Detection of selective sweeps in structured populations: a comparison of recent methods’, Molecular Ecology, 25(1), 89–103.

Vehovszky, Á., Farkas, A., Ács, A., Stoliar, O., Székács, A., Mörtl, M., and Győri, J. (2015) ‘Neonicotinoid insecticides inhibit cholinergic neurotransmission in a molluscan (*Lymnaea stagnalis*) nervous system’, Aquatic Toxicology, 167, 172–179.

Velthuis, H.H. and van Doorn, A. (2004) ‘The breeding, commercialization and economic value of bumblebees’, Solitary bees: Conservation, Rearing and Management in Pollination. Fortaleza: CE, Brazil, 135–149.

Velthuis, H.H. and Van Doorn, A. (2006) ‘A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination’, Apidologie, 37(4), 421–451.

Vierstraete, E., Verleyen, P., Baggerman, G., D’Hertog, W., Van den Bergh, G., Arckens, L., De Loof, A., and Schoofs, L. (2004) ‘A proteomic approach for the analysis of instantly released wound and immune proteins in *Drosophila melanogaster* hemolymph’, Proceedings of the National Academy of Sciences, 101(2), 470–475.

Vogel, G. (2017) ‘Where have all the insects gone?’, Science, 356(6338), 576-579.

Wada, J. and Kanwar, Y.S. (1998) ‘Characterization of mammalian translocase of inner mitochondrial membrane (Tim44) isolated from diabetic newborn mouse kidney’, Proceedings of the National Academy of Sciences, 95(1), 144–149.

Wagner, D.L. (2020) ‘Insect declines in the Anthropocene’, Annual Review of Entomology, 65, 457–480.

Walker, T. (2020) Pollination: The Enduring Relationship between Plant and Pollinator, Princeton University Press: New Jersey, USA.

Wall, B., Cahalane, A., and Derham, J. (2020) ‘Ireland’s Environment–An Integrated Assessment 2020’, Environmental Protection Agency: Wexford, Ireland, 460.

Walter, J. and Ley, R. (2011) ‘The human gut microbiome: ecology and recent evolutionary changes’, Annual Reviews of Microbiology, 65, 411-429.

Walter, P. and Ron, D. (2011) ‘The unfolded protein response: from stress pathway to homeostatic regulation’, Science, 334(6059), 1081–1086.

Walther, T.C. and Mann, M. (2010) ‘Mass spectrometry–based proteomics in cell biology’, Journal of Cell Biology, 190(4), 491–500.

Wang Liuhao, Wu Jie, Li Kai, Sadd Ben M., Guo Yulong, Zhuang Daohua, Zhang Zhengyi, Chen Yanping, Evans Jay D., Guo Jun, Zhang Zhigang, and Li Jilian (2019) ‘Dynamic Changes of Gut Microbial Communities of Bumble Bee Queens through Important Life Stages’, mSystems, 4(6), e00631-19.

Wang Yijin, Wang Wenshi, Xu Lei, Zhou Xinying, Shokrollahi Ehsan, Felczak Krzysztof, van der Laan Luc J. W., Pankiewicz Krzysztof W., Sprengers Dave, Raat Nicolaas J. H., Metselaar Herold J., Peppelenbosch Maikel P., and Pan Qiuwei (2016) ‘Cross Talk between Nucleotide Synthesis Pathways with Cellular Immunity in Constraining Hepatitis E Virus Replication’, Antimicrobial Agents and Chemotherapy, 60(5), 2834–2848.

Wang, Y., Allen, S.L., Reddiex, A.J., and Chenoweth, S. (2022) ‘Direct and indirect impacts of positive selection on genomic variation in *Drosophila* serrata’, bioRxiv.

Wang, Y., Zuber, R., Oehl, K., Norum, M., and Moussian, B. (2015) ‘Report on *Drosophila melanogaster* larvae without functional tracheae’, Journal of Zoology, 296(2), 139–145.

Wang, Z., Gerstein, M., & Snyder, M. (2009) ‘RNA-Seq: a revolutionary tool for transcriptomics’, Nature Reviews Genetics, 10(1), 57-63.

Warschefsky, E., Penmetsa, R.V., Cook, D.R., and Von Wettberg, E.J. (2014) ‘Back to the wilds: tapping evolutionary adaptations for resilient crops through systematic hybridization with crop wild relatives’, American Journal of Botany, 101(10), 1791–1800.

Weaver, D.B., Cantarel, B.L., Elsik, C.G., Boncristiani, D.L., and Evans, J.D. (2021) ‘Multi-tiered analyses of honey bees that resist or succumb to parasitic mites and viruses’, BMC Genomics, 22(1), 1–15.

Weber, E. (2013) '*Apis mellifera*: the domestication and spread of European honey bees for agriculture in North America'.

Weinberg, D. and Plowright, C.M.S. (2006) 'Pollen collection by bumblebees (*Bombus impatiens*): the effects of resource manipulation, foraging experience and colony size', Journal of Apicultural Research, 45(2), 22–27.

Weinhold, L., Ahmad, S., and Pardini, R. (1990) 'Insect glutathione-S-transferase: a predictor of allelochemical and oxidative stress', Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 95(2), 355–363.

Whitehorn, P.R., O'connor, S., Wackers, F.L., and Goulson, D. (2012) 'Neonicotinoid pesticide reduces bumble bee colony growth and queen production', Science, 336(6079), 351–352.

Whitney, K.D., Randell, R.A., and Rieseberg, L.H. (2006) 'Adaptive introgression of herbivore resistance traits in the weedy sunflower *Helianthus annuus*', The American Naturalist, 167(6), 794–807.

Whittaker, R.J. and Fernández-Palacios, J.M. (2007) Island Biogeography: Ecology, Evolution, and Conservation, Oxford University Press: Oxford, UK.

Whittington, R., Winston, M.L., Tucker, C., and Parachnowitsch, A.L. (2004) 'Plant-species identity of pollen collected by bumblebees placed in greenhouses for tomato pollination', Canadian Journal of Plant Science, 84(2), 599–602.

Widmer, A., Schmid-Hempel, P., Estoup, A., and Scholl, A. (1998) 'Population genetic structure and colonization history of *Bombus terrestris* s.l. (Hymenoptera : Apidae) from the Canary Islands and Madeira', Heredity, 81(5), 563-572.

Wilcock, H.R., Hildrew, A.G., and Nichols, R.A. (2001) 'Genetic differentiation of a European caddisfly: past and present gene flow among fragmented larval habitats', Molecular Ecology, 10(7), 1821–1834.

Wilfert, L., Gadau, J., and Schmid-Hempel, P. (2006) 'A core linkage map of the bumblebee *Bombus terrestris*', Genome, 49(10), 1215–1226.

Wilkins, A.S., Wrangham, R.W., and Fitch, W.T. (2014) 'The "domestication syndrome" in mammals: a unified explanation based on neural crest cell behavior and genetics', Genetics, 197(3), 795–808.

Wilkins, M.R., Pasquali, C., Appel, R.D., Ou, K., Golaz, O., Sanchez, J.-C., Yan, J.X., Gooley, A.A., Hughes, G., and Humphery-Smith, I. (1996) 'From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis', Biotechnology, 14(1), 61–65.

Williams, N.M., Mola, J.M., Stuligross, C., Harrison, T., Page, M.L., Brennan, R.M., Rosenberger, N.M., and Rundlof, M. (2019) 'Fantastic bees and where to find them: locating the cryptic overwintering queens of a western bumble bee', Ecosphere, 10(11), e02949.

Williams, P.H. (1998) 'An annotated checklist of bumble bees with an analysis of patterns of description (Hymenoptera: Apidae, Bombini)', Bulletin-Natural History Museum Entomology Series, 67, 79–152.

Williams, P.H., Araújo, M.B., and Rasmont, P. (2007) 'Can vulnerability among British bumblebee (*Bombus*) species be explained by niche position and breadth?', Biological Conservation, 138(3–4), 493–505.

Williamson, S.M. and Wright, G.A. (2013) 'Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees', Journal of Experimental Biology, 216(10), 1799–1807.

Wilson, E. O., & Brown Jr, W. L. (1953) 'The subspecies concept and its taxonomic application', Systematic Zoology, 2(3), 97-111.

Wilson, G.F. (1929) 'Pollination of hardy fruits: insect visitors to fruit blossoms', Annals of Applied Biology, 16(4), 602–629.

Wilson, K., Cotter, S.C., Reeson, A.F., and Pell, J.K. (2001) 'Melanism and disease resistance in insects', Ecology Letters, 4(6), 637–649.

Winbush, A., & Singh, N. D. (2021) 'Genomics of recombination rate variation in temperature-evolved *Drosophila melanogaster* populations', Genome Biology and Evolution, 13(1), evaa252.

Winfree, R., Williams, N.M., Dushoff, J., and Kremen, C. (2007) 'Native bees provide insurance against ongoing honey bee losses', Ecology Letters, 10(11), 1105–1113.

Winfree, R., Williams, N.M., Gaines, H., Ascher, J.S., and Kremen, C. (2008) 'Wild bee pollinators provide the majority of crop visitation across land-use gradients in New Jersey and Pennsylvania, USA', Journal of Applied Ecology, 45(3), 793–802.

Winter, K., Adams, L., Thorp, R., Inouye, D., Day, L., Ascher, J., and Buchmann, S. (2006) 'Importation of non-native bumble bees into North America: potential consequences of using *Bombus terrestris* and other non-native bumble bees for greenhouse crop pollination in Canada, Mexico, and the United States'. NAPPC: San Francisco, USA.

Withers, G.S., Fahrbach, S.E., and Robinson, G.E. (1993) 'Selective neuroanatomical plasticity and division of labour in the honeybee', Nature, 364(6434), 238–240.

WMO (2018) WMO Statement on the State of the Global Climate in 2017, World Meteorological Organisation (WMO): Geneva, Switzerland.

Wojda, I., Kowalski, P., and Jakubowicz, T. (2004) 'JNK MAP kinase is involved in the humoral immune response of the greater wax moth larvae *Galleria mellonella*', Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America, 56(4), 143–154.

Wolf, D.H. and Menssen, R. (2018) 'Mechanisms of cell regulation – proteolysis, the big surprise', FEBS Letters, 592(15), 2515–2524.

Wright, S. (1942) 'Statistical genetics and evolution'.

Wright, T.R. (1987) 'The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*', Advances in Genetics, 24, 127–222.

Wu, G., Fang, Y.-Z., Yang, S., Lupton, J.R., and Turner, N.D. (2004) 'Glutathione Metabolism and Its Implications for Health', The Journal of Nutrition, 134(3), 489–492.

Wu, Y., Zheng, Y., Chen, Y., Chen, G., Zheng, H., and Hu, F. (2020) 'Apis cerana gut microbiota contribute to host health though stimulating host immune system and strengthening host resistance to *Nosema ceranae*', Royal Society Open Science, 7(5), 192100.

Xiang, H., Liu, X., Li, M., Zhu, Y., Wang, L., Cui, Y., Liu, L., Fang, G., Qian, H., and Xu, A. (2018) 'The evolutionary road from wild moth to domestic silkworm', Nature Ecology & Evolution, 2(8), 1268–1279.

Xing, Z., Ma, W.K., and Tran, E.J. (2019) 'The DDX5/Dbp2 subfamily of DEAD-box RNA helicases', Wiley Interdisciplinary Reviews: RNA, 10(2), e1519.

