Ecology of the bumblebee gut microbiota

A thesis submitted for the degree of

Doctor of Philosophy

September 2018

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Declaration of Authorship

I, Sarah Walkington, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work and advice of others, this is clearly stated.

Signed: _____

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ACKNOWLEDGMENTS

Firstly, I would like to express my sincerest thanks to all my supervisors: Matt Heard, Mark Brown, Lindsay Newbold, Christopher van der Gast, and of course my Biobest Group CASE partners, Felix Wäckers and Annette van Oystaeyen. Thank you for the opportunity to undertake this PhD and for all your support these past four years.

Thank you as well to all the staff and students at the Centre of Ecology & Hydrology for being a massive source of help and encouragement. There are too many people to mention, but special thanks go to Charlie and Ilze for being there from the start and for all your support and friendship. I would also like to thank my Chiltern Wing officemates – Holly, Denny, and Elmer. You made me look forward to coming in to work every day, and I will miss you all!

I am immensely grateful to everyone who has helped me during my PhD, and for all the people I have had the opportunity to collaborate with. Special thanks go to Soon for your bioinformatics wizardry and Anna for help with the pollen barcoding. I would also like to thank Helen, Flavia, Carolin and the rest of the CEH Ecotoxicology team for sharing your time and expertise with me during the pesticide trial (Chapter 7).

As part of this PhD I also worked closely with the research and development team at Biobest Group NV, Belgium for the work in Chapters 5 and 6. I would like to thank Annette and Felix for hosting me, and all the staff in Westerlo for being so friendly and welcoming.

I am infinitely thankful to Clare, Lizzy, Suzie, and all my friends near and far for all your encouragement at the most stressful of times. But most of all, thank you mum and dad for supporting me tirelessly through everything I have set out to achieve. I would not be where I am today without you.

ABSTRACT

Bumblebees are key wild and commercial pollinators. However, there have been worldwide declines in these, and other pollinator species due to environmental stressors such as habitat loss, climate change, pesticide use and disease. A considerable percentage of human nutrition relies on animal pollinated crops, while over 80% of wildflower species require animal pollination. Maintaining healthy pollinator populations is thus both ecologically and economically important. To achieve this, we need a more comprehensive and holistic understanding of pollinator biology including evaluation of the role played by host-associated microorganisms, such as the gut microbiota. In order to harness the potential host benefits of these microbial communities we first need to understand their composition and how they develop within hosts and adapt to new environments and challenges.

Specifically, my thesis investigated the ecology of the gut microbiota in the globally important pollinator species, *Bombus terrestris*. I used manipulative experiments in the lab and field, combined with molecular screening, to explore how lifecycle and environmental factors affected gut microbiota abundance and diversity. I demonstrate, for the first time, clear adult/larval distinctions in gut microbiota and outline a preliminary model of assembly. I describe the overall community composition and show how this change in response to environmental perturbation. Core taxa were able to persist during gut remodelling (pupation), and after exposure to field-realistic pesticide doses, suggesting that they are highly adapted to their hosts and resilient to perturbation. There was also evidence for bacterial retention during queen diapause, and clear potential for vertical transmission of gut microbiota to affect future colony development. My results suggest that studies based solely on workers may not be a sufficient indicator for the 'microbial health' of the whole colony. Further it highlights that key microbiota could potentially improve commercial rearing practices and outcomes for species conservation initiatives.

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ABBREVIATIONS

AChR	Nicotinic acetylcholine receptor
AMPs	Antimicrobial peptides
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance analysis
ASV	Amplicon sequence variant
BLAST	Basic local alignment search tool
βME	(Beta) 2-Mercaptoethanol
bp	Base pair
Bt	*Bacillus thuringiensis*
CASE	Collaborative award in science and engineering
CEH	Centre for Ecology & Hydrology
DADA2	Divisive amplicon denoising algorithm
DGGE	Denaturing gradient gel electrophoresis
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EU	European Union
FISH	Fluorescent *in situ* hybridization
iTOL	Interactive tree of life
ITS	Internal transcribed spacer
LAB	Lactic acid bacteria
NGS	Next generation sequencing
OD	Optical density
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PEAR	Paired-end read merger
ppb	Parts per billion
proK	Proteinase K
PSS	Phenolic separation solution
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal database project
RH	Relative humidity
RR	Resource-rich
RP	Resource-poor
RT-qPCR	Real-time quantitative polymerase chain reaction
SBS	Sequencing-by-synthesis
SD	Standard deviation
SMS	Single molecule sequencing
SIMPER	Similarity percentage analysis
SSU	Small subunit
T-RFLP	Terminal restriction fragment length polymorphism
UV	Ultra violet

CHAPTER 1:

INTRODUCTION

1.1 GENERAL INTRODUCTION

Pollinators provide an essential ecosystem service to the Earth – namely pollination, which is required by over 80 percent of all flowering plant species (Ollerton *et al*. 2011). Pollination services are provided by animals, mainly insects, and the loss of such a service would have profound environmental and ecological consequences (Vanbergen *et al*. 2013). Yet, population declines of both wild and managed pollinators are reported globally (Potts *et al*. 2010). For instance, populations of honey bees in the USA declined by over half between 1947 and 2005 (van Engelsdorp *et al*. 2008), in Europe, a quarter of hives were lost between 1985 and 2005 (Potts *et al*. 2010) alongside significant declines in wild bee diversity and abundance.

Some of the main drivers leading to these population declines are a direct result of the intensification of agriculture practices since the 1940s (Robinson & Sutherland 2002). With these activities came a reduction in landscape and habitat diversity, and an increasing reliance on pesticides to maintain high crop yields (Tilman *et al*. 2002). Transportation and management of pollinators, such as honey bees and bumblebees, to provide pollination services has created health issues for these species that have also been linked to declines of wild bees through spill over of parasite and diseases loads (Colla *et al*. 2006; Goulson *et al*. 2015; Graystock *et al*. 2016). Lastly, climate change also appears to be a contributory factor, and has been linked to declines and contracted ranges in British bumblebee species (Williams *et al*. 2007) and is predicted to cause more declines in these species in the future (Dormann *et al*. 2008).

As a considerable percentage of our diet is dependent on animal-facilitated pollination (Klein *et al*. 2007), it is fundamentally important that we recognise the impacts that this may have on the future of global agriculture (Aizen *et al*. 2008). Increasing human populations will only escalate the food security challenges faced by the agricultural sector,

and intensify demands on wild bee populations and commercial growers to provide pollination services (Klein *et al*. 2007). In addition to conserving and enhancing wild pollinator communities, the domestication and culturing of key species will be vital to sustain growing agricultural demand (Aizen & Harder 2009).

Bees, along with other insect pollinators, are important for the maintenance of plant reproduction in natural and managed systems (Klein *et al*. 2007). Worldwide, insect pollination to crops is worth approximately €153 billion (Gallai *et al*. 2009) and in Europe 84% of crop species require animal-facilitated pollination (Klein *et al*. 2007). These important ecological and economic contributions, coupled with concerns over reported declines in pollinator populations, make conducting research into bee conservation and health all the more timely (Potts *et al*. 2010).

To fully comprehend these population declines and how to develop methods to improve pollinator health, it is essential to develop a thorough understanding of the whole organism. All animals, including bees, harbour diverse communities of microorganisms, referred to as microbiota (Engel *et al*. 2016). This is not to be confused with the microbiome, which includes the genetic information from all members of the microbiota (Turnbaugh *et al*. 2007). These host-associated communities, or microbiota, contribute towards an individual's 'extended' phenotype and understanding the role they play in normal homeostasis is vital to our comprehension of host organisms (Sommer & Bäckhed 2013). Gut microbiota are integral and often reflect changes in the host which might not otherwise be detected (Sommer & Bäckhed 2013).

Social insects, such as honey bees and bumblebees, harbour some of the most distinctive and consistent gut communities in the animal kingdom (Kwong & Moran 2016). As such, they offer a valuable model system for understanding the effects of different host-microorganism relationships, acquisition pathways, as well as the extent to which external environmental factors can influence them.

Here, this thesis explores (i) how the bacterial microbiota associated with bumblebee guts evolve throughout the host's lifecycle (from larval to adult stages); (ii) how acquisition is influenced by both natural and foraging environments and, (iii) how environmental stressors e.g. pathogens and toxic compounds or changes in diet affect microbiota community composition.

1.2 MICROBIAL COMMUNITY ANALYSIS

1.2.1 Microbial communities and gut microbiota

Bacteria are the most diverse and abundant organisms on the Earth, and hold an equally diverse array of functions (Nannipieri *et al*. 2003). It is estimated that there are up to 10^{10} bacterial cells in 1g of soil alone (Raynaud & Nunan 2014), where they live close to plant roots and play a critical role in organic matter decomposition and nitrogen cycling (Canfield *et al*. 2010). Bacteria are found throughout the environment and play hugely important roles in other biogeochemical cycles, such as fixing CO_2 to produce oxygen (Madsen 2011), while others are integral to animal mucosal surfaces, influencing host health and fitness.

A large proportion of host-associated microorganisms are located in the gastrointestinal tract as complex communities, or gut microbiota (Sommer & Bäckhed 2013). Research in many animals, particularly through medical studies, has demonstrated that the resident gut bacteria assists in many vital host functions, including protecting against pathogen infection (Round & Mazmanian 2009) and aiding nutritional processes such as digestion and synthesising beneficial molecules, e.g. vitamins (Maslowski & MacKay 2011). Disturbance to the gut community has been linked to numerous diseases, including obesity, inflammatory bowel disease, heart disease, as well as autoimmune diseases such as asthma and arthritis (Sommer & Bäckhed 2013).

The study of gut microbiota is a rapidly expanding area of microbiology, providing a leading edge for future advancements in medicine and technology. Understanding and harnessing knowledge about host gut microbiota and associated functions relating to host health could open up avenues for therapeutic treatments for many diseases (Engel & Moran 2013b; Sommer & Bäckhed 2013).

The extremely complex nature of the gut microbiome in vertebrates makes the use of model organisms important for furthering our knowledge in this field (Pernice *et al*. 2014). Insects in particular make excellent alternatives as they typically harbour gut communities with lower diversity, are easy to manipulate in the lab, and a wealth of genetic information is already available about their metabolic and immune pathways. This allows

researchers to theorise functional changes resulting from variations in gut the community (Pernice *et al*. 2014).

1.2.2 Methods of microbial community analysis

The difficulty in replicating the gut environment has long hindered cultivation-based study of microbiota diversity and resulted in a reliance upon morphological and biochemical methods of classification (Prosser *et al*. 2007; Pernice *et al*. 2014). It is estimated that only a fraction of bacterial communities can be cultured using traditional microbiological techniques, commonly referred to as 'the great plate count anomaly' (Staley & Konopka 1985).

The advent of so-called 'cultivation independent' methods using molecular techniques has significantly improved our capability to determine the composition of microbial communities from extracted environmental DNA (Forney *et al*. 2004). Observations of bacterial communities often use the 16S small subunit ribosomal RNA as the 'gold standard' to characterise prokaryotes (16S SSU rRNA; Woese & Fox 1977). This rRNA-encoding gene has many advantages, primarily its size (~1500 bp), which makes it large enough to provide valuable information, yet short enough to be easily sequenced (Wade 2002; Janda & Abbott 2007).

Researchers initially targeted the 16S SSU rRNA region using clone libraries to isolate and generate copies of DNA from mixed communities (Olsen *et al*. 1986). This proved to be a vital tool but its shortcomings lay in that the cost in both time and consumables limited the depth at which communities could be studied (Chandler *et al*. 1997). Further advances in molecular techniques have allowed increasingly more comprehensive analyses of microbial communities, such as the development of community fingerprinting techniques: (i) terminal restriction fragment length polymorphism (T-RFLP; Liu *et al.* 1997); and (ii) denaturing gradient gel electrophoresis (DGGE; Muyzer 1999). Both these methods work by physically separating fragments of the 16S rRNA gene to detect variations in the sequence among different members of a community (Prosser *et al*. 2007; Robinson *et al*. 2010). Although this can be useful for monitoring overall changes in microbial communities over time, they are limited in resolution (Frostegård *et al*. 1999;

Darling & Blum 2007). This is where emergent next-generation sequencing (NGS) technologies are advancing this research area (Buermans & den Dunnen 2014).

1.2.3 Application of Next Generation Sequencing (NGS) technologies

NGS technologies have rapidly improved sequencing capabilities, making them one of the most dependable techniques for analysing bacterial diversity (Prosser *et al*. 2007; Mardis 2017). Early NGS technologies, such as the Roche 454 or Illumina® platforms (*reviewed in* Mardis 2008, 2013, 2017), were based on 'sequencing-by-synthesis' (SBS) approaches. These radically changed microbial community analysis by allowing much deeper 'taxonomic' resolution than previously possible (Claesson *et al*. 2010). Although the read lengths produced are not as long as traditional Sanger sequencing, the depth of coverage means they can generate millions of reads by sequencing DNA fragments in parallel (Kircher & Kelso 2010; Liu *et al*. 2012). They also save time by avoiding the need for multiple steps for library construction and sequencing (Mardis 2013).

In bacterial community analysis, specific regions of the 16S rRNA gene are targeted to produce amplicons, for amplicon sequencing. One of the most popular and widely used NGS amplicon sequencing platforms currently is Illumina® MiSeq outlined in Fig. 1.1 (Mardis 2008, 2013; Wen *et al*. 2017). Briefly, this process starts with polymerase chain reaction (PCR) amplification of the DNA region of interest, which is then tagged with specific adapters that act as reference points during sequencing and analysis. This modified DNA is then loaded onto a specialised glass chip (flow cell) that acts as a 'microfluidic conduit' for amplification and sequencing. Along the bottom of the flow cell are hundreds of thousands of oligonucleotides that complement the attached adapters. DNA fragments attach to the flow cell and are amplified via bridge amplification, producing foci (clusters) for sequencing. Primers and modified nucleotides move into the flow cell, and at each step a nucleotide is added by polymerase, while unincorporated nucleotides are washed away. The flow cell is imaged to determine the base that was added after each round of synthesis by measuring the wavelength of the fluorescent tag, after which, the fluorescent groups are chemically cleaved and the process repeats. Both forward and reverse reads are generated, resulting in high-quality, alignable sequence data.

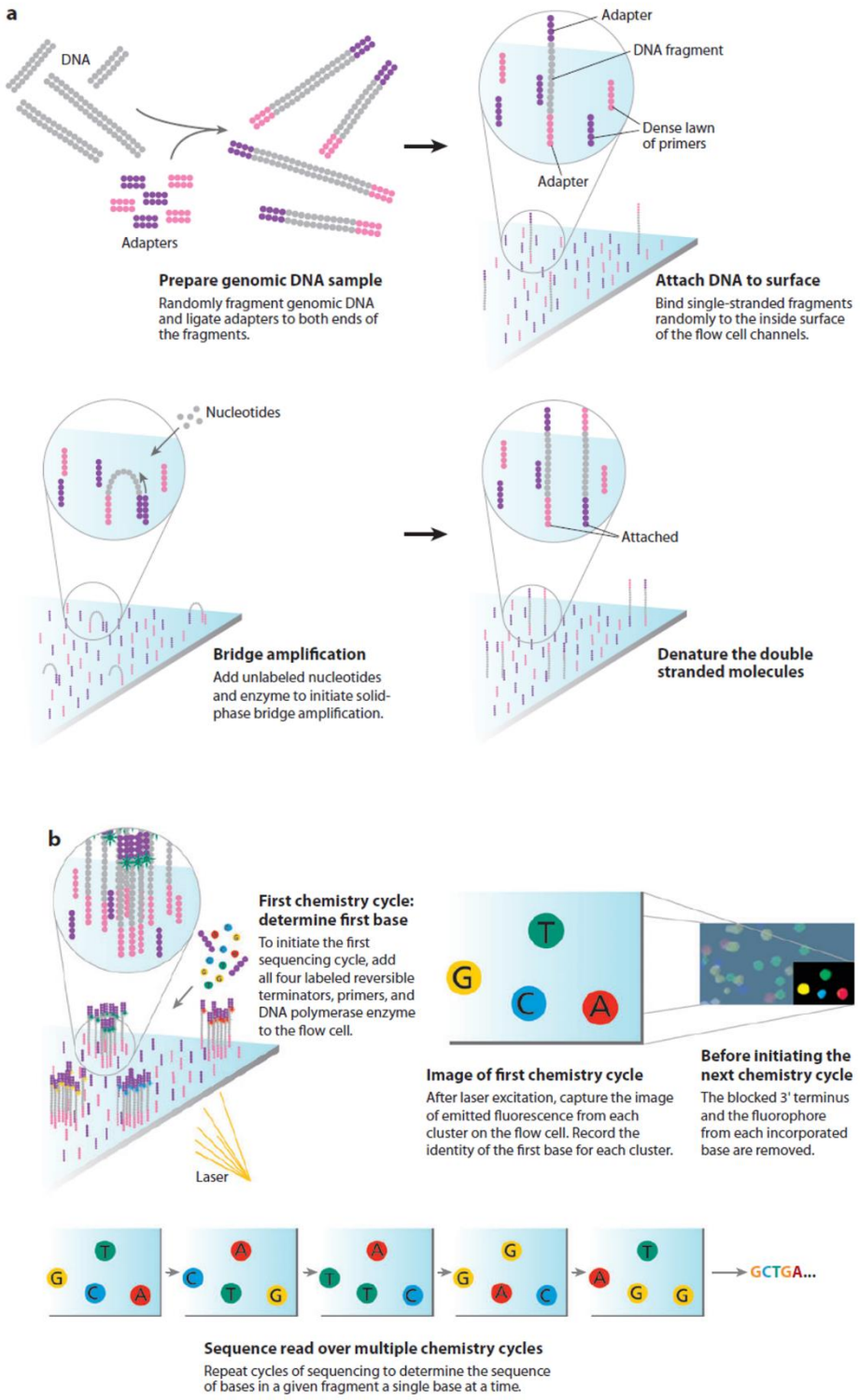

Figure 1.1 Overview of Illumina® sequencing-by-synthesis from Mardis (2008), from (a) preparation of library samples and cluster generation, to (b) imaging of the flow cell to determine base identities.

However, one of the biggest issues currently with NGS technologies (and previous community fingerprinting techniques) is that they are reliant on PCR, and therefore only represent the community that can be amplified (Mardis 2017). New third generation methods, such as the portable sequencer developed by Oxford Nanopore, are capable of directly sequencing complex genomes with minimal sample preparation, so eliminate enzyme-dependent amplification (Branton *et al*. 2008; Lu *et al*. 2016; Mardis 2017).

It is important to remember that the current NGS tools represent a snapshot in time. Especially within this field, technologies are constantly evolving and advancing with improvements in throughput (e.g. Illumina® HiSeq; Liu *et al.* 2012), and resolution from long-read single-molecule sequencing (SMS) platforms.

1.2.4 Computational analysis

The popularity of gene marker analysis coupled with improving NGS techniques and efficiency have led to a surge in the number and size of sequencing datasets published every year (Callahan *et al*. 2017). But disentangling biological variation from amplicon sequencing errors presents unique challenges in these increasingly large datasets (Kulkarni & Frommolt 2017), and algorithms designed to detect and correct (or exclude) critical errors as part of the downstream analysis of sequencing data (Luo *et al*. 2012).

One common way errors can be mitigated is by quality filtering and constructing closed reference Operational Taxonomic Units (OTUs) to partition sequencing data into bacterial taxonomy. In 16S metagenomics, the preferred method is to group sequences based on a fixed similarity threshold of ≥97% (Schloss *et al.* 2009; Caporaso *et al.* 2010; Edgar 2013), which was initially proposed by Stackebrandt & Goebel (1994), based on their findings that most bacterial strains had 97% 16S rRNA sequence similarity.

Closed reference OTU methods such as this are currently the standard practice for processing sequencing data, but the cut-offs used are gene-specific and often arbitrary (Callahan *et al*. 2017). Newer bioinformatics methods are starting to move to more flexible approach that takes inherent sequence differences into account, e.g. resolving amplicon sequencing data into amplicon sequence variants based on 100% sequence identity (ASVs).

ASV methods do not group sequences by thresholds, and instead identify biological sequences in the sample based partly on the assumption that real data will be observed

more than amplification and sequencing errors (Eren *et al*. 2013, 2015; Tikhonov *et al*. 2015; Callahan *et al*. 2016). ASVs appear to show increasing sensitivity and precision when compared to OTU methods, allowing better discrimination of ecological phenomena (Eren *et al*. 2013, 2015; Callahan *et al*. 2016, 2017).

1.2.5 Bees as a model organism

In gut microbiota analysis, as with many parts of biology, model organisms are used extensively to explain host-bacteria interactions. The honey bee (*Apis mellifera*) gut in particular has been studied by a worldwide community of researchers using a variety of different approaches (Engel *et al*. 2016). Their microbiota are relatively simple and well-characterised (Engel *et al*. 2012). In addition, some of the microbiota can be cultivated *in vitro*, hosts are easy to manage and rapidly reproduce, and their social behaviour allows for comparison with more complex mammalian systems (Koch *et al*. 2013; Kwong *et al*. 2014; Pernice *et al*. 2014). Consequently, they have provided major insights into understanding host metabolism, immune system functions, and host-pathogen interactions.

The majority of studies have focussed on the honey bee, while comprehensive studies of the microbiota of wild pollinators (e.g. solitary bees and bumblebees) are rare (Engel *et al*. 2016). Bumblebees are important wild, and managed pollinators, with species (e.g. *B. terrestris*) commercially bred for high value crop pollination (Garibaldi *et al*. 2014; Velthuis *et al*. 2006). As such, they bridge the gap between wild and managed agricultural pollinators, representing an important and overlooked area missing from existing honey bee studies. Like honey bees, bumblebees exhibit eusocial behaviours but have an annual lifecycle, allowing researchers to study bacterial population bottlenecks (e.g. during queen diapause) and the effects of microbiota turnover within the host (Martinson *et al*. 2011).

1.3 BOMBUS TERRESTRIS

1.3.1 Ecological and economic importance

Bombus terrestris (the buff-tailed bumblebee) is a major European pollinator, and is managed by commercial companies for agricultural pollination services to supplement wild pollinator populations (Velthuis *et al*. 2006).

Companies such as Biobest Group (Westerlo, Belgium), Koppert Biological Systems (Koppert B.V., the Netherlands), Global Horticulture (Beamsville, Ontario, CA), and Biobees Ltd. (New Zealand) have production facilities for bumblebee rearing, supplying Europe, Asia, North and South America, as well as Australasia.

Due to the many ecological, agronomic and economic benefits that they offer compared to honey bees, the use of bumblebees for agricultural pollination has strongly increased worldwide. They are highly efficient at pollinating fruiting trees in early spring when it is too cold for honey bees (Heinrich 1979), as well as crops that require 'buzz' pollination (sonication of the anthers to release pollen), such as cranberry, blueberry and tomato (Kearns & Inouye 1997). One of the major agricultural crops pollinated by bumblebees is the greenhouse tomato (*Lycopersicon esculentum*; Kevan *et al.* 1991; Morandin *et al.* 2001), due to their ability to function in the humid greenhouse conditions. As a result, tomato greenhouse growers make up approximately 95% of global bumblebee sales (Velthuis *et al*. 2006).

1.3.2 Life cycle and behaviour

Bombus terrestris are widely distributed, occurring in colonies of up to 350 workers headed by a single queen (Alford 1975). It is a holometabolous species, passing through four defined developmental stages prior to maturity (egg, larva, pupa, adult). Typically, development takes four to five weeks to pass from egg to adulthood, and throughout this period the larvae will shed its cuticle via ecdysis and pass through four instars (Alford 1975).

In temperate climates *B. terrestris* most commonly has an annual lifecycle, although in some habitats (e.g. urban; Stelzer *et al.* 2010) two colony cycles may be observed. Unlike honey bees where the whole colony hibernates, in bumblebees only mated queens overwinter (diapause) and emerge in early spring to establish new colonies (Alford 1975).

Individuals in an established *B. terrestris* colony can be grouped by the role they hold within the colony, or their caste (i.e. worker, male, and queen). Phenotypic differences between castes occur early in life during development, relating to genetics and diet (Kapheim *et al*. 2015). As developing larvae, queens receive additional nutrients to other castes and once they have matured and established their own colony, remain within the nest

to devote themselves to egg production. Males perform limited brood care in early life (Cameron 1985) before permanently leaving the nest and attempting to mate, while workers perform the vast majority of colony tasks, including caring for the brood and foraging. In contrast to *A. mellifera*, which divide and switch specialised tasks based on age ('age polyethism'; O'Donnell *et al.* 2000; Yerushalmi *et al.* 2006), task allocation in bumblebees seems to be related to phenotype. Smaller bees are more likely to engage in nest tasks, such as brood care, while larger bees forage (Goulson 2010). However, these roles are not completely fixed and colony tasks can switch depending on the current needs of the colony (Jandt & Dornhaus 2009).

1.3.3 Gut anatomy

In adult bumblebees the gut can be divided into three main components shown in Fig. 1.2a, which are: the stomodeum (foregut), mesenteron (midgut) and proctodeum (hindgut) (Alford 1975). The foregut comprises the mouth, oesophagus and the crop (or honey stomach). The crop is the main vehicle by which colony food stores are transported to the hive (Anderson *et al*. 2013). It is not involved in digestion but is instead primarily for the storage of liquids (water or nectar) and expands or contracts based on transportation needs. The crop ends at the proventriculus, a muscular part of the digestive tract that serves to grind food particles before entering the midgut (Bailey 1952), which it connects to by way of a 'valve-like door' that regulates the passage of food (Swingle 1927; Alford 1975).

The midgut is the primary site for digestion and absorption of food (Swingle 1927; Alford 1975). It is lined with finger-like projections called gastric caeca that provide additional surface area for enzyme secretion and the absorption of water (and other substances). Most enzymatic digestion of food occurs at the posterior end of the midgut (ventriculus). The ventriculus is also lined with microscopic projections (microvilli) that increase surface area for nutrient absorption (Terra *et al*. 1996). It is composed of a wide tube, approximately a quarter of the length of the gut, and lined with a delicate peritrophic membrane, which is continuously shed and replaced as particles pass through. This membrane secretes digestive enzymes and acts as a protective barrier to prevent microbial attachment and mechanical damage from food molecules and toxins (Santos & Serrão 2006; Chapman *et al.* 2012).

Between the midgut and the hindgut, a mass of slender tubes branches off throughout the centre of the abdomen, called the Malpighian tubules, which are critical to osmoregulation. They function to collect waste products for processing in the hindgut prior to excretion (Swingle 1927; Alford 1975).

The hindgut is the largest component of the bumblebee gut, and is made up of the ileum and rectum (Swingle 1927; Alford 1975). Like the midgut, the ileum is lined with a semi-permeable, peritrophic membrane designed to protect the delicate epithelial cells whilst allowing absorption of nutrients. Water and salts are absorbed in the rectum, and accumulated waste products are stored before being voided (Alford 1975).

By contrast, larvae have a simple, discontinuous gut in which the foregut and hindgut are not connected until just before pupation (Fig. 1.2b). All waste produced during development is accumulated until the entire gut system is reorganised at metamorphosis, when the entire larval gut and its contents are voided as a meconium (Alford 1975).

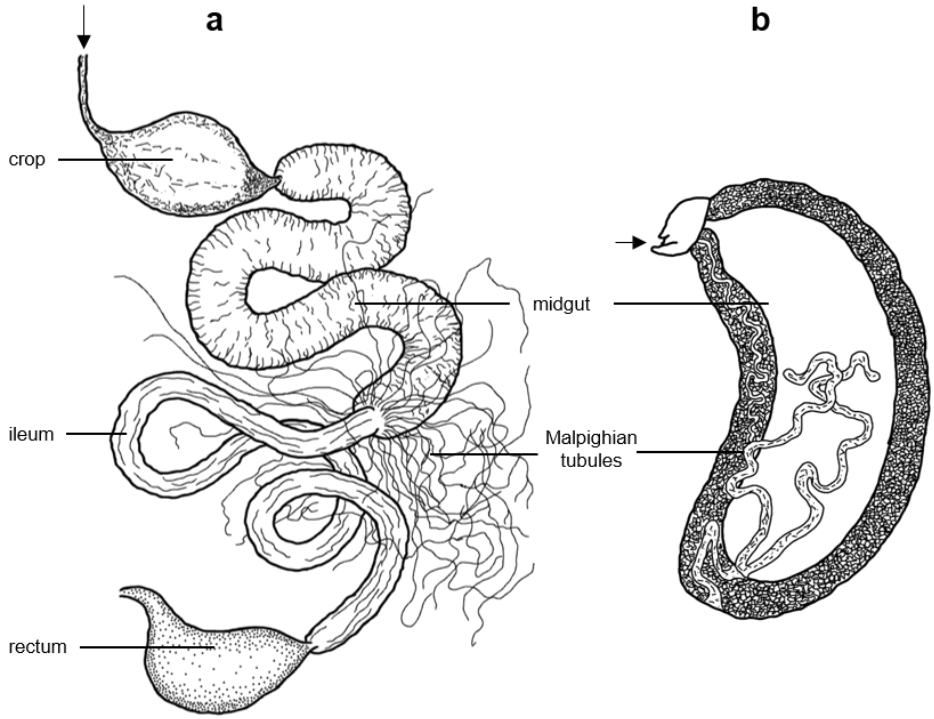

Figure 1.2 Adult (a) and larval (b) *Bombus terrestris* worker gut structures. Arrows denote start of gastrointestinal tract.

1.4 THE BUMBLEBEE MICROBIOME

1.4.1 Gut microbial diversity

The basic structure of the gastrointestinal tract is consistent across insects, though in certain taxa it is adapted for specialist feeding behaviours (Engel & Moran 2013b). As outlined in Fig. 1.3, the gut bacterial communities they contain are largely dominated by Proteobacteria (Alpha, Beta, and Gamma) as well as Bacteroidetes and Firmicutes (Colman *et al.* 2012).

Beetles and termites have higher gut microbiota diversity than that found in other insects, potentially due to their more compartmentalised guts rather than the environment or specialised diets (Dillon & Dillon 2004; Colman *et al*. 2012). Termites, in particular, have some of the best characterised gut mutualisms in the animal kingdom, as well as a wealth of information on nutrient provisioning via intracellular symbionts, e.g. recycling waste nitrogen into valuable nutrients (Engel & Moran 2013b).

Elsewhere in insects, few other species have such specific host-microbe associations, except for honey bees and bumblebees (Martinson *et al*. 2011; Koch *et al*. 2013). Typically, gut bacterial diversity in insects is much lower than that found in vertebrates (Colman *et al*. 2012; Pernice *et al*. 2014), but honey bees and bumblebees in particular harbour a uniquely restrictive gut community comprising just a handful of bacterial classes, while exhibiting high levels of strain diversity (Engel *et al*. 2012).

1.4.2 The core gut microbiota

Gut anatomy, as well as behaviour and life history traits can all influence the bacterial diversity of the bumblebee gut (Dillon and Dillon, 2004). Several studies have identified a simple, but specialised bacterial community restricted to adult honey bees and bumblebees consisting of eight core phylotypes, or nine bacterial species clusters (Mohr & Tebbe 2006; Cox-Foster *et al.* 2007; Martinson *et al.* 2011; Moran *et al.* 2012; Engel *et al.* 2012; Engel & Moran 2013a). Much of this bacterial diversity (Fig. 1.3b) is not shared with solitary bees, or found in the environment (Martinson *et al*. 2011; Koch *et al*. 2012; Cariveau *et al*. 2014), and make up the majority of the bacteria in adult workers regardless of location (95-99.9%; Kwong and Moran 2016).

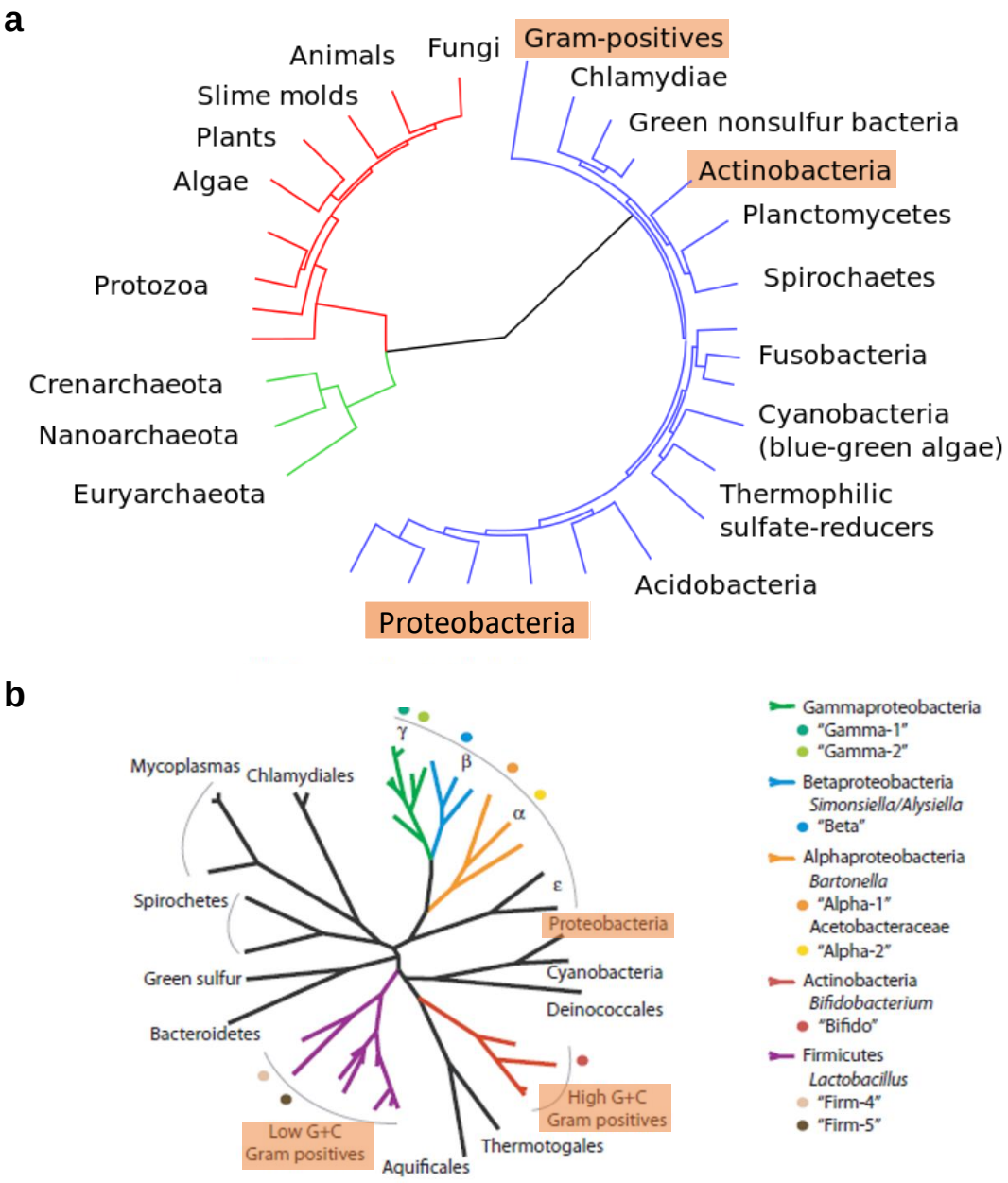

Figure 1.3 Highly resolved tree of life (a) based on Ciccarelli *et al*. (2006) showing the diversity of bacteria (blue) compared to archaea (green) and eukaryotic organisms (red). Image was generated using iTOL: Interactive Tree of Life (Letunic & Bork 2007). Bacteria present within the bumblebee and honey bee gut include Proteobacteria, Actinobacteria, and gram-positives Actinobacteria and Firmicutes, outlined in panel (b) adapted from Martinson *et al*. (2012).

However, core species are not the only bacteria present, and many non-core members contribute to the whole community composition, while also responding to external perturbation (Newbold *et al*. 2015). Some have even been implicated as potential pathogens due to their associations with colony collapse disorder in *A. mellifera* (e.g. *Bartonella apis*; Cornman *et al*. 2012; Raymann & Moran 2018), and others regularly detected include *Hafnia alvi* as well as *Enterobacter* and pathogenic *Serratia* spp. (Burritt *et al*. 2016).

Mohr & Tebbe (2006) were among the first to group bee gut core bacterial diversity into 'phylotypes' using partial sequence data extracted from guts of adults and larvae of three genera: *Apis*, *Bombus* and *Osmia*. They defined six core phyla as Alpha-, Beta-, and Gammaproteobacteria, Bacteroidetes and Firmicutes, before Martinson *et al.* (2011) further subdivided the Alpha- and Gammaproteobacteria phylotypes. Table 1.1. outlines the current accepted core microbiota proposed by Moran *et al.* (2012), of nine bacterial species clusters, only five of which have been found repeatedly in *Bombus* spp. (Martinson *et al*. 2011; Koch & Schmid-Hempel 2011a; Kwong & Moran 2013; Koch *et al*. 2013).

Despite their ubiquitous presence in honey bees and bumblebees, some bacterial species clusters exhibit host specificity (Koch and Schmid-Hempel, 2011a). Honey bees contain species that have not been found in bumblebees, including *Bartonella apis* and *Frischella perrara*, while '*Candidatus Schmidhempelia bombi*' (Martinson et al., 2014) and *Bombiscardovia coagulans* (Killer et al., 2010) have so far only been found in bumblebees. A notable example of host specificity is the genera *Gilliamella*, which has honey bee (*G. apicola*) and bumblebee-specific species (*G. bombicola*, *G. bombi*, *G. intestini*, and *G. mensalis*; (Praet et al., 2017). Other examples, including from *Lactobacillus* and *Bifidobacterium*, are outlined in Table 1.2 (Olofsson and Vásquez, 2008; Killer et al., 2009, 2011; Olofsson et al., 2014; Praet, Meeus, Cnockaert, Aerts, et al., 2015).

Taxa in the bumblebee gut also exhibit additional levels of specificity even at the strain level. For instance, *Snodgrassella alvi* isolated from *A. mellifera* cannot colonise the guts of *Bombus* spp., and vice versa (Kwong *et al.* 2014; Kwong & Moran 2015). In addition, strain level differences may even give rise to functional diversity, as identical 16S rDNA sequences may often differ at protein-coding loci resulting in different functional traits (Engel *et al.* 2012; Sabree *et al.* 2012; Kwong *et al.* 2014).