Yang, S.-Y., Han, M.-J., Kang, L.-F., Li, Z.-W., Shen, Y.-H., and Zhang, Z. (2014) 'Demographic history and gene flow during silkworm domestication', BMC Evolutionary Biology, 14(1), 1–8.

Yang, Y.-X., Yu, N., Zhang, J.-H., Zhang, Y.-X., and Liu, Z.-W. (2018) 'Induction of P450 genes in *Nilaparvata lugens* and *Sogatella furcifera* by two neonicotinoid insecticides', Insect Science, 25(3), 401–408.

Yerushalmi, S., Bodenhaimer, S., and Bloch, G. (2006) 'Developmentally determined attenuation in circadian rhythms links chronobiology to social organization in bees', Journal of Experimental Biology, 209(6), 1044–1051.

Ying, S.-H. and Feng, M.-G. (2019) 'Insight into vital role of autophagy in sustaining biological control potential of fungal pathogens against pest insects and nematodes', Virulence, 10(1), 429–437.

Yourth, C., Brown, M., and Schmid-Hempel, P. (2008) 'Effects of natal and novel Crithidia bombi (Trypanosomatidae) infections on *Bombus terrestris* hosts', Insectes Sociaux, 55(1), 86–90.

Yourth, C.P. and Schmid-Hempel, P. (2006) 'Serial passage of the parasite Crithidia bombi within a colony of its host, *Bombus terrestris*, reduces success in unrelated hosts', Proceedings of the Royal Society B: Biological Sciences, 273(1587), 655–659.

Yu, H.-S., Shen, Y.-H., Yuan, G.-X., Hu, Y.-G., Xu, H.-E., Xiang, Z.-H., and Zhang, Z. (2011) 'Evidence of selection at melanin synthesis pathway loci during silkworm domestication', Molecular Biology and Evolution, 28(6), 1785–1799.

Yu, K., Feng, M., Yang, G., Sun, L., Qin, Z., Cao, J., Wen, J., Li, H., Zhou, Y., Chen, X., Peng, H., Yao, Y., Hu, Z., Guo, W., Sun, Q., Ni, Z., Adams, K., and Xin, M. (2020) 'Changes in Alternative Splicing in Response to Domestication and Polyploidization in Wheat', Plant Physiology, 184(4), 1955–1968.

Yun, J.-H., Jung, M.-J., Kim, P.S., and Bae, J.-W. (2018) 'Social status shapes the bacterial and fungal gut communities of the honey bee', Scientific reports, 8(1), 1–11.

Zaluski, R., Bittarello, A.C., Vieira, J.C.S., Braga, C.P., Padilha, P. de M., Fernandes, M. da S., Bovi, T. de S., and Orsi, R. de O. (2020) 'Modification of the head proteome of nurse honeybees (*Apis mellifera*) exposed to field-relevant doses of pesticides', Scientific Reports, 10(1), 2190.

Zattara, E.E. and Aizen, M.A. (2019) 'Global bee decline', BioRxiv.

Zeller, U. and Göttert, T. (2019) 'The relations between evolution and domestication reconsidered - Implications for systematics, ecology, and nature conservation', Global Ecology and Conservation, 20, e00756

Zeuner, F.E., Manning, F.J., and Morris, S.F. (1976) 'A monograph on fossil bees (Hymenoptera: Apoidea)', Bulletin of the British Museum (Natural History) Geology, 27, 149–268.

Zhai, Z., Boquete, J.-P., and Lemaitre, B. (2018) 'Cell-specific Imd-NF-κB responses enable simultaneous antibacterial immunity and intestinal epithelial cell shedding upon bacterial infection', Immunity, 48(5), 897–910.

Zhang, C., Du, S., Liu, R., and Dai, W. (2022) 'Overexpression of Multiple Cytochrome P450 Genes Conferring Clothianidin Resistance in Bradysia odoriphaga', Journal of Agricultural and Food Chemistry, 70(25), 7636–7643.

Zhang, K., Shen, L., Wang, X., Yang, H., Zhang, X., Pan, G., Li, C., Ji, H., Abbas, M.N., and Li, C. (2021) 'Scavenger receptor C regulates antimicrobial peptide expression by activating toll signaling in silkworm, *Bombyx mori*', International Journal of Biological Macromolecules, 191, 396–404.

Zhang, X., Ni, Y., Guo, K., Dong, Z., Chen, Y., Zhu, H., Xia, Q., and Zhao, P. (2021) 'The mutation of SPI51, a protease inhibitor of silkworm, resulted in the change of antifungal activity during domestication', International Journal of Biological Macromolecules, 178, 63–70.

Zhang, Z., Peng, H., Yang, D., Zhang, G., Zhang, J., and Ju, F. (2022) 'Polyvinyl chloride degradation by a bacterium isolated from the gut of insect larvae', Nature Communications, 13(1), 5360.

Zhao, X., Yeh, J.Z., Salgado, V.L., and Narahashi, T. (2004) 'Fipronil is a potent open channel blocker of glutamate-activated chloride channels in cockroach neurons', Journal of Pharmacology and Experimental Therapeutics, 310(1), 192–201.

Zheng Hao, Nishida Alex, Kwong Waldan K., Koch Hauke, Engel Philipp, Steele Margaret I., and Moran Nancy A. (2016) 'Metabolism of Toxic Sugars by Strains of the Bee Gut Symbiont *Gilliamella apicola'*, mBio, 7(6), e01326-16.

Zheng, H., Powell, J.E., Steele, M.I., Dietrich, C., and Moran, N.A. (2017) 'Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling', Proceedings of the National Academy of Sciences, 114(18), 4775–4780.

Zheng, L., Michelson, Y., Freger, V., Avraham, Z., Venken, K.J., Bellen, H.J., Justice, M.J., and Wides, R. (2011) '*Drosophila* Ten-m and filamin affect motor neuron growth cone guidance', PLoS ONE, 6(8), e22956.

Zheng, X. (2013) 'A tutorial for the R Package SNPRelate', University of Washington: Washington, USA.

Zheng, X., Gogarten, S., Laurie, C., and Weir, B. (2020) 'Parallel computing toolset for relatedness and principal component analysis of SNP data', GitHub.

Zhou, G.-W., Yang, X.-R., Zheng, F., Zhang, Z.-X., Zheng, B.-X., Zhu, Y.-G., and Xue, X.-M. (2020) 'Arsenic transformation mediated by gut microbiota affects the fecundity of Caenorhabditis elegans', Environmental Pollution, 260, 113991.

Zhu, W., Smith, J.W., and Huang, C.-M. (2009) 'Mass Spectrometry-Based Label-Free Quantitative Proteomics', Journal of Biomedicine and Biotechnology, 2010, 840518.

Ziegler, R. and Van Antwerpen, R. (2006) 'Lipid uptake by insect oocytes', Insect biochemistry and molecular biology, 36(4), 264–272.

Zou, Z., Lopez, D.L., Kanost, M.R., Evans, J.D., and Jiang, H. (2006) 'Comparative analysis of serine protease-related genes in the honey bee genome: possible involvement in embryonic development and innate immunity', Insect Molecular Biology, 15(5), 603–614.

Chapter 8

Appendix

8.0 Appendix

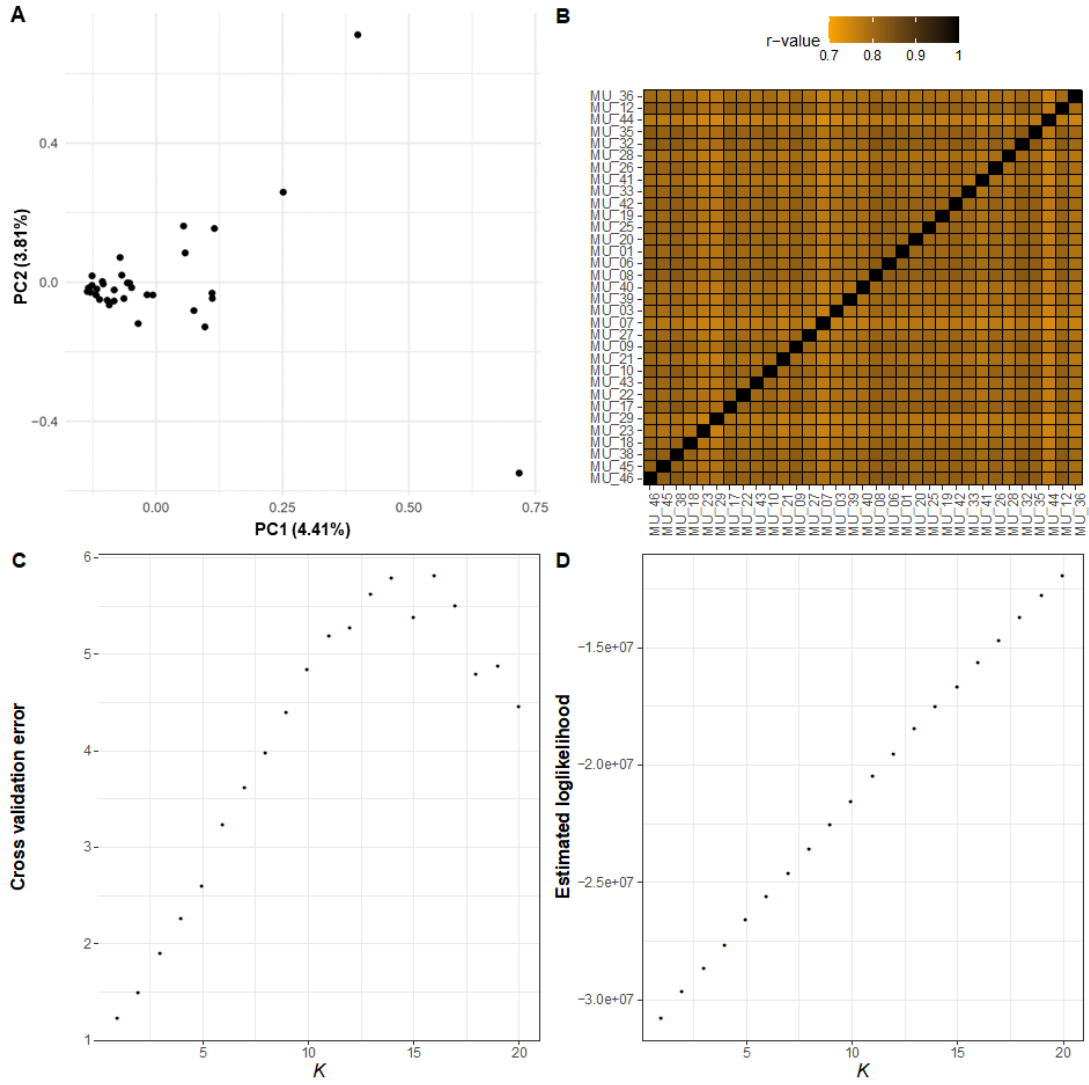


Figure A3.1 Population structure analysis of wild-caught *Bombus terrestris* bumblebees in Ireland. We examined population structure, relatedness and co-ancestry shared amongst collected males. (A) Scatterplot displaying the first two principal components (PC) for a principal component analysis based on a pruned SNP dataset. (B) As we lack genealogical information for sampled bumblebees, we performed an identity-by-state analysis to assess higher percentages of shared alleles amongst individuals which would suggest we sampled full siblings. Heatmap of the percentage of shared polymorphic sites (r-value) calculated between each sampled bumblebee is consistent with predictions based on sampling of non-related individuals. The names of each sample are provided on the x-axis. Scatterplots displaying the results of an ADMIXTURE-based analysis for (C) cross validation (CV) error rate and (D) estimated log-likelihood for each predicted *K* (1-20). The lowest calculated CV error rate was for *K* = 1 providing support for our sampled bumblebees originating from a single population.

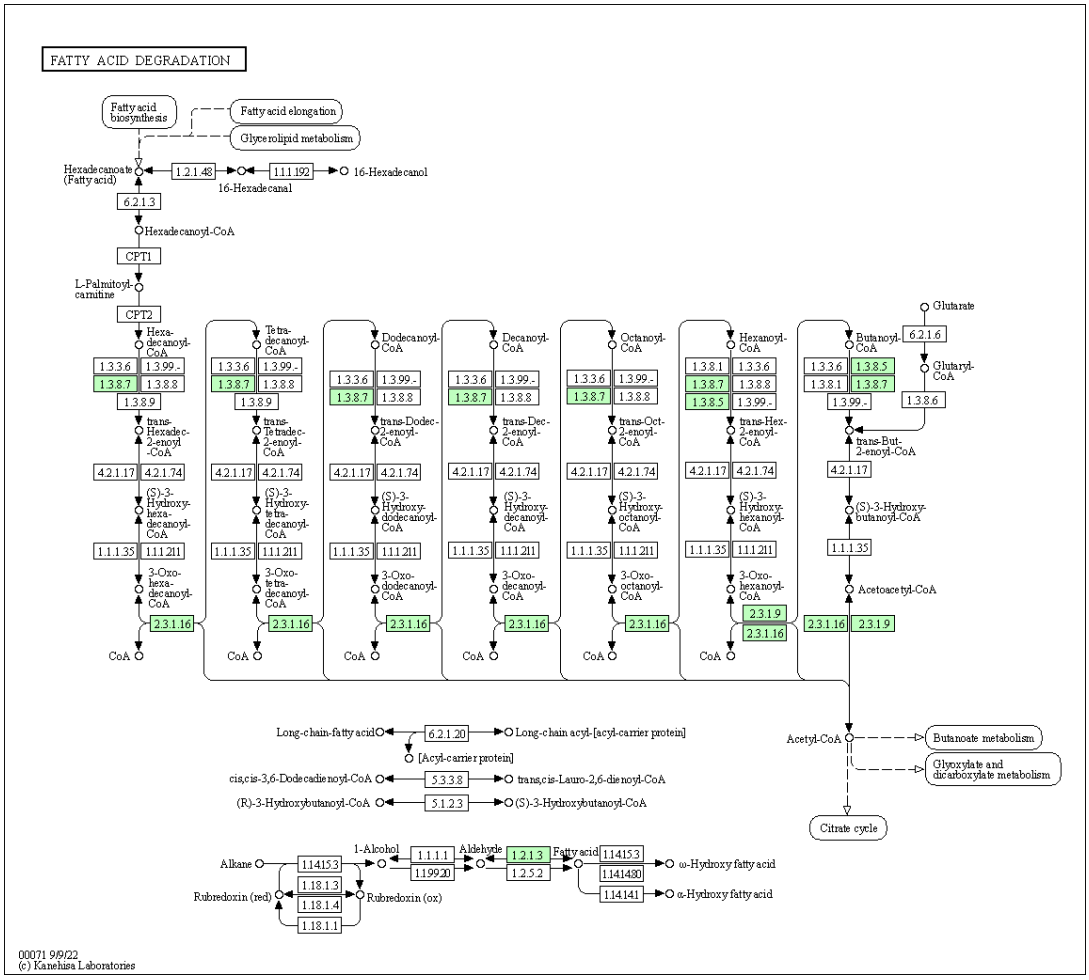


Figure A4.1 Enrichment in the 'fatty acid degradation' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of commercial *E. coli* infected *B. terrestris* workers in the commercial infected (CE) – wild infected (WE) fat body comparison. KEGG plot of proteins involved in fatty acid degradation and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

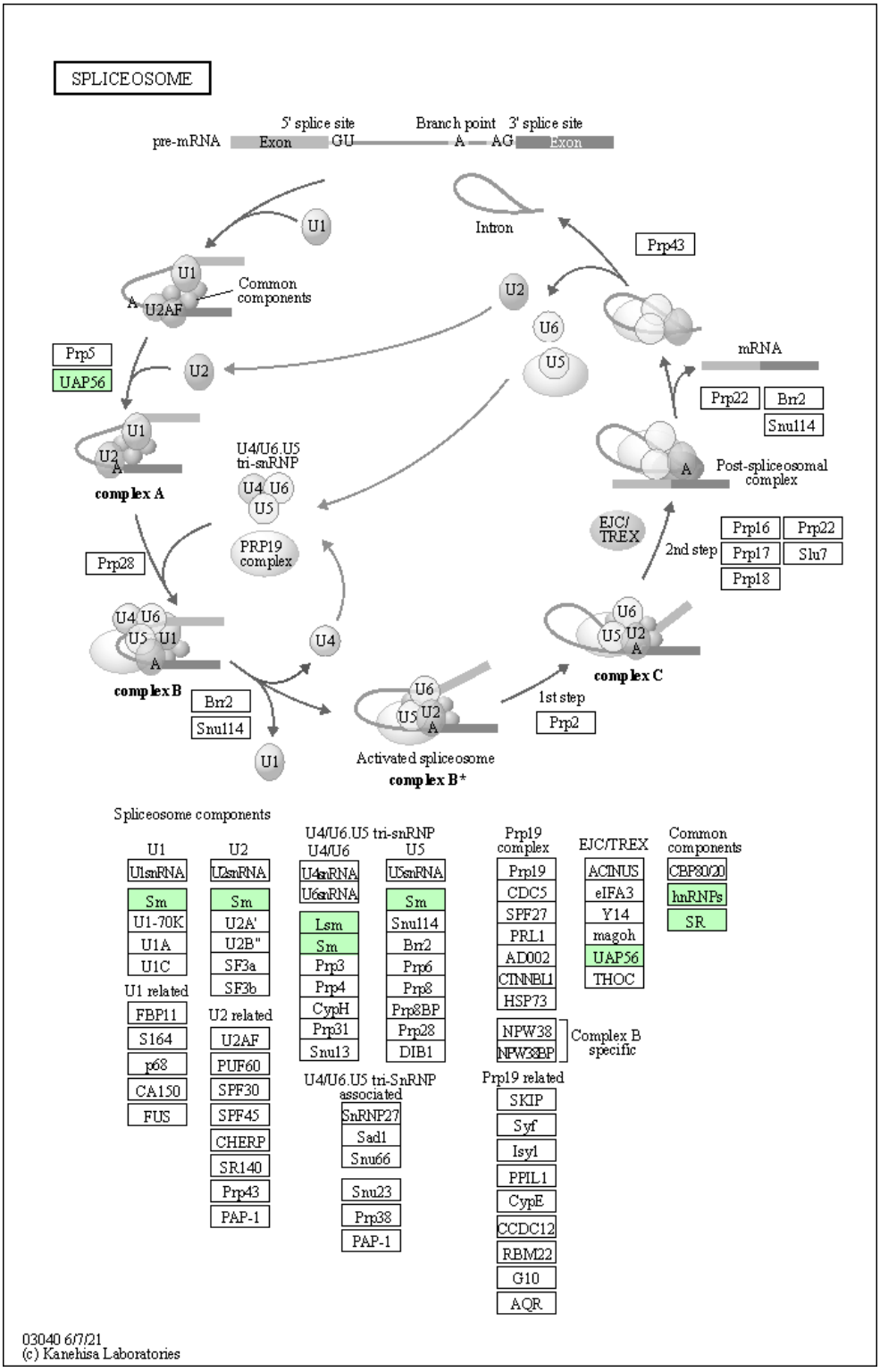


Figure A4.2 Enrichment in the 'spliceosome' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of commercial *E. coli* infected *B. terrestris* workers in the commercial infected (CE) – wild infected (WE) fat body comparison. KEGG plot of proteins involved in the spliceosome and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

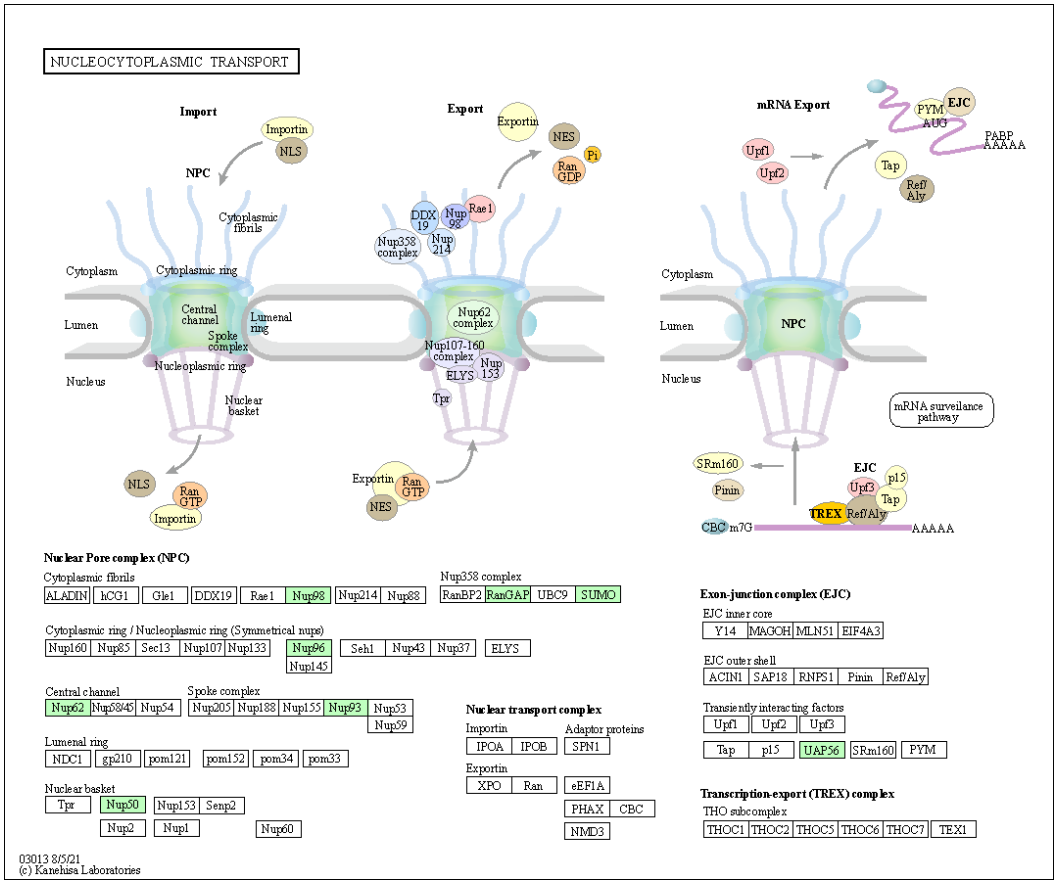


Figure A4.3 Enrichment in the 'nucleocytoplasmic transport' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of commercial *E. coli* infected *B. terrestris* workers in the commercial infected (CE) – wild infected (WE) fat body comparison. KEGG plot of proteins involved in nucleocytoplasmic transport and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