Table 1.1 Known core gut bacterial taxa present in *Apis mellifera* and *Bombus* spp. Genera highlighted (bold, underlined) are present in the nine core *A. mellifera* bacterial species clusters identified by Moran et al. (2012). These include *Bartonella apis*, *Parasaccharibacter apium*, *Snodgrassella alvi*, *Bifidobacterium* spp., *Gilliamella apicola*, *Frischella perrara*, and *Lactobacillus* spp.

Phylotype	Family	Genus	Host range	Typical abundance*	Notes	Reference
Alpha-1	Bartonellaceae	***Bartonella***	*Apis*	< 4%	Related to several ant-associated bacteria. Genus *Bartonella* also includes opportunistic intracellular parasites.	Kešnerová *et al*. (2016)
Alpha-2.1	Acetobacteriaceae	*Bombella* *Commensalibacter* *Gluconobacter* ***Parasaccharibacter***	*Apis*, *Bombus* *Apis, Bombus* *Apis* *Apis*	< 6%	Alpha-2.1 is related to *Gluconobacter* and Acetobacteriaceae, while Alpha-2.2 is related to *Saccharibacter floricola*, a bacterium associated with flowers.	Li *et al*. (2015, 2016); Yun*et al*. (2017) Kim *et al*. (2012); Li *et al*. (2015) Lambert *et al*. (1981) Corby-Harris *et al*. (2016)
Alpha-2.2		*Saccharibacter*	*Apis*			Veress *et al*. (2017)
Beta	Neisseriaceae	***Snodgrassella***	*Apis*, *Bombus*	0.6–39%	Sole species is *Snodgrassella alvi*. Cross feeding mechanism with *Gilliamella*.	Kwong & Moran (2013)
Bifido	Bifidobacteriaceae	***Bifidobacterium***	*Apis*, *Bombus*	15%	Some grow aerobically, but most are anaerobic or microaerophilic. Typical of animal gut communities, and have probiotic properties. Both *Apis*- and *Bombus* -specific species have been described.	Olofsson & Vásquez (2008) Praet *et al*. (2015) Killer *et al*. (2011)
Gamma-1	Orbaceae	***Gilliamella***	*Apis*, *Bombus*	0.6–30%	Both *Apis*- and *Bombus* -specific species of *Gilliamella* have been isolated and cultured. Cross-feeding mechanism with *S. alvi*.	Kwong & Moran (2013) Praet *et al*. (2017)
Gamma-2	Orbaceae	***Frischella***	*Apis*	13%	*Apis*-specific taxa isolated from the proventriculus of adult workers. Facultative anaerobe, but will not grow in fully aerobic conditions.	Engel *et al*. (2013)
***Lacto* Firm-4** ***Lacto* Firm-5**	Lactobacilliaceae	***Lactobacillus***	*Apis*, *Bombus*	20–99%	Like *Bifidobacterium*, *Lactobacillus spp*. are typical of animal gut communities and considered to have health benefits for the host. Both *Apis*- and *Bombus* -specific taxa have been isolated.	Olofsson et al. (2014) Killer et al. (2014a) Killer et al. (2014b) Valterova & Vlkova (2018) Praet et al. (2015b)

*In adult *Apis mellifera* worker (Moran *et al*. 2012)

1.4.3 Bacterial localisation

In common with many ecological systems, gut microbiota exhibit distinct spatial structure. As gut compartments vary in pH and nutrient availability the distribution of bacteria reflects the different niches that species are adapted to occupy (Engel & Moran 2013b). Our knowledge of gut bacteria localisation in bees stems from studies on *A. mellifera* using techniques such as fluorescence *in situ* hybridization (FISH) often combined with quantitative PCR (qPCR) to estimate the location and size of bacterial populations (Martinson *et al*. 2012). Given similarities in anatomy and diet between honey bees and bumblebees, it is likely they exhibit similar patterns of microbiota, but this has yet to be explored.

The anatomy of the bumblebee gut is outlined in Section 1.3.3, and many studies observing bacterial localisation in the *A. mellifera* gut have focussed on three distinct areas: the crop, the midgut, and the hindgut (ileum and rectum). When bacteria are ingested by the bee they first enter the crop, an extremely acidic environment, constantly being emptied and refilled, thus providing a great deal of disturbance to any taxa trying to occupy it. The crop (along with the midgut) contains the smallest populations of bacteria, since only very acidophilic bacteria can persist (Anderson *et al*. 2013). Nevertheless, a large, specialised community of lactic acid bacteria (LAB; Vásquez *et al.* 2012) survive here, as well as some bacteria found in nectar and hive materials, mostly species from Enterobacteriaceae and *Parasaccharibacter apium* (Anderson *et al*. 2013; Corby-Harris *et al*. 2014).

Similarly, few bacteria can persist in the midgut (approximately 1–4% of the total Beta, Firm-4, Firm-5, and Gamma microbiota; Martinson *et al.* 2011). Here not only do the presence of digestive enzymes make it a hostile, acidophilic environment, but the continuous shedding of the chitinous membrane (peritrophic matrix), also creates a very unstable substrate for bacterial colonisation (Terra *et al*. 1996; Kwong & Moran 2016).

The hindgut partitions into the ileum and the rectum, which differ markedly in microbial community composition. The ileum is the smaller of the two but its surface is highly folded, meaning that it contains an abundance of attachment sites (Terra *et al*. 1996). This, coupled with access to undigested nutrients make it an ideal location for bacterial colonisation (Martinson *et al*. 2012).

Table 1.2 Currently accepted nomenclature of host-specific gut bacterial taxa present in *Apis mellifera* and *Bombus* species.

	Family	Genus	Species	Reference
***Apis mellifera*-specific bacterial taxa**	Acetobacteriaceae	*Bombella*	*B. apis*	Yun *et al.* (2017)
		Parasaccharibacter	*P. apium*	Corby-Harris *et al.* (2016)
	Bartonellaceae	*Bartonella*	*B. apis*	Kešnerová *et al.* (2016)
	Bifidobacteriaceae	*Bifidobacterium*	*B. asteroides*	Olofsson & Vásquez (2008)
			B. coryneforme	
	Orbaceae	*Gilliamella*	*G. apicola*	Kwong & Moran (2013)
		Frischella	*F. perrara*	Engel *et al.* (2013)
	Lactobacillaceae	*Lactobacillus*	*L. apinorum*	Olofsson *et al.* (2014)
			L. mellifer	
			L. mellis	
			L. melliventris	
			L. kimbladii	
			L. helsingborgensis	
			L. kullabergensis	
			L. apis	Killer *et al.* (2014a)
***Bombus*-specific bacterial taxa**	Acetobacteriaceae	*Bombella*	*B. intestini*	Li *et al.* (2015, 2016)
	Bifidobacteriaceae	*Bifidobacterium*	*B. commune*	Praet *et al.* (2015a)
			B. actinocoloniiforme	Killer *et al.* (2011)
			B. bohemicum	
		Bombiscardovia	*B. coagulans*	Killer *et al.* (2010)
	Orbaceae	*Gilliamella*	*G. bombi*	Praet *et al.* (2017)
			G. bombicola	
			G. intestini	
			G. mensalis	
		'Candidatus Schmidhempelia'	*Ca. S. bombi*	Martinson *et al.* (2014)
	Lactobacillaceae	*Lactobacillus*	*L. bombi*	Killer *et al.* (2014b)
			L. bombicola	Praet *et al.* (2015b)

FISH microscopy techniques in the ileum have highlighted the importance of attachment and spatial organisation in the gut microbiota. Here *S. alvi* attaches directly to the gut wall, providing a layer above for *Gilliamella* species to adhere, resulting in a biofilm, or layer of bacterial species (Martinson *et al*. 2012; Powell *et al*. 2014).

The distal hindgut region (the rectum), is a relatively stable and nutrient-rich environment where the majority of the adult gut microbiota is located (Martinson *et al*. 2012). Here waste is stored before defecation, attracting a community of fermentative bacterial species, such as the *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, and *Bifidobacterium asteroides* (host-specific to *A. mellifera*). The geography of bacterial localisation in the worker gut is becoming clearer but the underlying mechanisms of colonisation are unknown. Through recent advances in metagenomics, we now know that some of the core taxa contain functional genes relating to cell adhesion and biofilm formation. However, more study is needed to understand these mechanisms (Engel *et al*. 2012; Kwong *et al*. 2014).

1.4.4 Differences in caste microbiota

Current understanding of the gut microbiota in bumblebees is based primarily on studies of mature workers; little is known about the microbial diversity within other members of the colony, such as reproductives or larvae. As discussed in Section 1.3.2, differences in gut morphology as well as behavioural, physiological and nutritional requirements between all members of a colony are likely to influence the gut microbiota (Kapheim *et al*. 2015).

However, little work on caste gut microbiota exists for bumblebees. Parmentier *et al.* (2018) provided limited data from a single *B. pascuorum* colony comparing adult workers and larvae, with the maternal queen. While a comprehensive investigation by Tarpy *et al.* (2015) on *A. mellifera*, observed the development of microbiota throughout the queen-rearing process. Although similar to workers, queen guts were much more variable between individuals and were dominated by Alphaproteobacteria phylotypes, potentially as queen larvae are fed from worker hypopharyngeal glands, which are associated with Alphaproteobacteria (Tarpy *et al*. 2015). While interesting for *A. mellifera*, it is currently unknown whether this finding is applicable to bumblebees, who do not perform trophallactic feeding (mouth-to-mouth transfer of food). New bumblebee colonies are

established annually by a single queen, likely putting the microbial community through a genetic bottleneck (Kwong *et al*. 2014). Microbiota retained from the queen through diapause and foraging during colony foundation is the primary source of microbiota early in colony development as environmental and horizontal transmission occurs when the first generation of workers leaves the nest to forage (Koch *et al*. 2013).

We know even less about male bumblebee gut microbiota. Investigations in *A. mellifera* suggest that though they contain much of the same bacteria as workers, they again differ significantly in relative proportions of certain taxa – much like queens, and again there is a need for a better understanding of male bumblebee gut microbial diversity and function (Kapheim *et al*. 2015). Future investigations should seek to examine the gut bacteria of reproductives and how they contribute to and are influenced by the whole colony microbiota.

1.4.5 Larval microbiota

Investigations into the gut microbiota within larvae have produced little and inconsistent information (Vojvodic *et al*. 2013). During bee metamorphosis the gut goes through extreme remodelling which is thought to severely disrupt or eliminate bacterial populations (Moll *et al*. 2001; Hakim *et al*. 2010). Antimicrobial peptides (AMPs) are produced during pupation (Russell & Dunn 1996), which led many culture-dependent studies to propose that the intestinal tract is sterile on emergence (Gilliam 1971; Gilliam & Prest 1987), and that bacteria recolonise the gut when exposed to the colony environment. These studies however, failed to take into account that not all bacteria can be cultured (Staley & Konopka 1985).

Martinson *et al.* (2012) found that *A. mellifera* larvae contained little if any bacteria, apart from some Alphaproteobacteria. However, they based their conclusions on localisation of just the Gamma-1 (*Gilliamella*), Beta (*Snodgrassella*), and Firm-5 (*Lactobacillus*) phylotypes. More robust sampling of *Apis*, *Bombus* and *Osmia* species found that late larval instars contained a diverse gut microbiota (Mohr & Tebbe 2006), and even detected small bacterial counts in pupae of *A. mellifera* (Hroncova *et al*. 2015).

Vojvodic *et al.* (2013) showed that the gut microbiota in *A. mellifera* larvae does not significantly differ from that found in adults. In adults, these core bacteria are mostly

associated with nutrient processing (*reviewed in* Kwong & Moran 2016), but could exhibit protective functions in the larvae (Vojvodic *et al*. 2013), who have reduced immune function and are often the target of disease (Wilson-Rich *et al*. 2009; Forsgren *et al*. 2010; Mattila *et al*. 2012). In contrast, recent work in bumblebees has identified distinctive differences between adult and larval gut communities (Parmentier *et al*. 2018). Larval gut communities appear to be dominated by Enterobacteriaceae and Lactobacillaceae, as well as low relative abundances of the core adult taxa, Neisseriaceae (*Snodgrassella*) and Orbaceae (*Gilliamella*). Given how important they are to the adult gut, their low abundance in larvae is significant, suggesting that the contrasting gut communities provide differing functional roles.

Clearly, there is a need for greater understanding of the gut microbiota of different developing stages in the bumblebee colony, in both natural and controlled (laboratory) conditions. The application of NGS techniques coupled with quantitative abundance data of bacterial counts could help to unravel this currently inconsistent area of research.

1.5 ACQUISITION AND TRANSMISSION OF GUT MICROBIOTA

1.5.1 Bacterial colonisation

Establishing a stable microbial community is critical for health and development (Sommer & Bäckhed 2013), so maintaining routes of beneficial microbiota acquisition and transmission is highly important. This is challenging for insect taxa that do not perform brood care (Royle *et al*. 2012), but eusocial species, such as bumblebees, have multiple potential pathways by which gut symbionts may be transmitted and acquired (Fig. 1.4).

Studies in *A. mellifera* using marked cohorts of workers have helped to characterise a timeline of bacterial development (Martinson *et al*. 2012; Powell *et al*. 2014). On emergence, the gut microbiota comprises small, fluctuating populations originating from the environment. They are not spatially structured through the gut regions but begin to increase in size in the initial few days following eclosion and increased exposure to the colony. By four to six days after emergence, core communities are present, and bacteria continue to be acquired during the worker lifetime (measured to day 30) (Powell *et al*. 2014).

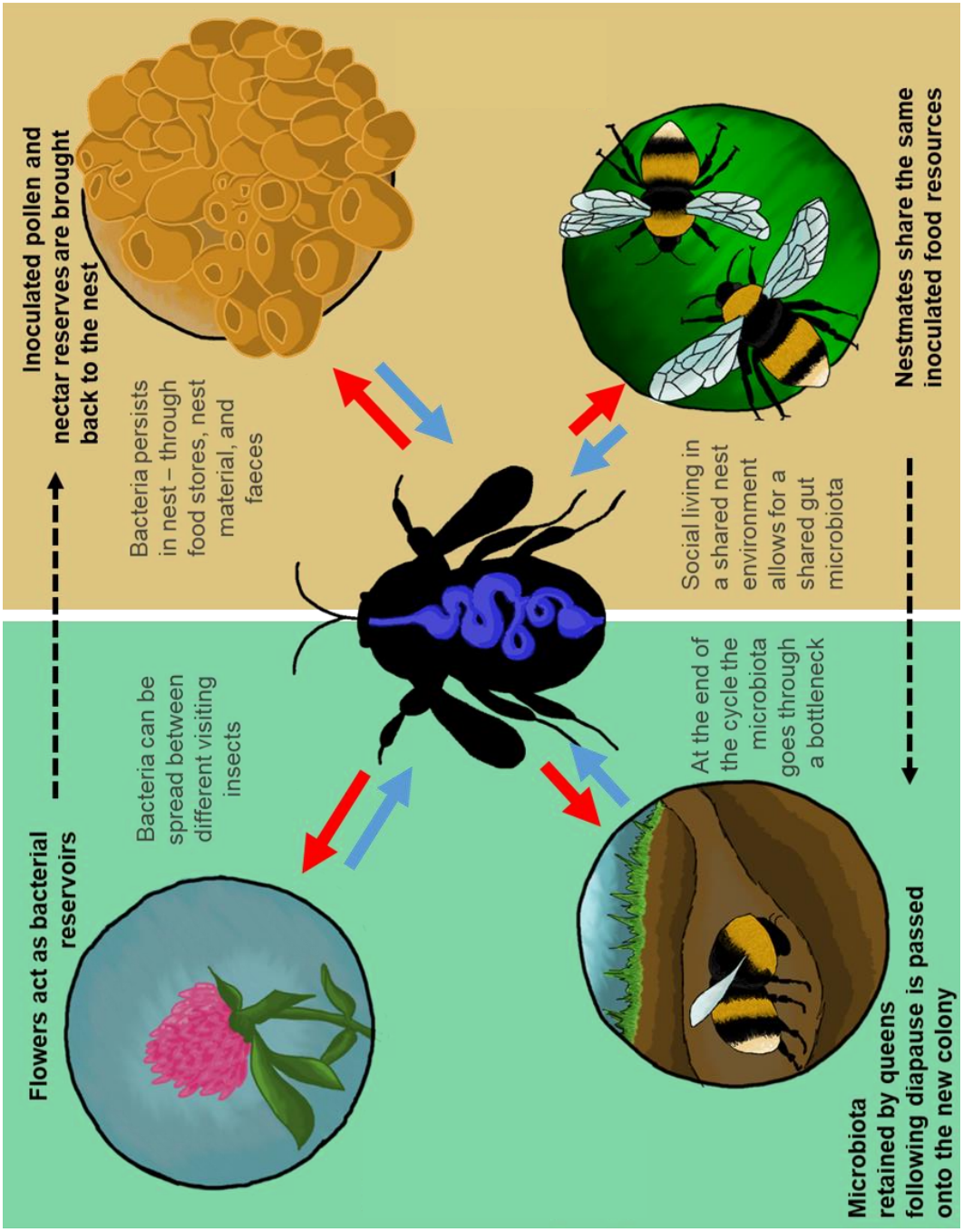

Figure 1.4 Proposed routes of gut microbiota transmission (red arrows) and acquisition (blue arrows) in bumblebees.

1.5.2 Social transmission

Sociality in bumblebees, along with communal living and shared food resources allows ample opportunity for horizontal (between workers) and vertical (maternal to offspring) transmission of gut bacteria (Billiet *et al*. 2017). We know little about the degree to which vertical and horizontal transmission influences community assembly within the *Bombus* gut, but sociality appears to be central to transmission of gut microbiota (Kwong & Moran 2016).

Experiments by Koch & Schmid-Hempel (2011b) showed that ingesting faeces from nest mates led to the establishment of microbiota comparable to that observed in healthy wild type bumblebees. Those without contact with the faeces of their nestmates, or that were fed cultured bacteria lacked this typical microbiota. Limited exposure to nestmates and hive material during adulthood in *B. terrestris* was found to reduce the relative abundance of Lactobacillaceae and Bifidobacteriaceae species (Billiet *et al*. 2017). But, there was no change in *Snodgrassella* (Neisseriaceae) and *Gilliamella* (Orbaceae) populations, suggesting these core taxa are associated with their host from early development and are not reliant on social behaviours to proliferate in the gut (Engel *et al*. 2012; Billiet *et al*. 2017).

Exposure to the social nest environment is also a key part of microbial acquisition and transmission. In *A. mellifera* colonies, Alpha-2 phylotypes commonly found in the crop are also present in honey and beebread (Anderson *et al*. 2013), acting as potential bacterial reservoirs. When honey bee pupae are removed from this environment and kept in sterile conditions they do not acquire typical microbial communities (Kwong *et al*. 2014; Powell *et al*. 2014), and even when partially exposed to social living (oral trophallaxis and hive material) workers develop uncharacteristic communities of non-core bacteria.

Communal living, trophallaxis between nestmates, and mother-offspring contact all appear to be a key determinant of symbiont acquisition in *A. mellifera* (Powell *et al*. 2014). Yet, in *Bombus* spp., foragers store the contents of their crop themselves and do not carry out trophallaxis to nestmates or larvae (Dornhaus & Chittka 2005), but still harbour similar gut bacterial taxa (Martinson *et al*. 2011). Clearly, behaviours such as trophallaxis are not always key for bacterial inoculation, but other aspects of communal living are. Therefore, it is important to understand what factors influence social transmission in bumblebees.

1.5.3 Environmental transmission

While sociality appears to be a key driver in gut microbiota assembly, other factors such as caste, behaviour, diet, and environment play a contributory role in shaping bacterial communities in other bee species (McFrederick *et al*. 2014). The persistence of bacterial communities in *Megalopta* (sweat bees), who exhibit both social and solitary behaviour, is promoted by environmental transmission (McFrederick *et al*. 2014). In wholly eusocial bees, like honey bees and bumblebees, exposure to different foraging environments does appear to alter the gut community of workers, though it mostly affects the rarer, transient members of the community (Newbold *et al*. 2015). Therefore, it is imperative to examine both the core (common) and rare bacterial taxa.

Foraging exposes bees to a multitude of bacterial populations that are associated with plants, via pollen, nectar, and the plant surface (phyllosphere), including acidophilic *Lactobacillus kunkeei*, a major fructophilic LAB (McFrederick *et al*. 2012; Anderson *et al*. 2013). Floral sources of bacteria are often shared by multiple species of pollinators (Campbell 1985), and pollinator exposure can itself change the chemical profile of nectar (e.g. nectar pH, sucrose-fructose balance; Aizenberg-Gershtein *et al.* 2013), thereby influencing the species that can grow. It is likely that resource sharing by closely related pollinators increases the probability of transfer bacterial symbionts and may explain the ubiquitous presence of *Gilliamella* and *Snodgrassella* in honey bee and bumblebee guts. Common gut bacterial phylotypes have also been identified in the guts of other insects, suggesting that floral resources could act as bacterial reservoirs (Moran *et al*. 2012).

Laboratory studies cannot mimic the complexity and constantly changing environment faced by wild foraging colonies (Newbold *et al*. 2015). Floral species not only grow and flower at different times in the season, but change daily in terms of floral resource quality (Prys-Jones & Corbet 1987). Koch *et al.* (2012) found that as the colony growth begins to slow in late summer there is a decline in bacterial diversity (visible in the last few weeks), possibly a result of symbionts acquired from the environment outcompeting core taxa. Similarly, in *A. mellifera* apiaries, changes in gut microbiota composition were observed throughout a season, highlighting the influence of changes in forage resources and diet; communities were more stable during the winter (Ludvigsen *et al*. 2015).

Table 1.2 Characteristics of the core gut bacterial taxa present in *Apis mellifera* and *Bombus* spp. including their inferred metabolism, substrates, products, and their potential functions within the host. Adapted from Bonilla-Rosso & Engel (2018). "n.d." is not determined.

Phylotype	Family	Genus	Host range	Gut region	Inferred metabolism						Substrates	Products	Function(s)
					E[a]	Cit[b]	Gly[c]	AA	Nuc	Vit			
Alpha-1	Bartonellaceae	*Bartonella*	*Apis*	n.d.	A	+	+	+	RY	+	Orotate, Quinic acid	n.d.	Possible positive effect on disease resistance.
Alpha-2.1	Acetobacteriaceae	*Bombella*	Bombus	Midgut	A	+	+	+	RY	+	n.d.	n.d.	n.d.
		Commensalibacter	*Apis* , *Bombus*	n.d.	U	U	U	U	U	U	n.d.	n.d.	Queen-specific (?)
		Gluconobacter	*Apis*	n.d.	U	U	U	U	U	U	n.d.	n.d.	n.d.
		Parasaccharibacter	*Apis*	Hive, Crop	A	+	+	+	RY	+	n.d.	n.d.	Possible positive effect on disease resistance.
Alpha-2.2		*Saccharibacter*	*Apis*	n.d.	U	U	U	U	U	U	n.d.	n.d.	Environmental (?)
Beta	Neisseriaceae	*Snodgrassella*	*Apis* , *Bombus*	Ileum	A	+	-	+	RY	+	Carboxylic acid, Orotate, Furoate	Kynurenic acid	Oxygen consumption in the ileum, biofilm formation.
Bifido	Bifidobacteriaceae	*Bifidobacterium*	*Apis* , *Bombus*	Rectum	F	-	+	+	RY	-	Flavonoid glycoside, Nucleosides, ω-hydroxy acids	Prostaglandins, Juvenile hormone derivatives	Triggers production of host-derived prostaglandins and juvenile hormone derivatives.
Gamma-1	Orbaceae	*Gilliamella*	*Apis* , *Bombus*	Ileum	F	-	+	+	R	+	Nucleosides, Quinic acid, Sugars	Organic acids, Aromatic compound degradation, Intermediates	Pectin degradation, cross-feeding with *S. alvi* , biofilm formation, metabolism of toxic sugars.
Gamma-2	Orbaceae	*Frischella*	*Apis*	Pylorus	F	-	+	+	R	+	Citrate, Nucleosides, Fuorate	Kynurenic acid	Triggers local immune response to induce scab phenotype. Results in deposition of melanin on the epithelium's cuticle lining.
***Lacto* Firm-4**	Lactobacilliaceae	*Lactobacillus*	*Apis* , *Bombus*	Rectum	F	-	+	-	Y	-	Flavonoid glycosides, Nucleosides, ω-hydroxy acids, Sugars	Aromatic compound degradation, Intermediates	Fermentation of waste pollen exines in the rectum. Possible metabolite transfer to host.
***Lacto* Firm-5**	Lactobacilliaceae	*Lactobacillus*	*Apis* , *Bombus*	Rectum	F	-	+	-	Y	-	Flavonoid glycosides, Nucleosides, Quinic acid, Citrate, Phenolamides, Sugars	Organic acids, Aromatic compound degradation, ω-hydroxy acids	

[a] Metabolism for energy production (A)erobic respiration, (F)ermentation, (U)nknown

[b] Citrate cycle: (+) pathway complete, (-) pathway incomplete

[c] Glycolysis: (+) pathway complete, (-) pathway incomplete

[d] Amino acids: most biosynthetic pathways present (+), or absent (-)

[e] Nucleosides: most genes present for pu(R)ine or p(Y)rimidine biosynthesis

[f] Vitamins: (+) most genes for B2, B3, B5, B6, B9, B12 biosynthesis present, or absent (-)

A substantial proportion of research on honey bee and bumblebee gut microbiota comes from studies of hosts reared in laboratories or highly managed settings. The microbiotas of indoor-reared *B. terrestris* are a subset of those found in wild counterparts, and often come from colonies that have had little or no exposure to natural environments for multiple generations (Meeus *et al*. 2015). Such approaches are useful in exploring interactions between core taxa, while controlling for biological variation (Meeus *et al*. 2015). However, more work into microbial transmission in naturalistic settings is needed to understand the context of observed effects and relate these findings to wild populations.

1.6 FUNCTIONS OF THE MICROBIOTA

1.6.1 Nutrition

In nature, pollen and nectar collected from flowers are the primary constituents of the bumblebee diet. In order to supplement their nutrient-poor diet, hosts are assisted in nutrient acquisition by commensal symbionts in the gut (Engel & Moran 2013b).

As outlined in Table 1.3, genomic data from honey bee gut microbiota has revealed potential functional roles, which are likely serving a similar purpose in bumblebees (Kwong & Moran 2016; Kešnerová *et al.* 2017; Bonilla-Rosso & Engel 2018). Many of these bacteria are suitably adapted to thrive in the bee gut and contain genes encoding carbohydrate-related functions, such as fermentation, as shown in Fig. 1.5 (*reviewed in* Kwong & Moran 2016). *Gilliamella* in particular, is able to metabolise carbohydrates, including toxic sugar strains (Zheng *et al.* 2016), as well as encode enzymes to digest the structural sugar, pectin. Breaking down pectin is critical to digestion of pollen into monosaccharides (Engel *et al*. 2012), which the host cannot do itself (Meeus *et al*. 2013).

S. alvi instead has a different metabolic niche to the other core bacteria. It encodes an alternative citrate cycle and has genes for carboxylate transport, but contains none of the genes for glycolysis (Bonilla-Rosso & Engel 2018). The diversification of metabolic niches appears to be crucial in allowing these bacteria to coexist in the gut. In fact, *Gilliamella* and *Snodgrassella* exhibit a syntrophic interaction (or cross-feeding) in which fermentation by *Gilliamella* produces lactic acid, acetate, and formate, which are all oxidised by *S. alvi* (Kwong *et al*. 2014).

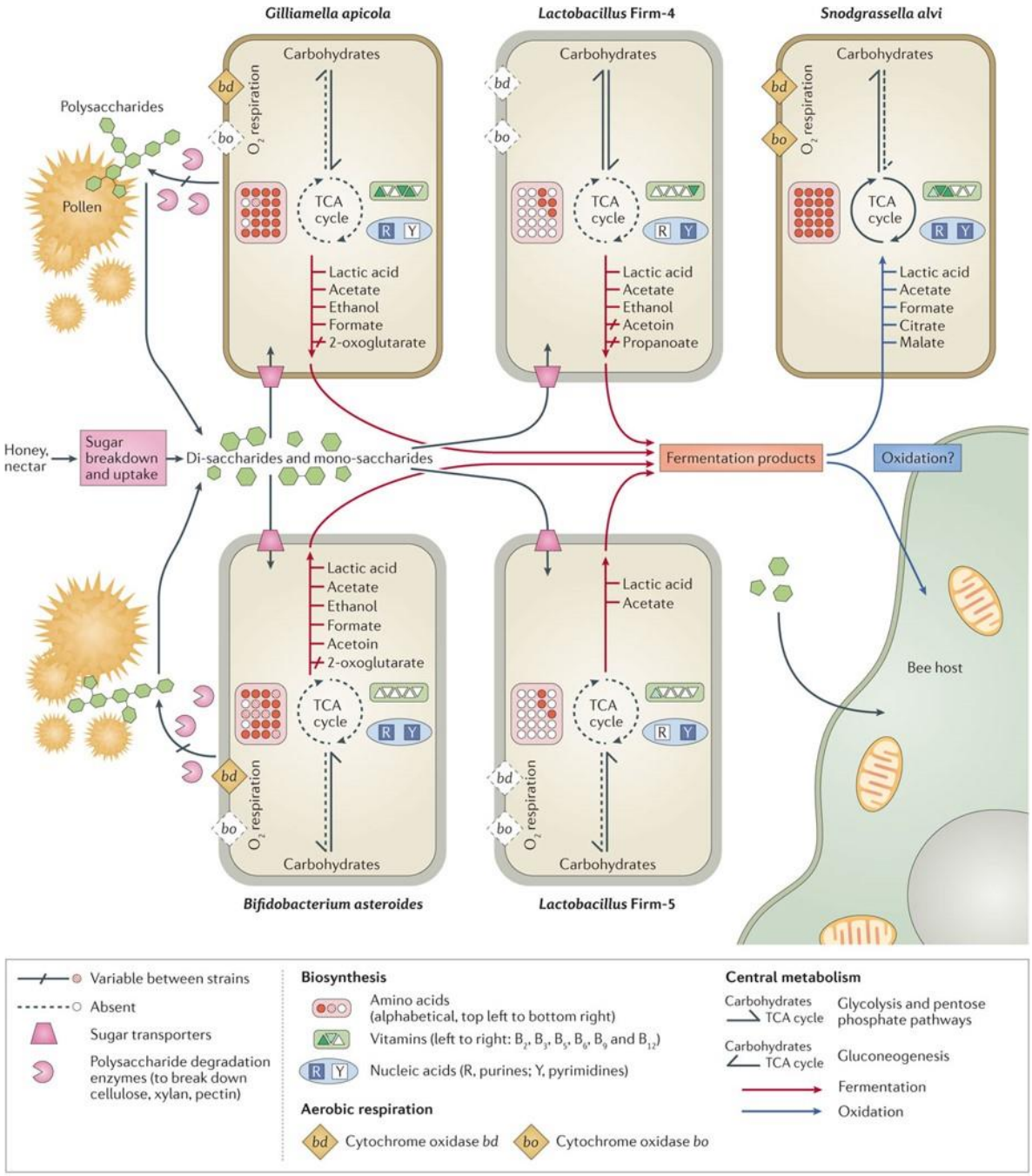

Figure 1.5 Summary of metabolic functions of key bacterial taxa inferred from genomic, metagenomic and metatranscriptomic studies as well as experiments with cultured bacterial strains (from Kwong & Moran 2016). N.B. *Bifidobacterium asteroides* is specific to *Apis mellifera*.

An interesting aspect of *S. alvi* is its localisation to the gut wall of the ileum (Section 1.4.2), where oxygen concentrations in the guts peak (Egert et al., 2005; Brune, 2014). *S. alvi* grows optimally under microaerophilic conditions oxidising carboxylates to produce energy (Kwong & Moran 2013; Kwong *et al*. 2014). Here it produces an oxygen gradient in the ileum, making conditions more favourable for the fermentative taxa such as, *Gilliamella* and *Lactobacillus* (Zheng *et al*. 2017)

It is thought that the resulting products produced by these reactions are potentially absorbed by the host, since the presence of core microbiota in the honey bee worker gut has been associated with faster host weight gain (Zheng *et al*. 2017). Gaining adequate nutrition is of paramount importance to host health as poor diets and starvation are associated with times of stress in colonies often lead to pathogen outbreak, symptomatic of decreasing immune response and host fitness (Evans & Schwarz 2011; Brunner *et al*. 2014). Therefore, it is imperative to understand the role of these integral organisms under different environmental exposure and foraging pressures.

1.6.2 Defence and immunity

The gut environment, while an interface for nutrition acquisition, is also a site of immune reaction. In humans, the balance of microbiota is crucially important for health and fitness of the host and any disturbance is associated with disease (Lozupone *et al*. 2012). The same may be true for bumblebees, where damage to gut microbiota has implicated the presence of invading pathogens (Vásquez & Olofsson 2009).

The most notable example of the protective function is from experiments on germ-free bumblebee workers. Koch & Schmid-Hempel (2011b) reared workers without gut microbiota and challenged them with the trypanosome parasite, *Crithidia bombi*, and found that they were much more susceptible to infection than workers with an intact gut microbiota. A negative correlation between the presence of *Gilliamella* spp. and *C. bombi* furthered this work, supporting the protective role that core bacterial species play in the host (Cariveau *et al*. 2014). Culture-based studies have also hinted towards the inhibitory affect that some *Lactobacillus* and *Bifidobacterium* spp. have on other microorganisms (Forsgren *et al*. 2010; Vásquez *et al*. 2012; Butler *et al*. 2013; Killer *et al*. 2014).

To protect the host, gut microbiota can also trigger immune system responses, and induce AMPs in the haemolymph (Casteels *et al*. 1989; Casteels *et al*. 1990; Casteels *et al*. 1993; Danihlík *et al*. 2015). Kwong *et al.* (2017) also demonstrated that non-pathogenic microbiota can affect AMP abundance in the haemolymph, indicating that this is a systemic (whole organism) immune effect, rather than a local one, to help prime hosts against pathogens.

The composition and effectiveness of the microbiota is a critical factor in the fight against infection. In response to pathogens, both the host and its constituent microbiota adapt and can evolve together, resulting in changes to the phenotype and overall fitness of the host (the hologenome theory; Zilber-Rosenberg & Rosenberg 2008). Therefore, to truly understand the impacts of disease on host gut microbiota will involve switching from studying the impacts of individual pathogens to a more holistic approach that considers the whole gut community, and its host (Schwarz *et al*. 2015).

1.6.3 Detoxification

Parasites and disease are not the only threat to pollinator species. Bumblebees, as important pollinators of agricultural crops, have increased risk of exposure to pesticide crop treatments, despite being a non-target organism. They are generalist pollinators, and collect floral resources from a variety of sources, potentially being exposed to a multitude of detrimental chemicals in the field that could impact the immune function of the host and its microbiota (Evans & Schwarz 2011).

There is now a growing appreciation of the damaging effects of broad-spectrum and systemic pesticides on pollinator health and efficiency. Neurotoxic neonicotinoid pesticides are some of the most common pest control chemicals in use globally. They have been linked to deficiencies in learning and motor functions (Decourtye *et al*. 2004), colony growth and queen production (Whitehorn *et al*. 2012) as well as overall fitness (Rundlöf *et al*. 2015; Woodcock *et al*. 2017).

Currently, there are no studies on the action of pesticides on the gut microbiota in *Bombus* spp., but recently published work by Raymann *et al.* (2018) found little effect of a class of neonicotinoids (imidacloprid), on the microbiota of *A. mellifera*, even while host mortality increased. These findings corroborated work in adult *D. melanogaster*, which

found that imidacloprid exposure resulted in no significant change to the bacterial community (Daisley *et al*. 2017). Although imidacloprid exposure resulted in an increase in honey bee mortality this was not associated with microbiome dysbiosis. Rather, exposed bees were found to be more susceptible to *Serratia* infection, possibly caused by down regulation of the immune system by the pesticide (Brandt *et al*. 2016).

Other biological control organisms, such as *Bacillus thuringiensis* (*Bt*), target the insect digestive system directly and disrupt it by producing spores. Babendreier *et al.* (2007) hypothesised that the gut microbiota of honey bees could act as an indicator of pesticide exposure and tested the effects of insecticides (including *Bt*-maize pollen) on bacterial species abundance. They found no significant impact on bacterial communities until they administered a lethal dose, suggesting that the gut community may be able to tolerate exposure to sub lethal insecticides and is not as sensitive as previously thought. Contradictory findings were found in gypsy moth larvae exposed to *B. thuringiensis*, where the insecticide was only effective in larvae with intact midgut bacteria (Broderick *et al*. 2006). The relationship between the insecticide and the gut microbiota needs to be explored further and has important ramifications for novel and more critically, targeted pest control systems.

Understanding the impact of pesticides (and pathogens) on these gut communities requires further insight into the structure and particularly the function of these bacteria, to better assess how fitness may be affected, including additional work on the long-term impacts of exposure. A hologenomic approach (Section 1.6.2), examining the host and its constituent microbiome would be beneficial to underpin the functional changes that can be inferred from changes to the gut microbial community.

1.7 RESEARCH AIMS AND APPROACH

Social insects, such as *B. terrestris*, are emerging as powerful model systems for gut microbiota research: their microbiota is simple, well characterised, and their social behaviour allows for comparison to more complex systems. Declines in pollinator populations have also encouraged research into the assembly, diversity and function in gut microbiota in response to pathogens and disease. Considerable research has been conducted

in the closely related species *A. mellifera*. However, differences in life history and social behaviour between honey bees and bumblebees limits the extent to which these findings can be generalised. This makes it essential to investigate specifically the gut communities of bumblebees and the potential factors that may influence them, including how behavioural, physiological, nutritional and environmental differences between colony members may affect the relationship between host and microorganism. Bumblebees also bridge the gap as a model wild species and a commercial pollinator, so promoting healthy bee populations could increase their efficacy. Understanding the role of microbial communities is highly integral both in terms of optimising bumblebee rearing and in assuring that colonies perform well as pollinators, and thus is of significant commercial relevance.