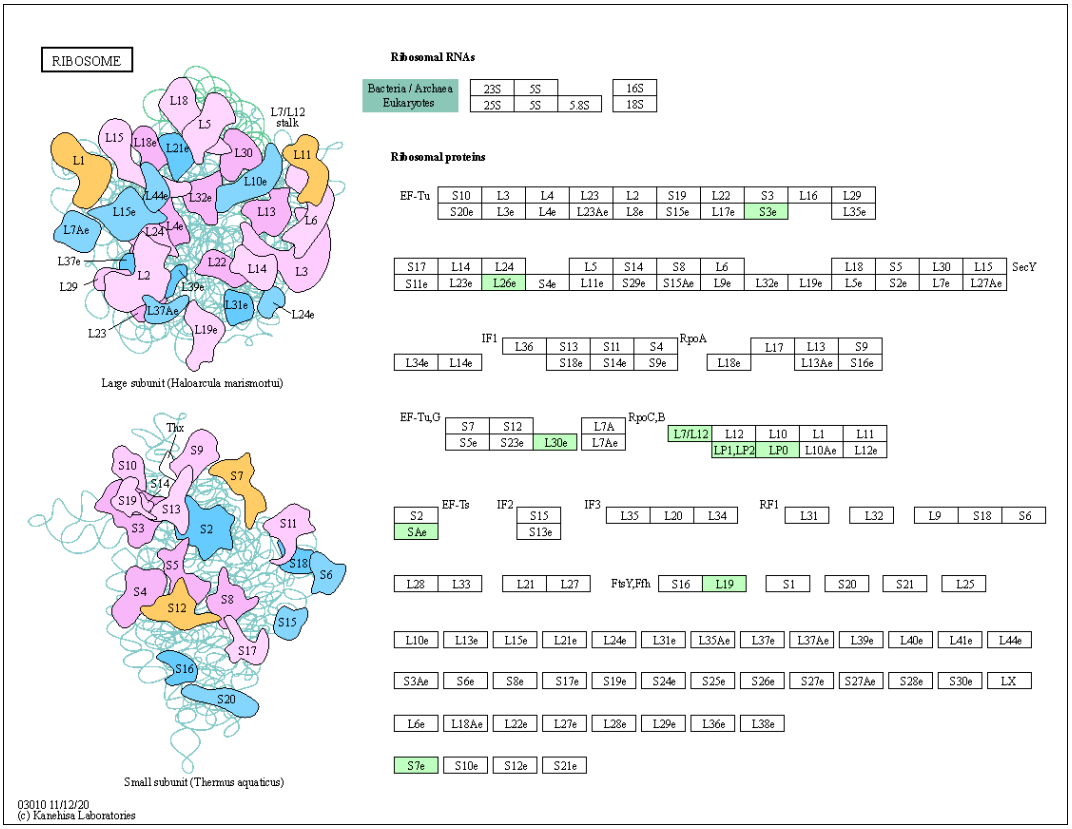


Figure A4.4 Enrichment in the 'ribosome' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of wild *E. coli* infected *B. terrestris* workers in the commercial infected – wild infected fat body comparison. KEGG plot of proteins involved in the ribosome and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

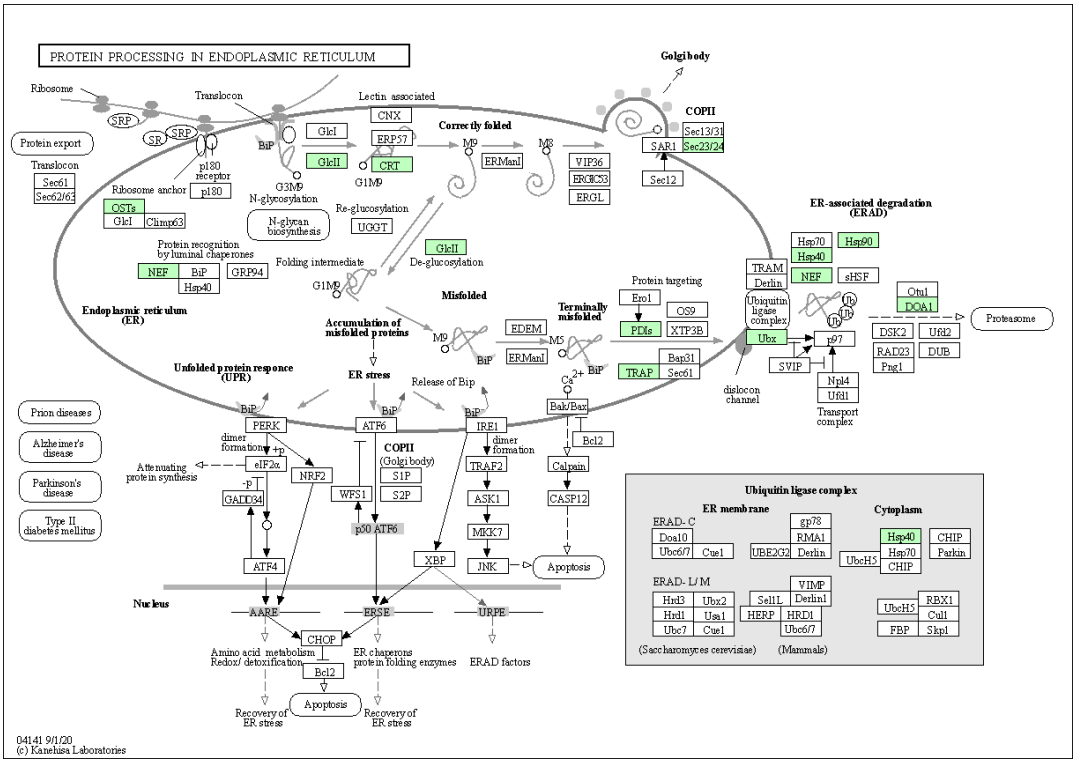


Figure A4.5 Enrichment in the 'protein processing in the endoplasmic reticulum' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of wild *E. coli* infected *B. terrestris* workers in the commercial infected – wild infected fat body comparison. KEGG pathway for protein processing in the endoplasmic reticulum and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

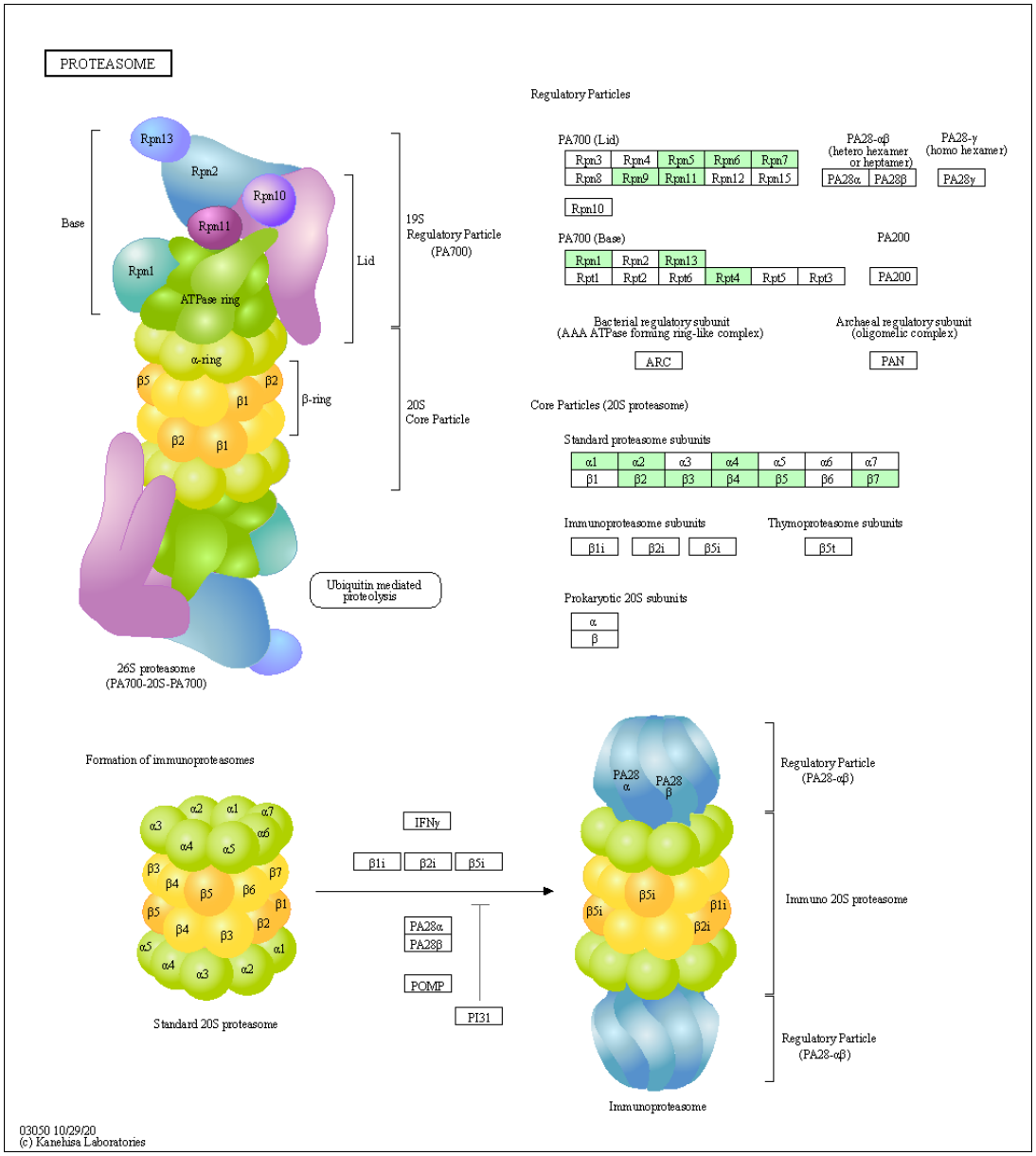


Figure A4.6 Enrichment in the 'proteasome' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of wild *E. coli* infected *B. terrestris* workers in the commercial infected (CE) – wild infected (WE) fat body comparison. KEGG plot of proteins involved in the proteasome and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

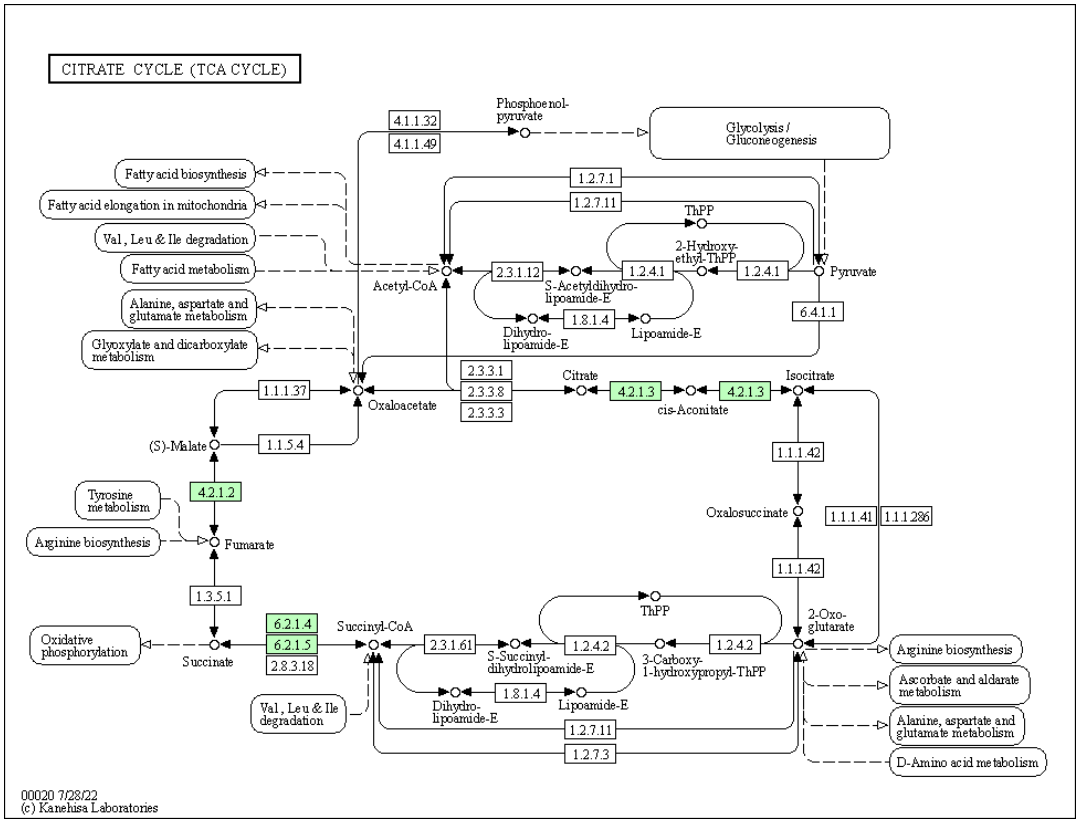


Figure A4.7 Enrichment in the 'TCA cycle' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of commercial Ringer control *B. terrestris* workers in the commercial Ringer control – wild Ringer control fat body comparison. KEGG plot of proteins involved in the TCA cycle and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

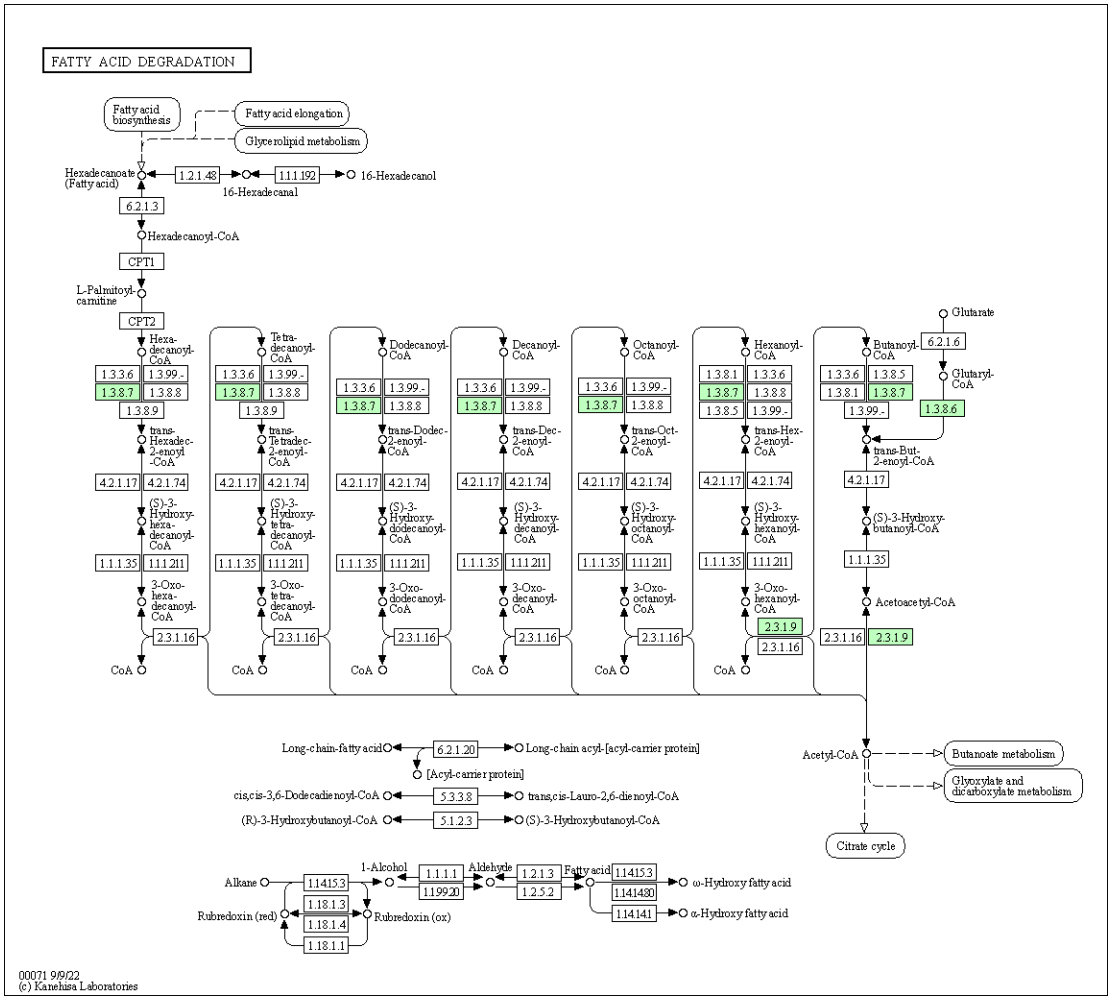


Figure A4.8 Enrichment in the 'fatty acid degradation' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of commercial Ringer control *B. terrestris* workers in the commercial Ringer control – wild Ringer control fat body comparison. KEGG plot of proteins involved in fatty acid degradation and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

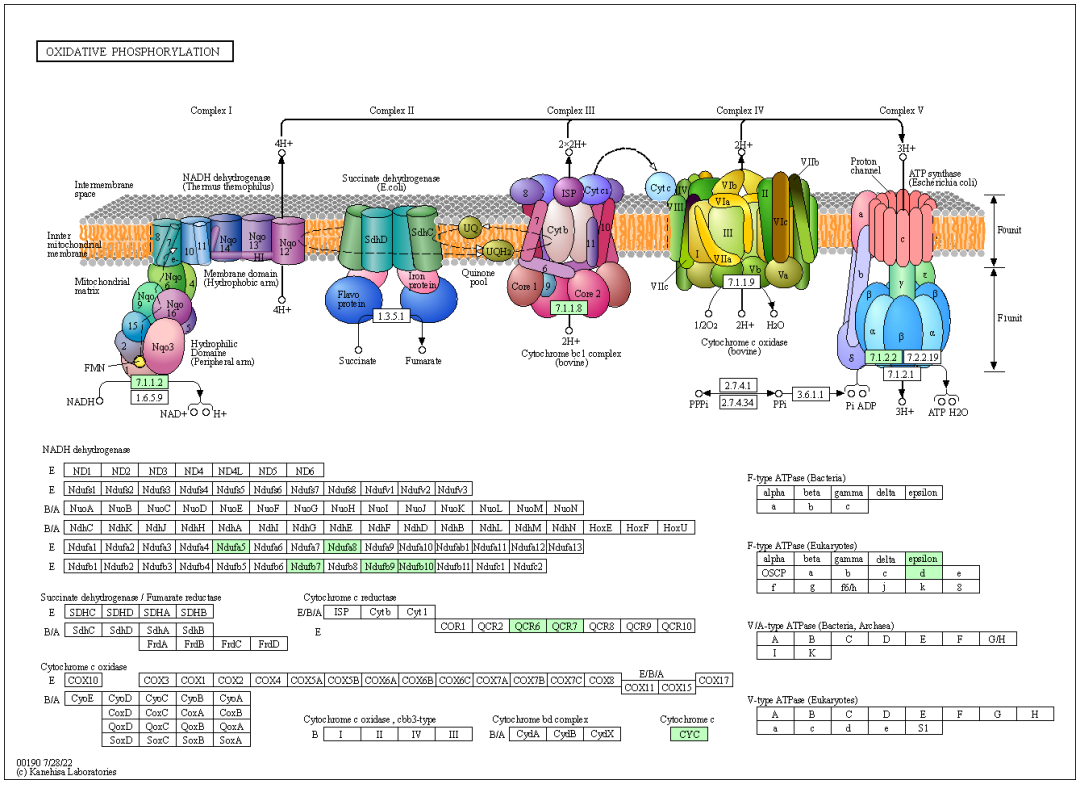


Figure A4.9 Enrichment in the 'oxidative phosphorylation' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of commercial Ringer control *B. terrestris* workers in the commercial Ringer control – wild Ringer control fat body comparison. KEGG plot of proteins involved in oxidative phosphorylation and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

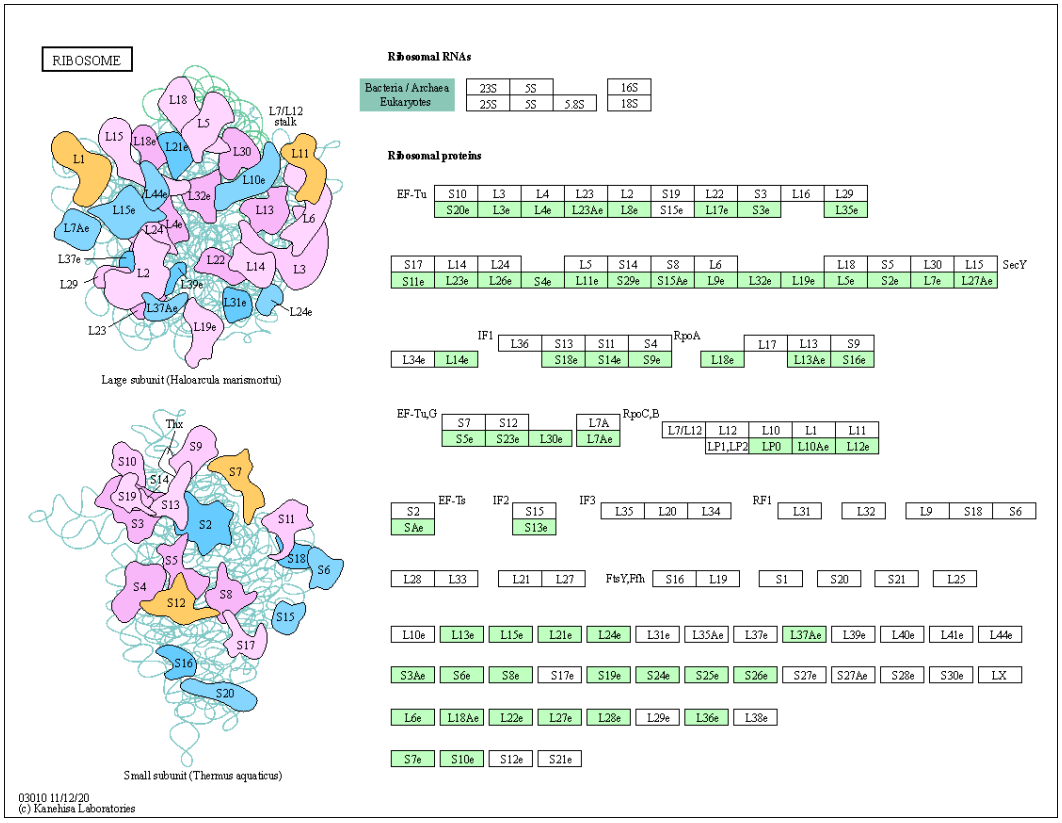


Figure A4.10 Enrichment in the 'ribosome' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of wild Ringer control *B. terrestris* workers in the commercial Ringer control – wild Ringer control fat body comparison. KEGG plot of proteins involved in ribosome and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