Our current knowledge of the bumblebee gut microbiota has been largely based on classical microbiology and molecular techniques. The application of the latest NGS technologies provides a greater depth of analysis than previously achievable, increases taxonomic (and functional) resolution level and provides more holistic descriptions, of gut microbial communities (Buermans & den Dunnen 2014). For this thesis, I used the 16S SSU rRNA gene (the 'gold standard' used to characterise prokaryotic microbiota) to determine the bacterial community within *B. terrestris* and place these organisms within their ecological context. My research was driven by the following questions:

1. *Do gut microbiota profiles differ between individuals of different castes, reflecting their roles within the colony?*
2. *Are microbial gut communities stable or variable over the whole colony cycle/foraging cycle?*
3. *How does gut microbiota composition change during queen diapause and does this affect health and colony foundation success?*
4. *How does access to different food sources affect the gut microbiota?*
5. *Does the gut microbiota give bees protection against challenges from widely used pesticides?*

1.8 THESIS STRUCTURE

The following chapter summarises the methodological approaches used in this project including the experimental locations, colony maintenance, the collection and preparation of samples, and laboratory protocols. Chapters 3–7 report the findings of experiments investigating the ecology of gut microbiota in *B. terrestris*, starting with aspects concerning the whole colony as a community (Chapters 3, 4) before drilling down to examine variables at the individual level (Chapters 5–7).

Chapter 3 describes the variation in key gut community composition between *B. terrestris* castes and developmental stages and provides a holistic view of gut microbiota assembly based on colonies located in both natural and artificial environments. Chapter 4 explores temporal shifts in the gut microbiota in castes and larvae when challenged with two types of field environments compared to colonies left in an artificial laboratory setting. Then Chapter 5 completes our understanding of gut microbiota during the *B. terrestris* lifecycle, investigating how gut microbiota before, during and after diapause affects the health of the founding queen and the overall success of colony foundation. Chapter 6 evaluates the impacts of different pollen diet mixtures on colony development; using regularly sampled workers to investigate how pollen diet and nutrition affects their gut microbiota, with potential implications for commercial bumblebee rearing. The final research chapter applies what we know about 'healthy' gut bacterial communities to investigating a fundamental concern addressing pollinator research, how pesticide exposure is affecting host health. The thesis concludes with a summary and evaluation of the main findings and their implications for wild and commercially managed bumblebee populations, and highlights areas of interest for future research.

CHAPTER 2:

MATERIALS AND METHODS

2.1 STUDY SPECIES AND COLONY MAINTENANCE

2.1.1 Bombus terrestris (ssp. audax)

The native UK bumblebee *Bombus terrestris audax* is used as the study organism throughout this thesis, and any reference to *B. terrestris* refers to this subspecies. It is a short-tongued bumblebee, and in temperate regions typically follows an annual colony cycle, though has been observed to have two colony cycles (Stelzer *et al*. 2010). New queens enter hibernation in autumn and emerge in spring to search for nesting sites and establish new colonies that can reach sizes of up to three hundred 'daughter' workers (Alford 1975).

Bumblebees are increasingly reared commercially for agricultural pollination to supplement wild pollinator populations (Velthuis *et al*. 2006). Over the last 20 years, there has been an increase in the number of bumblebees managed for commercial pollination of agricultural crops (e.g. soft fruits, tomatoes) in the UK, reaching over 5000 hives *B. t. audax* and over 16,000 *B. t. terrestris* and *B. t. dalmatinus* (Defra 2014). Bumblebees therefore bridge the gap between model wild species and commercial pollinator, so understanding the role of microbial communities is highly relevant both in assuring pollinator efficiency and in terms of optimising commercial bumblebee rearing.

2.1.2 Colony maintenance

All *B. terrestris* samples, except where noted, were supplied by the production facility, Biobest Group NV, Belgium. All colonies were reared following standard procedures, which included the use of helper workers to promote queen egg-laying (Sladen 1912). All colonies, except where specified, were fed a standardised diet of gamma-irradiated pollen and BIOGLUC® (Biobest Group NV, Belgium) *ad libitum*.

In line with protocols at Biobest Group, full *B. terrestris* colonies were monitored during development in Chapters 3, 4 and 6 to observe the variation present in

full size colonies. Chapter 7 used queenless micro-colonies to accurately monitor the effects of pesticide dosing on individual workers. Micro-colonies were comprised of three related sister workers removed directly from one of twelve parent colonies under red light (Section 2.4.2).

2.1.3 Colony nutrition

Biobest Group feed their bumblebees a sterile diet to prevent disease transmission. Their proprietary 50% w/v sugar water solution, BIOGLUC® (Biobest Group NV, Belgium), is a floral nectar substitute comprised of fructose-glucose syrup, saccharose, water, and preservative agents: propylene glycol (E1520), methyl-*p*-hydroxybenzoate (E218), and propyl-*p*-hydroxybenzoate (E216).

In Chapter 6, six irradiated *Apis mellifera*-collected pollen diets were fed to developing colonies to assess their impact on the gut microbiota of workers. Sterilisation was carried out using gamma irradiation (15 kGy; STERIS AST, Etten-Leur, the Netherlands) prior to arrival on site. To make monofloral diets pollen pellets were then hand-sorted by colour to produce visually monofloral batches (Hodges 1974; Kirk 2006), and plant taxa were confirmed via DNA sequencing of the ITS2–4 region (Sickel *et al.* 2015; Section 2.7.2).

2.2 EXPERIMENTAL LOCATIONS

2.2.1 Field sites

The field sites (B–G) used in Chapters 3 and 4 were in heterogeneous agricultural land around Brightwell Baldwin, UK (51.6444° N, −1.0573° W), separated by a minimum of 0.5 km (Fig. 2.1). Sample sites were selected to provide a broad range of environmental variation and consisted of a mixture of farmland margins and wildflower grassland. To estimate environmental quality sample sites were classified as either ‘resource-rich’ (RR; sites B, E, F), or ‘resource-poor’ (RP; sites C, D, G). RP sites were typically largely grass-dominated agricultural field margins or monoculture plots (e.g. *Hordeum vulgare* and *Triticum aestivum*), while RR sites were previously under entry-level stewardship schemes and contained nectar-rich plants such as *Lotus corniculatus*, *Trifolium* and *Onobrychis* spp. Site B was mown during the trial and was therefore reclassified to RP (Section 4.2.2). Wild post-diapause queens (Chapter 5) were also collected from sites C, D, E and F.

Figure 2.1 Six experimental field sites used in Chapters 3 and 4. Colonies were located in areas highlighted as resource-rich (RR; red) or resource-poor (RP; blue). Above are photographs of the typical landscape and floral composition of these sites.

In addition, colonies were covered by white corrugated plastic cardboard to protect from direct sunlight, and securely positioned in Gabion cages to avoid interference from predators (e.g. badgers, *Meles meles*). Foraging colonies were located at least 50–100 m apart to minimise potential worker drift. This is likely to be prevalent since entrances are shorter and more visible in artificial nest boxes than naturally occurring nests (Birmingham & Winston 2004; O'Connor *et al*. 2013).

Figure 2.2 Colonies were placed out of direct sunlight, usually in hedgerows (when available), covered by white corrugated plastic cardboard in Chapters 3 and 4.

2.2.2 Centre for Ecology & Hydrology (CEH) insectary

Indoor control colonies in Chapters 3 and 4, and the experimental micro-colonies used in Chapter 7 were housed on site within the CEH insectary. This is a controlled temperature room set to ambient conditions, 25 ± 2°C and 60% ± 5 relative humidity (RH) under constant darkness mimicking commercial rearing procedures. To prevent exposure to bacteria originating from the insectary/prior housed insects, all surfaces and floors were disinfected with Virkon® (Day-Impex Ltd.), followed by 70% ethanol before experimentation.

2.2.3 Biobest Group, NV.

Three months of this research project were spent embedded with the research and development team of my CASE partner, Biobest Group NV, providing a unique opportunity to work at the interface of academic research and industry. For Biobest

Group, understanding the role of microbial communities is highly relevant both in terms of optimizing bumblebee rearing and in assuring that bumblebee colonies perform well as pollinators. While research in the field has previously identified the key constituents in the gut microbiota of *B. terrestris,* what is not clear is the role they play in pollen digestion and how they react to differing nutritional inputs, or lack thereof (e.g. during diapause).

Here I conducted two trials between October and January 2018 analysing the impacts of commercial rearing procedures on the host gut microbiota: one focussing on the impacts of different irradiated pollen diets (Chapter 6), and the second observing gut microbiota throughout queen diapause (Chapter 5).

2.3 SAMPLE COLLECTION AND PREPARATION

2.3.1 Identifying developmental stages

The investigation outlined in Chapter 3 aimed to explore potential gut microbiota changes between life stages and castes within *B. terrestris*. Twelve distinct categories were selected to provide developmental variation present within a typical bumblebee colony. The stages selected are outlined in Appendix 1, and included eggs, larval instars, pupae, newly emerged adults, reproductive castes (males and queens) and workers.

Once samples were identified and catalogued, they were weighed, and anatomical measurements were collected as metadata to supplement gut microbiota analysis for every bee sampled. Digital callipers were used to measure various body measurements in mm, including body length, intertegular distance (Cane 1987), and head width (Hagen & Dupont 2013). Foraging activity/age was also assessed in samples used in Chapters 3 and 4, based on wing wear following a method outlined in Mueller & Wolf-Mueller (1993).

2.3.2 Gut removal

All sampled individuals were stored at −80°C for at least 48 h to ensure they had all been euthanized prior to gut dissection. Samples were rinsed in 70% ethanol and then phosphate-buffered saline (1xPBS), to minimise cross contamination.

To dissect adults, the bee was divided at the intersection separating the thorax and abdomen with a disposable sterile blade (Swann-Morton™). It was useful to make

the incision at an angle parallel to the base of the thorax. This helped to cleanly separate the thorax from the abdomen and keep the crop intact. The abdomen was teased apart by removing the abdominal segments with sterile curved forceps (No.7 Superfine; Watkins & Doncaster). Great care was taken to avoid non-gut tissues, including fat bodies and the ovaries (Fig. 2.3a), and once the whole gut was removed, it was again rinsed in 70% ethanol followed by 1xPBS. Clean, excised guts were placed in sterile DNA extraction plates and stored at −20°C until needed.

In early developmental stages larvae have a simple discontinuous gut, which takes up most of the host body (Fig 2.3b). When dissecting the gut, the head of each larva was removed using a sterile blade and the abdominal cuticle was peeled back with sterile forceps to reveal the intact gut. The gut was then rinsed in ethanol (70%) and then 1xPBS before being placed into a sterile extraction plate and stored at −20°C until needed.

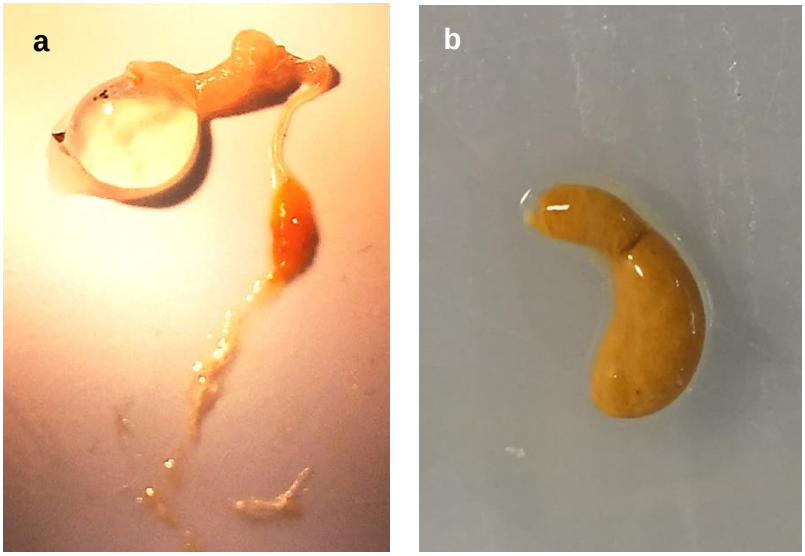

Figure 2.3 Dissected (a) adult and (b) third instar larval *Bombus terrestris audax* worker guts.

2.4 PESTICIDE TOXICITY TESTING

2.4.1 Chemical dosing

The pesticide assay in Chapter 7 exposed *B. terrestris* workers to a series of sub-lethal concentrations of clothianidin (0.026, 0.016. 0.01, 0.006, 0.004 µg/ml) in sterile sucrose

solution (Heard *et al*. 2017). Clothianidin was obtained as analytical grade pesticide standard (PESTANAL®, Sigma-Aldrich®), and experimental dosages were produced via serial dilution in water from the highest concentration of clothianidin. Stocks solutions were then used to spike a 50% w/v sucrose solution (molecular biology grade, Sigma Chemicals).

2.4.2 Treatment pots

Pesticide assays were conducted using a prototype cage developed by researchers at CEH for controlled dosing of insects (Heard & Hesketh 2017). This modular plastic cage comprises a feeding chamber with a ventilated lid, and a base to hold a dose applicator as displayed in Fig. 2.4. Spiked sucrose solutions were supplied in disposable 50 ml Luer centric syringes (Latex and silicone oil free) modified to provide an approximate 3 mm diameter drinking hole.

Figure 2.4 Modular plastic cages used for clothianidin dosing of workers (Chapter 7) developed by Heard & Hesketh (2017).

2.5 BACTERIAL CULTURING

2.5.1 Cultures and growth conditions

Freeze-dried cultures of *Bombus* gut bacterial isolates were acquired from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). These included cultures of

Snodgrassella alvi (DSM 104735; Kwong & Moran 2013) and three *Gilliamella* species described in Praet *et al.* (2017). *Gilliamella* species (*G. bombi* DSM 104030; *G. bombicola* DSM 104085; and *G. intestini* DSM 104029) were grown in Tryptone Soya Broth (Oxoid), while *S. alvi* was grown in Trypticase Soy Broth (Thermo Fisher-Scientific). All strains were cultured in deep-well plates covered with AeraSeal™ film (Excel Scientific, Inc.) in total darkness for 2–3 days at 37°C on a shaker.

2.5.2 Experimental assay

Bacteria were challenged with exposure to clothianidin and optical density (OD) of cultures was measured to estimate growth rates. A BioTek Synergy HT plate reader was used to measure OD at 600 nm every 6–12 h for 1 week. Bacterial suspensions of 20 µl were added to 180 µl of culture broth or 180 µl broth spiked with 0.004–0.026 µg/ml clothianidin. Samples were plated up in triplicate into a 96-well plate, including duplicate controls consisting of culture broth or broth with clothianidin (at each of the five doses) to check for contamination. Plates were incubated at 37°C in total darkness on a shaker.

2.6 DNA EXTRACTION

2.6.1 Pilot study: Lysis optimisation

To optimise DNA recovery, it was important to improve every stage of the DNA extraction protocol, to test the impact of different lysis buffers, bead beating methods, and additional reagents (e.g. proteinase K) on the DNA extracted and further downstream analysis.

These three factors were tested on different castes to select the best performing combination for the variety of samples that need to be processed (Fig. 2.5). Array 1 tested PowerSoil® Bead Beating solution, and array 2 tested the efficiency of PowerMag® Microbiome buffer. Both arrays tested each buffer in combination with garnet (~0.7 mm; Mobio Laboratories, USA) or fine glass beads (150–212 µm; Sigma-Aldrich, USA), and with or without the addition of proteinase-K (20 mg/ml; Macherey-Nagel, Düren, Germany).

DNA extraction products were amplified using primers based on the universal primer sequence 63F (5′–CAGGCCTAACACATGCAAGTC–3′; Marchesi *et al.* 1998) and 530R (5′–GTATTACCGCGGCTGCTG–3′; Tyler *et al.* 1995) to target the 16S

rRNA gene. PCR amplifications consisted of an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of: 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 1 minute with a final 10-minute extension at 72°C. Resulting PCR products were cleaned using the ZR-96 DNA Clean-up Kit™ (Zymo Research, USA) following the manufacturers recommended protocol. Ultra-pure DNA was then digested using MSP1 restriction enzyme (Promega, USA) designed to cleave C^CGG sites in preparation for T-RFLP (Terminal restriction fragment length polymorphism) sequencing.

T-RFLP sequence data was visualised using GeneMarker® (SoftGenetics LLC., USA) and size was determined against the control GeneScan 600LIZ® size standard (Thermofisher Scientific). The data highlighted considerable peaks, suggesting that the PCR products amplified well. Final extraction buffer mechanical lysis was determined to be the PowerMag® buffer, garnet beads and proteinase K as this provided highest DNA concentration and T-RFLP peak diversity.

2.6.2 Bee gut DNA extraction

In Chapters 3 and 4 gut bacterial DNA was extracted using an optimised protocol (Section 2.6.1; Appendix 2) combining PowerMag® Microbiome RNA/DNA and PowerSoil®-htp 96 Well Soil DNA isolation kits (Mobio Laboratories, USA). Cleaned samples were placed into individual wells of a PowerSoil® Bead Plate along with 650 µl of warmed PowerMag® Microbiome Lysis Solution/2-Mercaptoethanol (βME) and 5 µl proteinase-K (20 mg/ml; Macherey-Nagel, Germany), before continuing with the PowerSoil®-htp 96 Well Soil DNA Isolation Kit recommended protocol. A blank well (extraction negative) was added to each plate to check for cross contamination. DNA was eluted and frozen at −20°C in elution buffer until after sequencing, and then at −80°C for long term storage.

The PowerMag® Microbiome RNA/DNA buffer was no longer available commercially from Chapter 5 onwards, so DNA extraction from adult gut samples were performed using PowerSoil®-htp 96 Well Soil DNA isolation kits (Mobio Laboratories, USA) following the manufacturer's recommended protocol. Although DNA recovery was slightly lower with this method, no diversity or abundance differences were detected using T-RFLP.

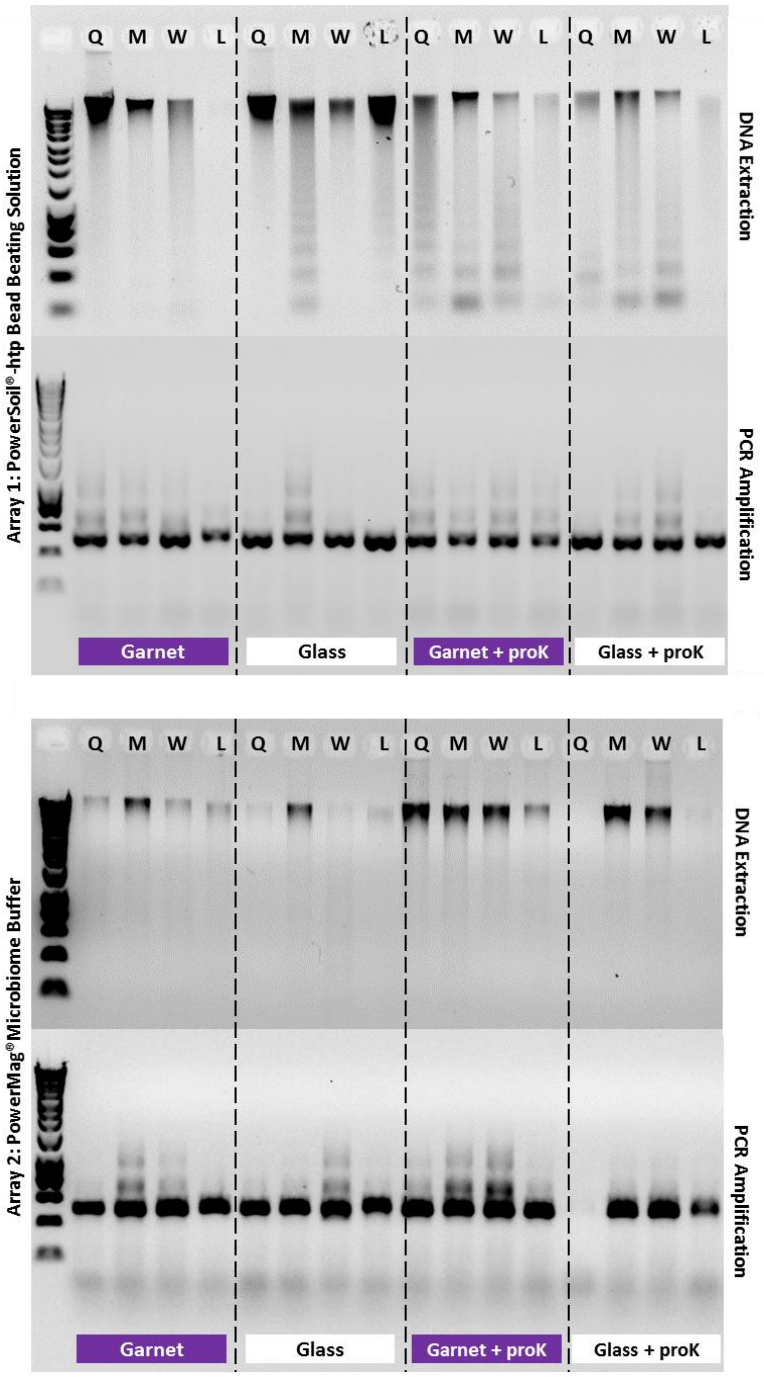

Figure 2.5 Lysis optimisation gel electrophoresis for samples of queens (Q), males (M), workers (W) and larvae (L). Arrays tested one of two buffer solutions (PowerSoil®-htp Bead Beating Solution or PowerMag® Microbiome) on DNA recovery and PCR amplifcation using garnet or glass bead methods, with or without proteinase K (proK). Overall, PowerMag® Microbiome Buffer with garnet beads and proK was judged to provide optimal DNA recovery and PCR amplification.

2.6.3 DNA quantification

NanoDrop™ 8-Sample Spectrophotometer (Thermofisher Scientific) was used to approximate DNA extract concentrations and sample quality by measuring absorbance at 280nm, 260nm, and 230nm. A260/280 ratio was used to determine protein contamination, while the A260/230 ratio indicated the presence of organic contaminants (e.g. phenol).

The caste analyses in Chapter 4 involved samples that had very low biomass (e.g. eggs and 1st and 2nd larval instars), resulting in DNA extractions that failed to visualise on a 1% agarose gel due to low DNA concentration. To improve yield and further downstream analyses, aliquots of all samples were taken for a template DNA plate and diluted with PCR grade water to make up samples at ~20 ng/µl concentration. Samples that did not reach this threshold were concentrated down from their eluted volume of 100 µl to 50 µl using an Eppendorf® Concentrator 5301 (Eppendorf AG, Germany), and then subsampled for template DNA. Original samples were put in long term storage at −80°C, while the template DNA was used in all downstream analyses.

2.6.4 Pollen DNA extraction

In Chapter 6, samples from each pollen diet were sequenced to confirm the composition of plant pollen species. DNA was extracted from representative samples of each diet using an optimised DNeasy® PowerPlant® Pro Kit (Qiagen, Hilden, Germany). Approximately 100 mg of homogenised pollen was added to individual bead beating tubes containing 0.5 ml of mixed size glass beads (50:50 ratio of 1.0 and 0.1 mm; Sigma-Aldrich, Missouri, United States). Samples were incubated with 5 µl of proteinase K (>600mAU/ml) at 65°C for 30 minutes before adding 40 µl Phenolic Separation Solution (PSS). For the remainder of the extraction the manufacturer's recommended protocol was followed.

2.7 Polymerase Chain Reaction (PCR)

Two approaches to sequencing were applied, one-step (for bacterial 16S amplicons based on Kozich *et al.* 2013), and due to challenges amplifying the ITS2–4 region, a two-step approach was used for pollen barcoding. The latter more closely follows the Illumina® MiSeq workflow.

2.7.1 One-step PCR

PCR reactions were carried out in an aseptic UV cabinet with a sterile 96 well PCR microplate using a Mastercycler® nexus (Eppendorf AG, Germany). Approximately 20 ng of template DNA was amplified with Q5® high-fidelity DNA polymerase (New England Biolabs, UK) each with a unique dual-index barcode primer combination (Kozich *et al*. 2013). The total reaction volume of 50 µl contained: 10 µl 5x Q5® Reaction buffer, 10 µl High GC buffer, 1 µl dNTPs (~10mM), 24.5 µl PCR grade water, 0.5 µl Q5® high-fidelity DNA polymerase, 2 µl MiSeq primers and 2 µl of template DNA.

Individual PCR reactions employed 30 cycles of an initial 30 seconds 98°C denaturation step, followed by annealing phase for 30 seconds at 55°C, and a final extension step lasting 90 seconds at 72°C. Primers used were based on the universal primer sequence 341F (5′−CCATCTCATCCCTGCGTGTCTCCGACTCAG–3′) and 806R (5′−GCTGCCT CCCGTAGGAGT–3′) to target the V3–V4 hypervariable regions of the 16S rRNA gene. PCR products were held at 4°C then stored at −20°C. Negative extraction controls, and positive and negative controls were added to each PCR run to check contamination and efficiency.

Successful PCR amplification was confirmed via gel electrophoresis using 1% agarose gel electrophoresis stained with ethidium bromide, at 85V for 45 minutes with Hyperladder™ 1kb (Bioline, UK) as a size marker. Resulting gels were imaged with a Gel Doc™ XR^+ Imager using Image Lab™ software (Bio-Rad Laboratories, USA).

2.7.2 Two-step PCR

Two-step PCR reactions were used to amplify the ITS2–4 region in DNA extracted from pollen pellets for pollen barcoding (Chapter 6). Before two-step PCR, extracted DNA was cleaned with the ZR–96 DNA Clean-up Kit™ (Zymo Research, California, USA) to remove any PCR inhibitors. PCR was performed in 50 µl reactions, containing 10 µl 5x buffer (New England Biolabs, Hitchin, UK), 0.5 µl each of the forward and reverse primers, 1 µl 10 nM dNTPs, 0.5 µl Q5® polymerase, 35.5 µl PCR grade water and 2 µl template DNA (~ 10 ng/µl). The first PCR reaction for gene specific amplification also added on the Illumina® adapters (in bold), and used primers based on Sickel *et al.* (2015): ITS2F (5′−AATG ATACGGCGACCACCGAGATCTACACXX XXXXXXCCTGGTGCTGGT**ATCTGGATTAGATACCCTGGTA**−′3) and ITS4R

(5′−CAAGCAGAAGACGGCATACGAGATXXXXXXXXAGTCAGTCAGCC**CTCG ACRRCCARGCANCACCT**−′3). PCR conditions consisted of an initial denaturation of 30 seconds at 98ºC, followed by 20 cycles of: 10 seconds at 98ºC, 10 seconds at 50ºC and 20 seconds at 72ºC. A final extension of 10 minutes at 72ºC was also included to complete the reaction. All resultant PCR products went through an additional clean-up step to remove potential PCR inhibitors and were eluted in 40 µl dH_2O (ZR-96 DNA clean up kit; Cambridge Bioscience).

Purified PCR product was used in the second PCR reaction to add Illumina® TruSeq adapters (IT) primers: ITF SA/B501−8 and ITR SA/B701−12. PCR was performed in 50 µl reactions, containing 10 µl 5× buffer, 1 µl 10 mM dNTPs, 5 µl primer mix (IT array A–D), 20 µl DNA template, 0.5 µl Q5® polymerase and 13.5 µl PCR grade water. PCR condition consisted of an initial denaturation of 30 seconds at 98ºC, followed by 10 cycles of 10 seconds at 98ºC, 20 seconds at 62ºC and 30 seconds at 72ºC. A final extension of 2 minutes at 72ºC was also included to complete the reaction.

2.7.3 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was used to estimate absolute copy numbers of 16S rRNA genes to complement the high-throughput sequencing data in Chapters 5 and 7. The Femto™ Bacterial DNA Quantification Kit (Zymo Research, USA) was used to amplify bacterial DNA in triplicate using kit supplied primers. Approximately 2 µl of template DNA was used in reaction volumes of 10 µl. DNA standards (1 µl per reaction) and negative PCR grade water controls were also added, and the whole plate was placed in a LightCycler® 480 (Roche Molecular Systems, Inc.) following the manufacturer's recommended thermocycling parameters. Absolute gene copy numbers were calculated from the thermocycler, based on the known input values of the standard curve DNA.

2.8 NEXT GENERATION SEQUENCING AND BIOINFORMATICS

2.8.1 Library preparation and construction

Constructing mixtures of 'tagged' or 'bar-coded' DNA for sequencing is an important requirement for the efficient use of next-generation sequences in applications where limited sequence data are required per sample. Multiplex PCR products are run simultaneously but are normalised to control for over or under sequencing of a specific

sample. There are still inbuilt biases in MiSeq sequencing itself (i.e. PCR amplification) but it is important to minimise these errors so that samples are not unequally sequenced. PCR products were normalised using the SequelPrep™ Kit (Thermofisher Scientific) following the manufacturers recommended protocol. Each plate of samples was individually normalised and pooled into a 2 ml LoBind tube (Eppendorf AG, Germany).

The contents of each 2 ml tube were concentrated down to ~250 µl for gel purification. Pooled samples were run on a 1% agarose gel for 50 mins at 85V. Gel was visualised using visible blue light on a Dark Reader® Transilluminator (Clare Chemical Research, USA) to avoid damaging the DNA with ultra violet (UV) light. DNA bands were carefully excised from the gel using a sterile scalpel and placed into a 2 ml LoBind tube before following the remainder of the QIAquick® Gel Extraction kit protocol (Qiagen, USA). Samples were quantified using a Qubit® Fluorometer and the dsDNA high sensitivity assay kit (Life Technologies).

2.8.2 Illumina® MiSeq

Resulting 16S libraries were sequenced on an Illumina® MiSeq (Illumina Inc., San Diego, CA, USA) at varying concentrations (see individual research chapter methods for details) along with a 10% addition of an Illumina generated PhiX control library. Sequencing runs following the protocol described by Kozich *et al.* (2013) generated 2 x 300 bp reads using V3 chemistry.

2.8.3 Bioinformatic analyses: OTU method

In Chapters 3, 4 and 7, the raw sequencing data produced was analysed using a pipeline of bioinformatics tools developed by Hyun Soon Gweon. These consisted of two major computational processes (i) a pre-processing stage for quality control and alignment of raw sequence data; and (ii) an identification stage to assign operational taxonomic unit (OTU) identity.

Sequenced paired-end reads were joined using PEAR (Stamatakis *et al*. 2014), quality filtered with FASTX tools (Hannon 2010), then filtered to a minimum length of 300 bp. The presence of PhiX and adaptors were checked and removed with BBTools (Bushell 2017), and chimeras were identified and removed with VSEARCH_ UCHIME_REF (Rognes *et al*. 2016) using Greengenes Release 13_5 (at 97%) (DeSantis *et al*. 2006). Sequences that only occurred once (‘singletons’) were removed

and the resulting sequences were clustered into operational taxonomic units with VSEARCH_CLUSTER (Rognes *et al*. 2016) at 97% sequence identity (Tindall *et al*. 2010). Unless stated otherwise, default parameters were used for the steps listed.

2.8.4 Bioinformatic analyses: ASV method

New bioinformatics approaches became available during this research project, and whilst none of the previous findings or work are invalidated, the raw sequencing data produced by Chapters 5 and 6 were analysed using a different suite of tools. The curated Divisive Amplicon Denoising Algorithm pipeline (DADA2; Callahan *et al.* 2016) became the preferred pipeline (Appendix 10) as it is more intuitive and does not require an expert bioinformatician to maintain. It also able to run within R, containing tools to merge, denoise, and remove chimeras from paired-end fastq files prior to analysis (Callahan *et al*. 2016).

One advantage of this method is that since ASVs represent real, independent biological data, they can be reused across marker-gene studies and replicated in future data sets, as long as they sequence the same region of DNA using the same primer set (Callahan *et al*. 2017). In Chapter 5, this allowed me to compare lab-reared queens with wild-caught samples previously sequenced from another MiSeq run.

For all data produced and analysed using both OTU and ASV methods resultant data was processed to remove kit contamination, by processing negative control samples (Salter *et al*. 2014; Glassing *et al*. 2016). Singletons and sequences that did not correspond to bacterial taxonomy were also removed.

2.8.5 Bacterial taxonomic classification

Representative sequences for each OTU were taxonomically assigned using the RDP Classifier with the bootstrap threshold of 0.8 or greater (Wang *et al*. 2007) based on taxonomy from the Greengenes Release 13_5 (full) (DeSantis *et al*. 2006). On occasion, taxa were left unresolved to sufficient taxonomic resolution, suggesting that the Greengenes database may not have optimal coverage for bee microbiota species. Other databases were trialled to a similar effect, including Ribosomal Database Project (RDP; Wang *et al.* 2007), the SILVA ribosomal RNA database (Quast *et al*. 2013), and GenBank (Clark *et al*. 2016). While incredibly useful, these databases often have little or no peer-review process and sequences are often deposited unlabelled (e.g. GenBank),

or at the other extreme contain curated databases that undergo high levels of quality control before being deposited, resulting in a limited amount of reference sequences (e.g. SILVA and RDP; Park *et al.* 2012). Instead, taxonomies were further corroborated using Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1990) and EzBioCloud database (Yoon *et al*. 2017), with the identity of unknown OTUs selected based on >99% matches. However, taxonomic assignment for bee microbiota classification is sensitive to training set (Newton & Roeselers 2012), and given the shortness of sequences generated by sequencing, taxonomic identities should be considered putative.

2.9 STATISTICAL ANALYSES

Samples were removed from analyses if they had fewer than 1000 sequence reads, because Fisher's alpha (log series alpha) was used to quantify sample alpha diversity, and it is independent of sample size when $n > 1000$ (Magurran 2004). To assess the quality of the data used for diversity assessments, rarefaction curves were used to confirm that sequencing depth was sufficient for each sample (Appendix 3). After quality filtering, data were rarefied to an equal sequencing depth using the 'rrarefy' function R:VEGAN (Dixon 2003). Community composition and beta diversity analyses were then conducted using Bray-Curtis dissimilarity values generated in PAST (Hammer *et al*. 2001).

Univariate and multivariate analyses were performed in the base R statistics package, SPSS, and PAST, with differential expression analyses conducted in the Microsoft Excel add-in software XLSTAT (Addinsoft, Paris, France). More detail for individual analyses can be found within individual research chapter methods.

CHAPTER 3:

CHARACTERISING DEVELOPMENT OF CASTE-SPECIFIC MICROBIOTA

3.1 INTRODUCTION

Microorganisms colonise the guts of a wide range of animal species where they can be pathogenic, commensal or beneficial (aiding digestion, defence and overall fitness of the host) (Robinson *et al.* 2010; Ezenwa *et al.* 2012; Engel & Moran 2013a). The importance of these microorganisms and their functional role spans topics within medicine, agriculture, and ecology (Engel & Moran 2013a). However, studying these communities within vertebrate hosts is often difficult due to the high complexity and diversity of microbiota present (Pernice *et al*. 2014). Insect microbiota are typically much less diverse, and so they offer a valuable model for understanding the effects of different host-microorganism relationships, acquisition pathways, and environmental factors on bacterial communities (Engel & Moran 2013a; Pernice *et al.* 2014).

Declining insect pollinator populations have led to research into the assembly, diversity and function of gut microbiota in response to pathogens and disease (Engel & Moran 2013a). In particular, eusocial bees have been found to harbour a distinctive gut microbiota, composed of a relatively simple, but specialised community dominated by several 'core' phylotypes consistently present regardless of geographic location (Mohr & Tebbe 2006; Cox-Foster *et al.* 2007; Martinson *et al.* 2011; Engel *et al.* 2012; Moran *et al.* 2012; Engel & Moran 2013b). A major limitation to our current understanding is that studies are almost exclusively on workers from *Apis mellifera* and *Bombus* spp., with little research investigating gut microbial diversity between reproductive castes or developmental stages (Kapheim *et al*. 2015; Tarpy *et al*. 2015). This may be in part due to the colony structure, where there is an abundance of workers, fewer males, only one queen, and larvae that, while abundant, are low in biomass making representative sampling

challenging. As bees are ecologically and economically important for the maintenance of crop production and biodiversity (Klein *et al*. 2007; Gallai *et al*. 2009), there is a clear need to improve our understanding of the impacts of life history, host gut morphology, and behavioural traits (e.g. foraging activity) on the development of bee gut microbiota (Kapheim *et al*. 2015).

Bumblebees (*Bombus* spp.) undergo extensive morphological changes throughout their development and during caste differentiation. A large part of the bumblebee lifespan is devoted to maturing into an adult (Alford 1975) yet little is known about the microbiota they harbour throughout this phase. Preliminary molecular work on late larval instars in eusocial (*Apis, Bombus*) and solitary (*Osmia*) bee spp. (Mohr & Tebbe 2006) has begun to challenge the assumption that the larval gut is sterile which arose from earlier, culture-based, microbial assessments (Gilliam 1971). Additionally, sampling of a single *B. pascuorum* colony has suggested differences between worker and larval gut microbiota composition, further challenging this view (Parmentier *et al*. 2018).

Similarly, how environmental change affects the gut community is still largely unclear (Newbold *et al*. 2015). Exposure to environmental resources has been shown to alter the microbiota of eusocial bees (Newbold *et al*. 2015; Parmentier *et al*. 2016). Bacteria on plants and flowers are able to persist in a dormant state within the environment (McFrederick *et al*. 2012; Anderson *et al*. 2013), and since floral resources are frequently shared by diverse insect assemblages (Herrera 2018) it is likely that they may act as reservoirs prior to gut bacterial transmission (Moran *et al*. 2012). The shared presence of social bee gut bacteria phylotypes in both *Apis* and *Bombus* spp. as well in the guts of other insects (Moran *et al*. 2012) highlights the need to investigate the roles of horizontal and vertical transmission of microbiota in a more naturalistic setting.

This study aimed to develop a model of microbiota assembly throughout the *B. terrestris* lifecycle, and provide a holistic view of the bacterial community, while highlighting a natural pathway of microbial colonisation. It quantitatively describes the gut community variance between castes and explored (i) the impacts of physiological and caste differences on gut microbiota; and (ii) how exposure to environmental sources of bacteria (via foraging) affects host gut microbiota.

3.2 MATERIALS AND METHODS

3.2.1 Study species

Bombus terrestris is a short-tongued bumblebee native throughout Europe. In temperate regions it typically follows an annual colony cycle; new queens enter hibernation in autumn and emerge in spring to search for underground nest sites and found new colonies. Each colony may produce up to three hundred daughter workers that forage from spring to summer for nectar and pollen to rear new workers, queens and males (Alford 1975). The species is reared commercially for fruit and vegetable pollination.

3.2.2 Field sites and sample processing

Commercially reared colonies of *Bombus terrestris audax* (Biobest Group NV, Belgium) were reared on a diet of sterile, gamma-irradiated pollen and BIOGLUC® (Biobest Group NV, Belgium). Colonies reaching an average size of *c*. 10–15 workers were monitored for one week to ensure that they were healthy and developing normally prior to inclusion in the study. A total of 35 colonies were housed in six field sites and one sterile, climate-controlled insectary. Field sites (separated by a minimum of 0.5 km) were located in a farmed landscape around Brightwell Baldwin, UK (51.6444° N, −1.0573° W). Sites consisted of a mixture of farmland margin and wildflower grassland, near heterogeneous landscape, chosen especially to provide a broad range of variation in habitat forage quality (Section 2.2.1). At each site five colonies were placed 50–100 m apart to minimise potential worker drift; a concern when using artificial nest boxes due to shorter entrances and increased visibility (Birmingham *et al*. 2004; O'Connor *et al*. 2013). Colonies were placed out of direct sunlight, covered by white corrugated plastic cardboard, and securely positioned in Gabion cages to prevent predator interference (e.g. *Meles meles*). Five control colonies were housed in a controlled temperature room on a day/night cycle at 25 ± 2°C and 60% ± 5 RH and fed on a standardised diet of commercial (irradiated) pollen and BIOGLUC® *ad libitum*.

At regular intervals between June and August 2015 one colony from each field site and a control colony were selected randomly and destructively sampled. Samples were taken across multiple time points to provide a holistic overview of the microbiota across

colony development. At each sample point, colony boxes were sealed and stored at −80°C within 2h of removal from the field. Boxes were left for at least 48 h to ensure all individuals had been euthanized. Whole frozen colonies were carefully dismantled and sorted into twelve different castes/developing stages: eggs; 1-4 larval instars; pupa; newly emerged worker; worker; newly emerged male; male; new queen; and mature queen (Appendix 1). Of the 35 colonies used in this experiment, 33 survived to be sampled, where a representative 10% subset of each caste/developmental stage per colony was extracted for molecular sequencing.

3.2.3 DNA isolation and sequencing

Gut bacterial DNA was extracted using an optimised DNA extraction protocol combining PowerMag® Microbiome RNA/DNA and PowerSoil®-htp 96 Well Soil DNA isolation kits (Mobio Laboratories, Carlsbad, CA, USA). To minimise cross contamination between gut contents and environment, samples were first washed in 70% ethanol and vortexed to surface sterilise and remove attached particles (Banfalvi 2016). All samples were then rinsed in phosphate buffered saline (1xPBS) to remove trace amounts of ethanol whilst maintaining cell integrity prior to dissection. Intact eggs, with whole guts dissected from larvae and adults and negative controls were then placed into individual wells of a PowerSoil® Bead Plate following the optimised protocol outlined in Appendix 2.

Approximately 20 ng of template DNA from each sample was amplified using Q5® high-fidelity DNA polymerase (New England Biolabs, Hitchin, UK) with unique dual-index primers barcodes (Kozich *et al*. 2013) to target the V3–V4 hypervariable regions of the 16S rRNA gene. Full details are outlined in Section 2.7.1 and resulting PCR products were normalised using the SequelPrep™ Normalization Plate (96) Kit (Thermofisher Scientific). Once pooled, amplicon libraries consisting of ~550 bp amplicons spanning the V3−V4 hypervariable regions of the 16S rRNA gene were gel purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and quantified with a Qubit® Fluorometer using a dsDNA high sensitivity assay kit (Life Technologies). The resulting 16S library was sequenced at a concentration of 6.4 pM with a 0.6 pm Illumina generated PhiX control library. Sequencing runs were performed on an Illumina MiSeq using V3 chemistry producing 2 x 300 bp (Illumina Inc., San Diego, CA, USA).

3.2.4 Sequence analysis

Full details of the sequence analyses conducted on this data are outlined in Section 2.8.3. Following this, further controls were added to minimise kit contamination. To do this, negative control samples were processed and included into analyses and potential false positives were removed (Salter *et al*. 2014; Glassing *et al*. 2016). Sequences that did not correspond to bacterial taxonomy (e.g. chloroplasts, mitochondria, Archaea, or Eukaryota), and those that only occurred once in the dataset were removed from all downstream analysis.

After filtering low abundance OTUs and probable kit contaminants a total of 930,610 sequences were retained, representing 789 OTUs. These sequences were distributed across 415 samples (Table 3.1; Appendix 4), but some OTUs were left unresolved past family level, suggesting that the Greengenes Release 13_5 (full) database may not have optimal coverage for bee microbiota species. Taxonomies were further corroborated using Basic Local Alignment Search Tool (Altschul *et al*. 1990) and EzBioCloud database (Yoon *et al*. 2017), with identity of unknown OTUs selected based on > 99% matches. However, given the shortness of sequences generated by sequencing (~300 bp), all OTU identities should be considered putative.

3.2.5 Statistical analysis

Rarefaction curves were used to confirm that the MiSeq sequencing depth was sufficient to provide a good description of the alpha diversity within each sample (Appendix 3). The number of reads did not affect the number of operational taxonomic units (OTUs) detected suggesting that communities were completely, or near completely sampled. Data was rarefied to an equal sequencing depth to match that of the lowest read count (=2273 sequences) using ‘rrarefy’ function in R:VEGAN package (Dixon 2003). This subset was used in subsequent analyses.

Bacterial community diversity was estimated using Fisher’s alpha (log series alpha) as the response variable since it is independent of sample size (Magurran 2004). In initial investigations there was no significant difference in Fisher’s alpha diversity between larval instars ($\chi^2 = 6.15$, $p = 0.10$, $n = 150$) so they were combined into a single category (‘larva’) for further analyses.

To investigate bacterial composition, a frequency table of OTUs in each sample was screened and converted to a Bray-Curtis dissimilarity matrix using PAST v3.11 (Hammer *et al*. 2001). In accordance with previous microbiome studies, the component taxa within each host caste and developmental stage were separated into common and rare groupings (Hedin *et al*. 2015). 'Common' OTUs were defined as those present in the upper quartile of sample occupancy with >75% across all samples (Appendix 5).

Kruskal-Wallis, one-way ANOVA and univariate statistics were carried out using the base statistics package in R version 3.3.2. Rarefication was performed using the VEGAN package in R (Dixon 2003), and exploratory analyses in R: Phyloseq (McMurdie & Holmes 2013). Principal component analysis (PCA) and similarity of percentages (SIMPER) analysis were performed using PAST with Bray-Curtis as the underpinning community similarity measure. Differential expression analyses were conducted using the XLSTAT (v2017.2, Addinsoft, Paris, France).

3.3 RESULTS

3.3.1 Caste differences in gut microbiota

From the 789 bacterial OTUs identified, 29 were present in at least one individual from every caste and developmental stage examined. On average 45.1 ± 42.2 OTUs (± 95% confidence interval) were detected per individual, with significant differences in average OTU count between each mature caste/developmental stage (Table 3.1; $\chi^2 = 59.65$, $p < 0.0001$, $n = 391$). Fisher's alpha diversity index was significantly different between all mature castes and developmental stages ($\chi^2 = 59.95$, $p < 0.0001$, $n = 391$). Further pairwise comparisons revealed that worker and male bacterial communities were significantly different from queens, and developmental stages. Queens, on average, had the highest diversity index overall (12.3), followed by pupae (12.1), larvae (11.9) and eggs (7.8), compared to workers and males with the lowest (4.5 and 5.2, respectively; Fig 3.1a, b).

Table 3.1 Summary data from castes and developmental stages of *B. terrestris* in both control (C) and foraging (F) treatments, including brief metadata, average OTU counts for each caste/stage. OTUs associated with a caste/stage or treatment are calculated by examining the whole caste/stage population. Differences between treatments have been calculated by comparing shared and unique OTUs for each caste/stage.

Variable	Egg		Larva		Pupa		Worker		Male		Queen	
	C	F	C	F	C	F	C	F	C	F	C	F
Sample size (*n*)	3*	12*	35	115	14	43	34	73	21	13	4	24
Mass (g)	0.007	0.014	0.112	0.144	0.272	0.294	0.198	0.162	0.252	0.240	0.722	0.828
Head width (mm)	-	-	1.24	1.36	4.14	4.15	3.67	3.57	3.76	4.11	5.04	5.48
Wing length (mm)	-	-	-	-	-	-	12.11	11.01	13.31	14.08	14.99	16.46
Average OTU count	28	44	65	54	70	53	26	27	34	39	58	50
OTUs associated with caste/stage	266		752		614		399		439		453	
OTUs associated with each treatment	60	253	584	701	432	542	238	354	254	302	152	421
OTUs shared between treatments	47		533		344		193		117		120	
Treatment-unique OTUs	13	206	51	168	72	182	45	161	137	185	32	301

*Clutches of eggs (~approx. 6 individual eggs per sample)

As an overview of bacterial community dynamics, principal component analysis (PCA) was performed on larvae, pupae, and mature adult castes (Fig. 3.2). This highlighted clear differences in bacterial composition between workers and males against developing stages and queens, indicating that immature stages and queens have more diverse and variable communities.

The contribution of individual taxa to the overall dissimilarity between developmental stages and castes was further explored by similarity percentage analyses (SIMPER; Appendix 6). The OTUs that contributed most to the overall dissimilarity included *Gilliamella bombicola*, *Snodgrassella alvi*, and two species putatively identified as *Lactobacillus apis* and *Enterobacter aerogenes* (*Klebsiella aerogenes*). Both *G. bombicola* and *S. alvi* had a high relative abundance in workers (23.2% and 20.0%, respectively) and males (19.8%, 17.2%). Meanwhile *E. aerogenes* was much more abundant in larval and pupal guts (14.4% and 16.6%, respectively) and new queens (20.9%), in contrast to mature queen guts, which were dominated by *Lactobacillus apis* (19.4%).

3.3.2 Typical caste microbiota and common OTUs

A common set of fourteen ubiquitous OTUs were found across mature castes and developing worker stages, regardless of treatment. These OTUs were resolved to taxa from

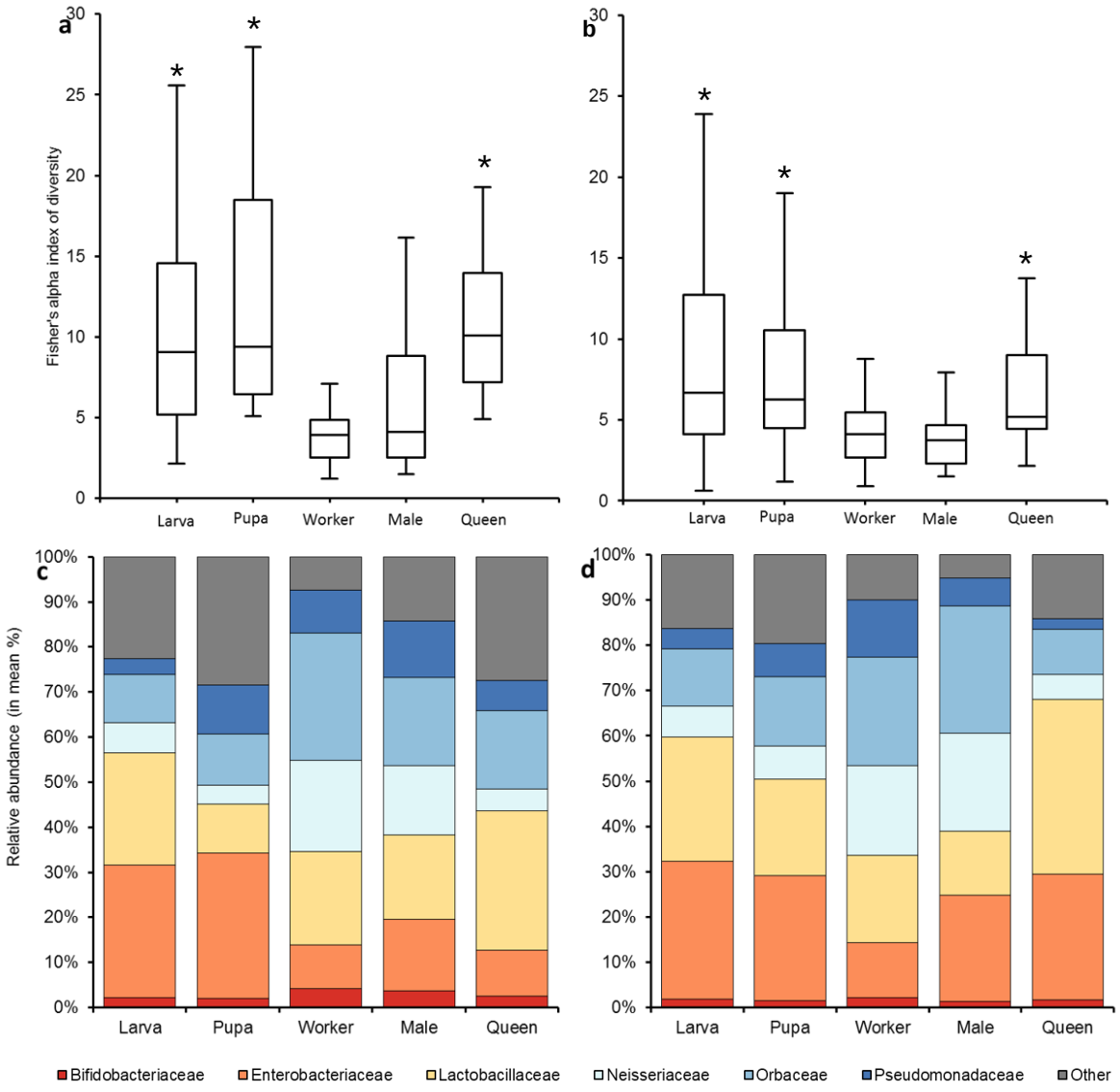

Figure 3.1 Comparisons of diversity and composition characteristics between stages and castes between treatments (control and foraging). (a) Fisher's alpha diversity in foraging colonies. (b) Fisher's alpha diversity in control colonies. (c) The relative abundance (% mean) of the six common bacterial families present in foraging colonies. (d) The relative abundance (% mean) of the six common bacterial families present in control colonies. Significance at the $p < 0.05$ denoted by an asterisk (*).

six bacterial families, and typically made up between 60–80% of the total sequence abundance. They included *Bombiscardovia coagulans* (Bifidobacteriaceae); *E. aerogenes* (Enterobacteriaceae); *Lactobacillus* spp. (Lactobacillaceae); *S. alvi* (Neisseriaceae); *G. bombicola* (Orbaceae); and *Ventosimonas* spp. (Pseudomonadaceae). Adult caste microbiota varied in their relative proportions of these six bacterial families (Fig. 3.1c, d).

In workers, the gut microbiota was primarily composed of the dominant families, Orbaceae (23.2 ± 3.4%) and Neisseriaceae (20.0 ± 3.7%), which contain the key social bee bacterial species, *G. bombicola* and *S. alvi*, respectively. Lactobacillaceae (19.7 ± 8.7%), Pseudomonadaceae (11.7 ± 5.2%), Enterobacteriaceae (11.4 ± 9.5%), and Bifidobacteriaceae (2.81 ± 1.23%) were also consistently present, but in much lower abundances. Newly emerged workers (<2 days old) were similar in composition to mature workers, with slight differences in the proportions of *Lactobacillus* spp.

Similarly, male gut microbiota was dominated by Orbaceae (22.9 ± 8.1%), and Neisseriaceae (17.7 ± 6.2%), followed by Lactobacillaceae (17.0 ± 13.8%), Pseudomonadaceae (10.1 ± 9.0%), Enterobacteriaceae (18.8 ± 24.4%), and Bifidobacteriaceae (2.8 ± 2.3%). In contrast, newly emerged males had much higher levels of *S. alvi* (39.9 ± 17.5%), *Saccharibacter floricola* (5.2 ± 10.0%) and *Lactobacillus* spp. (19.2 ± 36.7%), albeit with more variance.

In mature queens, almost 84% of total gut diversity was comprised of the six families present in both workers and males, but with an additional four low abundance families: Commonadaceae, Bradyrhizobiaceae, Enterococcaceae, and Oxalobacteraceae. Unlike other adult stages in the *Bombus* colony, queen guts were dominated by Lactobacillaceae (37.8 ± 25.7%) and by Enterobacteriaceae (25.2 ± 26.1%), while the key species *G. bombicola* and *S. alvi* each had <10% relative abundance. Mature queen gut communities were comparable with new (daughter) queens but had lower mean abundances of *G. bombicola* (9.8 ± 4.9% compared to 16.7 ± 28.9%), *E. aerogenes* (7.6 ± 4.1% compared to 20.9 ± 37.6%) and *Pediococcus acidilactici* (0.3 ± 0.3% compared to 16.0 ± 31.1%) but had a higher mean abundance of *Lactobacillus apis* (19.3 ± 9.5% compared to 9.6 ± 10.7%).

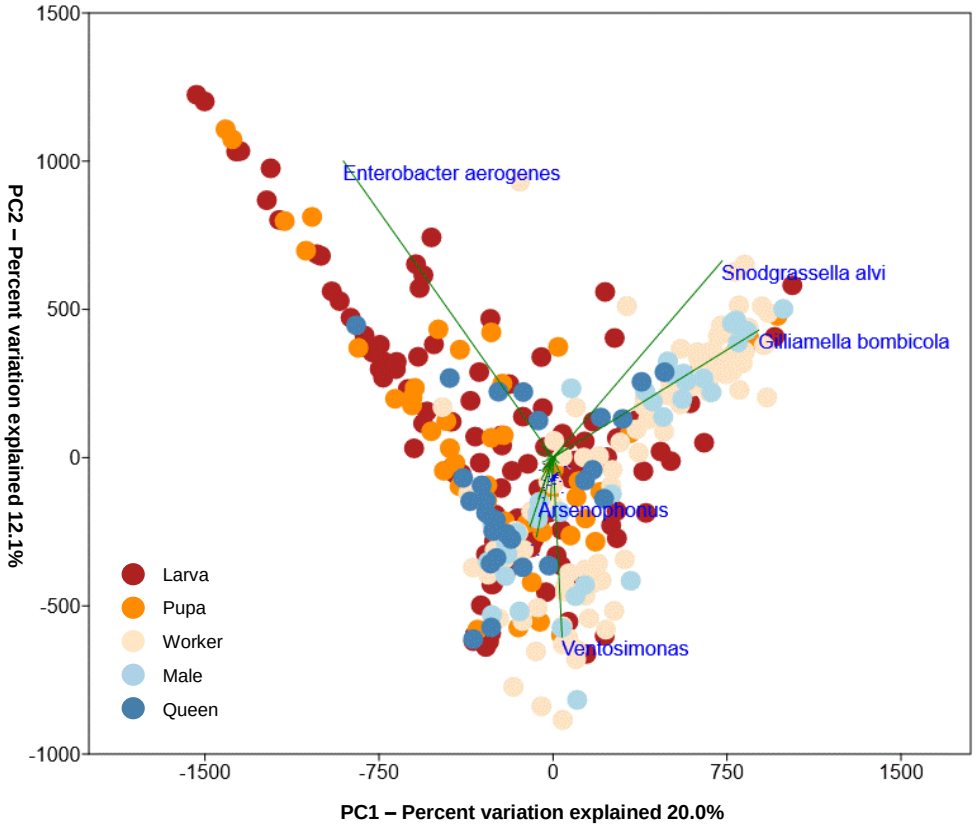

Figure 3.2 Principal Component Analysis (PCA) of gut microbiota data for larvae (red), pupae (orange), workers (beige), males (light blue), and queen (dark blue) *Bombus terrestris* individuals. A biplot highlights in blue the taxa that are most responsible for differences in gut microbiota community structure, including *Enterobacter aerogenes*, which is typically associated with larvae, and opposite, the adult-associated *Gilliamella bombicola* and *Snodgrassella alvi*. A SIMPER analysis on all castes and developmental stages (Appendix 6) suggests that the overall average dissimilarity between samples =79.96%.

3.3.3 Proposed model of gut microbiota assembly

The data suggested some clear, broad patterns of community assembly from all life stages throughout worker development and proposing a model of gut microbiota assembly (Fig. 3.3). Beginning at egg laying, it was established that eggs within controlled and natural environments contained on average 41 ± 16.9 OTUs, consisting of taxa predominantly from Enterobacteriaceae and Lactobacillaceae families, in particular, *E. aerogenes* (22.8 ± 15.9%), with *G. bombicola* and *S. alvi* also present in low abundance (8.4 ± 6.0% and 3.3 ± 3.3%, respectively).

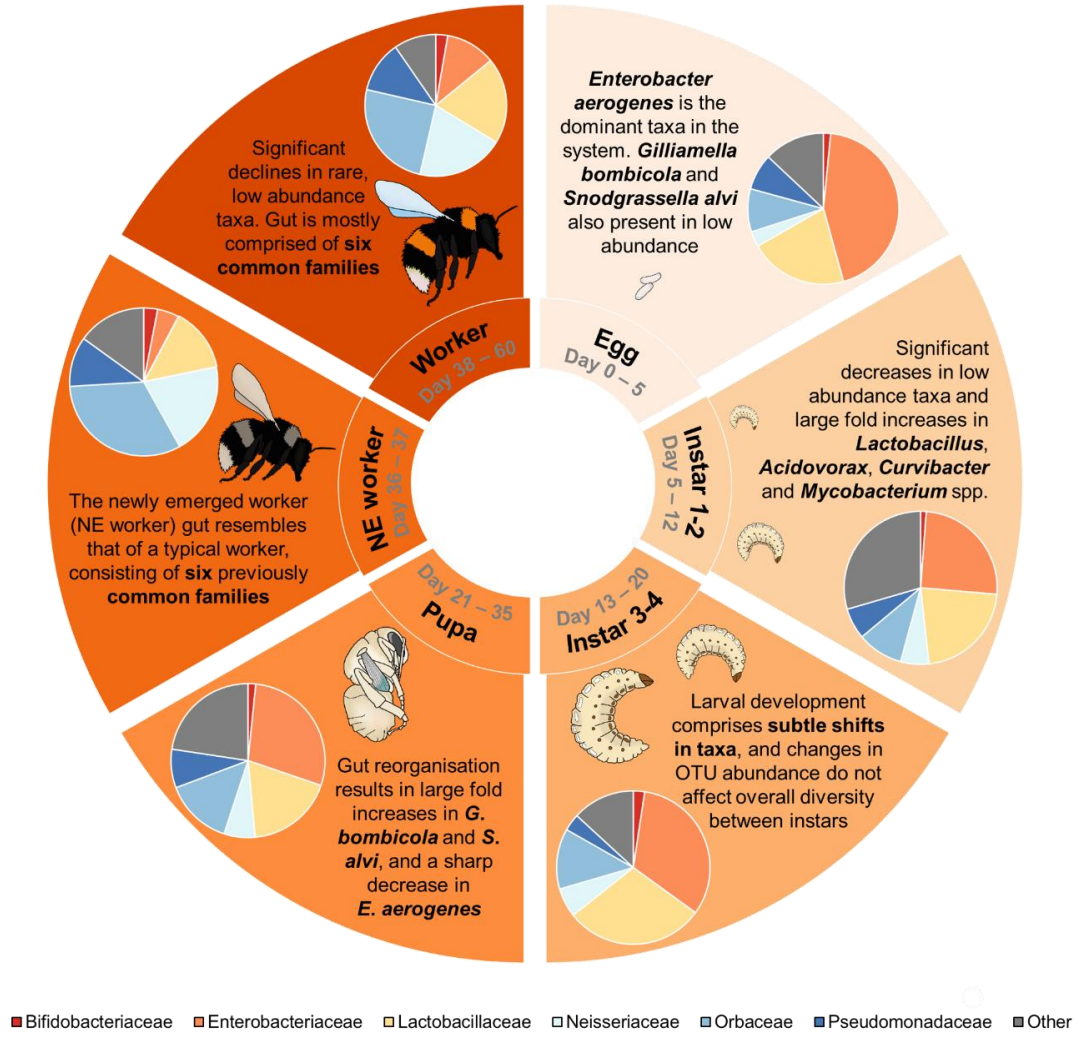

Figure 3.3 Overview of the life cycle and gut microbiota development in *Bombus terrestris* workers, from egg to mature adult. Pie charts represent mean percentage relative abundance of bacterial classes present in each developmental stage.

Larval development saw significant decreases in detection of low abundance taxa (<1% relative abundance) with large fold increases in *Lactobacillus*, *Acidovorax*, *Curvibacter* and *Mycobacterium* spp., resulting in high alpha diversity. The number of OTUs increased as the egg developed into a larva, reaching a peak at the first instar 76.0 ± 24.3. By the third instar, OTU detection decreased by 38.1%, before increasing 36.7% at instar four, and continuing to increase as the larva developed into a pupa. Despite these changes in OTU detection, overall diversity between instars did not differ significantly (χ^2 = 6.15, p = 0.10, n = 150).

At the pupal stage, the relative abundances of two major *Lactobacillus* spp. (9.6 ± 4.4%) decreased along with other rarer taxa from Firmicutes and Actinobacteria. At this stage, the relative abundance of *E. aerogenes* decreased significantly (t = 5.31, p < 0.001, n = 74) corresponding with large fold increases in the main constituents of the worker microbiota, *G. bombicola* and *S. alvi*. Diversity significantly decreased between pupa and newly emerged workers (t = 3.25, p = 0.001, n = 74).

The newly emerged worker gut resembled that of a typical worker, consisting of the six previously identified families, but in the relatively brief time an adult worker matures (1–2 days), there was a major shift in composition characterised by significant declines in detection of rarer low abundance taxa (Fig. 3.4).

3.3.4 Environmental impacts on microbiota

The influence of environmental sources of bacteria (via foraging) on host gut microbiota was first analysed using Fisher's alpha diversity to test for differences between colonies confined to the lab and those foraging freely in the field. Overall, there was no significant difference in alpha diversity between the two treatments (t = 0.22, p = 0.83, n = 376). A two-way ANOVA analysis of developing stages and mature castes also indicated that there was no significant difference in diversity between control or foraging treatments ($F_{(1, 375)}$ = 7.44, p = 0.86, n = 376). Further exploration of the common gut taxa also showed that there were no significant differences in the relative abundance of the common species *G. bombicola* and *S. alvi* between control and foraging treatments (t = 1.23, p = 0.22, n = 376; t = 0.81, p = 0.42, n = 376, respectively).

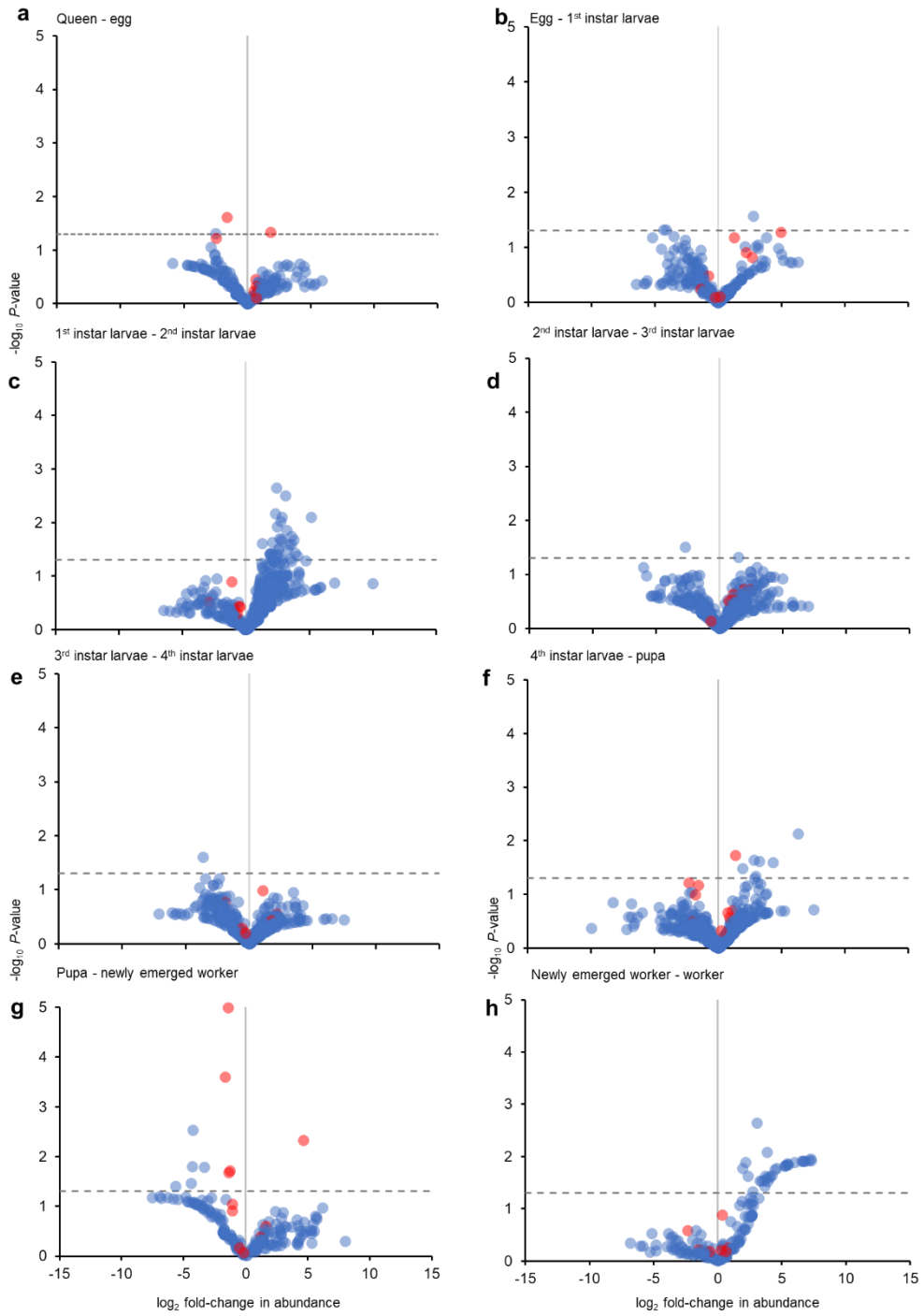

Figure 3.4 Changes in relative abundances of individual gut microbiota taxa in *B. terrestris* worker development from egg laying by queen (a), larval instars (b – f), pupa (g), and emergence as a mature worker (h). Shown are the log fold-changes in relative abundance of common (red) and rare (blue) taxa, with positive and negative values representing increases and decreases in relative abundance through development. Significance (p = 0.05) is shown by the dashed line.

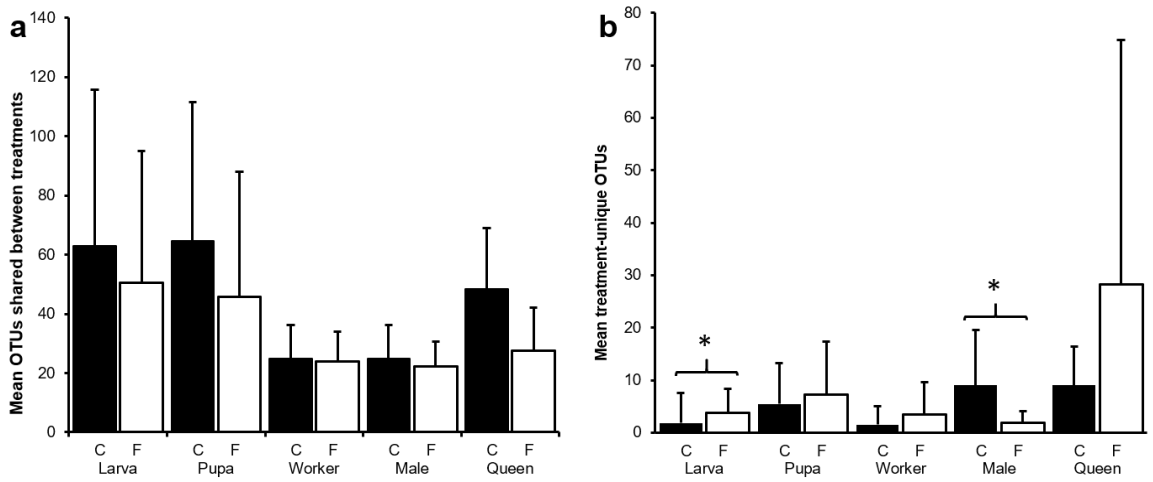

Figure 3.5 Comparisons of associated and unique OTUs between larvae, pupae, workers, males and mature queens in *Bombus terrestris* reared in controlled laboratory conditions (C) and allowed to forage (F). (a) Mean number of shared OTUs between treatments at each caste/developmental stage; (b) mean number of treatment-unique OTUs present in each caste/developmental stage. Error bars indicate + 1 standard deviation (SD), and asterisks denote significant differences at the $p < 0.05$ level between treatments for the same caste/stage.

The shared abundance of OTUs between castes and treatments is outlined in Table 3.1. Two-way ANOVA analyses indicated that overall there was a difference in the presence of shared OTUs between treatments ($F_{(1,\ 375)} = 4.34$, $p = 0.04$, $n = 376$) where control individuals tended to share more OTUs on average. There was also a significant different between stage/caste ($F_{(4,\ 375)} = 14.46$, $p < 0.001$, $n = 376$), indicating that larvae and pupae in both treatments had more shared OTUs than mature castes (Fig 3.5a).

While overall bacterial diversity and the abundance of shared OTUs were not affected by treatment type, the number of unique OTUs was (Fig 3.5b). Examination of treatment effects on unique OTU presence within each caste stage (i.e. OTUs identified as 'treatment-unique' for a caste) showed that individuals from foraging colonies generally contained more treatment-unique OTUs than control colonies, except for males. This difference was statistically significant in larvae ($t = -2.14$, $p = 0.03$, $n = 150$), where there were over three times as many unique OTUs present in larvae from foraging colonies. In males, however, the opposite was found. Those in control colonies tended to have more treatment-unique OTUs ($t = 3.01$, $p = 0.006$, $n = 34$). Queens had 301 OTUs unique to

foraging treatments compared to just 32 in the control treatment, though this was not statistically significant ($t = -1.88$, $p = 0.07$, $n = 28$). Queens in colonies that foraged had on average 50 unique OTUs compared to 58 in their control counterparts. There was a high degree of variation among individuals, with many treatment-unique OTUs present in individual queen samples.

Overall, control colonies had 11 OTUs unique to their treatment, compared to 79 OTUs in foraging colonies. These OTUs belonged to the bee microbiota 'phyla' Bacteroidetes, Deltaproteobacteria, Firmicutes, Alphaproteobacteria, Acidobacteria, Betaproteobacteria, Gammaproteobacteria, and Actinobacteria. Both treatment types had similar proportions of unique OTUs within each phylum, except Actinobacteria, which constituted 8.9% of the unique OTUs in foraging colonies, compared to 2.6% in control colonies. There was a significant difference in the number of treatment-unique OTUs per sample ($t = -4.26$, $p < 0.0001$, $n = 376$), in which samples from foraging colonies had over twice as many treatment-unique OTUs per sample.

3.4 DISCUSSION

This study proposes the first model of gut microbiota assembly and development within the pollinator *Bombus terrestris*. It provides insights into the development of the host bacterial community, highlighting a set of fourteen ubiquitous OTUs found across all castes and developing stages, regardless of whether bees had access to natural foraging or not. Significant differences in gut microbial community composition and diversity were identified throughout colony development and between castes. Microbiota typically comprised the same six bacterial families, but these varied in relative proportions during development. In addition, bacterial DNA was recovered from every caste and developmental stage, which meant that the subtle changes in diversity and composition from eggs to larval instars, through pupation and to adult emergence could be monitored.

Throughout worker development, microbiota assembly followed a clear pattern, shifting from a diverse bacterial community to a more conserved set of core microbiota after adult emergence. There did not appear to be any differences in gut diversity or composition between larval instars in bumblebees contrary to what has been published for

the honey bee (Vojvodic *et al*. 2013). Instead, within the larval instars, the major taxa within the gut remained relatively stable, suggesting subtle shifts in gut microbiota throughout development. In *A. mellifera*, larvae have defined microbial communities at different instars, possibly due to dietary and behavioural differences between the two bees. Briefly, *A. mellifera* larvae receive nutrition from nurse bees via trophallaxis. In early stages this is comprised of royal jelly, which is antiseptic (García *et al*. 2010), but later this diet is supplemented with honey, nectar and pollen (Beetsma 1985). Bumblebee larvae however, are fed a liquid mixture of pollen and nectar regardless of age (Sladen 1912; Michener 1974), which appears to prevent the bacterial succession pattern exhibited in *A. mellifera*.

The dominant taxon throughout larval development was putatively identified as *Enterobacter aerogenes*, and its ubiquitous presence and high abundance in the larval gut suggests an important functional role that is not required in adults (Parmentier *et al*. 2018). Many species within the class Enterobacteriaceae contain facultative anaerobes involved in the fermentation of sugars and nitrogen metabolism (Anderson *et al*. 2011; Kakumanu *et al*. 2016). It is well reported that diversity and structure of the microbial gut community is influenced and maintained by the host diet (Colman *et al*. 2012; Blum *et al*. 2013), which supports the theory that dietary differences between larvae and adults could account for differences in gut communities. *B. terrestris* larvae feed primarily on protein-rich pollen for growth and development, in contrast to workers that mostly subsist on high levels of carbohydrates (Stabler *et al*. 2015). *E. aerogenes* appears well suited to take advantage of this niche within the larval gut. In addition to being a facultative anaerobe, it can utilise a variety of different sugars (Martinez-Porqueras *et al*. 2013) and has a short doubling time (Tanisho 1998), which may allow it to proliferate to such an extent. Dietary requirements and gut physiology are likely affecting the gut communities within different *B. terrestris* castes, and this distinct adult/larval difference clearly highlights the need for further investigation into the functions of larval gut microbiota.

In contrast to work in honey bees queens, bumblebee queens were more diverse than workers (the opposite was seen in Tarpy *et al.* 2015); they were also not dominated by Alphaproteobacteria. Indeed, superficially, queens appeared to have a similar microbial community to larvae, characterised by *Enterobacter* and *Lactobacillus* spp., though it was

typically more diverse and contained additional Bradyrhizobiaceae. Like larvae, queens rely on a protein-rich diet for egg laying and remain within the colony once the first generation of workers is established. This may explain some of these similarities, though the greater abundance of *Lactobacillus* spp. could potentially be due to consuming more nectar (Anderson *et al*. 2013). However, there were striking compositional differences between new and mature queens. The gut microbiota of new queens was comprised of more *Enterobacter* spp. than mature queens, while the latter had nearly twice as many *Lactobacillus* spp. than their daughters. In *A. mellifera*, queen microbiomes were demonstrated to change throughout maturity (Tarpy *et al*. 2015). It is likely that the shift in new queens from being relatively more distinct and idiosyncratic at emergence, with convergence to a more conserved composition as they mature, is due to interactions with the colony and nest materials, in addition to consuming a more mixed diet.