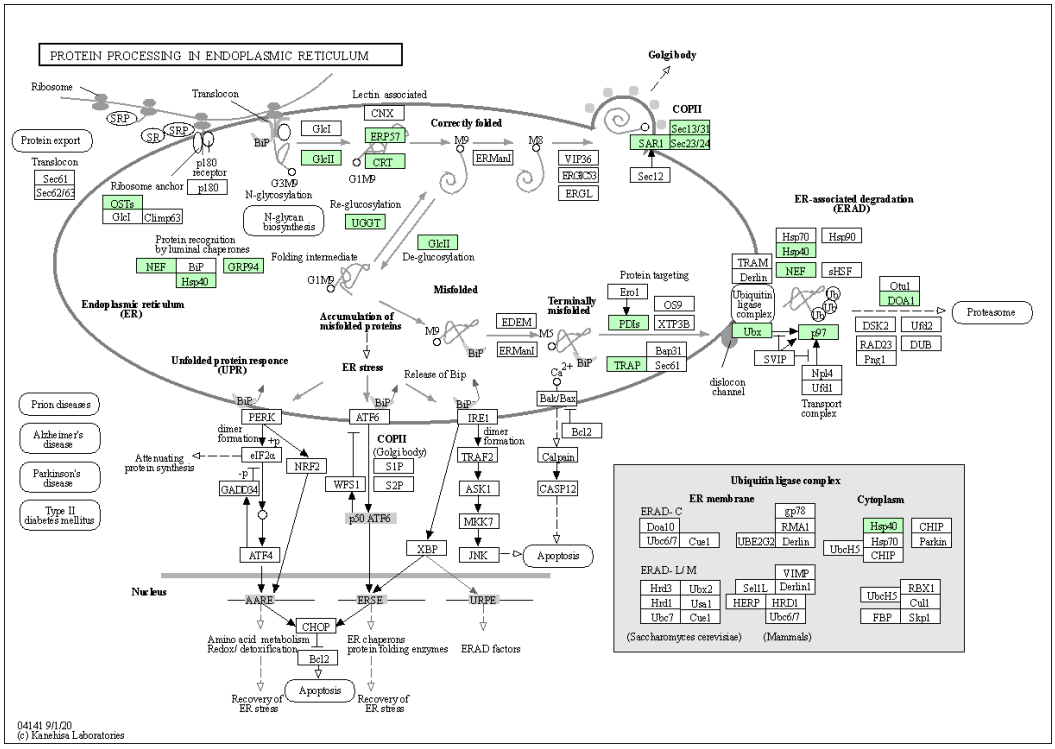


Figure A4.11 Enrichment in the 'protein processing in the endoplasmic reticulum' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of wild Ringer control *B. terrestris* workers in the commercial Ringer control – wild Ringer control fat body comparison. KEGG plot of proteins involved protein processing in the endoplasmic reticulum and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

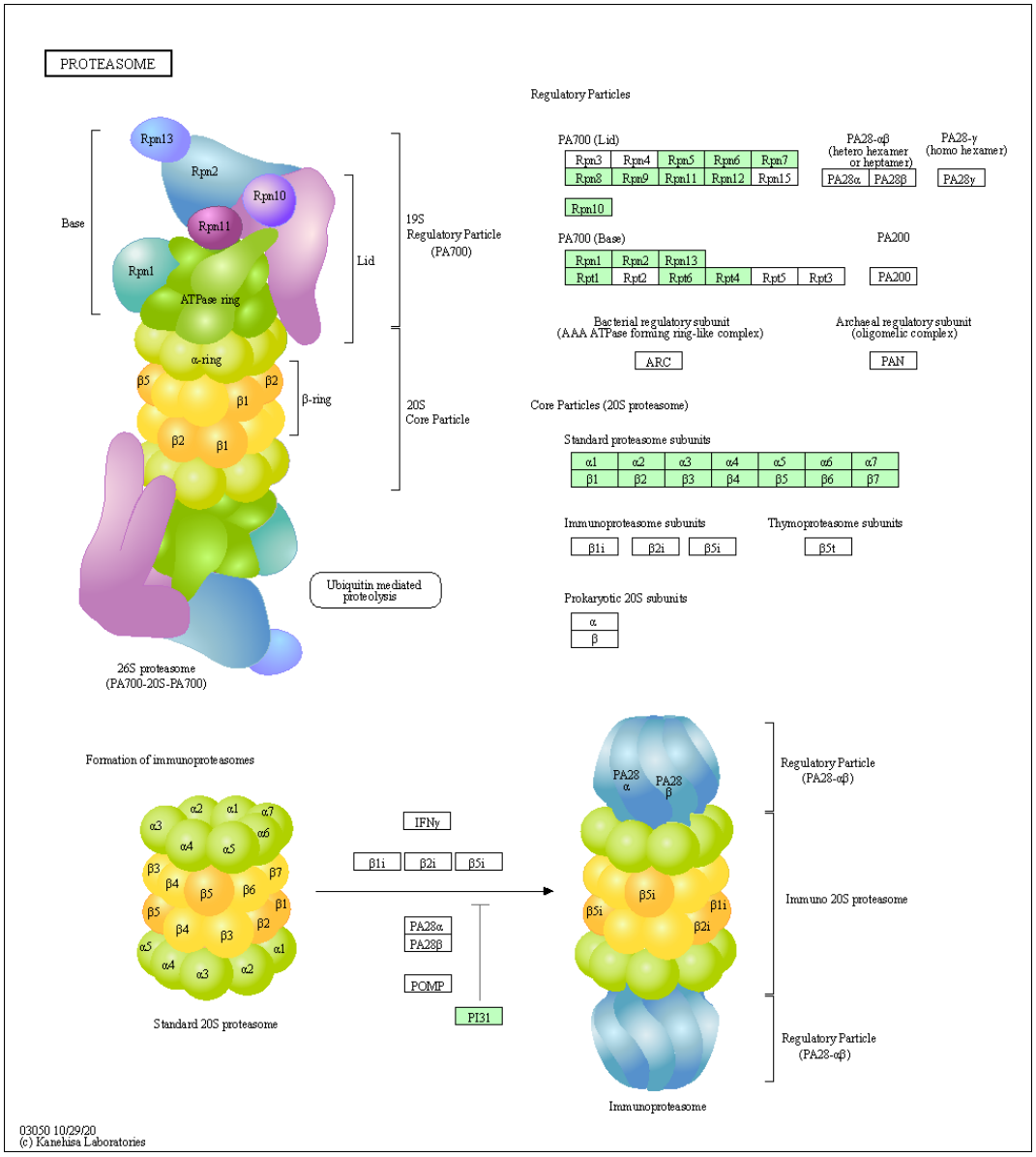


Figure A4.12 Enrichment in the 'proteasome' KEGG pathway by SSDA proteins increased in abundance in the fat bodies of wild Ringer control *B. terrestris* workers in the commercial Ringer control – wild Ringer control fat body comparison. KEGG plot of proteins involved proteasome and SSDA proteins enriched for this pathway (highlighted in green) as determined by Blast KOALA analysis.

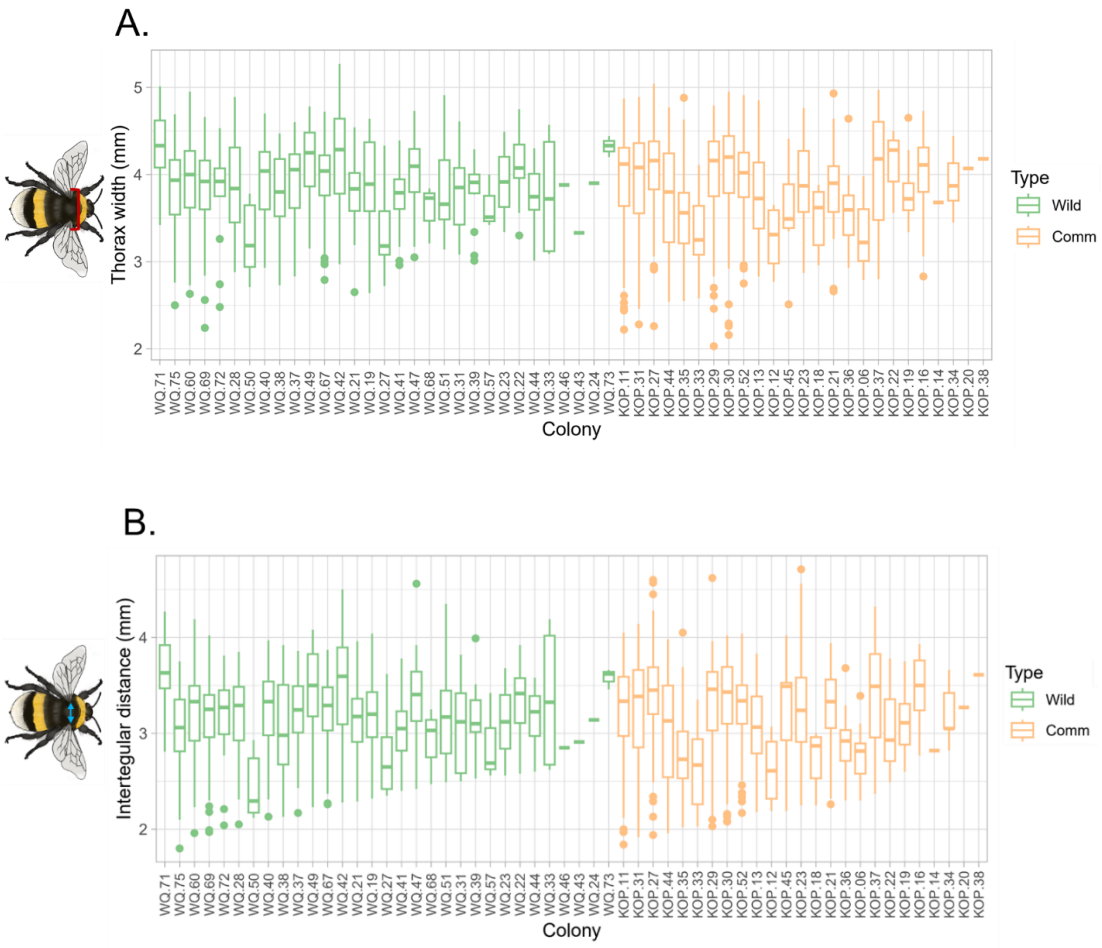


Figure A5.1 Wild and commercial colony worker size measurements by individual colony. (A) Thorax width (mm) data and (B) intertegular distance (mm) for each worker-producing colony, with group indicated by green (wild) and orange (commercial) colour. Median values are represented by thick horizontal lines, each box represents the interquartile range and whiskers mark the min and max values in each group. Individual circles are outliers in each data set.