While key elements of the microbiota were shared throughout all members of the colony, presumably via horizontal transmission (Koch & Schmid-Hempel 2011b), there was also some evidence for potential vertical transmission of bacteria. Egg samples were found to contain *Gilliamella* and *Snodgrassella* spp., and corroborated a previous study that eggs contained amplifiable bacterial DNA (Long-long *et al*. 2014). Though it is unlikely to be environmental (nest) contamination as samples were surface sterilised in 70% ethanol and vortexed to remove nest material before processing (Banfalvi 2016), this cannot be ruled out definitively. However, this finding is especially noteworthy, as these typical gut microbiota species have also been found in queen ovaries (Billiet 2016), and maternal transmission of beneficial symbionts is not unheard of in other insect taxa (Douglas 1989). The presence of these key bacteria at such an early developmental stage (and prior to feeding) suggests that they may be maternally inherited from the queen through egg laying and are able to persist within an individual throughout its lifetime.

It is clear that the majority of the microbiota found across castes and development stages comprised highly conserved OTUs that were relatively unaffected by foraging context. While overall gut diversity was not significantly affected by exposure to the natural environment, it did influence the number of unique OTUs. Individuals from foraging colonies typically contained more treatment-unique OTUs than in the controls, which may be a result of horizontal transmission (Newbold *et al*. 2015). It would be

interesting to know if the identity of the rarer microbiota influences overall host health more than diversity. In general, many of the rarer OTUs belonged to the same phyla as the common OTUs, which could suggest some degree of taxonomic redundancy within the gut communities that could increase resilience of their function. However, one striking difference was the presence of *Pediococcus acidilactici* in daughter queens from foraging colonies. *P. acidilactici* has been tested as a potential probiotic in *A. mellifera*, and was observed to be beneficial directly after bee emergence (Kaznowski *et al*. 2005). Its potential role in bumblebees will require further investigation to establish its exact function within the gut, but what is highly significant is that this taxon was only isolated in daughter queens from the foraging treatment.

Only larval guts were significantly impacted when their colony was exposed to foraging, resulting in an increase in the numbers of treatment-unique OTUs, presumably due to increased diet diversity from foraged pollen. Pollen is able to harbour a variety of bacterial species, while the carbohydrate-rich adult diet of foraged nectar supports a more limited, acidophilic community (Anderson *et al*. 2013). Workers are exposed to both food types as foragers but by having an already established gut microbiota (Koch & Schmid-Hempel 2011a) the probability of acquiring new taxa appeared to be reduced (e.g. through 'priority effects'; Young *et al.* 2001). In contrast, normal larval development is continuously changing the gut environment (Alford 1975) perhaps making it more susceptible to colonisation.

Overall, this description of microbiota assembly throughout *B. terrestris* development highlights a typical pathway of microbial colonisation, and how key gut community composition differs between *B. terrestris* castes. Four dominant species (*Gilliamella bombicola*, *Snodgrassella alvi*, *Lactobacillus apis* and *Enterobacter aerogenes*) were most common during worker development and were ubiquitous in colonies reared in both natural and artificial settings, suggesting their importance to the host. Foraging was not found to be a key contributor to gut microbiota diversity, but access to external sources of bacteria appeared to influence the rare microbiota in flux. Clearly, caste and developmental stage influence bumblebee gut microbiota, so to maximise the value of this model system it is crucial that we understand how development shapes and forms the gut microbial community.

CHAPTER 4:

CASTE-SPECIFIC GUT MICROBIOTA RESPONSES TO FORAGING

4.1 INTRODUCTION

Establishment and persistence of beneficial microbiota is highly important for host health and survival (Sommer & Bäckhed 2013) as host-associated bacteria may aid critical functions such as nutrient acquisition (Warnecke & Hugenholtz 2007), pathogen defence (Dillon *et al*. 2005), and resistance to toxic compounds (Kikuchi *et al*. 2012; Ceja-Navarro *et al*. 2015). Bacterial population stability therefore plays a central role in host health, ensuring that the presence of advantageous symbionts and their associated functions are retained by the host throughout its lifetime (Coyte *et al*. 2015). Numerous factors can influence stability of microbial populations, including health and age of the host, gut morphology, and the availability of nutrients (Dillon & Dillon 2004; Anderson *et al*. 2011; Martinson *et al*. 2011). However, our understanding of a 'healthy' or 'core' microbiota can be biased by sample method, environment and the time of collection (Pollock *et al*. 2018).

In gut microbiota analysis, as with many parts of biology, model organisms are used extensively to explain host-bacteria interactions (Pernice *et al*. 2014). Social bees in particular make a powerful model system, containing a simple, well-characterised, but specialised community made up of a small number of core bacteria (Mohr & Tebbe 2006; Cox-Foster *et al.* 2007; Martinson *et al.* 2011; Moran *et al.* 2012; Engel *et al.* 2012; Engel & Moran 2013a). They exhibit sociality and communal living allowing us to extrapolate to higher, more complex organisms (Koch *et al*. 2013; Kwong *et al*. 2014), and as important pollinator species, the maintenance of host health is of critical importance for both the conservation of biodiversity and food security (Klein *et al*. 2007).

The development of bee gut microbiota at the individual level is shaped by host phenotype and gut morphology, along with nutrition and exposure to external sources of

bacteria (e.g. via foraging) (Dillon & Dillon 2004; Martinson *et al*. 2011; Newbold *et al*. 2015). At the broader colony population scale, seasonal fluctuations in foraging plant populations may influence microbiota abundance and diversity through differential sugar and protein composition. In addition, this may then impact interactions between flower visitors with different phenologies (Ludvigsen *et al*. 2015; Anderson & Ricigliano 2017). In addition general variability in colony life (e.g. poor nutrition) can result in gut dysbiosis and increase susceptibility to pathogens and disease (Maes *et al*. 2016).

Currently, our understanding of the stability of bacterial populations in *Bombus* is unclear (Cariveau *et al*. 2014). Much work has stressed the conserved nature of the bee gut microbiota across geographical locations (Martinson *et al*. 2011; Moran *et al*. 2012), yet shifts in microbiota have been observed when moving colonies from restricted to diverse foraging environments (Newbold *et al*. 2015). Similarly, Cariveau *et al.* (2014) found a decline in worker gut bacterial diversity towards the end of summer, possibly resulting from environmental symbionts outcompeting and becoming more dominant than preferred core taxa. These studies suggest that the core taxa are largely unchanged, but a proportion of the microbiota, made up of rarer taxa, are flexible and change in relation to the environment and time potentially as a result of dietary changes (Chapter 3; Newbold *et al.* 2015).

There is considerable evidence that the diversity and structure of the gut microbiota is influenced and maintained by the host diet (Colman *et al*. 2012). By foraging, bees can obtain bacteria from the floral resources in the environment (Anderson *et al*. 2013), but as floral species grow and flower at different times in the season, and also change daily in terms of floral resource quality (Prys-Jones & Corbet 1987) the amount of bacterial diversity they come into contact with is likely to change. Research in *Drosophila* found that acquisition and persistence of bacteria relied upon repeated inoculation via diet, implying that that food sources can act as bacterial reservoirs in the establishment of the microbiome (Blum *et al*. 2013), as well as suggesting some bacteria are transient when nutrients are unavailable.

The current view of microbiome composition is temporally static, ignoring seasonal dynamics. However, in an annual bee species, like the bumblebee, this may alter the success of colony founding, as well as growth and fitness throughout the season. Here this

study aimed to observe microbiota development in commercially reared colonies allowed to forage in natural environments of differing resource quality. As previously described in Chapter 3, there are distinct differences in composition between castes and larvae, so the scope of this investigation was broadened to incorporate major colony constituents (larvae, workers, males and queens) to explore both temporal and environmental impacts of foraging on the whole colony.

4.2 MATERIALS AND METHODS

4.2.1 Study species

Bombus terrestris is a short-tongued bumblebee, widely distributed throughout Europe and occurring in colonies of up to 350 workers headed by a single queen (Alford 1975). It typically follows an annual lifecycle in temperate habitats, although winter cycles are becoming increasingly common (Stelzer *et al.* 2010). This species is a generalist pollinator and can be successfully bred in captivity making it especially important in commercial bumblebee production where it is reared for fruit and vegetable pollination (Velthuis *et al*. 2006).

4.2.2 Field sites and sample processing

A total of 35 commercially reared colonies of *Bombus terrestris audax* (Biobest Group NV, Belgium) were obtained early in development at a size of *c*. 10–15 workers. Colonies were fed on a diet of gamma-irradiated pollen supplemented with BIOGLUC® (Biobest Group NV, Belgium), and monitored for one week to ensure normal development prior to experimental manipulation.

Colonies were distributed equally between six experimental field sites and one indoor control as previously described in Sections 2.2.1 and 3.2.2. Sites were categorised by sample quality, into either ‘resource-rich’ (RR; pollen/nectar-rich wild flowers), and ‘resource-poor’ (RP; grass-dominated field margins). Control colonies were housed in a controlled temperature room on a day/night cycle (25 ± 2°C and 60% ± 5 RH) with *ad libitum* irradiated pollen and BIOGLUC®.

Colony size, weight and general condition were monitored weekly throughout the experiment. At regular intervals, one colony from each field site and a control colony were

selected at random and destructively sampled. All samples were euthanized through storage at −80°C within 2 h of collection from the field. Sites were sampled at 2, 3, 5, 7 and 8 weeks (hereafter referred to as T_2, T_3, T_5, T_7 and T_8) after the initiation of the study (between 25th June – 6th August 2015), when colonies were aged 5, 6, 8, 10 and 11 weeks, respectively. Time points were skewed towards the start and end of colony development to capture any potential microbiota shift expected to occur in the early stages of colony initiation with the first generations of workers. Once euthanized, whole colonies were weighed inside the nest box to estimate colony size and development before and nest weight was calculated by deducting the box weight from the from the whole colony weight. After colonies were dismantled and sorted into larvae, workers, males and queens (Appendix 1), anatomical measurements were taken from samples prior to dissection, in addition to estimations of worker age and foraging activity based on wing wear (Mueller & Wolf-Mueller 1993).

All but two of the 35 colonies used in this experiment survived to their sample point. Colonies that failed had become infested with the specialist predator, *Aphomia sociella* (wax moth), which consumed the *Bombus* brood and food stores. Both colonies were in RP sites (B and G). At the start of the trial, site B had been categorised as RR but was mown four weeks into the experiment, greatly reducing its floral diversity. For this reason, it was reclassified as resource-poor from T_5 onwards. From the surviving colonies, 33 were processed for gut microbiota analysis, where a representative 10% subset of larvae, workers and males were extracted per colony for molecular sequencing in addition to colony queens, resulting in 319 individual samples.

4.2.3 DNA isolation and sequencing

Gut bacterial DNA was extracted from sterilised whole bee guts using an optimised PowerSoil®-htp 96 Well Soil DNA Isolation Kit protocol, outlined in Section 2.6.2 and Appendix 2. From these samples, 20 ng of template DNA was amplified with unique dual-code barcodes, normalised using the SequelPrep™ Normalization Plate (96) Kit (Invitrogen) (Section 2.7.1; Kozich *et al.* 2013), and then gel-purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), before being quantified with a Qubit® Fluorometer using a dsDNA high sensitivity assay kit (Life Technologies). The resulting

amplicon library consisting of ~550 bp amplicons spanning the V3–V4 hypervariable regions of the 16S rRNA gene, was then sequenced at a concentration of 6 pM with a 10% PhiX addition on an Illumina MiSeq using V3 chemistry (Illumina Inc., San Diego, CA, USA).

4.2.4 Sequence analysis

Resulting raw sequence reads were processed and analysed using the sequencing analysis pipeline outlined in Section 2.8.3 using default parameters. Potential kit contamination was controlled for in subsequent checks on the data (Salter *et al*. 2014; Glassing *et al*. 2016), and 'singleton' sequences and non-bacterial taxa were also removed.

After rarefaction and filtering, 715,264 of the total sequences were retained, representing 737 operational taxonomic units (OTUs) distributed across 319 samples (Table 4.1). Since many OTUs were left unresolved further than phylum classification, additional taxonomic classification was performed with the Basic Local Alignment Search Tool (Altschul *et al*. 1990) and EzBioCloud database (Yoon *et al*. 2017). Identity was assigned based on >99% matches of short DNA fragments so OTU identities should still be considered putative.

4.2.5 Statistical analysis

Rarefaction curves confirmed that MiSeq sequencing depth was sufficient to capture the alpha diversity within each sample (Appendix 3). Data were rarefied to the lowest read count depth (=2273 sequences) in R:VEGAN with the 'rrarefy' function (Dixon 2003), and this subset was used in all subsequent analyses.

Alpha diversity was quantified using Fisher's alpha (log series alpha), and to investigate bacterial composition, a frequency table of OTUs in each sample was screened and converted to a Bray-Curtis dissimilarity matrix using PAST (Hammer *et al*. 2001). Larval instars were again grouped together as 'larva' and component taxa within each host caste and developmental stage into 'common' (sample occupancy with >75% across all samples) and 'rare' groupings (Hedin *et al*. 2015).

Univariate statistics were performed in R (v3.3.2), with multivariate analyses in PAST (v3.08) and XLSTAT (v2018.2, Addinsoft, Paris, France) package for Microsoft Excel.

4.3 RESULTS

4.3.1 Adult/larval gut microbiota responses to the environment

Mature caste and larval gut microbiota was sampled from developing *Bombus terrestris* colonies that had been challenged with natural and artificial environments over a period of two months. A total of 737 bacterial OTUs were identified, spanning mature castes and larvae. Initial analyses highlighted significant adult/larval differences in average OTU counts ($\chi^2 = 59.12$, $p < 0.0001$, $n = 319$), with pairwise comparisons indicating that larvae tended to have on average more OTUs than workers and males ($p < 0.001$), as did queens when compared to workers ($p < 0.01$). Similarly, there were significant differences in Fisher's alpha diversity between larvae and adults ($\chi^2 = 59.38$, $p < 0.001$, $n = 319$). Specifically, larvae and pupae were significantly more diverse than workers and males regardless of treatment ($p < 0.001$), and again, queens were significantly more diverse than workers ($p < 0.01$).

Table 4.1 Summary data from castes and developmental stages of *B. terrestris* in both control (C), resource-rich (RR), and resource-poor (RP) foraging treatments, including brief metadata.

Variable	Larva			Worker			Male			Queen		
	C	RR	RP	C	RR	RP	C	RR	RP	C	RR	RP
Sample size (*n*)	35	58	56	34	35	38	21	4	9	4	10	14
Mass (g)	0.112	0.119	0.153	0.198	0.139	0.165	0.252	0.240	0.241	0.722	0.819	0.835
Head width	1.24	1.32	1.42	3.67	3.53	3.61	3.76	3.94	4.17	5.04	5.404	5.53
Wing length	-	-	-	12.11	10.58	11.42	13.31	14.45	13.93	14.99	17.20	15.93
Average OTU count	65	43	65	26	28	27	34	28	22	57	52	60
OTUs shared between time points	8	35	97	19	27	27	-	-	-	-	-	-
OTUs associated with each treatment	576	565	580	238	254	263	250	69	85	151	379	337
OTUs shared between treatments		416			130			46			96	
Treatment-unique OTUs	43	28	34	45	58	63	165	10	12	31	1	8

The potential interaction between caste/stage and habitat were investigated to see if there were any differences in gut microbiota diversity. Gut diversity in adults was unaffected by habitat quality (Fig. 4.1), but differed significantly in larvae ($\chi^2 = 6.77$, $p = 0.03$, $n = 150$). In both the indoor control (C) and resource-poor (RP) treatments, larval guts contained more diverse gut communities when compared the resource-rich (RR) sites ($p < 0.05$).

Pairwise Bray-Curtis dissimilarity values were calculated to characterise the distribution of microbiota for larvae and castes between the three habitat types. Non-multidimensional scaling (NMDS) were performed to visualise differences in gut communities between treatments, and time points (Fig. 4.2). Overall, larval gut microbiota in the T_2 and T_8 time points appeared to cluster closely compared to T_3–T_7 samples. This was also observed in C and RR treatments, though was not apparent in RP larvae.

Additional similarity of percentage analyses (SIMPER; Appendix 7) were used to assess the contribution of individual taxa to the overall dissimilarity. Characteristic microbiota species *Enterobacter aerogenes, Gilliamella bombicola, Lactobacillus apis* and *L. bombicola*, amongst others, contributed greatly to the overall dissimilarity between habitat types. In addition, *Enterococcus faecalis* was largely unobserved in larvae from RR colonies, but present in those from C and RP. While *Gilliamella bombicola* and *L. kunkeii* were more abundant in RR larvae compared to the other two treatments. Similarly, there were differences between the lab-reared and foraging colonies. For instance, *Providencia vermicola* was primarily found in field colonies, while conversely, *Pseudocitrobacter anthropi* appeared more abundant in the indoor colonies.

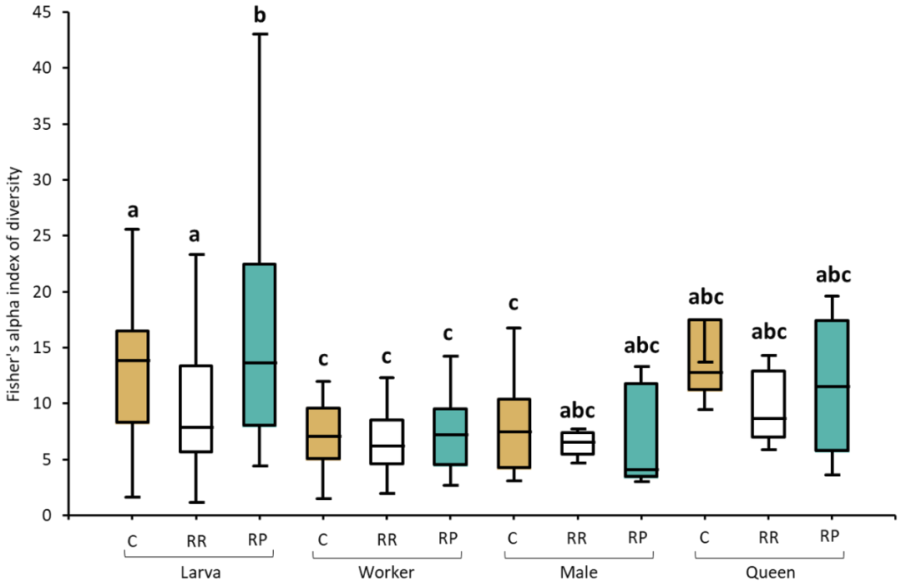

Figure 4.1 Fisher's alpha diversity across all time points in larvae, workers, males and queens between treatments C (indoor control; gold), RR (resource-rich; white), and RP (resource-poor; turquoise). Significant differences between treatments/stages calculated by Kruskal-Wallis at the $p < 0.05$ level and are denoted by letters, i.e. 'a' is significantly different to 'b'.

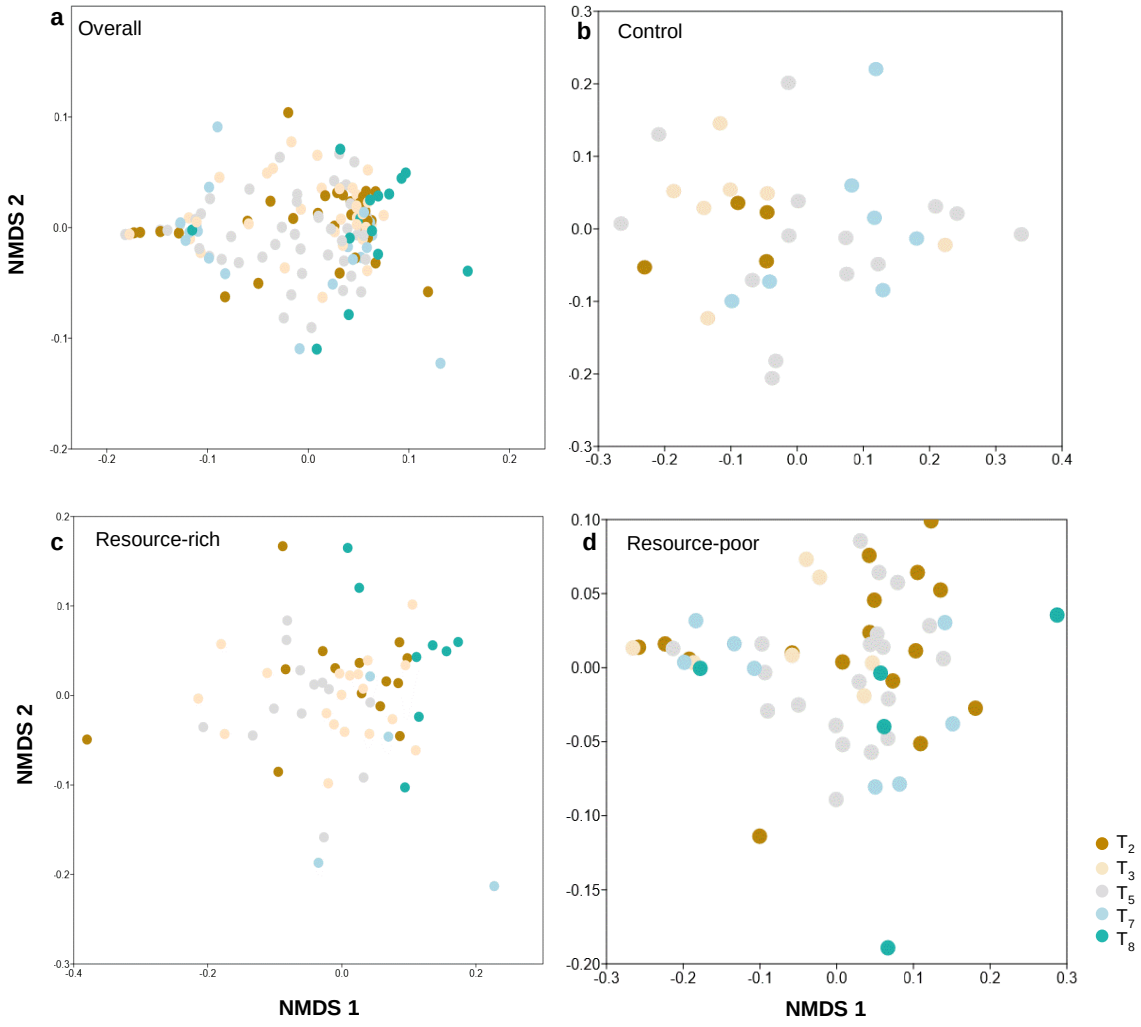

Figure 4.2 Non-multidimensional scaling analysis (NMDS) of larval gut microbiota between different sample time points (T_2 – gold, T_3 – beige, T_5 – light grey, T_7 – light blue, and T_8 – turquoise). Panel (a) shows all larval samples, while (b–d) display NMDS plots for control (C), resource-rich (RR) and resource-poor (RP), respectively. Additional SIMPER analyses (Appendices 7 and 8) further explore the taxa that contribute most to the dissimilarity between treatments and time points in larvae.

4.3.2 Longitudinal impacts of foraging on gut microbiota

There was a significant effect of time point on Fisher's alpha diversity of the gut microbiota (χ^2 = 23.17, p < 0.001, n = 319), where the final time point (T_8) had significantly lower diversity compared to T_2, T_5 and T_7 (p < 0.05). After examining each caste individually, the reproductive castes showed no significant difference in diversity over time, while workers showed borderline significance (χ^2 = 9.90, p = 0.049, n = 107), but the differences between time points were no longer significant after Bonferroni-correction. Additionally, larvae also showed significant differences in diversity over time (Fig. 4.2; χ^2 = 23.26, p < 0.01, n = 150). Larval guts from T_8 were significantly less diverse compared to earlier in the colony season (p < 0.05).

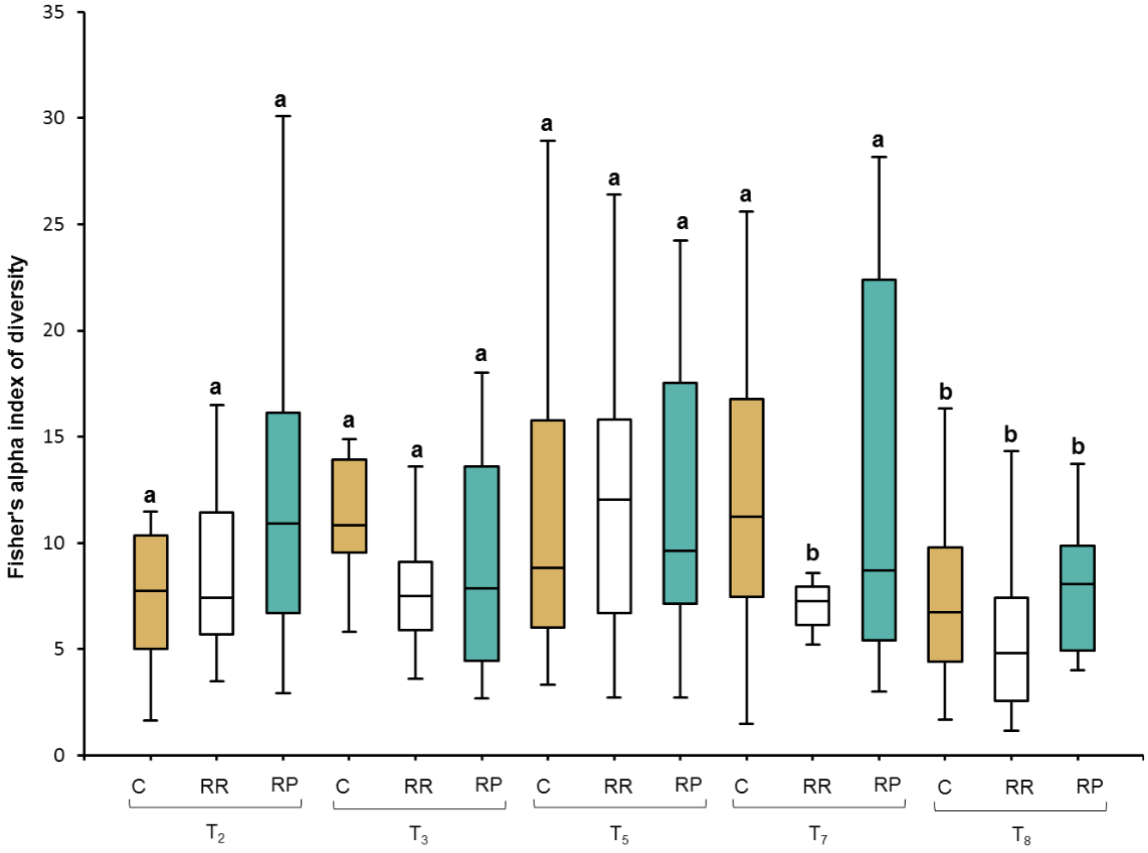

Figure 4.3 Comparison of Fisher's alpha diversity across all time points in larvae between treatments C (indoor control; gold), RR (resource-rich; white), and RP (resource-poor; turquoise). Error bars represent +1 standard deviation (SD). Significant difference between time points and treatments are denoted by different letters.

After evaluating the effects of habitat type in larvae, it was apparent that there was no significant difference in diversity between time points in both the indoor control or RP treatment. However, larvae in the RR treatment did respond temporally (χ^2 = 20.71, p < 0.001, n = 59), with gut microbiota between T_2–T_5 significantly more diverse than at T_8 (p < 0.01). The impact of time on RR larval diversity was present in both common (χ^2 = 21.64, p < 0.001, n = 64) and rare OTU totals (χ^2 = 22.86, p < 0.001, n = 64).

Interestingly, when comparing larval composition between treatments and over time (Fig. 4.3) the dominant genera at each time appear much more variable than in workers. Worker guts usually contain the key genera *Gilliamella*, *Snodgrassella*, and *Lactobacillus*, often in similar proportions, while in larvae the dominating taxa *Gilliamella*, *Enterobacter* and *Lactobacillus* vary much more in their relative proportions between treatments and time points.

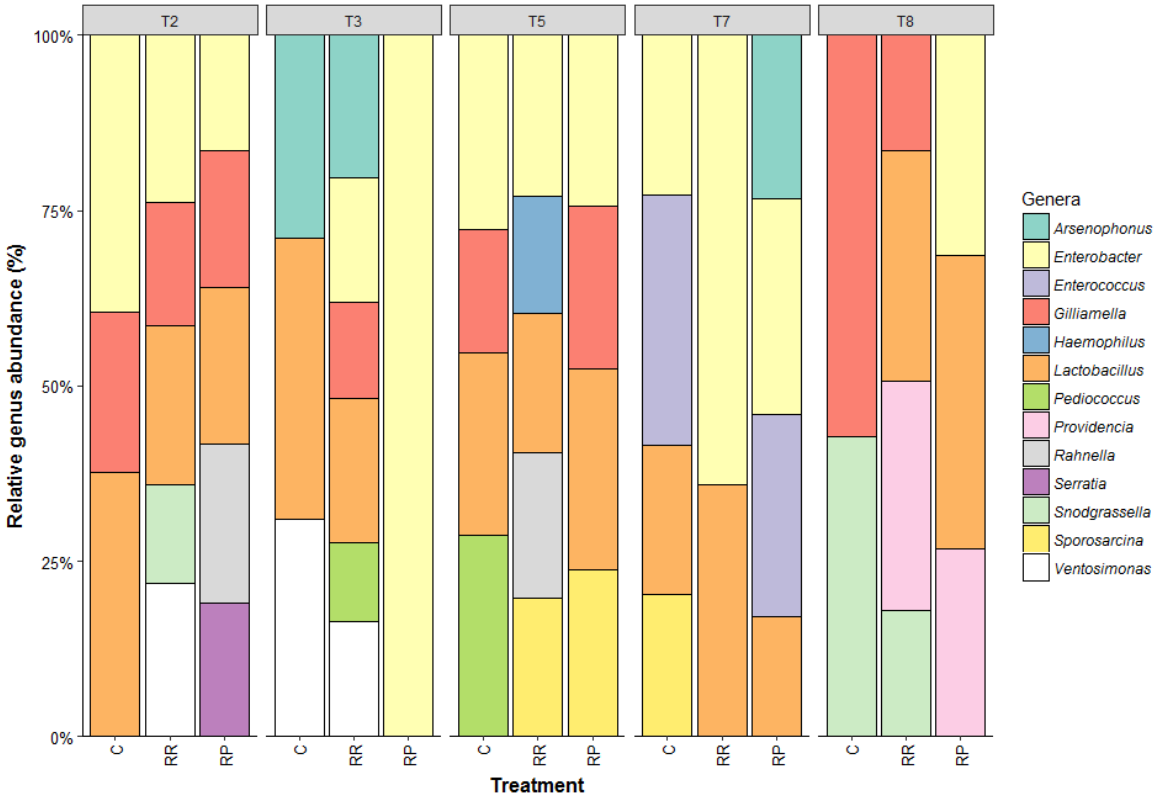

Figure 4.4 Comparison of average larval gut microbiota between each treatment over time (T2 – T8). Highlighted is the variation in relative abundance (% mean) of genera present in at least 50% of the larval samples sequenced.

SIMPER analyses (Appendix 8) indicated that the taxa contributing most to this dissimilarity between time points included *E. aerogenes*, *G. bombicola*, *Snodgrassella alvi*, as well as *Lactobacillus* species. What was particularly striking were the differences in relative abundances between T_7–T_8. The dominant taxa in larvae, *E. aerogenes*, steadily increased in abundance throughout the colony season and peaked at 38.98 ± 39.91% before there was a decrease in abundance to 0.52 ± 0.54%. Other decreases were observed in *Lactobacillus*, *Arsenophonus* and *Enterococcus* spp., while in RR colonies at T_8 there was a substantial increase in an OTU putatively identified as *Providencia vermicola*, from 0.02 ± 0.03% to 33.25 ± 31.24% in relative abundance. Along with this, the two key taxa *G. bombicola* and *S. alvi* both increased from 8.32 ± 9.41% to 22.49 ± 14.89% and 3.33 ± 5.3% to 17.97 ± 12.43% mean relative abundance, respectively.

4.3.3 Comparisons between lab and field colonies

Fisher's alpha diversity was used to test for differences between colonies confined to the lab and colonies foraging freely in both field site conditions. There was no significant difference in Fisher's alpha diversity between the indoor control (A) and six field sites (B–G) tested ($\chi^2 = 6.28$, $p = 0.39$, $n = 319$), between field sites ($\chi^2 = 6.06$, $p = 0.30$, $n = 225$) or within the treatment groups (RR sites: $\chi^2 = 1.40$, $p = 0.50$, $n = 117$; RP sites: $\chi^2 = 7.13$, $p = 0.07$, $n = 111$). When all sites were pooled into their respective treatments, there was also no significant difference in gut microbiota diversity ($\chi^2 = 5.25$, $p = 0.07$, $n = 319$).

At the end of the experiment, the average colony size across all treatments was 212.8 g, an increase of 156.2 g from the average starting weight of 56.6 g at T_0. The smallest colony at T_7 weighed 50.3 g from a resource-poor habitat, whilst the largest was 358.6 g from the insectary control. All treatments increased in size over the course of the experiment (Appendix 9) with the indoor controls increasing more than both field colonies, as expected. Nest weight (used as a proxy for colony development) was significantly lower in RR (61.7 g) and RP (77.6 g) compared to the control nests (256.5 g) ($\chi^2 = 10.86$, $p < 0.01$, $n = 33$).

However, when comparing colonies measurements from the two field treatments, there was no evidence that colony characteristics, such as weight, population, and measures

of productivity (e.g. larval cells, honeypots, production of males) were significantly different between field treatments or correlated with bumblebee gut microbial diversity.

4.3.4 Shared and unique OTUs

Analysis of shared and treatment unique OTUs demonstrated that, in addition to the seven common OTUs, a further 416 OTUs were shared across treatments in larvae. Interestingly, the allocation of rare OTUs was highest in the C treatment (43 OTUs; 7% of the total OTUs present in this treatment) when compared with the RR (28 OTUs; 5%) and RP field treatments (34 OTUs; 6%), but when grouped together, larvae from field colonies contained 134 unique OTUs. Two-way ANOVA analyses showed that both treatment ($F_{(2,149)} = 3.88$, $p = 0.02$, $n = 150$) and time point ($F_{(2,149)} = 2.03$, $p = 0.09$, $n = 150$) were significantly affecting the number of shared OTUs in larvae. However, habitat-unique OTUs (i.e. those in just either control or field treatments) were only significantly affected by treatment ($F_{(2,149)} = 3.94$, $p = 0.22$, $n = 150$).

In workers, there were six common OTUs, and 130 shared between treatments. When focussing on the rarer taxa, the number of treatment unique OTUs was highest in the RP treatment (63 OTUs; 24%), followed by RR (58 OTUS; 23%) and C (45 OTUs; 19%). Both field treatments combined contained 158 OTUs not found in control workers. A two-way ANOVA of shared OTUs across treatments and time points highlighted again a borderline statistical significant difference temporally in workers ($F_{(2,106)} = 2.478$, $p = 0.049$, $n = 107$), while habitat-unique OTU presence was unaffected.

4.4 DISCUSSION

Over a period of two months, whole *Bombus terrestris* colonies were sampled intensively to investigate how different habitat challenges affected the development of gut microbiota in castes and developing stages through time. This study provides insights into the temporal stability of bacterial populations in adults and larvae, highlighting a critical adult/larval difference in response. This suggests that the larval gut microbiota is much more unstable over time compared to adult *Bombus* castes. Significant differences in gut diversity were also identified between different field treatments, with colonies in resource-poor sites producing larvae with more diverse gut microbiota. Overall, core gut microbiota was

largely unchanged between sites, while the rare (environmental OTUs) showed most variation.

For the duration of this study, only larval gut microbiota varied significantly over time and between habitat types, when compared to adult *Bombus* castes. Larvae contained more diverse gut communities, appearing to reiterate the adult/larval gut microbiota distinction previously highlighted in Chapter 3 and Parmentier *et al.* (2016). Not only does this suggest that gut communities of adults and larvae differ, but that they also respond differently to environmental and temporal stimuli.

As previously explored in Chapter 3, adults and larvae differ markedly in diet and gut structure. *B. terrestris* larvae feed primarily on protein-rich pollen for growth and development (Stabler *et al*. 2015) and do not excrete any waste products until metamorphosis, so anything they ingest is retained within the gut (Alford 1975). This provides the gut microbiota with a simple, consistent gut structure and ample food resources, promoting a diverse gut community. Adults, by contrast, have a compartmentalised gut, and their diet is largely comprised of carbohydrates (as well as pollen for egg-laying in queens) (Stabler *et al*. 2015). The dietary dependence of larvae on pollen may make them more likely to be impacted by seasonal trends in floral resources than adults are. In terms of bacterial colonisation, pollen is a more suitable growth medium for a much wider range of bacteria than acidophilic nectar, so any change to the foraging environment (e.g. habitat destruction) could change the spread, diversity, or survival of bacteria within floral resources (Anderson *et al*. 2013). Yet, if sources of pollen are causing an increase in diversity in larvae, it appears that workers were unaffected, despite gathering this pollen for the nest. It may be that while workers are exposed to these resources when foraging, their established gut microbiota buffers against colonisation by new taxa (Koch & Schmid-Hempel 2011b), making them less prone to perturbation than developing larvae.

Larval gut diversity was significantly different between the three treatments tested: indoor control (C), resource-rich (RR), and resource-poor (RP). One of the most surprising results of this study was that gut diversity differed between the two field treatments, where RP larvae harboured more diverse gut microbiota than their RR and C cohorts. Exposure to external sources of forage has been shown to affect the non-core gut community in indoor-reared workers (Newbold *et al*. 2015), and it was expected that a similar effect would be

found in comparisons of larvae from control and field sites, but not necessarily between field treatments.

Other than larval gut microbiota diversity, no other defining characteristics (except worker head width) significantly differed between RR and RP colonies. The size of an individual had no bearing on their gut community diversity (Koch *et al*. 2012), nor did the predicted diversity of their diet (McFrederick & Rehan 2016). Given that sites were less than a kilometre apart, located in mixed agricultural land with other food sources within flying distance (e.g. gardens), it is likely that some RP workers may have foraged away from their monoculture sites resulting in a more diverse diet than anticipated (Knight *et al*. 2005; Osborne *et al*. 2008). However, diversity of pollen diet has not been shown to correlate with gut bacterial species richness (McFrederick & Rehan 2016). In poor resource quality environments however, there may have been a greater number of interactions between individuals and species with limited resources (Evans *et al*. 2017). Flowers may be acting as bacterial reservoirs in the environment (Moran *et al*. 2012) and interactions on the same resources could result in an increase in bacterial transmission between foraging insects.