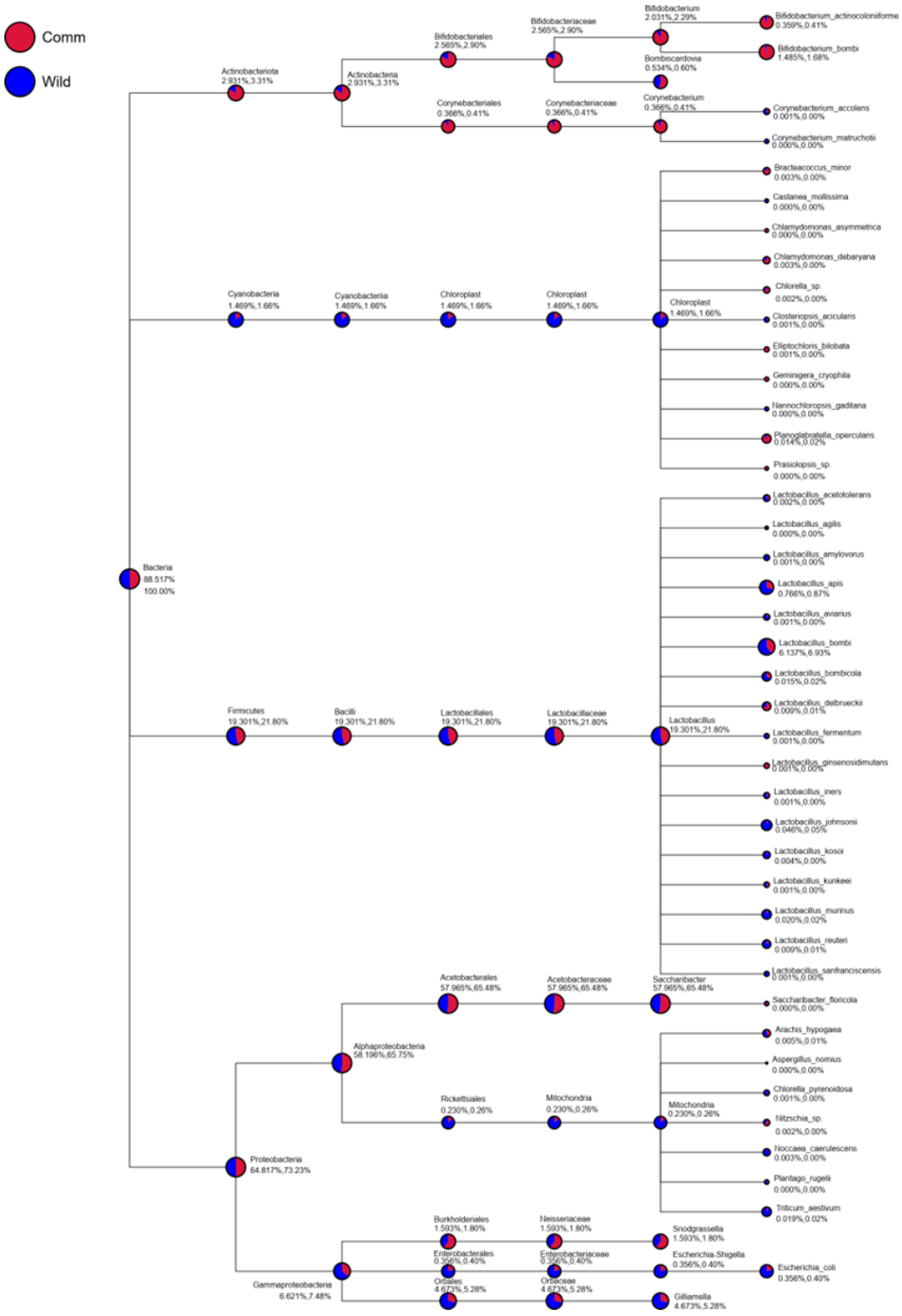


Figure A5.2 Taxonomy tree on wild and commercial bacterial microbiomes. The top ten genera in each group were used in the generation of a group taxonomy tree for species of bacteria found within the guts of lab-reared lines of wild and commercial lineage *B. terrestris* workers. Size of circles indicated the relative abundance of the species. First number underneath the taxonomic name is the percentage of the whole taxon and the second number is the percentage in the selected taxon. Colours represent the relative proportion of OTUs in each group to reach each level of taxonomy.

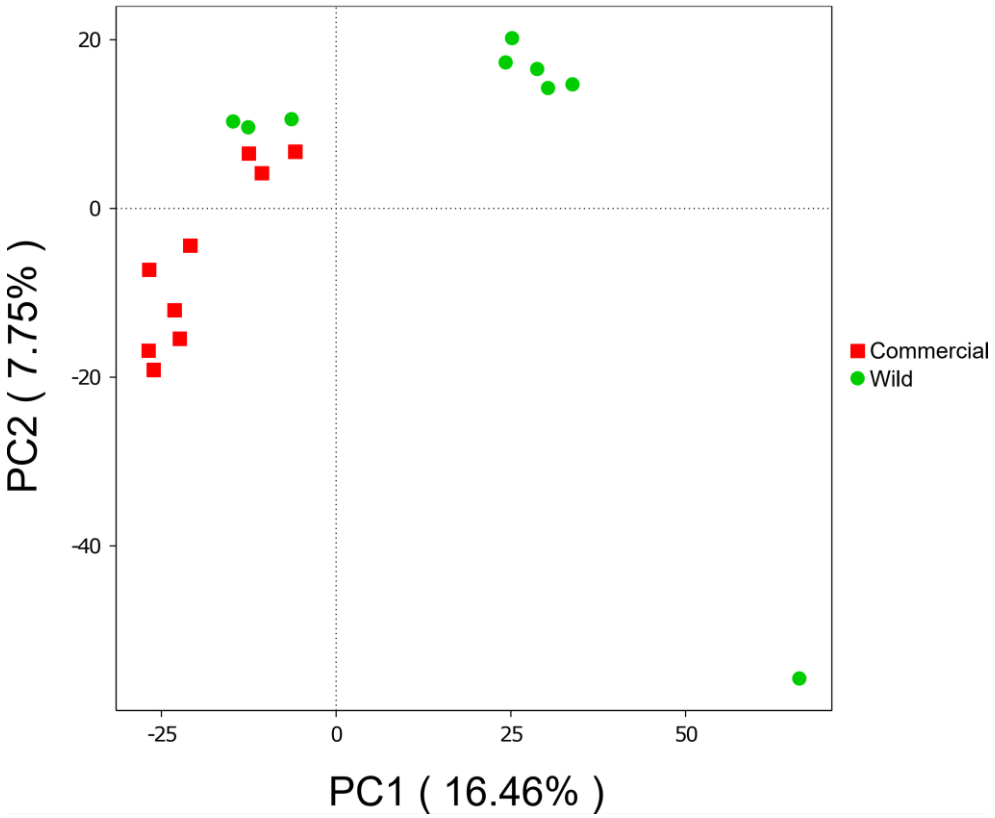


Figure A5.3 Principal component analysis on the bacterial communities of lab-reared lineages of wild and commercial *B. terrestris* workers. PCA on all wild and commercial microbiome samples to visualise variability and differences in community composition between groups of wild and commercial *B. terrestris* worker bacterial gut microbiomes.

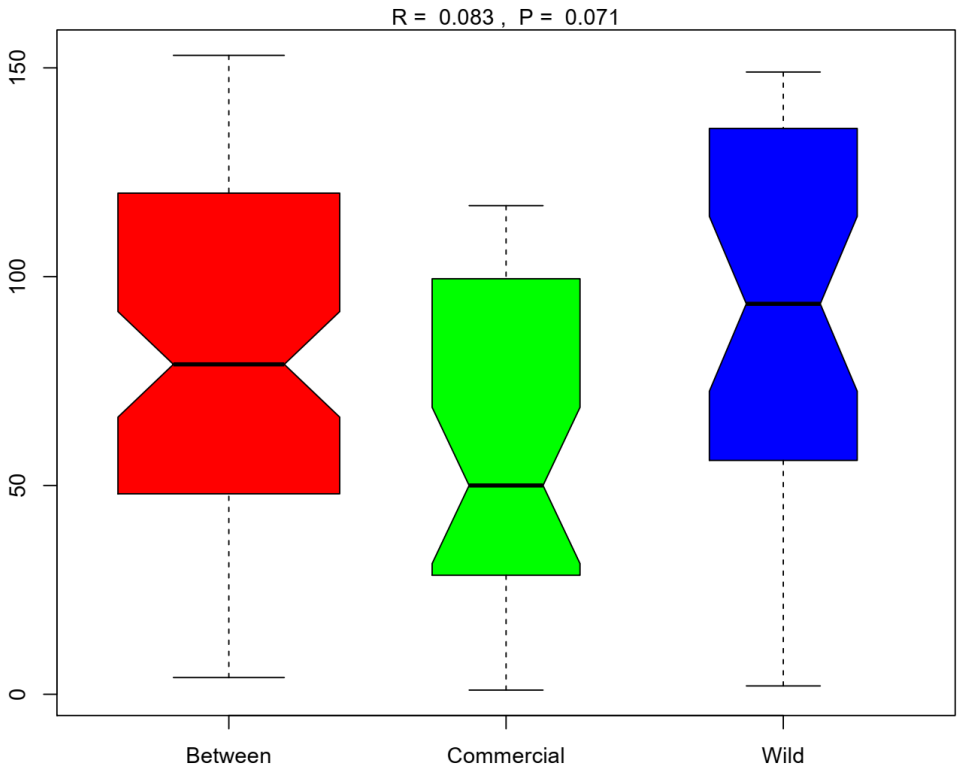


Figure A5.4 Visualisation of ANOSIM results on wild and commercial *B. terrestris* worker bacterial microbiome groups. ANOSIM tests whether variation among groups is larger than the variation within groups. Y-axis represents the rank value, and x-axis shows variability between groups and within commercial and wild groups.

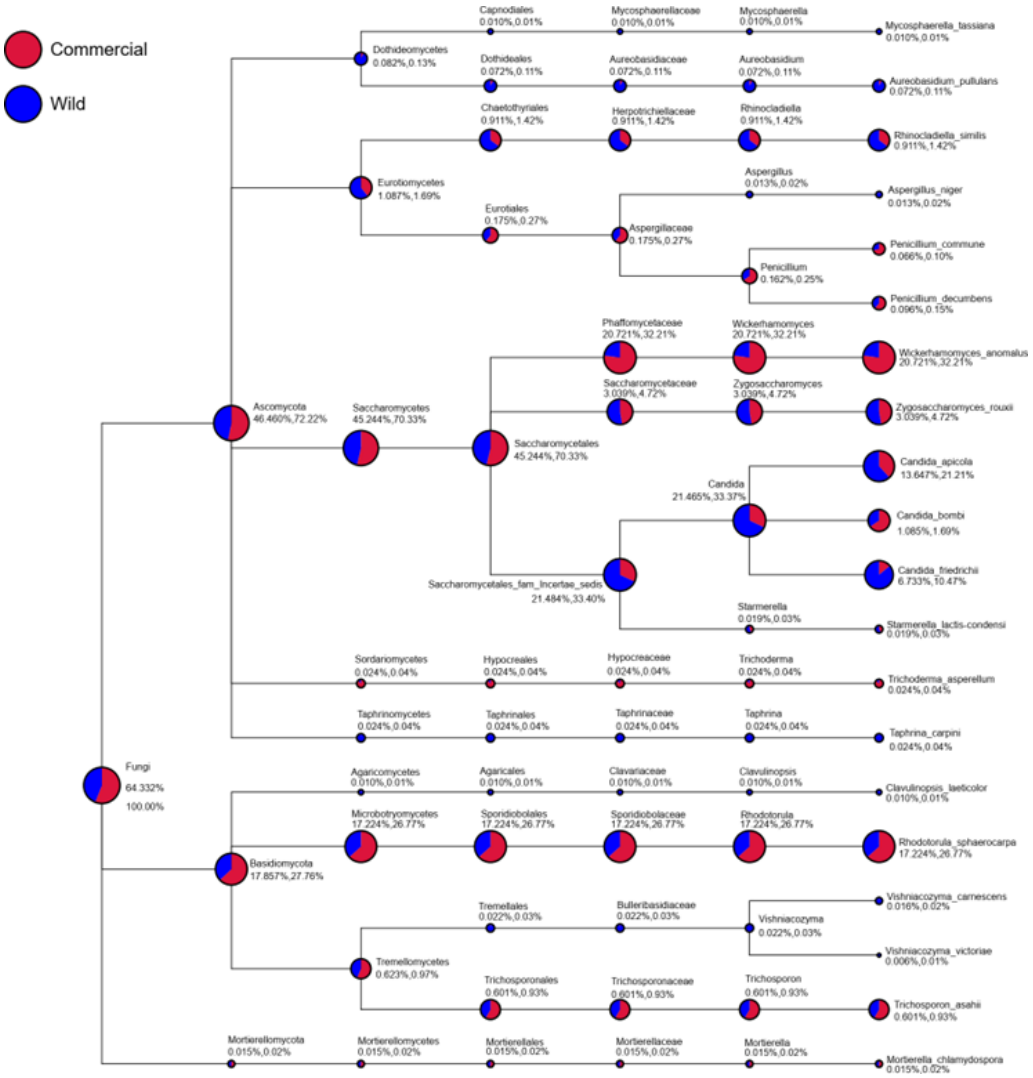


Figure A5.5 Taxonomy tree on wild and commercial fungal microbiomes. The top ten genera in each group were used in the generation of a group taxonomy tree for species of fungi found within the guts of lab-reared lines of wild and commercial lineage *B. terrestris* workers. Size of circles indicated the relative abundance of the species. First number underneath the taxonomic name is the percentage of the whole taxon and the second number is the percentage in the selected taxon. Colours represent the relative proportion of OTUs in each group to reach each level of taxonomy.

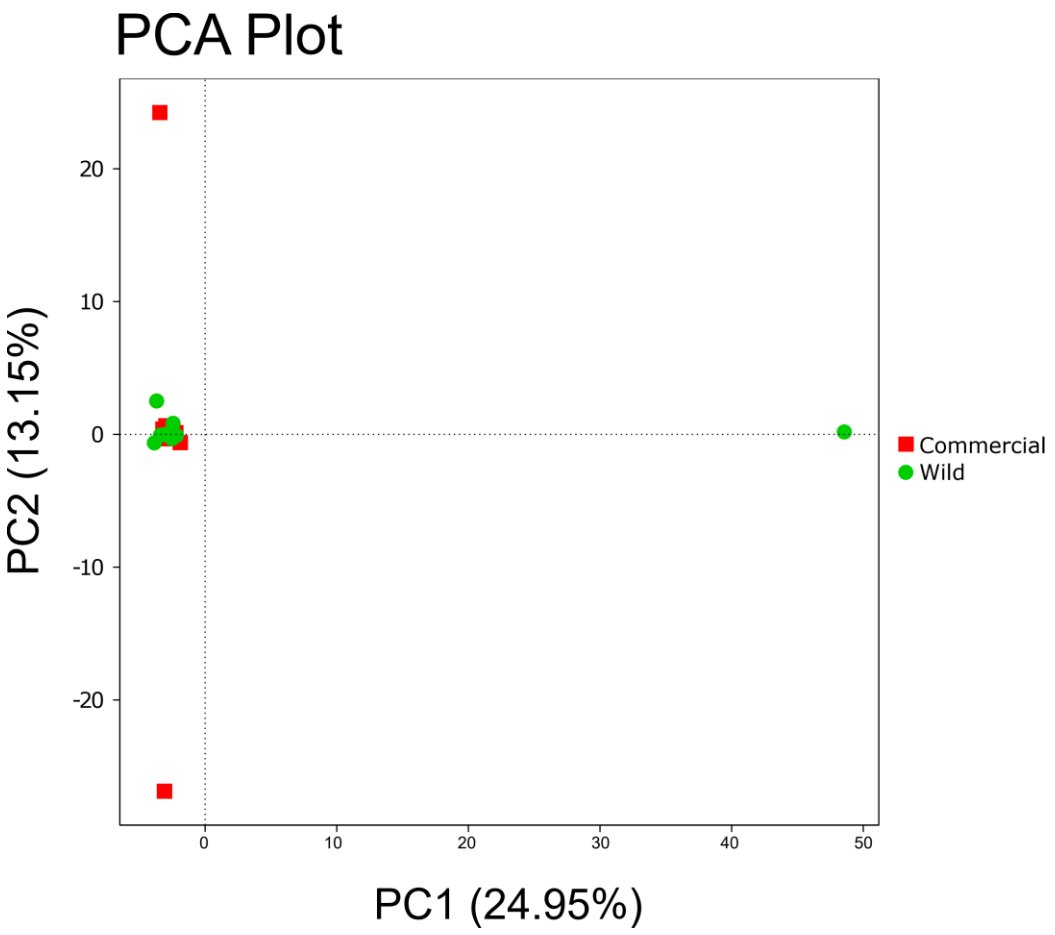


Figure A5.6 Principal component analysis on the fungal communities of lab-reared lineages of wild and commercial *B. terrestris* workers. PCA of all wild and commercial microbiome samples to visualise sample variability and differences in community composition between groups of wild and commercial *B. terrestris* worker fungal gut microbiomes.

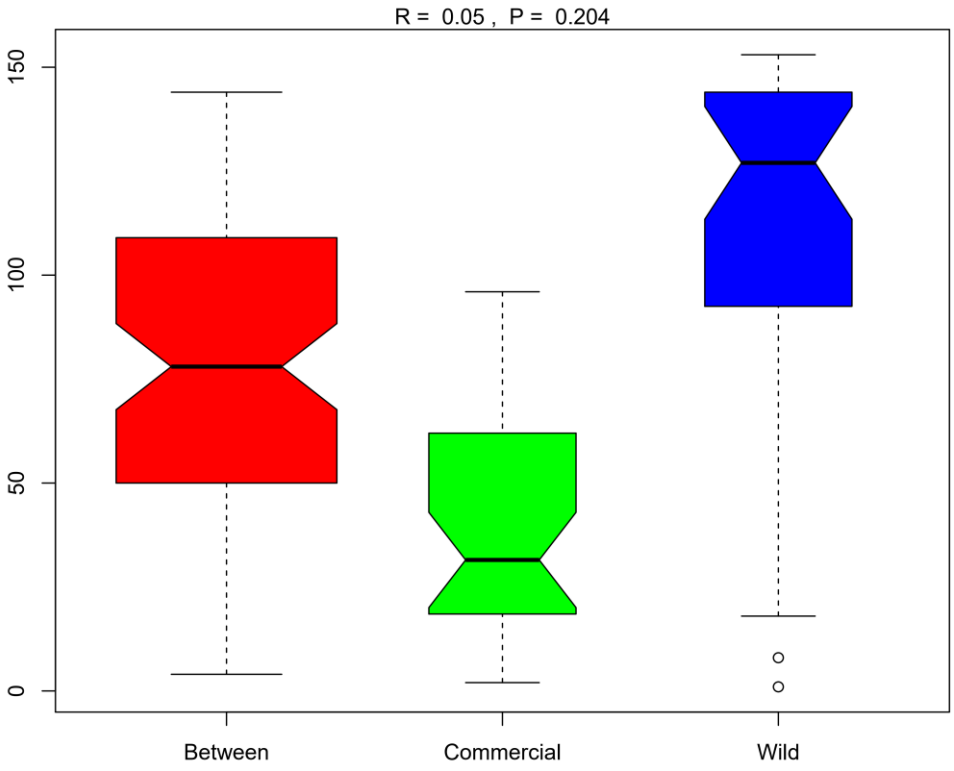


Figure A5.7 Visualisation of ANOSIM results on wild and commercial *B. terrestris* worker bacterial microbiome groups. ANOSIM tests whether variation among groups is larger than the variation within groups. Y-axis represents the rank value, and x-axis shows variability between groups and within commercial and wild groups.

The following appendix files and tables are provided on the OneDrive link below:

https://maynoothuniversity-my.sharepoint.com/:f:/g/personal/sarah_larragy_2019_mumail_ie/EtnXh1HVfHZCh-HOp4wd9aYBTL9yECiDSYRtclDz9ru_cA?e=i8sCZD

Chapter 1

Appendix file 1.1 All-Ireland Pollinator Plan How to Guide 7: Guidelines for users of imported bumblebee colonies

Chapter 2

Table A2.1 Male *B. terrestris* sample collection and sequencing information

Table A2.2 Results of ADMIXTURE analysis on Irish, British, commercial, German and Turkish *B. terrestris* samples

Table A2.3 F_{ST} analyses results for Irish – British, Irish – commercial and British – commercial comparisons

Table A2.4 XP-nSL analyses results for Irish – British, Irish – commercial and British – commercial comparisons

Table A2.5 GO term enrichment analyses results on SNPs of interest from F_{ST} analyses

Table A2.6 GO term enrichment analyses results on SNPs of interest from XP-nSL analyses

Chapter 3

Table A3.1 Sampling information for wild-caught bumblebees.

Table A3.2 Nucleotide diversity analyses of canonical immune genes.

Table A3.3 Gene Ontology term enrichment analysis on canonical immune genes in regions of significantly low diversity.

Table A3.4 Extended haplotype homozygosity analysis of canonical immune genes.

Table A3.5 Copy number variation analysis for canonical immune genes.

Table A3.6 Episodic selection analyses for canonical immune genes.

Chapter 4

Table A4.1 SSDAs from pair-wise comparisons and hierarchical clustering analysis of wild and commercial clothianidin exposed and control *B. terrestris* worker brain proteomes

Table A4.2 Venn analysis of SSDA proteins from wild and commercial *B. terrestris* brain samples

Table A4.3 STRING enrichment analysis on groups of SSDA proteins from wild and commercial *B. terrestris* brain samples

Table A4.4 TopGO enrichment analysis on groups of SSDA proteins from wild and commercial *B. terrestris* brain samples

Table A4.5 SSDAs from pair-wise comparisons and hierarchical clustering analysis of wild and commercial *E. coli* infected and Ringer control *B. terrestris* worker fat body proteomes

Table A4.6 Venn analysis of SSDA proteins from wild and commercial *B. terrestris* fat body samples

Table A4.7 STRING enrichment analysis on groups of SSDA proteins from wild and commercial *B. terrestris* fat body samples

Table A4.8 TopGO enrichment analysis on groups of SSDA proteins from wild and commercial *B. terrestris* fat body samples

Chapter 5

Table A5.1 Lab-reared *B. terrestris* queen collection, mating and diapause information

Table A5.2 Lab-reared *B. terrestris* wild and commercial colony success rates

Table A5.3 *B. terrestris* lab-reared wild and commercial colony growth rates

Table A5.4 Developmental time of first worker from lab-reared *B. terrestris* wild and commercial colonies

Table A5.5 Worker size data from lab-reared *B. terrestris* wild and commercial colonies

Table A5.6 Sucrose consumption of lab-reared *B. terrestris* wild and commercial colonies

Table A5.7 Wild and commercial worker bacterial microbiome relative abundances, alpha diversity estimates, community structure statistics and statistically significant differences in bacterial genera and species.

Table A5.8 Wild and commercial worker fungal microbiome relative abundances, alpha diversity estimates, community structure statistics and statistically significant differences in bacterial genera and species.