Throughout the foraging season, larval gut bacterial diversity generally increased to a peak in mid-July (T_5) then declined at the start of August (T_8), when floral resources availability may have been lower (Hicks *et al*. 2016). The bacteria that contributed most to this observed decline in diversity included *Enterobacter aerogenes* and *Providencia vermicola*. At the final sample point, *E. aerogenes* relative abundance decreased dramatically from being the dominant larval taxon in the gut, and instead was replaced by *P. vermicola*. The novel species, *P. vermicola*, was first isolated from *Steinernema thermophilum*, an entomopathogenic nematode (Somvanshi *et al*. 2006) but it is action in the *Bombus* gut remains unclear. If pathogenic, this infection could be causing gut dysbiosis in the larval host, as has been seen in honey bee workers infected with *Nosema* (Diaz *et al*. 2018). Although *P. vermicola* was found in both field-foraging treatments, it was more abundant overall in RR T_8 larvae. The dominance of *Providencia* in these samples dramatically reduced the diversity of larval gut microbiota in this group, as it appeared to have taken over the niche normally occupied by the common larval taxa. This could prevent them from carrying out their host-associated beneficial functions (e.g.

biofilm formation to trap invading pathogenic bacteria) and reduce host fitness (Raymann & Moran 2018). Normally, diseased larvae would be removed from the nest by nest bees, but towards the end of the annual colony cycle the queen switches from laying workers to focussing on producing reproductive castes (males and new queens), which inevitably results in decreasing worker populations and a reduction in brood care (O'Donnell *et al*. 2000). There is little work on bumblebee larval pests and diseases compared to that on honey bees (McMenamin *et al*. 2018) so understanding the signs of infection and how this can disrupt the gut microbiota, may suggest solutions for improving pollinator health that could be highly beneficial to bumblebee producers and for helping wild populations.

The composition and effectiveness of microbiota is likely to be a critical factor in the fight against pathogens, especially for larvae who have severely reduced immune function and are often the target of disease (Wilson-Rich *et al*. 2009; Forsgren *et al*. 2010; Mattila *et al*. 2012). Pathogen infection can disrupt microbiota directly or indirectly via the immune response and subsequently affect the present commensal bacteria (Hamdi *et al*. 2011), and thereby affect host health. While studies of failing honey bee colonies have not picked up significant declines in diversity (Cox-Foster *et al*. 2007; Cornman *et al*. 2012), in *Bombus*, studies with workers have shown increased microbial diversity (at colony level) in response to infection with the gut parasite, *Crithidia bombi* (Koch *et al*. 2012). This potentially could be the result of some cooperative behaviour between the gut bacteria and the parasite or, more likely, that the parasite disturbs the microbiota allowing more species to colonize. It appears that the presence of core taxa, not just diversity (which may include non-core species) may well be the real driver of protection for the host (Cariveau *et al*. 2014).

The findings here further solidify the adult/larval distinction in gut microbiota previously outlined in Chapter 3 and show that not only do adults and larva contain different assortments of bacterial taxa, but that their microbiota respond differently to perturbation. Larvae appear much more susceptible to colonisation and pathogen infection so could therefore be used as indicators to provide information about the health of the whole colony. In light of this, future investigations of bumblebee gut microbiota should explore whether larval gut microbiota could be used as a vitally important tool of study for understanding the microbial health of colonies and the microbial diversity of foraging sites.

CHAPTER 5:

MICROBIOTA THROUGHOUT QUEEN DIAPAUSE

5.1 INTRODUCTION

Diapause, a period of suspended development, is an important but energetically costly process (Hahn & Denlinger 2007) allowing animals to survive harsh seasonal conditions in a pre-programmed state of arrest. It is a key life strategy in many insects, and is often a crucial part of the reproductive life cycle (Tauber *et al*. 1986) allowing an individual to synchronize their life cycle to times better suited for growth and reproduction (Hahn & Denlinger 2011).

During this process, host functions do not completely cease (Hahn & Denlinger 2007), instead temporal patterns of gas exchange, gene expression, and regulation of metabolic processes all continue in reduced or upregulated levels, depending on the needs of the animal (Andrewartha 1952; Denlinger 2002; Koštál 2006). All non-essential functions cease and to reduce energy consumption, redundant tissues, such as the gut, deteriorate and must recover after diapause (de Kort 1990; Hahn & Denlinger 2007).

Animal guts harbour a wide range of microorganisms that can be highly beneficial, supporting digestion, pathogen defence and homeostasis functions (Engel & Moran 2013b; Sommer & Bäckhed 2013). However, during diapause as the animal fasts, a major energy source for the microbiota is eliminated (Duman & Patterson 1978; Carey & Assadi-Porter 2017). Increased competition for limited food resources likely plays a role in bacterial community dynamics. In some animals, beneficial microbes could persist by entering dormancy themselves or die off when resources are low allowing the host organism to acquire new environmental microbes immediately after emergence (Pereira & Berry 2017).

Many temperate insects with over-wintering strategies play key ecological and economic roles, including the wild bumblebee, *Bombus terrestris*, a key pollinator of crops and wildflowers (Klein *et al*. 2007). Queen diapause is integral to its annual lifecycle; only the young, fertilised queens over-winter, so survival during diapause is crucial to the

success of the future colony (Alford 1969). Under natural conditions, diapause can last between six and nine months but in managed breeding programmes this is shortened to around 10-15 weeks depending on the species, mating technique used, and commercial needs (Gosterit & Gurel 2009). As such a significant ecological and economic species, understanding host health and efficiency during this process is a major challenge.

The bumblebee gut is an important model in microbiota research (Robinson *et al*. 2010; Engel & Moran 2013b), and while there is some information on the gut communities in queens (Chapter 3; Parmentier *et al.* 2018), little is known about the community throughout the queen lifecycle. In honey bees, a defined model of bacterial colonisation has been shown through the queen rearing process (Tarpy *et al*. 2015) – but as honey bees do not undergo diapause, there is of yet little knowledge on how this process shapes the queen gut, her health and her future colony success (Rangel *et al*. 2016).

Koch *et al*. (2013) found evidence that some common gut taxa can persist in queens after undergoing diapause, but that *Gilliamella* populations are more likely to be lost than *Snodgrassella*. Still, how the rest of the gut community is affected is unclear. At diapause, the *Bombus* colony passes through a population bottleneck, where potentially the initial microbiota to colonise workers will largely have come from the mother queen (Koch *et al*. 2013). During diapause the gut tissues degrade and when coupled with a lack of food, it is likely that the gut microbiota will be detrimentally affected – though how this could affect the queen on emergence is unclear. Queens in the wild must obtain sufficient fat reserves in order to survive diapause (Holm 1972), and even in commercial settings, under optimum conditions, a proportion of the queens that survive fail to found colonies (Beekman *et al*. 1998) but in the absence of disease it is unclear why.

This study followed a related cohort of queen *Bombus terrestris audax* within a commercial bumblebee breeding facility (Biobest Group NV, Belgium) before, during and after undergoing diapause. Destructive queen samples were taken at regular intervals to investigate how gut microbiota changes over the course of commercial rearing and may affect overall success of colony foundation. This study aimed to (i) assess the impacts of diapause on gut microbiota, and (ii) examine potential differences in post-diapause microbiota between healthy and poor lab-reared queens.

5.2 MATERIALS AND METHODS

5.2.1 Study species

The buff-tailed bumblebee (*Bombus terrestris*) is a short-tongued bumblebee, widely distributed across Britain, and occurring in colonies of up to 350 workers (Alford 1975). Colonies are annual, with only the queens (reproductive females) able to survive the winter months in a state of diapause. After mating, young queens go into diapause while the males and the maternal colony die in late summer (Beekman & Van Stratum 2000). After emergence, the queens establish new colonies and produce reproductive males and females to continue the cycle the next year.

B. terrestris is commonly reared for fruit and vegetable pollination, where global food producers rely on insect pollination throughout the year (Klein *et al*. 2007). Bee breeding facilities achieve this by using artificial hibernation techniques in which queens typically undergo a shorter hibernation period of between 10–15 weeks at 4°C (Röseler 1985; Velthuis *et al*. 2006). This provides an excellent model system in which to examine the impact of diapause on microbiota in insects in a controlled setting. In this study, the subspecies *B. t. audax* was used, which is the native *Bombus terrestris* subspecies in the UK and Ireland.

5.2.2 Sample collection

Forty queens from separate colonies were selected at random from a larger cohort entering a commercial breeding cycle. These queens were sampled before diapause (before and after mating), during diapause at three different time points and after diapause (both successful founding queens and unsuccessful founding queens, hereafter referred to as 'healthy' and 'poor', respectively) at the bumblebee breeding facility, Biobest Group NV in Westerlo, Belgium, following standard mass rearing procedures. Throughout the pre- and post-diapause phase queens were fed on a standardised diet of gamma-irradiated pollen (15 kGy; STERIS AST, Etten-Leur, the Netherlands) supplemented with the commercial sugar solution BIOGLUC® (Biobest Group NV, Belgium).

At each sampling point, queens were euthanized through storage at −80°C. A baseline of virgin queen samples was taken aged 6–9 days old. These queens were collected

from their maternal colony shortly (max. 2 days) after eclosing and placed into a new plastic box until queens reached the age for mating. Remaining queens were mated with males at the optimal mating ages of both sexes in specially designed cages (Biobest Group NV, Belgium), immediately after which point another sample was taken. Another sample was taken five days later, before entering diapause (pre-diapause), which for this study kept queens at a lower temperature before entering diapause (Salt 1961; Zachariassen 1985; Clark & Worland 2008). Samples were then taken 1, 5 and 10 weeks into artificial diapause. Mated queens were stored in cold chambers with a constant temperature of 3°C and minimum relative humidity of 80%. Remaining queens were awoken after 10 weeks of diapause, and colony founding success was assessed six weeks after emergence from diapause. At the end of these six weeks, mother queens of normally developing colonies ('healthy', defined as rapid colony foundation and worker production) and of poorly developing colonies ('poor', defined as no egg laying) were sampled and compared to assess correlations between queen quality and gut microbiota.

Wild post-diapause *B. t. audax* queens ('wild') were also collected to examine to how the gut microbiota of free foraging queens after diapause differs to that of lab-reared queens. These additional queens were collected in early May 2015 while apparently nest searching in the farmed landscape around Brightwell Baldwin, UK (51.6444° N, −1.0573° W). Queens were collected from sites of differing resource outlined in Section 2.2.1, including three queens from two poor resource sites (RP; grass-dominated) and three queens from two resource-rich sites (RR; florally diverse). After identification, queens were sealed inside collection pots and anaesthetised on ice. These pots were then stored at −80°C within 2 h of removal from the field until queens had been euthanized.

5.2.3 DNA isolation and sequencing

Whole queen guts were dissected using sterile apparatus and sterilised in 70% ethanol and then phosphate-buffered saline (1xPBS). Great care was taken to avoid non-gut tissues, including fat bodies and the ovaries. Dissected guts were placed into individual wells of a PowerSoil® Bead Plate, and DNA was extracted following the PowerSoil®-htp 96 Well Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA) recommended protocol. To fully homogenize all queen gut tissues an additional bead-beating step (10 mins at speed

20) was required. From the extracted DNA, approximately 20 ng of template DNA from each sample was amplified and sequenced following the protocol in Section 2.7.1 at a concentration of 5 pM with a 10% addition of an Illumina generated PhiX control library.

5.2.4 Quantitative PCR (qPCR)

In addition to high-throughput sequencing, quantitative qPCR was used to estimate absolute copy numbers of 16S rRNA genes among a subset (n = 27; three per sample point) of the sampled queens. Bacterial DNA was amplified using the Femto™ Bacterial DNA Quantification Kit (Zymo Research, USA, using approximately 2 µl of template DNA, to 1 µl of standards and 1 µl PCR grade water to act as negative controls. Reaction volumes of 10 µl were prepared in triplicate and placed in a LightCycler® 480 (Roche Molecular Systems, Inc.) under the manufacturer's recommended thermocycling parameters. Absolute gene copy numbers were calculated based on the known input values of the standard curve DNA, and all amplification after 33 cycles was removed from the analysis as background.

5.2.5 Sequence analysis

Resultant sequenced paired-end reads were processed using the DADA2 pipeline (Section 2.8.4; Appendix 10; Callahan *et al.* 2016). Due to the quality of the reverse reads generated, only the forward reads were processed. These were trimmed to 290 bp before fragment mapping, and amplicon sequence variant (ASV) identity was assigned using the Greengenes Release 13_5 (at 97%) (DeSantis *et al*. 2006). Since many ASVs were still left unresolved below phylum classification, additional taxonomic classification was performed with the Basic Local Alignment Search Tool (Altschul *et al*. 1990) and EzBioCloud database (Yoon *et al*. 2017), with identity of unknown ASVs selected based on > 99% matches. Given that sequences were based on short fragments (~290 bp) of only forward reads, taxa were resolved to Genus, when possible, and ASV identities should be considered putative.

Further checks on the sequence data were added to control for kit contamination, including removing singletons, sequences that were not bacterial taxa and potential false positives (Salter *et al*. 2014; Glassing *et al*. 2016).

5.2.5 Statistical analysis

From the 49 queen samples sequenced, six samples were removed from further analysis due to poor read counts (<1000), including three wild and three lab-reared queens. The remaining samples (*n* = 43*)* were rarefied to an equal sequencing depth (=11,032 sequences) in R using the VEGAN package ('rrarefy' function; Dixon 2003). After rarefaction and filtering, a subset of 474,369 sequence reads (126 ASVs) was used in subsequent analyses. Before rarefaction there was no significant difference in the number of ASVs between queen sample points (χ^2 = 8.79, *p* = 0.36, *n* = 43), and rarefaction curves were used to confirm that sequencing depth had sufficiently captured the alpha diversity within each sample (Appendix 3).

Fisher's alpha (log series alpha) was used to quantify the alpha diversity of queen gut microbiota samples, and composition was investigated on normalised ASV frequency data that had been converted to a Bray-Curtis dissimilarity matrix using PAST v3.08 (Hammer *et al*. 2001). Then, univariate statistics (Kruskal-Wallis, one-way ANOVA) were performed using the base statistics package in R version 3.3.2. Additional microbiome community analyses carried out using the R:Phyloseq package (McMurdie & Holmes 2013) with multivariate analyses performed using PAST v3.08 using Bray-Curtis index of similarity as the underpinning similarity measure.

5.3 RESULTS

5.3.1 Gut microbiota changes throughout queen aging and diapause

From the 126 ASVs identified, 13 were present in queens from all rearing stages, and 11 from all queens sampled in this study (commercial and wild; Table 5.1). On average 20.7 ± 3.2 ASVs (± 95% confidence interval) were detected per queen, with significantly more ASVs found at the later time points (χ^2 = 16.75, *p* = 0.02, *n* = 37). Fisher's alpha diversity index was significantly different between commercial queen rearing stages (Fig. 5.1; $F_{(7,29)}$ = 4.05, *p* < 0.01, *n* = 37). Tukey HSD post-hoc analyses indicated that after 10 weeks of diapause (3.2 ± 0.7) queen gut microbiota was more diverse than before diapause (virgin – pre-diapause stages: 1.7 ± 0.2; *p* < 0.01) as well as in both lab-reared 'poor' (1.6 ± 0.2; *p* = 0.001) and 'healthy' post-diapause groups (2.1 ± 0.4; *p* = 0.03).

Table 5.1 Summary data from commercially-reared and wild-caught *Bombus terrestris audax* queens, including brief metadata, and average ASV counts at each sampled stage.

Variable	Virgin	Mated	Pre-diapause	1 Week	5 Weeks	10 Weeks	Healthy	Poor	Wild
Sample size (*n*)	5	5	5	5	5	3	5	4	6
Mass (g)	0.8999	0.8860	0.6962	0.8365	0.7200	0.7908	0.8947	0.7959	0.6989
Head width (mm)	5.33	5.54	5.53	5.78	5.69	5.59	5.75	5.48	5.45
Wing length (mm)	17.986	17.524	16.60	18.05	17.45	18.28	17.67	17.64	18.12
Gut weight (g)	0.1481	0.1118	0.1018	0.0962	0.2445	0.1680	0.1534	0.1672	-
Average ASV count	14	16	15	17	20	26	18	14	44
Associated ASVs	26	29	28	30	36	41	33	25	84
Shared ASVs	9	11	10	11	12	12	9	9	-
Unique-ASVs	0	2	0	1	5	7	5	0	-
Shared with 'Wild'	15	14	15	17	21	25	13	14	-

To characterise the distribution of microbiota, pairwise Bray-Curtis dissimilarity values were calculated for each of the queens sampled. Analysis of similarity (ANOSIM; Table 5.2) tests highlighted significant differences in bacterial composition throughout the artificial diapause process, and additional similarity percentage (SIMPER; Table 5.3) analyses demonstrated that the dissimilarity throughout artificial rearing appeared to be a result of changes in abundance of the major taxa in the system, *Gilliamella*, *Snodgrassella*, *Lactobacillus*, *Schmidhempelia*. During artificial diapause there were declines in *Snodgrassella* and a slight increase in *Sphingomonas* by week 10, when gut microbiota alpha diversity increases.

5.3.2 Genus level dynamics affected by diapause

Communities present across the biological replicates at each stage were characterised to determine the gut microbiota in queens throughout rearing. Throughout diapause, between 89–99% of the queen gut microbiota community comprised just a handful of genera. The three main genera: *Gilliamella* (mean 48.22%, range 3.35–90.82%), *Lactobacillus* (mean 4.33%, range 0.09–24.02%), and *Snodgrassella* (mean 36.23%, range 5.89–57.49%). However, the relative abundance of these genera differed between individuals (Fig. 5.2) and additional genera (*Bombiscardovia, Kingella, Schmidhempelia, Sphingomonas, and Ventosimonas*) were occasionally present.

In the early samples from the trial, queen gut microbiota was characterised by high counts of *Gilliamella* and *Snodgrassella* ASVs, with relatively low levels of *Lactobacillus* detected (Fig. 5.3). As queens stopped feeding, prior to entering diapause, the abundance of *Lactobacillus* decreased from 7.52% to 0.33% representing a log fold change of 4.49.

The levels of *Lactobacillus* detected in queen guts differed significantly over time (χ^2 = 20.93, p = 0.002, n = 33), remaining at a significantly low level from mated to mid-diapause (5 week) queens (p < 0.001). In addition to these low levels of *Lactobacillus*, between mated to 10-week diapause queens the relative abundances of *Snodgrassella* and *Gilliamella* appeared to be negatively correlated (R^2 = 0.51, p < 0.001).

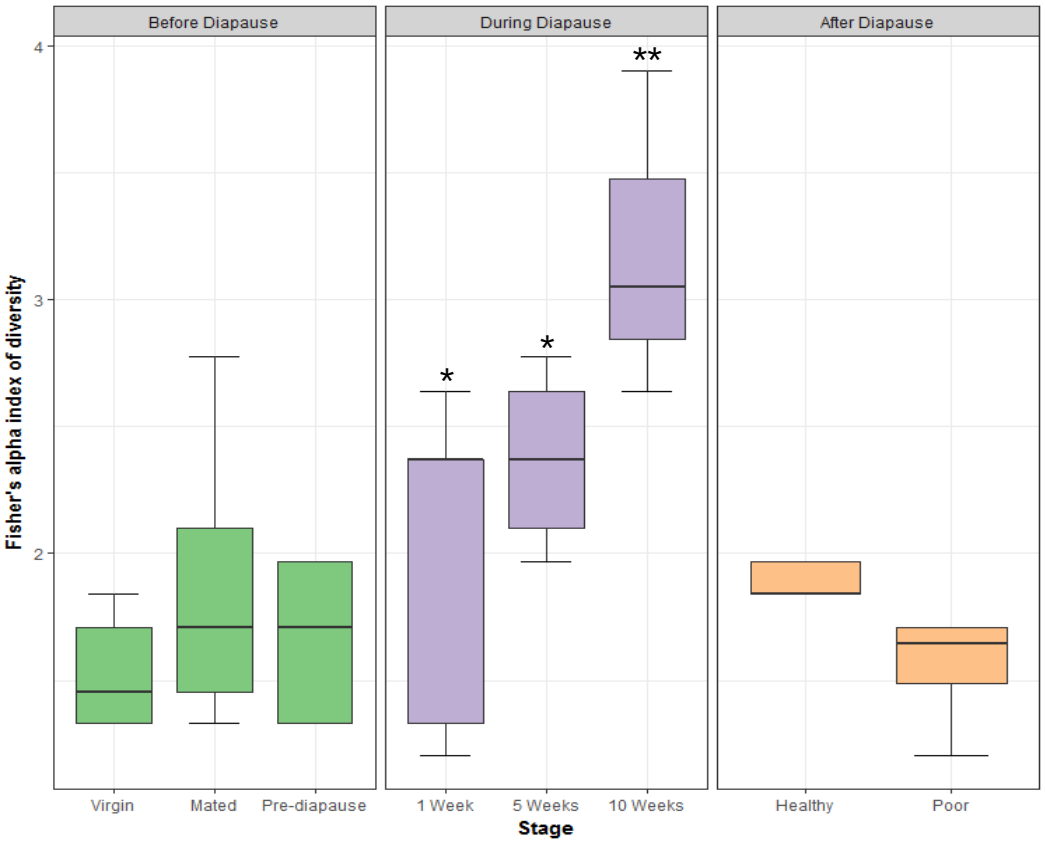

Figure 5.1 Fisher's alpha diversity of queen gut microbiota before, during and after diapause. Significance is denoted at the p < 0.05 level (*) and p < 0.01 level (**).

Table 5.2 Analysis of similarity (ANOSIM) of sampled queens through commercial rearing. Values generated using the Bray-Curtis measure of similarity, with the ANOSIM test statistic (R) shown below the diagonal and corresponding probability (*p*) shown above. Those with an asterisk and highlighted in bold font indicate that the two compared groups are significantly different at the $p < 0.05$ level.

Table 5.2	Virgin	Mated	Pre-diapause	1 Week	5 Weeks	10 Weeks	Healthy	Poor
Virgin	-	0.178	0.182	0.104	0.056	0.222	0.060	0.275
Mated	0.088	-	0.985	0.173	0.301	0.698	0.050	0.473
Pre-diapause	0.144	-0.192	-	0.473	0.754	0.722	**0.007***	0.302
1 Week	0.168	0.136	0.004	-	0.792	0.497	**0.032***	0.422
5 Weeks	0.164	0.052	-0.108	-0.088	-	0.835	**0.038***	0.201
10 Weeks	0.139	-0.097	-0.118	0.005	-0.210	-	0.087	0.345
Healthy	0.204	0.380	0.368	0.268	0.264	0.374	-	**0.008***
Poor	0.044	-0.025	0.025	0.019	0.113	0.093	0.475	-

Table 5.3 Similarity of percentages analysis (SIMPER) of *B. terrestris* queen gut microbiota during the commercial rearing. Overall average dissimilarity based on Bray-Curtis (=79.81%). A cut-off of > 1% contribution was used, so the list of taxa is not exhaustive. ASV identities are based on ~290 bp sequences and should therefore be considered putative.

Table 5.3			Mean % Relative Abundance								Cont. %	Cuml. %
Class	**Family**	**Genus**	**Virgin**	**Mated**	**Pre-diapause**	**1 Week**	**5 Weeks**	**10 Weeks**	**Healthy**	**Poor**		
Betaproteobacteria	Neisseriaceae	*Snodgrassella*	33.30	17.40	15.70	29.30	24.90	28.50	44.90	25.50	18.63	18.63
Gammaproteobacteria	Orbaceae	*Gilliamella*	11.30	20.90	25.70	30.70	26.80	25.00	23.20	18.20	17.47	36.10
Betaproteobacteria	Neisseriaceae	*Snodgrassella*	10.90	28.20	19.30	1.56	2.24	6.10	0.06	10.70	14.37	50.47
Gammaproteobacteria	Orbaceae	*Gilliamella*	22.00	9.23	10.80	16.20	7.13	8.41	7.45	28.40	12.38	62.85
Gammaproteobacteria	Orbaceae	*Gilliamella*	0.95	6.86	10.70	10.40	20.40	6.59	7.97	3.25	11.49	74.34
Gammaproteobacteria	Orbaceae	*Schmidhempelia*	3.30	5.98	15.40	0.23	12.50	6.37	0.01	0.01	8.72	83.06
Bacilli	Lactobacillaceae	*Lactobacillus*	7.52	0.33	0.27	0.57	0.25	4.72	11.30	0.40	4.35	87.41
Gammaproteobacteria	Orbaceae	*Gilliamella*	1.11	4.20	0.65	0.40	2.85	3.32	0.10	6.31	2.88	90.28
Gammaproteobacteria	Orbaceae	*Gilliamella*	0.00	2.52	0.10	3.42	0.00	0.50	0.99	2.00	1.80	92.08
Bacilli	Lactobacillaceae	*Lactobacillus*	4.45	1.02	0.81	0.61	0.44	1.72	0.99	0.05	1.72	93.80
Gammaproteobacteria	Orbaceae	*Gilliamella*	0.00	2.50	0.16	2.82	0.00	0.56	1.05	1.87	1.66	95.46
Betaproteobacteria	Neisseriaceae	*Kingella*	0.00	0.00	0.00	2.74	1.08	0.00	0.00	2.79	1.35	96.81

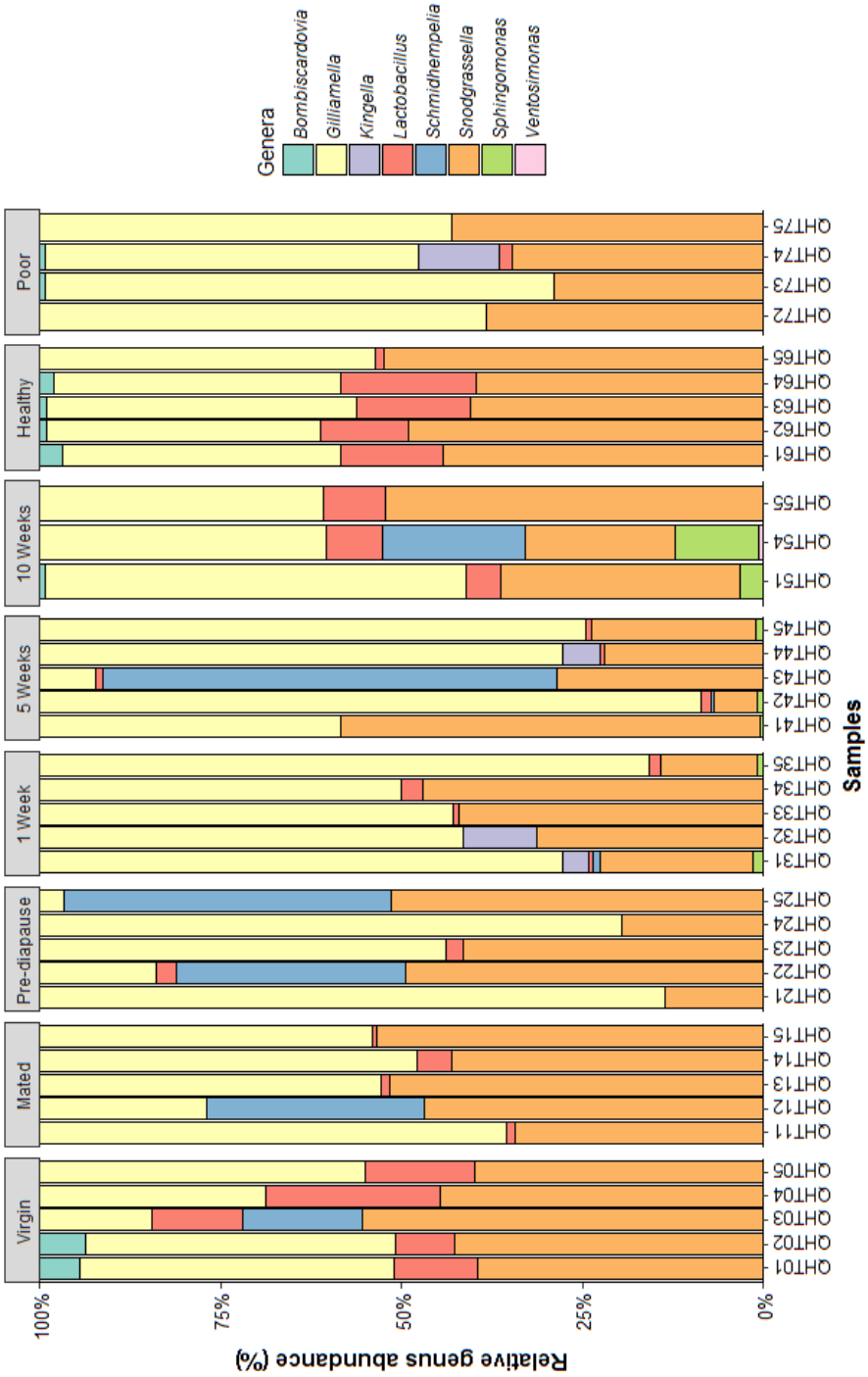

Figure 5.2 Individual variation in gut microbiota composition of genera (mean % relative abundance) between samples of lab-reared *B. terrestris* before, during and after diapause.

Throughout diapause, between 89–99% of the queen gut microbiota community was comprised of just a handful of genera: *Gilliamella*, *Snodgrassella*, *Schmidhempelia*, as well the rarer taxa, *Lactobacillus* and *Sphingomonas*. *Snodgrassella* detection increased at 5 weeks, as *Gilliamella* declined, and at 10 weeks of diapause there was a significant increase in diversity, along with increases in the relative abundances of *Lactobacillus* (6.94%), and the previously undetected *Sphingomonas* (6.37%).

During artificial rearing there was also a significant decline in bacterial copy number between sample points (Fig. 5.4; $\chi^2 = 14.79$, $p = 0.04$, $n = 25$). Pre-diapause queens harboured larger bacterial communities compared to queen during and after artificial diapause ($p < 0.05$), and as queens progressed through this rearing process, bacterial copy number decrease. The community fell to its lowest size after 10 weeks of diapause. Six weeks following emergence, 'healthy' post-diapause queens had slightly higher bacterial populations than 'poor' queens but were still an order of magnitude lower than pre-diapause counts.

5.3.3 Environment influences post-diapause gut microbiota

There were distinct differences in gut microbiota between lab-reared queens classified as 'healthy' and 'poor'. Healthy queens showed a slight decline in *Gilliamella* relative abundance (39.75%) but increases in *Snodgrassella* (45.01%) and *Lactobacillus* (11.32%). Poor queens, however, showed an increase in *Gilliamella* (58.16%), a slight increase in *Snodgrassella* (36.19%), but there was very little detection of *Lactobacillus* in the gut (0.04%). Both groups of commercial post-diapause queens had low detection of *Sphingomonas* and *Schmidhempelia* (0.01–0.12%), which had constituted ~10% of the queen gut at 10 weeks of diapause.

As expected, analyses of alpha diversity of all post-diapause queens a significant difference in diversity between the two lab-reared groups when compared to 'wild-caught' queens ($\chi^2 = 12.49$, $p = 0.002$, $n = 15$).

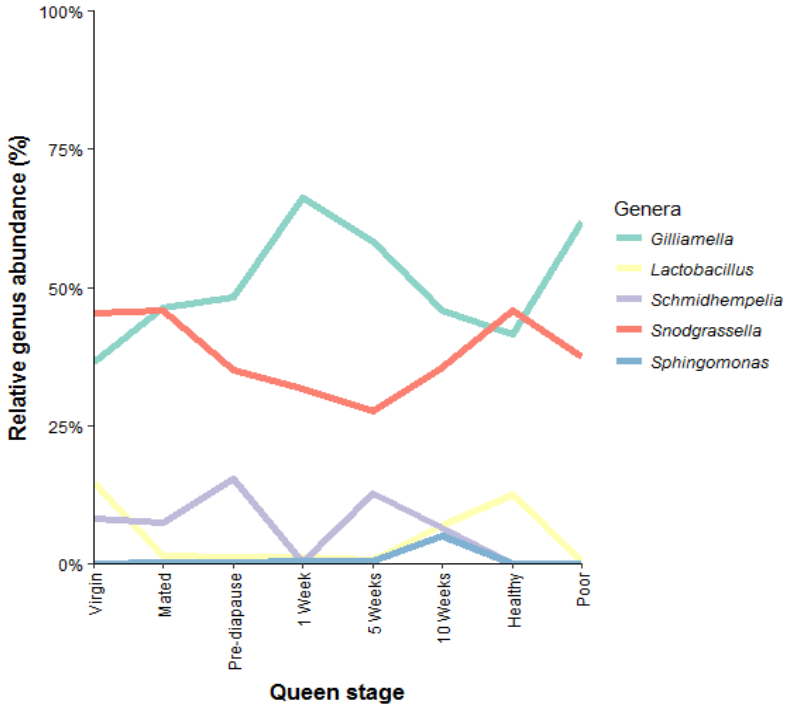

Figure 5.3 Trends in relative abundance of key bumblebee gut genera (*Gilliamella*, *Lactobacillus*, *Schmidhempelia*, *Snodgrassella*, and *Sphingomonas*) before during and after diapause. Calculated from 16S amplicon sequencing data.

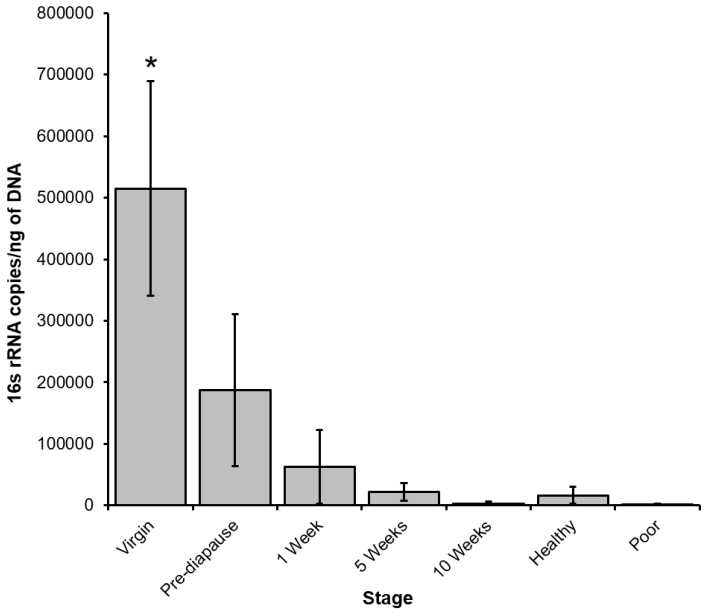

Figure 5.4 Bacterial count data in lab-reared queens before and during diapause based on 16S rRNA copies. Significant differences denoted by * are at the $p < 0.05$ level.

ANOSIM tests also highlighted significant differences in bacterial composition between the post-diapause queen groups (Table 5.2). When compared to the other two groups, wild queen microbiota contained numerous environmental ASVs from Enterobacteriaceae, Lactobacillaceae, and Ventosimonaceae (Fig. 5.5). There was a significant difference in within groups Bray-Curtis similarity between queen rearing stages ($\chi^2 = 24.29$, $p < 0.01$, $n = 84$), where both lab-reared post-diapause groups had higher similarity than wild queens ($p < 0.05$) and healthy queens exhibited higher similarity than during all commercial rearing stages ($p < 0.05$).

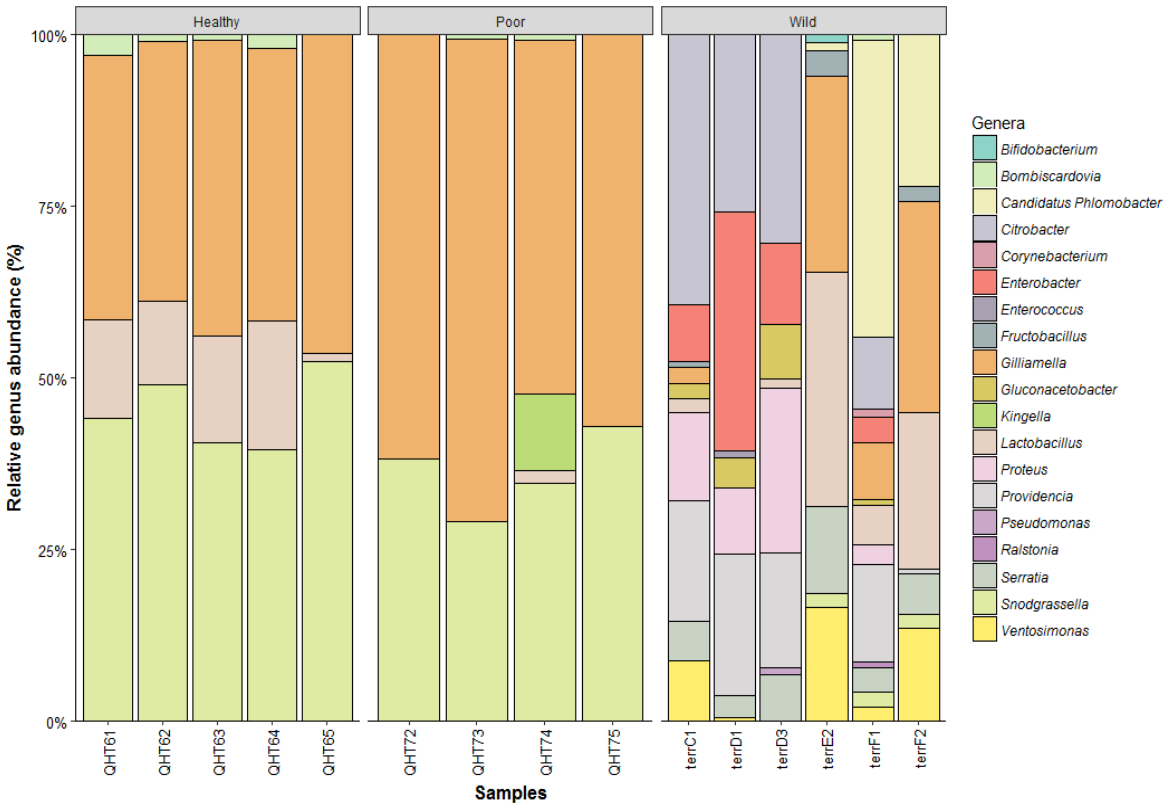

Figure 5.5 Comparison of gut microbiota composition (percentage relative abundance) of 'healthy', 'poor' and 'wild' post-diapause *B. terrestris* queens. Both lab-reared groups show similar compositions but differ in relative abundance of *Lactobacillus* spp. Wild queen composition appears to separate by site (terrC and terrD = resource-poor; terrE and terrF = resource-rich).

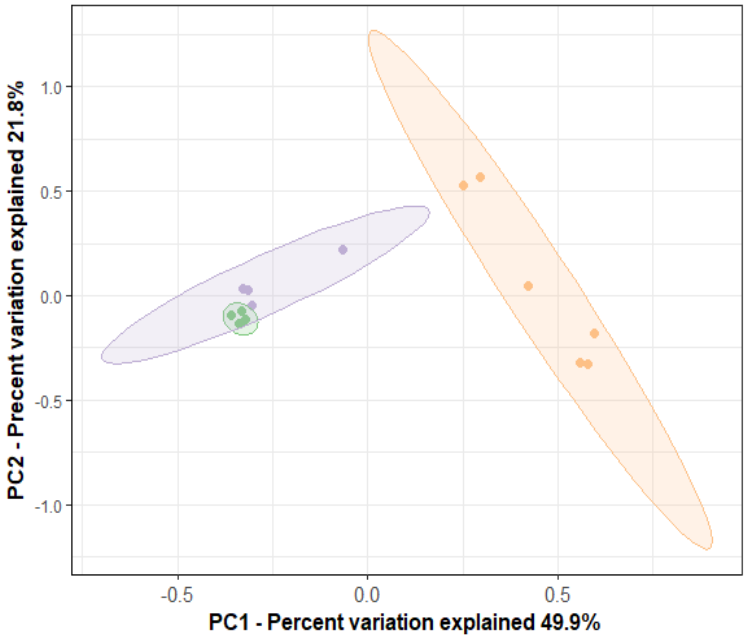

Figure 5.6 Principal Coordinates Analysis (PCoA) highlights significant separation of wild (orange), 'healthy' (purple), and 'poor' (green) post-diapause queens based on relative ASV abundance data.

To further visualise similarities and determine the differences observed across post-diapause queens, a principal components analysis was performed (Fig. 5.6). Sample clustering was strongly influenced by the environment, such that lab-reared queens clustered separately to wild queens (the first component accounted for 49.9% of the total variance, while the second was 21.8%). Lab-reared queens also clustered by quality, with poor queens appearing as a subset of the more varied healthy queens. Wild queens also showed more variance within their group, than either healthy or poor groups.

SIMPER analyses were performed to assess the contribution of individual taxa to the overall dissimilarity between post-diapause queens (Table 5.4). Healthy queens had a higher relative abundance of *Lactobacillus* sp. (11.3 ± 6.3%) compared to 'poor' (0.4 ± 0.7%) and wild queens (0.1 ± 0.2%). In poor queens a substantial proportion of dissimilarity stemmed from the presence of a *Kingella* sp. (2.69 ± 5.5%), which was not detected in healthy or wild queens. This ASV was isolated from only one individual and was not present in any other post-diapause queen samples, so it is likely not representative of the treatment group.

Table 5.4 Similarity of percentages analysis (SIMPER) of post diapause *B. terrestris* queen gut microbiota. Given is the percentage mean abundance, with the overall average dissimilarity based on Bray-Curtis (=81.79%). Percentage contribution (Cont.%) is the mean contribution divided by the mean across samples. A cut-off of >1% contribution was used, so the list of taxa is not exhaustive and cumulative percentage (Cuml.%) does not add up to 100%. ASV identities are based on ~290 bp sequences and should therefore be considered putative.

			Mean % Relative Abundance			Cont. %	Cuml. %
Class	**Family**	**Genus**	**Healthy**	**Poor**	**Wild**		
Betaproteobacteria	Neisseriaceae	*Snodgrassella*	44.90	25.50	0.58	16.89	16.89
Gammaproteobacteria	Orbaceae	*Gilliamella*	23.20	18.20	1.59	15.08	31.97
Gammaproteobacteria	Orbaceae	*Gilliamella*	7.45	28.40	0.73	10.87	42.84
Gammaproteobacteria	Enterobacteriaceae	*Citrobacter*	0.00	0.00	14.10	10.25	53.10
Bacilli	Lactobacillaceae	*Lactobacillus*	11.30	0.40	0.12	8.81	61.90
Betaproteobacteria	Neisseriaceae	*Snodgrassella*	0.06	10.70	0.06	6.49	68.40
Gammaproteobacteria	Orbaceae	*Gilliamella*	7.97	3.25	0.78	3.30	71.70
Bacilli	Lactobacillaceae	*Lactobacillus*	0.02	0.01	7.99	2.83	74.53
Gammaproteobacteria	Enterobacteriaceae	*Proteus*	0.00	0.00	7.67	2.45	76.98
Gammaproteobacteria	Enterobacteriaceae	*Candidatus Phlomobacter*	0.00	0.00	6.96	1.62	78.60
Bacilli	Lactobacillaceae	*Lactobacillus*	0.00	0.00	6.25	1.54	80.14
Gammaproteobacteria	Orbaceae	*Gilliamella*	0.10	6.31	2.88	1.40	81.54
Gammaproteobacteria	Enterobacteriaceae	*Providencia*	0.00	0.00	5.93	1.35	82.89
Gammaproteobacteria	Enterobacteriaceae	*Enterobacter*	0.00	0.00	5.53	1.30	84.19
Gammaproteobacteria	Ventosimonadaceae	*Ventosimonas*	0.01	0.00	4.07	1.26	85.45
Gammaproteobacteria	Enterobacteriaceae	Unknown	0.00	0.00	3.77	1.25	86.69
Gammaproteobacteria	Enterobacteriaceae	*Enterobacter*	0.00	0.00	3.47	1.19	87.89
Bacilli	Lactobacilliaceae	*Lactobacillus*	0.00	0.00	2.41	1.13	89.01
Gammaproteobacteria	Enterobacteriaceae	*Gluconacetobacter*	0.00	0.00	2.34	1.00	90.01

Additionally, there were interesting differences observed between post-hibernation gut microbiota in the wild queen cohort. Queens sampled from resource-poor sites (terrC1, terrD1, terrD3) contained higher amounts of *Enterobacter* and *Citrobacter* species, while queens from resource-rich sites (terrE2, terrF1, and terrF2) had higher relative abundances of '*Candidatus Phlomobacter'*, *Ventosimonas*, *Gilliamella* and *Snodgrassella*.

Anatomical measurements (Appendix 11) taken from each queen indicated that there were no significant differences in weight, head width, or intertegular distance between post-diapause queens reared in artificial settings, or between reared and wild queens, although queens classified as 'poor' tended to be smaller than the healthy cohort.

5.4 DISCUSSION

High-throughput sequencing of the gut microbiota of *Bombus terrestris audax* queens during commercial rearing revealed differing responses by the major gut taxa to food deprivation during host diapause, reflecting their metabolic specialisms. Though the core microbiota were retained during diapause, the bacterial community declined, suggesting that queen gut microbiota may be more prone to opportunistic colonisation soon after emergence. These gut communities therefore, may be highly influenced by the post-diapause environment.

Throughout artificial rearing, queen gut microbiota was dominated by three main genera: *Gilliamella*, *Snodgrassella*, and *Lactobacillus*. This simple, conserved gut community was generally consistent with previous findings of the core microbiota in artificially-reared *B. terrestris* workers (Meeus *et al*. 2015), however Bifidobacteriaceae (*Bombiscardovia*), which was present in workers, did not appear particularly prevalent in queens. When it did appear, it showed variance among individuals, as did *Schmidhempelia*, which occurred in high relative abundance in some queen guts but was absent in others.

The queens sampled for this study had much more simple gut communities than found previously (Chapter 3). In Chapter 3, in established colonies in both lab and field settings, queen guts were dominated by Lactobacillaceae and by Enterobacteriaceae populations, with low levels of *Gilliamella* and *Snodgrassella*. In contrast, *Gilliamella* and *Snodgrassella* were found to be the dominant taxa throughout queen rearing in this study.

However, these differences are not too surprising. Queens undergoing diapause do not feed, so are unlikely to contain environmentally-acquired *Enterobacter* species and diet-associated *Lactobacillus* species (Anderson *et al*. 2013). As the gut tissues atrophy during diapause (de Kort 1990), it appears that all but the most tolerant and persistent species can remain in the gut.

The seemingly intolerant environment faced in the diapausing gut was further demonstrated by the decline in bacterial population. Bacterial count data showed a sharp decline in the microbiota population when queens ceased feeding and transitioned into diapause. Six weeks after diapause, the queen gut community had not yet recovered to its pre-diapause population size, suggesting that recovery is a long-term process. In mammals, winter fasting has been found to have lasting effects on the gut community, and even after two weeks of refeeding the gut microbiota is still not fully restored to its summer composition (Carey *et al*. 2013; Dill-McFarland *et al*. 2014).

However, the findings of this study showed that some gut microbiota do persist in queens throughout diapause, in agreement with previous work by Koch *et al.* (2013). In addition, there was also evidence for their suggestion that gut populations of *Gilliamella* are more likely to be lost than *Snodgrassella*. Specifically, during the 10 weeks of diapause, the three temporal sample points showed a decrease in *Gilliamella*, while *Snodgrassella* levels fluctuated. As *Gilliamella* is one of the major sugar fermenters in the bee gut (Zheng *et al*. 2017) it was anticipated that removal of its primary food source during diapause would adversely affect its growth and reproduction. To avoid the formation of ice crystals during diapause, the content of the gut (but not necessarily the crop) are voided (Duman & Patterson 1978) so this environment is severely depleted. In the ileum and rectum, *Gilliamella* competes with *Bifidobacterium* and *Lactobacillus* spp. to ferment the same sugars. *Snodgrassella*, however, utilises an alternate metabolic pathway to oxidise the products of these fermentative species. *Gilliamella* and *Snodgrassella* form a biofilm in the ileum, and while there is evidence of syntrophy (or cross-feeding) between them, the latter is not reliant on fermentation products solely from *Gilliamella* for survival (Kwong *et al*. 2014). The biofilm they produce likely functions to protect the host from parasite invasion (Engel *et al*. 2012) so a reduction in *Gilliamella* populations during diapause could impair this protective layer, leading the emerging queen to be more susceptible to pathogens. It

would be useful if future work could examine whether the bacteria retained by the queen gut are live and continue to contribute to this protective function, using methods such as real-time quantitative PCR (RT-qPCR) to monitor transcription rates in bacterial cells.

Throughout diapause, queen microbiota appeared to be more variable, with additional rarer taxa appearing to make a niche within the gut while common taxa declined. In particular, detection of *Sphingomonas* increased with alpha diversity of the whole gut community. It appears likely that nutrient scarcity in the gut may have reduced populations of some of the more established bacterial taxa (e.g. *Gilliamella*), thereby allowing small colonies of opportunistic, rarer taxa to occupy newly available niches within the gut. Species such as *Sphingomonas* are metabolically versatile, utilise a wide range of naturally occurring compounds, and thrive in nutrient-poor conditions (Balkwill *et al*. 2006), which makes them ideally suited to this environment.

The detection of *Lactobacillus* in the gut was one of the main differences highlighted in comparisons of gut microbiota between 'healthy' and 'poor' post-diapause queens. Poor queens harboured little or no *Lactobacillus*, and had an especially species-poor microbiota, consisting predominantly of *Gilliamella*, *Snodgrassella*, some *Bombiscardovia*, and one sample with low levels of *Lactobacillus*. This queen also contained a bacterium putatively matched to the *Kingella* genus, which although not commonly found in *Bombus*, is a close relative of *Snodgrassella alvi* (~94% 16S rRNA identity; Kwong & Moran 2013). The difference in detection of *Lactobacillus* spp. between poor and healthy queens appears especially significant given the evidence for their probiotic benefits to bee health and colony performance (Evans & Lopez 2004; Audisio & Benítez-Ahrendts 2011; Pătruică & Mot 2012). Dietary supplementation with lactobacilli is affordable and practical, and could potentially improve post-diapause queen health, although further investigation is required.

When comparing all post-diapause queens, it was apparent that those sampled from the wild exhibited more diverse gut bacterial communities than their lab-reared counterparts. All groups shared the key genera *Gilliamella*, *Snodgrassella*, and *Lactobacillus*, but these were in much lower relative abundance in wild queens. Prior work has highlighted the importance of host environment to the gut microbiota, and clear differences have been found between lab-reared and wild *Bombus* workers (Chapter 3;

Newbold *et al.* 2015). However, the results of this study could suggest that post-diapause queen microbiota may be more responsive to the environment than previously observed in workers, possibly reflecting their differences in life history.

Bumblebees have an annual colony cycle, where new queens undergo diapause and re-emerge in the spring to found their colonies, while workers and the remainder of the maternal nest die in late summer (Beekman & Van Stratum 2000). During diapause, the queen gut microbiota faces a highly competitive and challenging environment with limited nutrient resources. Workers, and their associated microbiota, do not face this prolonged period of winter fasting so their microbiota remains relatively constant, albeit with some seasonal fluctuations (Chapter 4; Ludvigsen *et al.* 2015).

Between queens, the striking differences in post-diapause gut microbiota appeared to be predominantly a result of differences in colony founding success (i.e. healthy vs. poor) and the environment. In the wild, queens diapause underground putting them in contact with the high bacterial diversity in the soil (Torsvik *et al*. 1990), at which point, after 7–8 months of diapause (Free & Butler 1959) their gut microbiota is depleted and more susceptible to colonisation from opportunistic taxa that the host comes into contact with. Commercially reared queens, however, were reared in controlled environments, fed sterile diets, and had a much shorter diapause (~3 months). The stability of this environment was reflected in the relative stability of the gut community.

My direct comparison of wild and lab-reared queens should be interpreted with caution, due to unknown characteristics about wild queen diapause (nesting site, diet, length of diapause, etc.). However, when partitioning wild queens by the sites in which they were found, it was clear that wild queens foraging in rich-resources contained more plant-associated bacteria, including '*Candidatus Phlomobacter*' as well as *Ventosimonas*, an endosymbiont first characterised in ants (Lin *et al*. 2016). They also had increased relative abundances of *Gilliamella* and *Snodgrassella*, contrasting with the gut communities of queens foraging in poor-resource sites, which contained mostly *Enterobacter* spp. (often associated with disease; Sanders Jr. & Sanders 1997).

Overall, this characterisation of gut microbiota through bumblebee queen diapause demonstrates the stability and resilience of the major gut taxa and improves our understanding of gut bacterial responses to food deprivation. Three main genera:

Gilliamella, *Snodgrassella*, and *Lactobacillus* were detected throughout commercial rearing and diapause, but their responses to low nutrient conditions varied, resulting from differing metabolic niches in the gut. When contrasted to wild post-emergence queens, lab-reared samples harboured less gut microbiota diversity, suggesting the importance of the host environment in the initial stages of nest foundation. Post-diapause communities also appeared to have increased susceptibility to opportunistic colonisation, but further investigation is needed. Still, these findings suggest possible avenues of research for dietary supplementation for queen health and highlight new questions about whether maternal gut microbiota influences the resulting workers and overall colony success.

CHAPTER 6:

POLLEN DIETS AND GUT MICROBIOTA

6.1 INTRODUCTION

Since domestication efforts intensified during the 1960s, the use of bumblebees for agricultural pollination has become a significant industry worldwide, producing over two million colonies a year (Velthuis *et al*. 2006; Graystock *et al*. 2016). As generalist pollinators, they offer many ecological, agronomic and economic benefits compared to honey bees. They are less sensitive to temperature, meaning that they can pollinate in both hot and humid greenhouses, and outside in cooler temperatures (Heinrich 1979), and they perform 'buzz' pollination on crops, such as blueberry and tomato (Kearns & Inouye 1997).

To prevent disease outbreak, commercially produced bumblebees are reared indoors without access to natural resources (Meeus *et al*. 2014). For this reason, bees are prevented from foraging naturally and their diet is supplemented with imported pollen. Approximately 500 tonnes of honey bee-collected pollen is needed to support colonies in rearing facilities, making them reliant on pollen supplies all year round (Goulson 2013a).

However, there are different nutritional requirements between bumblebees and honey bees, with bumblebees preferring pollens with high protein content and essential amino acids (Leonhardt & Blüthgen 2012). Commercial pollens can vary greatly with supplier and season, from largely monofloral batches (e.g. Oilseed rape) to polyfloral mixtures (e.g. wildflowers) (Rasmont *et al*. 2005) as a result, they are often not optimal for bumblebee rearing and development. To mitigate these issues, bumblebee producers typically mix pollens to provide more balanced diets to alleviate a lack of nutritional requirements as well as reducing the presence of toxic compounds that can occur in monofloral diets (Eckhardt *et al*. 2014).

Typically, pollen quality is assessed by measuring the protein content (Buchmann 1986), which is highly variable between plant species (Roulston *et al*. 2000; Vanderplanck *et al*. 2014). The importance of protein and amino acid content of pollen for bumblebee and

honey bee colony development has been intensively studied and confirmed (e.g. de Groot 1953; Tasei & Aupinel 2008, Moerman *et al.* 2015, Vaudo *et al*. 2016) but it is conceivable that other micronutrients, such as sterols (Moerman *et al*. 2017) and vitamins (Nation & Robinson 1968), also play an important role.

Diet choice can also influence the biochemistry of the gut and regulates how the gut microbial community develops within the host (Colman *et al*. 2012; Blum *et al*. 2013; Pernice *et al*. 2014). There are many studies of dietary effects on the animal microbiome, particularly in humans and other mammals, but these highly complex communities can often be difficult to interpret clearly (Wang *et al*. 2017). Instead, bees make a good model system because their gut microbiota is simplistic and there is a wealth of information on their nutritional needs (Moerman *et al*. 2015, 2017; Stabler *et al*. 2015). For instance, diets high in fat or carbohydrates are not beneficial for colony development (Vaudo *et al*. 2016), while those high in proteins and essential amino acids are (Moerman *et al*. 2015). However, there is limited information on the impact of diet composition on the gut microbiota, though high protein diets appear to result in low community richness (Billiet *et al.* 2016).

The bumblebee gut microbiota is important for many aspects of host functioning, but in particular is associated with protection against pathogens (Koch & Schmid-Hempel 2011b) and host development, particularly with faster host weight gain (Zheng *et al*. 2017). Establishing a stable microbiota is therefore critical for host health and development, so gaining adequate nutrition to support the host and its microbiota is of paramount importance (Sommer & Bäckhed 2013). Poor diets and starvation can lead to pathogen outbreak, symptomatic of decreasing immune response and host fitness resulting from microbiota dysbiosis (Evans & Schwarz 2011; Brunner *et al*. 2014; Maes *et al*. 2016). This is highly relevant to the bumblebee-rearing industry, where bees are reared in the absence of natural food resources and have a limited exposure to (beneficial) environmental microorganisms. Bumblebees reared this way only harbour a subset of the gut microbiota of those found in wild counterparts, so may lack other protective functions (Meeus *et al.* 2015; Chapter 5).

Whether the gut microbiota of lab-reared bumblebees can be impacted by different rearing practices is potentially important for sustainable production methods. This study aimed to look at the effects of pollen diet composition on the development of the conserved

gut microbiota of indoor-reared *Bombus terrestris* workers during commercial rearing. Here, colonies were fed with different pollen diets, both monofloral and polyfloral, and workers were sampled at different time points that corresponded to different colony sizes to investigate whether (i) horizontal transmission (between nest mates) is important to the abundance and persistence of beneficial gut microbiota, (ii) key gut microbiota populations change through colony development, and (iii) type of pollen diet affects the composition of the gut microbiota.

6.2 MATERIALS AND METHODS

6.2.1 Study species

Bombus terrestris is a short-tongued bumblebee native throughout Europe. Colonies are annual, founded by a single queen following hibernation and can produce up to three hundred 'daughter' workers. In the wild, workers forage from spring to summer for nectar and pollen to rear new workers, queens and males (Alford 1975), but in commercial settings, producers rely heavily on controlled and sterile conditions (Meeus *et al*. 2014), and prevent worker foraging so colony pollen diets consist of honey bee-collected pollen.

6.2.2 Colony rearing and pollen diets

A total of 120 post-diapause *Bombus terrestris audax* queens were placed into individual boxes within the commercial bumblebee breeding facility, Biobest Group NV (Westerlo, Belgium). Queens were randomly assigned to one of six gamma-irradiated pollen diets to mimic commercial rearing procedures (supplier details commercial in confidence), resulting in 20 replicates for each treatment. Within two days an unrelated callow worker (newly emerged, <1 day old) was added within to encourage queen egg laying, and colonies were monitored daily for egg laying behaviour.

Diets consisted of largely monofloral and polyfloral mixtures (Table 6.1) blended with 15% BIOGLUC sugar solution (Biobest Group NV, Belgium). Pollen was sterilised using gamma irradiation at a minimal dose of 15 kGy (STERIS AST, Etten-Leur, the Netherlands). Three of the diets were received as nearly monofloral pollen (V_{mono}, R_{mono}, and P_{mono}) and they were further hand sorted by colour (Hodges 1974; Kirk 2006) to produce a monofloral composition. Diet C_{mono} was not sorted by colour as it appeared

homogeneous in colour and the consistency of the pollen pellets was too grainy to sort by hand. These were compared to two polyfloral pollens, including both a natural wildflower pollen (FL_{poly}), and one that was created by mixing all monofloral types (V_{mono}, R_{mono}, P_{mono}, $C_{mono} = M_{poly}$).

6.2.3 Sample collection

The trial ran for 70 days (19th October–28th December 2017). Development of all colonies was followed for the whole trial, and after 5–6 weeks of development, five colonies per treatment were selected for repeated worker sampling. Only colonies that were developing normally were selected, i.e. with healthy larvae and little or no males produced early on. The first sample of three workers (T_1) was taken when colony size reached *ca*.10 workers, a sample of three workers at T_2 at a colony size of 30–40 workers, and seven T_3 samples were taken when colony size exceeded 70 workers. Some colonies failed to develop and so were sampled up to T_2 or sampled before T_1. All queens fed with diet C_{mono} failed to found colonies due to larval mortality (i.e. the diet was not suited for normal bumblebee development).

At the end of the trial (day 70) colonies were euthanised and populations sizes were counted. Colonies were scored according to commercial criteria, as follows: L2 (queen did not lay eggs); L3 (only males produced); L6 (workers and males produced simultaneously); XSmall (<30); small (30–69), medium (70–110), large (111–150), and extra-large (>151).

6.2.4 Bacterial DNA isolation and sequencing

Bees were surface sterilised prior to dissection by submersion in 70% ethanol followed by vortexing in phosphate buffered saline (1xPBS). This removed trace amounts of ethanol whilst maintaining cell integrity. Once removed, intact whole guts were placed into individual wells of a PowerSoil®-htp 96 Well Soil DNA isolation kit (Mobio Laboratories, Carlsbad, CA, USA) and gut bacterial DNA was extracted following the manufacturer's recommended protocol. Approximately 20 ng of template DNA per sample was amplified and sequenced following the protocol in Section 2.7.1 at a concentration of 5 pM with an additional 10% PhiX Illumina generated control library.

Table 6.1 Description of diets with estimated nutritional information, including estimated protein content. Family level taxonomic identity is based on BLAST searches using a ~355 bp fragment of the ITS2–4 region and should be considered putative.

Label	Diet Type	Description	Diet Composition		Avg protein content (%)*
			Main families	% Rel Abn	
V	Monofloral	Hand sorted by colour. Dominant species was expected to be Blackberry/Bramble	Rosaceae	60.85	20.00
			Asteraceae	16.89	20.65
			Brassicaceae	8.80	21.80
			Apiaceae	4.25	25.35
			Plantaginaceae	3.14	*n.d.*
R	Monofloral	Hand sorted by colour. Dominant species was expected to be Oilseed Rape (OSR)	Brassicaceae	43.89	21.80
			Fabaceae	13.93	31.50
			Papaveraceae	12.56	19.10
			Rosaceae	11.95	20.00
			Apiaceae	8.20	25.35
P	Monofloral	Hand sorted by colour. Dominant species was expected to be Opium Poppy	Brassicaceae	30.53	21.80
			Apiaceae	27.64	25.35
			Papaveraceae	19.62	19.10
			Rosaceae	6.18	20.00
			Fabaceae	4.57	31.50
C	Monofloral	Unsorted pollen. Dominant species was expected to be Cistus.	Asteraceae	44.31	20.65
			Fabaceae	8.12	31.50
			Brassicaceae	7.34	21.80
			Boraginaceae	7.21	*n.d.*
			Plantaginaceae	4.56	*n.d.*
FL	Polyfloral	Unsorted pollen. Expected to be a typical wildflower mixture.	Fabaceae	24.50	31.50
			Brassicaceae	16.45	21.80
			Rosaceae	13.36	20.00
			Orobanchaceae	11.70	*n.d.*
			Papaveraceae	10.26	19.10
M	Polyfloral	Mixture of equal parts of the four monofloral diets. Most similar to the pollen diets used by Biobest Group			

*Based on Hanley *et al.* (2008), Forcone *et al.* (2011), and Liolios *et al.* (2015)

6.2.5 Pollen DNA extraction

High throughput sequencing of the ITS2–4 region was used to confirm the composition of the different pollen diets (Chen *et al*. 2010; Sickel *et al*. 2015). DNA was extracted from representative samples of each diet using an optimised incubation step in addition to the DNeasy® PowerPlant® Pro Kit (Qiagen, Hilden, Germany). Full details are covered in Section 2.6.4, but briefly, 100 mg of homogenised pollen was incubated with 5 µl of proteinase K (>600mAU/ml) at 65°C for 30 minutes, then 40 µl of Phenolic Separation Solution (PSS) was added. After incubation, the manufacturer's recommended protocol was followed.

6.2.6 Pollen barcoding

Extracted pollen DNA was cleaned with a ZR-96 DNA Clean-up Kit™ (Zymo Research, California, USA) before performing two-step PCR reactions targeting the ITS2–4 region (Section 2.7.2).

Resulting PCR products were normalised with the SequalPrep™ Kit (Thermofisher Scientific), pooled, concentrated and then gel purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Gel purified DNA was quantified with a Qubit® Fluorometer and the dsDNA high sensitivity assay kit (Life Technologies) to produce equimolar amplicon libraries. These were sequenced at a concentration of 5 pM with a 10% addition of an Illumina generated PhiX control library. Sequencing runs were performed on an Illumina MiSeq using V3 chemistry producing 2 x 300 bp (Illumina Inc., San Diego, CA, USA).

All pollen samples were processed within the central facility at CEH as part of the National Honey Archive scheme.

6.2.7 Sequence analysis

Resultant sequence paired-end reads were processed using the DADA2 pipeline (Callahan *et al*. 2016) producing amplicon sequence variants (ASVs), as described in Section 2.8.4. This was followed with further quality controls, including removing kit contaminants, singleton sequences, and non-bacterial sequences (Salter *et al*. 2014; Glassing *et al*. 2016). Additional ASV taxonomic classification was performed with EzBioCloud database (Yoon *et al*. 2017) and the Basic Local Alignment Search Tool (Altschul *et al*. 1990), with ASVs identity based on >99% matches. However, given the shortness of the sequences (~300 bp) and that only the forward reads were processed, taxa were resolved to Genus level when possible, but ASV identities should be considered putative.

Sequences from pollen barcoding were based on preliminary pilot analyses targeting a ~355 bp fragment length of the ITS2–4 region. They were processed, denoised, and filtered in DADA2 like the 16S sequencing data, but taxonomy assigned solely using BLAST (Altschul *et al*. 1990). Current databases for pollen identification often lack sufficient depth for taxonomic assignment (Sickel *et al*. 2015), and because of potential

fungal co-amplification (Cheng *et al*. 2016) taxonomic classification was carried out to family-level.

6.2.8 Statistical analysis

From the 297 worker samples taken, thirty were removed from further analysis due to poor read counts (>1000). All remaining samples (n = 268) were rarefied to the lowest read count (=5,050) using the 'rrarefy' function in R: VEGAN (Dixon, 2003). After rarefication and filtering, 1,345,461 sequence reads were retained, representing 225 ASVs. Rarefaction curves confirmed that the sequencing depth was sufficient to provide a good description of the alpha diversity within each sample (Appendix 3), and this subset was used in all further analyses.

Gut community and pollen sample diversity was quantified by Fisher's alpha (log series alpha) as it is independent of sample size (Magurran 2004). Pollen taxa Simpson's dominance (D) index was calculated from Simpson's diversity (1-Simpson index; D = sum($(n_i/n)^2$) where n_i is number of individuals of taxon i).

Bacterial ASV frequencies were converted to a Bray-Curtis dissimilarity matrix using PAST v3.11 (Hammer et al., 2001) to investigate microbiota composition, and statistical analyses using Kruskal-Wallis, one-way ANOVA and other univariate statistics were carried out using the base statistics package in R version 3.3.2. Principal Coordinates analysis was performed in R using the Phyloseq package (McMurdie & Holmes 2013). Analysis of similarity (ANOSIM) and similarity of percentages (SIMPER) analysis were performed using PAST (v3.11). Bray-Curtis index of similarity was used as the underpinning community similarity measure for both ANOSIM and SIMPER tests.

6.3 RESULTS

6.3.1 Pollen diet affects microbiota genus level dynamics

From the 225 bacterial ASVs identified, 32 were present in at least one individual from each pollen diet. The ASVs identified belonged to 50 genera, with only 17 of these however, present in 5% or more of the total sampled workers. On average 14.8 ± 0.7 ASVs (±95% confidence interval) were detected per individual and there were significant differences in average ASV count between diets (χ^2 = 29.62, p < 0.001, n = 268). ASV

counts tended to be higher in workers fed V_{mono} and M_{poly} diets ($p < 0.01$), and these workers also contained the most diet-specific ASVs, with 0.4 ± 0.23 and 0.49 ± 0.16, respectively ($\chi^2 = 11.71$, $p < 0.001$, $n = 268$).

Two-way analysis of variance (ANOVA) analyses showed that overall, Fisher's alpha index of diversity was significantly different between treatments (Fig. 6.1; $F_{(4,267)} = 6.57$, $p < 0.001$, $n = 268$), but not overall between sample points ($F_{(4,267)} = 2.22$, $p = 0.11$, $n = 268$). However, there did appear to be an interaction between these two variables ($F_{(8,267)} = 1.74$, $p < 0.01$, $n = 268$). Gut microbial diversity was significantly lower in workers fed on R_{mono} (1.45 ± 0.20; $p < 0.01$) and FL_{poly} diets (1.60 ± 0.17; $p < 0.05$) compared to the M_{poly} (2.17 ± 0.19) and Rosaceae diets (V_{mono}; 2.00 ± 0.19).

Gut communities present in workers of each diet were characterised to determine the common microbiota (Fig. 6.2). Averaged across all treatments and sample points, the gut microbiota was dominated by the genera *Gilliamella* (mean 49.81 ± 2.02%),

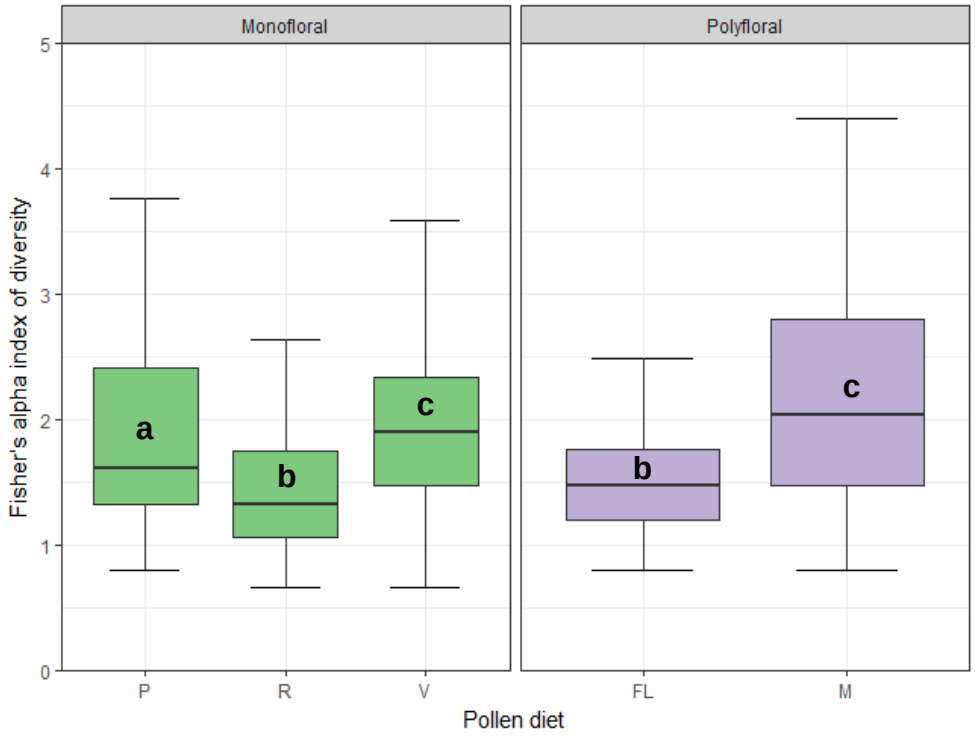

Figure 6.1 Fisher's alpha diversity of gut microbiota in workers sampled from each of the five pollen diet treatments (monofloral = green, polyfloral = purple) averaged across all time points. Significance between diets (denoted by different letter) at the $p < 0.05$ level.

Snodgrassella (mean 28.11 ± 1.54%), and *Lactobacillus* (mean 20.53 ± 1.71%), which were present in 100% of workers sampled. When the data was partitioned into treatments sample points, the common taxa also included *Bombiscardovia* (mean 0.83 ± 0.17%) and *Sphingomonas* spp. (mean 0.22 ± 0.09%). Between treatments, there were no significant differences in the relative abundance of *Gilliamella* (χ^2 = 3.91, p = 0.42, n = 268), *Snodgrassella* (χ^2 = 7.40, p = 0.12, n = 268), or *Sphingomonas* species (χ^2 = 7.80, p = 0.08, n = 268). The relative abundance of *Lactobacillus* did differ between the diet treatments (χ^2 = 9.96, p = 0.04, n = 268), where relative abundance was significantly higher in workers fed both the P_{mono} and M_{poly} diets, compared to those on V_{mono} ($p < 0.05$). In addition, there was a significant difference in the relative abundance of *Bombiscardovia* (χ^2 = 20.54, $p <$ 0.001, n = 268), with relative abundance significantly higher in workers fed diet M_{poly} compared to those on R_{mono} and P_{mono} diets ($p < 0.05$).

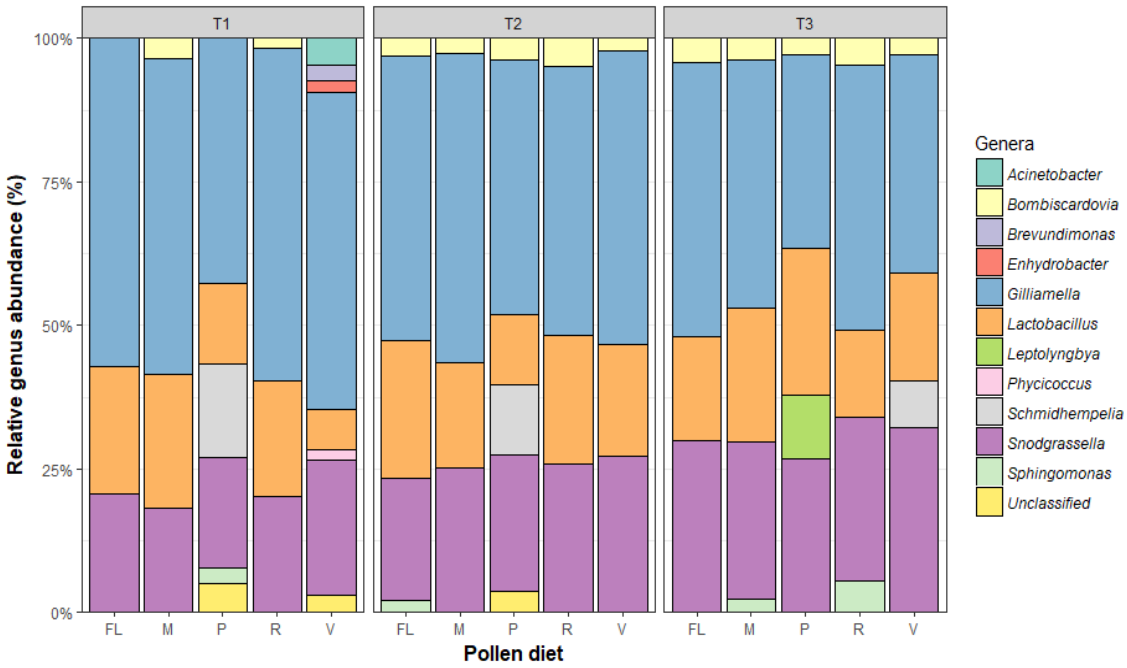

Figure 6.2 Average gut microbiota composition between workers fed different pollen diets. Samples were taken as colonies increased in size (T_1 = *ca*. 10 workers; T_2 = 30–40 workers; and T_3 = >70 workers) and shown a relatively consistent microbiota between diets and over time.

ANOSIM tests were performed to further characterise the microbiota, using pairwise Bray-Curtis dissimilarity values (Table 6.2). Significant differences in bacterial composition were highlighted between workers from the five diets and showed that diet M_{poly} was significantly different to diets V_{mono}, R_{mono} and P_{poly} ($p < 0.05$), and diet FL_{poly} differed significantly to diet P_{mono} ($p < 0.01$). Additional analysis of how composition was affected by diet was carried out using similarity percentage (SIMPER; Table 6.3) analyses of the workers followed throughout differential feeding. This highlighted that multiple ASVs corresponding to *Gilliamella*, *Snodgrassella*, *Lactobacillus*, and *Bombiscardovia* species contributed most to the dissimilarity in gut microbiota between treatments, primarily the taxa that make up the artificially-reared *Bombus* workers.

6.3.2 Diet and temporal effects on gut communities

Fisher's alpha diversity fluctuated between sample points for each diet treatments throughout the trial (Fig. 6.3). At T_1 (when colonies contained ~10 workers), there was a significant difference ($\chi^2 = 11.34$, $p = 0.02$, $n = 61$), with R_{mono} workers less diverse than their cohorts fed on the V_{mono} diet ($p < 0.01$). By T_2 (30–40 workers), both M_{poly} and P_{mono} workers were significantly more diverse than the R_{mono} cohort ($\chi^2 = 17.22$, $p < 0.01$, $n = 79$; $p < 0.001$). At the final sample point (T_3; >70 workers), M_{poly} and V_{mono} diets were both significantly more diverse than the FL_{poly} cohort ($\chi^2 = 23.49$, $p < 0.0001$, $n = 128$; $p < 0.0001$).

Diversity also fluctuated within each diet treatments as colonies increased in size. There were significant differences in gut microbiota diversity in diets R, P, and FL ($\chi^2 = 8.67$, $p = 0.01$, $n = 32$; $\chi^2 = 8.08$, $p = 0.02$, $n = 68$; $\chi^2 = 8.72$, $p = 0.01$, $n = 41$, respectively).

Colonies feeding on diet R generally showed an increase in gut microbiota diversity by the final sample point ($p < 0.001$), while those on diets P_{mono} and FL_{poly} fluctuated. Diversity decreased significantly between T_2 and T_3 in diet P_{mono} ($p = 0.01$), while in diet FL, diversity was significantly lower by T_3 compared to T_1 ($p < 0.01$).

Variance in diversity was low, ranging between 1.27 ± 0.35 (T_2, diet R_{mono}) and 2.52 ± 0.53 (T_2, diet P_{mono}). Despite this small variance however, there were significant differences between sample points of each diet ($\chi^2 = 55.29$, $p < 0.0001$, $n = 268$).

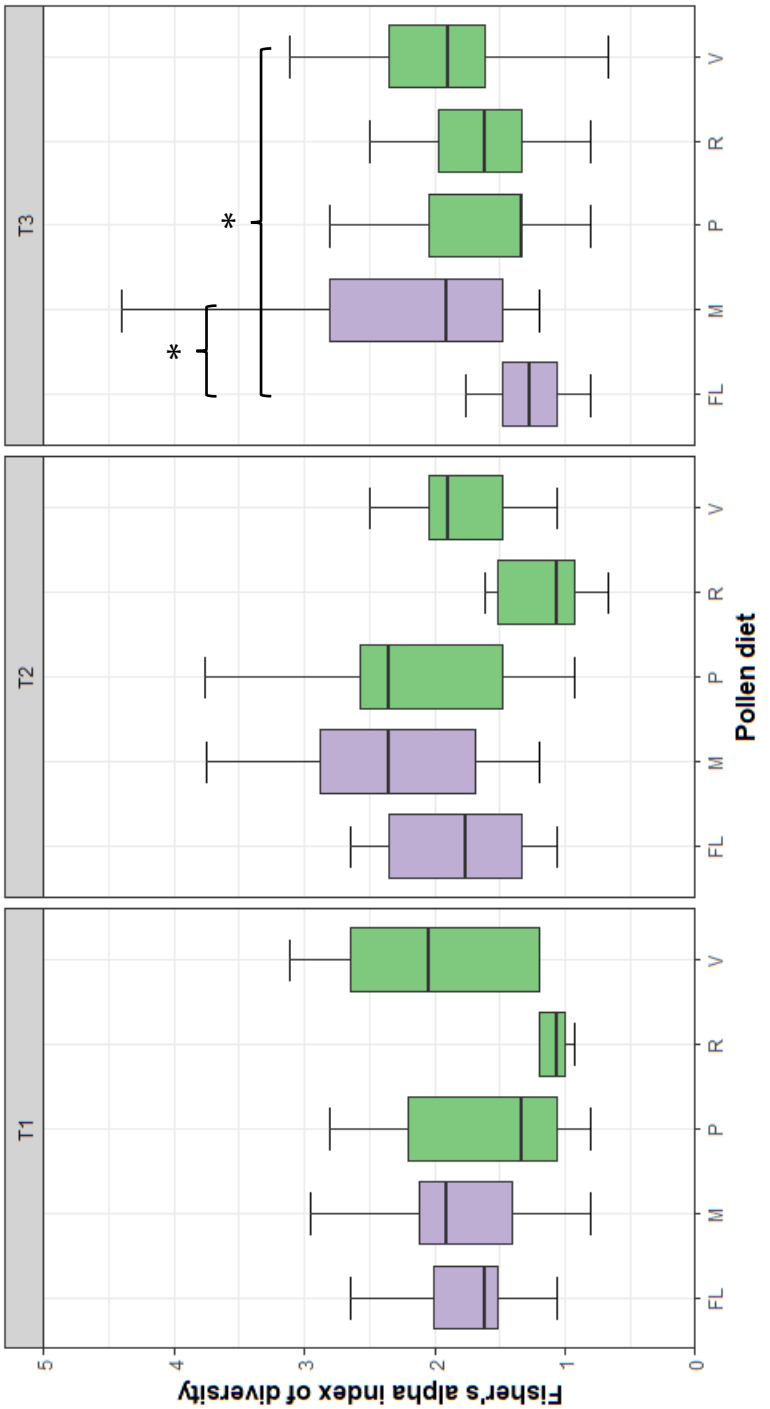

Figure 6.3 Fisher's alpha diversity between pollen diet treatments and sample points. Monofloral diets (V, R, and P) are shaded in green, and polyfloral diets (M and FL) are shaded in purple. Significant differences (*) at the $p < 0.05$ level.

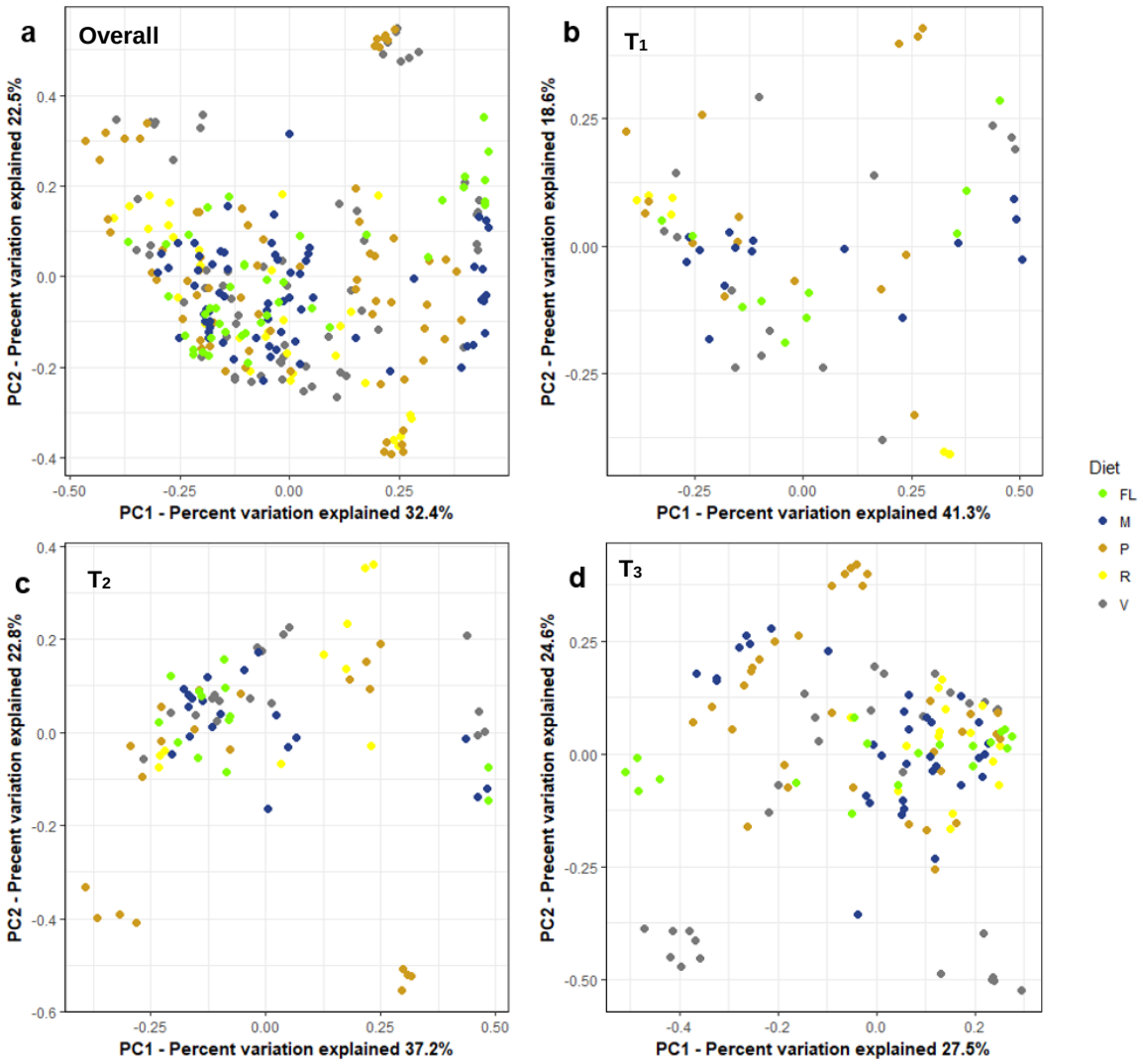

Figure 6.4 Principal Coordinates Analysis (PCoA) of worker gut microbiota between different pollen diets (FL_{poly} – green, M_{poly} – blue, P_{mono} – gold, R_{mono} – yellow, and V_{mono} – grey). Panel (a) shows all sample points, while (b) – (d) display PCoAs for sample points T_1 – T_3, respectively.

When looking at whole gut communities overall (including common and rare taxa) worker microbiota appeared to become more clustered in certain diets as the colonies increased in size (Fig. 6.4). However, these trends were not uniform for treatments, as for some diets samples clustered separately to the rest of their treatment types, such as P_{mono} (at T_2; Fig 6.4 c) and V_{mono} (at T_3; Fig. 6.4 d).

Table 6.2 Analysis of similarity (ANOSIM) of workers fed one of the five commercial pollen diets. Values were generated using the Bray-Curtis measure of similarity, with the ANOSIM test statistic (R) shown below the diagonal with the probability (*p*) above. Those highlighted in bold font with an asterisk indicate significance ($p < 0.05$).

Table 6.3 Similarity of percentages (SIMPER) analysis of worker gut microbiota between the five different commercial diets. Given is the percentage mean abundance, with the overall average dissimilarity between samples =55.41%. N.B Only >1% contribution taxa are shown, and ASV identities are based on ~290bp fragments and should be considered putative.

Table 6.2	V	R	P	M	FL
V	-	0.754	0.783	**0.000***	0.842
R	-0.028	-	0.998	**0.022***	**0.008***
P	-0.009	-0.087	-	**0.000***	1.000
M	0.093	0.091	0.063	-	0.071
FL	-0.023	0.077	-0.070	0.045	-

Table 6.3

Class	Family	Genus	% Mean abundance V	R	P	M	FL	Av. dis.	Cont. %	Cuml. %
Gammaproteobacteria	Orbaceae	*Gilliamella*	29.30	37.50	29.10	32.70	37.00	12.69	22.90	22.90
Betaproteobacteria	Neisseriaceae	*Snodgrassella*	21.90	28.30	20.70	22.50	24.60	8.33	15.03	37.93
Bacilli	Lactobacillaceae	*Lactobacillus*	9.82	12.30	10.90	4.82	16.10	6.14	11.08	49.01
Gammaproteobacteria	Orbaceae	*Gilliamella*	7.00	0.97	5.70	8.05	9.14	5.09	9.18	58.18
Gammaproteobacteria	Orbaceae	*Gilliamella*	7.14	4.65	4.68	4.99	2.93	3.62	6.53	64.71
Bacilli	Lactobacillaceae	*Lactobacillus*	1.56	7.13	5.05	6.44	1.06	3.51	6.33	71.04
Betaproteobacteria	Neisseriaceae	*Snodgrassella*	8.01	0.00	5.91	0.69	0.02	3.15	5.68	76.72
Gammaproteobacteria	Orbaceae	*Gilliamella*	3.90	4.08	4.84	3.17	3.21	2.90	5.23	81.95
Bacilli	Lactobacillaceae	*Lactobacillus*	1.14	0.01	2.52	6.50	1.06	2.29	4.13	86.08
Bacilli	Lactobacillaceae	*Lactobacillus*	1.50	0.96	3.30	2.54	1.46	1.79	3.23	89.32
Betaproteobacteria	Neisseriaceae	*Snodgrassella*	1.52	0.72	1.92	2.28	1.37	1.34	2.42	91.73
Actinobacteria	Bifidobacteriaceae	*Bombiscardovia*	0.72	0.85	0.45	1.35	0.72	0.62	1.12	92.85

Specifically, workers in the final sample point of the FL_{poly} diet had consistently lower gut microbiota diversity compared to workers in V_{mono}, and M_{poly} diets ($p < 0.01$), and were significantly lower than R_{mono} T_3, P_{mono} T_2, and earlier FL_{poly} samples ($p < 0.05$).

The common genera also showed trends in relative abundance over time but differed between some diets (Fig. 6.5). Typically, most diets showed similar trends as colonies increased in size during the trial. When pooled by diet, the dominant taxa had weak, linear trends over time, with a decrease in *Gilliamella* ($R^2 = 0.10$, $p < 0.0001$) and increase in *Lactobacillus* ($R^2 = 0.02$, $p = 0.03$). Levels of *Sphingomonas* fluctuated at low levels between treatments and over time, while *Bombiscardovia* appeared to be ubiquitous in worker guts from T_2 onwards.

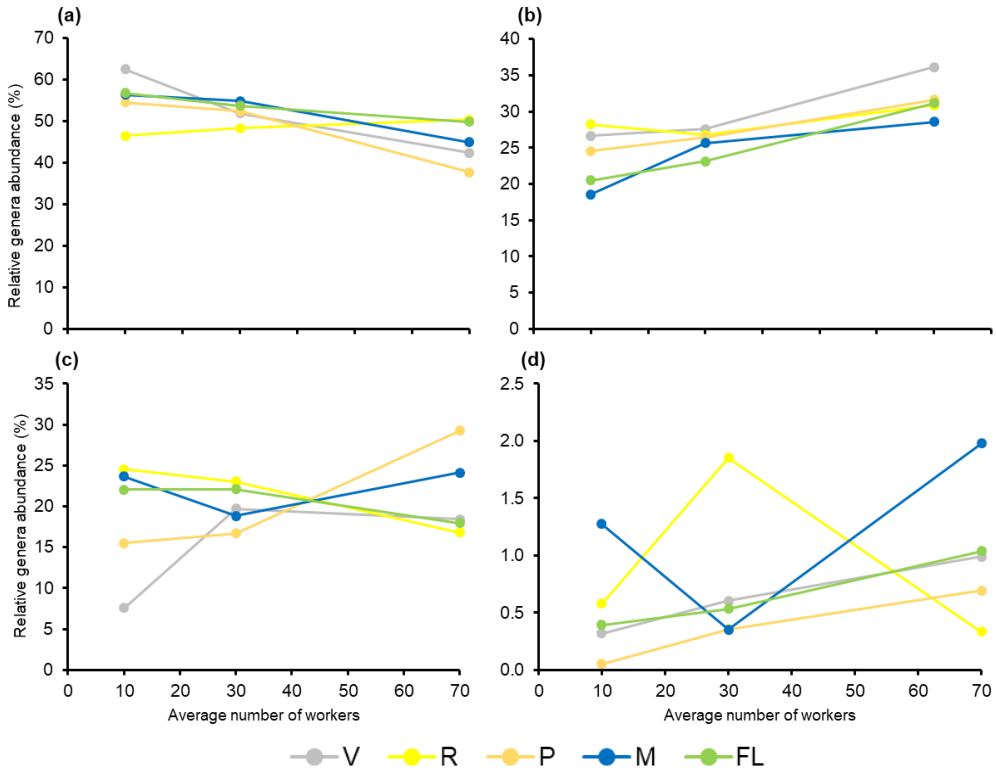

Figure 6.5 Trends in relative abundance of the four main *Bombus* genera, (a) *Gilliamella*, (b) *Snodgrassella*, (c) *Lactobacillus*, and (d) *Bombiscardovia* between different pollen diets (FL_{poly} – green, M_{poly} – blue, P_{mono} – gold, R_{mono} – yellow, and V_{mono} – grey). Relative abundance of genera is plotted against worker population size.

6.3.3 'Monofloral' and 'polyfloral' pollen diets

Diets V_{mono}, R_{mono}, C_{mono}, and P_{mono} consisted of commercially available pollens that were sold as 'monofloral'. They were further sorted by colour to reduce plant species diversity in each diet, which was then confirmed using high throughput sequencing, with samples from each sorted pollen diet found to be more diverse than expected. Fisher's alpha diversity ranged between 2.93 (diet P_{mono}) and 8.53 (diet C_{mono}), and composition analyses highlighted that 'monofloral' diets were more diverse than anticipated (Fig. 6.6), though, sorting by hand did reduce richness (Appendix 12). Simpson's dominance index (D; scaled from 0 to 1) indicated that C_{mono}, M_{poly} and FL_{poly} diets ranged between 0.07 – 0.08, indicating taxa were almost equally present. The other diets were less equally distributed, but still not highly dominated by one plant family (P_{mono} = 0.13; R_{mono} = 0.15), except for diet V_{mono} (0.24), which was largely comprised of Rosaceae pollen.

The diets that produced the colonies with the highest yields (P_{mono} and M_{poly}) were also observed to have similar compositions, largely comprised of plant taxa from Apiaceae, Brassicaceae, Fabaceae, Papaceraceae and Rosaceae, and were both almost 'equally polyfloral'. Fisher's alpha diversity showed that diet C_{mono} by comparison was the most diverse but did not produce any viable larvae. It had a high relative abundance of Asteraceae, so could still be classed as monofloral.

Averages of pollen protein content (%) for each family were estimated from existing literature (Table 6.1). Based on these data, and the relative abundance of the major families, the pollen diet with the highest 'quality' was R_{mono} (20.82 ± 4.42% protein content), while diet FL_{poly} had the lowest quality (15.94 ± 5.60% protein content).

6.3.4 Diet impacts on worker production

Before starting the trial, queen weight was measured to determine any characteristics that may advantage some queens over others, but there was no significant difference between any of the diets ($F_{(5,119)}$ = 0.50, p = 0.66, n = 120). During the experiment, 19.2% of queens across all pollen diets did not lay eggs, but of those that did, the majority (~75%) did not produce colonies of over 20 workers before the end of the trial. Queens fed diet C_{mono} did not produce any viable larvae and so this treatment was terminated shortly after the start of the trial.

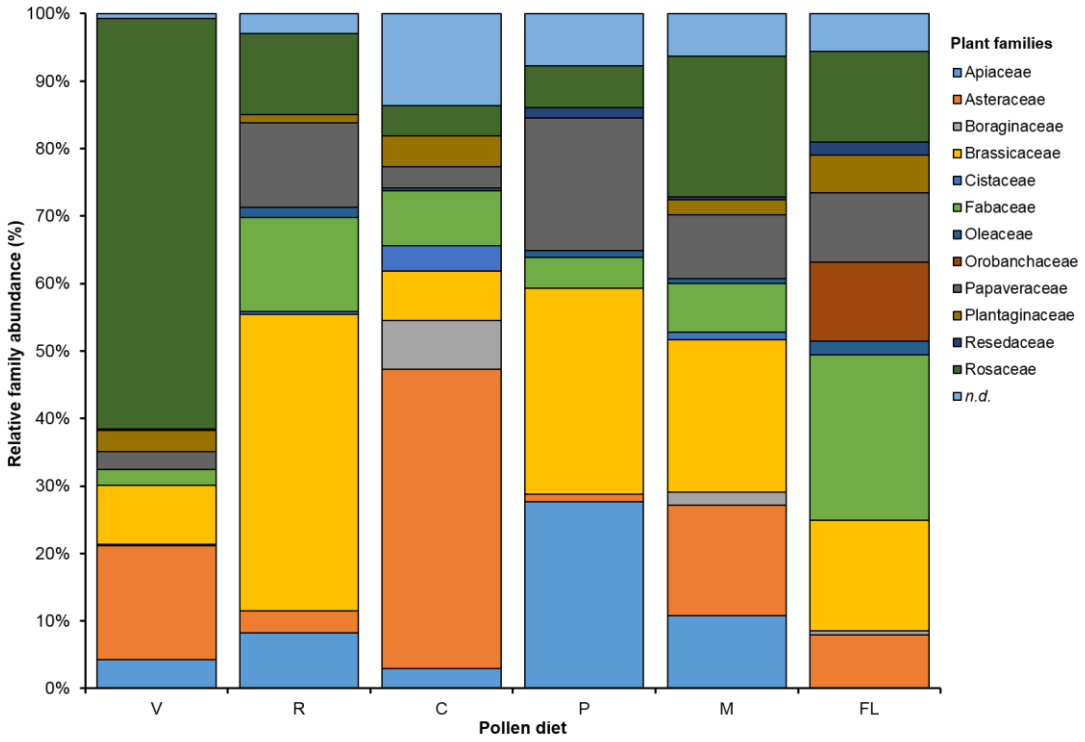

Figure 6.6 Average plant family composition of commercial pollen diets used in the trial determined through analysis of plant ITS gene.

Queens did successfully establish new colonies in the remaining diets, and typically laid eggs 9.3 ± 0.8 days after the start of the trial. Queen weight did not significantly impact the final number of workers (R^2 = 0.01, p = 0.13), or correlate with the time to start egg laying (R^2 = 0.02, p = 0.09) or the emergence of the first workers (R^2 = −0.01, p = 0.81).

On average, the first generation of workers emerged 36–39 days into the trial, first in the V_{mono} and M_{poly} diets at 27–28 days. There was a significant difference in the numbers of workers and pupae produced (χ^2 = 11.27, p = 0.02, n = 100; χ^2 = 13.54, p = 0.01, n = 100, respectively), with diet P_{mono} significantly higher than diets R_{mono}, V_{mono} and FL_{poly} ($p < 0.05$)

The final count at the end of the trial (day 70), was used to calculate the overall worker population of each colony. In addition, colonies were scored according to those that were suitable for commercial use (Section 6.2.3). Of the 120 started queens only 20% produced commercially viable colonies (>70 workers), with diets M_{poly} and P_{mono} producing the most sellable colonies with 40% and 50%, respectively (Fig. 6.7; Table 6.4).

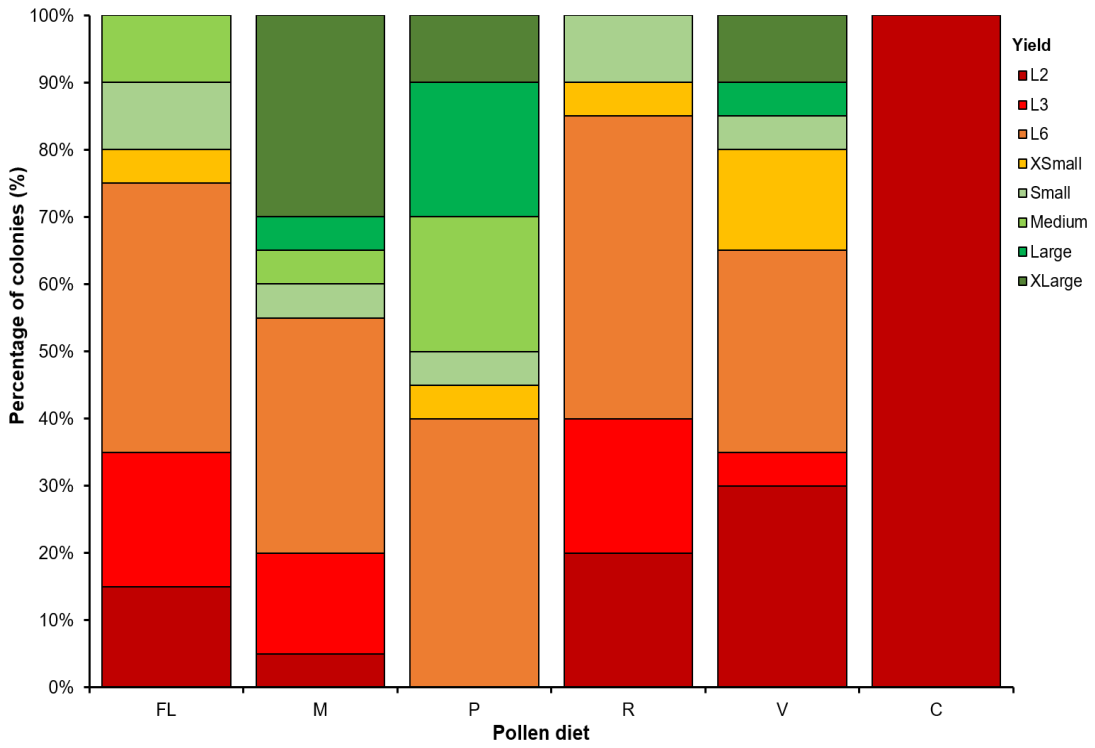

Figure 6.7 Total worker production (score) for colonies reared on each commercial diet. Worker population was scored based on size and composition of colony. L2 (no eggs produced); L3 (only males produced); L6 (workers and males produced simultaneously); XSmall (>30); Small (30–69); Medium (70–110); Large (111–150); and XLarge (>151).

Table 6.4 Summary of final colony data including scores based on worker population size, data on the pollen diets and dominance of plant family taxa.

Diet	Commercially-viable nests	Pollen diversity	Dominance	Avg worker population*	Colony score							
					L2	L3	L6	XSmall	Small	Med	Large	XLarge
V	3	4.96	0.24	159	6	1	6	3	1	0	1	2
R	2	5.46	0.15	90	4	4	9	1	0	2	0	0
P	10	2.93	0.13	129	0	0	8	1	1	4	4	2
C	0	8.53	0.07	0	20	0	0	0	0	0	0	0
M	8	3.72	0.10	166	1	3	7	0	1	1	1	6
FL	2	4.76	0.08	95	3	4	8	1	2	2	0	0

*Based on commercially-viable nests (>70 workers)

6.4 DISCUSSION

This study followed worker gut microbiota in twenty-three *Bombus terrestris audax* colonies fed a range of irradiated commercial diets over a period of two months. Workers were sampled from colonies at multiple points, reflective of colony size, to analyse if gut microbiota is influenced by nutritional content in otherwise sterile pollen diets. Diet was found to affect microbiota diversity, as well as colony productivity (i.e. number of workers), however, the overall microbial diversity did not appear to correlate with colony size. Instead, the consistency of bacterial species in worker guts reflected the stability and sterility of the environment and the sterilized commercial pollen diet (Biobest Group NV, Belgium).

Throughout the trial, the common bacterial species remained relatively constant between all diets and sample points tested. This subset community commonly found in lab-reared bumblebee workers, consisted mainly of *Gilliamella*, *Snodgrassella*, *Lactobacillus*, and *Bombiscardovia*, though *Schmidhempelia* appeared relatively rare in samples from this study in comparison to Meeus *et al.* (2015). By comparison to previous work (Chapters 3, 4) the gut community of workers reared indoors is severely restricted, and the fact that the gut community was largely comprised of the same genera, regardless of pollen diet, suggests that these bacteria can establish in the worker gut with limited horizontal transmission from food resources, the environment, and nest mates, which could indicate that vertical transmission may play a role in acquisition of key components of the gut microbiota (Koch *et al.* 2013).

It seems reasonable to propose that the gut microbiota likely originated from the queen (and potentially the helper worker). In commercial settings, near-sterile conditions are used, i.e. sterilised pollen/sugar syrup, sterile nest boxes, all housed within enclosed rearing chambers. Populations in these facilities have had no natural environmental contact for multiple generations (Meeus *et al*. 2015), so external sources of bacteria are very limited.

Queens themselves were placed inside in a sterile colony box following emergence from diapause, with a depleted gut microbiota (Chapter 5), followed by helper workers with an immature gut microbiota, which contains both key genera and additional rarer taxa

(Chapter 3). Both individuals share food resources and build the nest for the first generation of workers, inoculating the nest with bacteria (Anderson *et al*. 2013; Billiet *et al*. 2017), which is then transmitted to rest of the colony – though vertical transmission from the queen may also occur (Chapter 3; Koch *et al.* 2013).

Transmission of the gut microbiota appears, therefore, to be highly conserved. The typical gut community was present in workers when colonies had 10, 30 and 60^{+} workers, suggesting that once acquired, these taxa require little horizontal reinforcement. Other genera, such as *Bombiscardovia* were found ubiquitously in workers sampled above a size of 30 workers, which may suggest that this rarer taxon takes longer to be acquired and requires more propagation through the colony (through social contact or shared food resources).

Increased propagation via an increase in colony size, also did appear to influence certain taxa. *Snodgrassella*, *Lactobacillus*, and *Bombiscardovia* spp., which all appeared to increase in relative abundance according to colony size (though diet R_{mono} was often the exception). In contrast, *Gilliamella* appeared to decrease, suggesting that these taxa can increase in the gut as a result of the shift in *Gilliamella* abundance.

Though the tested diets produced similar compositions of gut microbiota genera, there were differences in microbiota diversity between diets and sample points. Of all the treatments, diet R_{mono} (predominantly Brassicaceae) produced colonies with the lowest yield and the lowest gut microbiota diversity, despite being estimated to have the highest protein content. Billiet *et al.* (2016), also showed that high protein diets result in low community richness, and the workers fed on this diet showed increases in *Gilliamella*, and decreases in *Lactobacillus* as colonies developed, which contrasted trends observed in other diets. However, only protein content was estimated for these diets, which did not appear to be the main nutritional factor needed for adequate colony development. Clearly other compositional factors (e.g. sterols, protein:lipid ratios) play a role in both colony development, and using protein content is not sufficient (Vaudo *et al.* 2016b).

Diversity of pollen diet did not necessarily correlate with colony success (i.e. worker population size), as shown by comparisons in alpha diversity between the diets. Although the pollens were much less ‘monofloral’ than expected, most diets contained clear dominant plant families, except P_{mono}, which made it more similar in composition to

the two polyfloral diets. Both P_{mono} and M_{poly} produced the most commercially viable colonies, and comparisons of diet composition between them highlighted similar relative abundances of Brassicaceae, Fabaceae, and Rosaceae. Though FL_{poly} was also highly diverse and had a low dominance index it did not produce as many viable colonies. It appears likely that both P_{mono} and M_{poly} contained the necessary proteins, amino acids and other micronutrients for healthy bumblebee development compared to the other diets (Moerman *et al*. 2015), suggesting that plant diversity is not important, but that the right assortment of essential nutrients are present (Moerman *et al*. 2017).

In addition, these two diets (P_{mono} and M_{poly}) also exhibited higher relative abundances of certain genera, including *Lactobacillus* spp., suggesting a beneficial role of this bacterium in the host and colony in general. There is already some indication of the probiotic potential of *Lactobacillus* spp. for improving bee health and colony functioning (Evans & Lopez 2004; Audisio & Benítez-Ahrendts 2011; Pătruică & Mot 2012), and its detection in healthy post-diapause queens in Chapter 5, suggests that the presence of *Lactobacillus* in the gut may be a good indicator of general health.

Commercial bumblebee producers should consider tailoring practices to optimise the host gut microbiota as well as worker population size, focussing on maintaining the core, functional taxa, *Gilliamella*, *Snodgrassella*, *Bombiscardovia*, and *Lactobacillus* spp. (Billiet *et al*. 2016). It appears from these findings that diets rich in Brassicaceae, Fabaceae, and Rosaceae species can improve colony population size and increase the relative abundance of beneficial gut microbiota. In the interests of optimising commercial bumblebee breeding, and to promote the health of wild populations, it is important to understand how different diets impact these functional taxa and feeding bees sufficiently optimised diets could confer sufficient protection when they perform their functional pollination roles.

CHAPTER 7:

HIGH NEONICOTINOID INSECTICIDE EXPOSURE DISRUPTS BUMBLEBEE GUT MICROBIOTA

7.1 INTRODUCTION

Current agricultural practices have become increasingly dependent on insecticides to maintain high crop yields (Tilman *et al*. 2002), but there is a growing appreciation of the extent to which broad-spectrum and systemic pesticides have negative impacts on biodiversity and the ecosystem services they provide (Stanley *et al*. 2015). Their use has been implicated as a key factor contributing to continuing global declines of wild and managed pollinator populations. Such declines have implications for the future of sustainable crop pollination and terrestrial biodiversity (Vanbergen *et al*. 2013).

Neonicotinoid pesticides in particular, have seen a major global increase in use over the last decade (Elbert *et al*. 2008; Mullin *et al*. 2010; Jeschke *et al*. 2011; Van der Sluijs *et al*. 2013; Goulson 2015). Concerns about their impact on non-target organisms have resulted in a complete ban on outdoor use of three classes of neonicotinoid (imidacloprid, clothianidin and thiamethoxam) within the European Union (EU). Despite this, however, neonicotinoids continue to be used outside the EU, and persist as environmental residue, particularly around mass flowering crops such as oilseed rape (Woodcock *et al*. 2018).

Neonicotinoids are water soluble compounds and easily absorbed by plants where they are spread throughout all tissues via the vascular system, providing extensive protection against herbivorous insects. However, neonicotinoids may also be expressed in pollen and nectar stores and ingested by non-target pollinating insects, such as honey bees and wild bees (Elbert *et al*. 2008; Bonmatin *et al*. 2015). They act as neurotoxins, disrupting the nicotinic acetylcholine receptor (nAChR), which can result in abnormal behaviour, paralysis and death (Matsuda *et al*. 2001; Tomizawa & Casida 2005). Both laboratory and semi-field studies on bumblebees have demonstrated that exposure to even

sublethal levels of neonicotinoids can impair learning and motor functions (Feltham *et al*. 2014; Gill & Raine 2014), reduce colony growth and queen production (Whitehorn *et al*. 2012; Baron *et al*. 2017) and decrease overall colony fitness (Rundlöf *et al*. 2015).

While the effects of pesticides on bees have been investigated extensively, as yet there has been little investigation of their action on bee gut microbiota. The insect microbiome is well known for its importance to host health and critical function with numerous studies exploring the importance of key taxa for metabolism (*reviewed in* Kwong & Moran 2016), host weight gain (Zheng *et al*. 2017), pathogen resistance (Koch & Schmid-Hempel 2011b), and immunity (Kwong *et al*. 2017). Social bees in particular have characteristically simple, yet specialised gut microbiota (Mohr & Tebbe 2006; Cox-Foster *et al.* 2007; Martinson *et al.* 2011; Moran *et al.* 2012; Engel *et al.* 2012; Engel & Moran 2013a), making them ideal subjects to study the effects of pesticides, both for their economic value and use as models for more complex organisms (Koch *et al*. 2013; Kwong *et al*. 2014; Pernice *et al*. 2014).

Comparative pesticide studies have found species-specific differences between social bees (Rundlöf *et al*. 2015; Heard *et al*. 2017). It appears that honey bees are able to clear ingested neonicotinoids faster than bumblebees and have more resilience to pesticide impacts on population parameters (Cresswell *et al*. 2014). One recent study tested the effects of imidacloprid on *Apis mellifera* gut microbiota and found increased mortality in workers, but did not find a link to changes in the gut community (Raymann *et al*. 2018). Few studies, however, have examined the impacts of pesticide toxicity on wild bees, despite the critical agricultural pollination services they provide.

This experiment explored the impacts of sublethal doses of the neonicotinoid insecticide, clothianidin on the microbiome of the bumblebee *Bombus terrestris audax* worker. Standard toxicological tests typically measure exposure over short time frames (less than 96 h), but here this study used a relatively long term, exposure assay (240 h) to test if there were time dependent effects of exposure on: (i) bacterial community structure and, (ii) if this altered individual worker bee behaviours and growth rates of some of the core members of the community, *Gilliamella* and *Snodgrassella* species.

7.2 MATERIALS AND METHODS

7.2.1 Study species

Bombus terrestris is a short-tongued bumblebee, widely distributed throughout Europe and occurring in colonies of up to 350 workers headed by a single queen (Alford 1975). In temperate climates, it most commonly has an annual lifecycle, although in some habitats (e.g. urban, Stelzer *et al.* 2010) two colony cycles may be observed. It is a generalist pollinator that has been domesticated to provide pollination for high value horticultural crops, where it provides a valuable ecosystem service to many wildflower species and agricultural crops, but is vulnerable to exposure from various harmful agrochemicals (Potts *et al*. 2010).

7.2.2 Experimental micro-colonies

Twelve colonies of *Bombus terrestris* (ssp. *audax*) were obtained from Biobest Group NV (Westerlo, Belgium), each containing a single queen and approximately 50 workers. From these replicate queenless micro-colonies (n = 100) were established, each comprised of three sister workers originating from the same parent colony. Workers were removed directly from their parent colony under red light using long, sterile forceps before being randomly allocated to plastic assay pots (Section 2.4.2). Each pot comprised a plastic cage with a ventilated lid, and a base to hold a 50 ml Luer centric syringe (Latex and silicone oil free). Syringes were loaded with dosed sucrose solution and modified to provide a 3 mm diameter-drinking hole.

7.2.3 Pesticide dosages and sample collection

Clothianidin was obtained as analytical grade pesticide standard (PESTANAL®) from Sigma-Aldrich®. Five experimental dosages were produced via serial dilution in water from the highest concentration of clothianidin (0.026, 0.016. 0.01, 0.006, 0.004 μg/ml). These dosages were known to be sublethal (Heard *et al*. 2017), and were then added to a 50% w/v sucrose solution (molecular biology grade, Sigma Chemicals). An additional treatment of 100% w/v sucrose was used as a negative control.

Micro-colonies were randomly assigned to one concentration of clothianidin and allowed to feed *ad libitum* for the duration of the trial. They were kept in a controlled

temperature room at 25 ± 2°C and 60% ± 5 RH under constant darkness (except during data collection). Worker mortality was assessed three times a day during the first 96 hrs of exposure, and then daily for the remainder of the experiment. Any deceased workers were removed from the treatment pot and stored at −80°C. Sucrose consumption was measured daily by weight over the whole exposure period. Baseline samples of the average gut microbiota within each parent colony were taken to assess inter-colony variation prior to experimental manipulation and dosing. The whole experiment ran for 10 days, with individual micro-colony pots assigned to one of three exposure periods: 24 (T_1), 96 (T_2) and 240 hrs (T_3). Exposure periods were assigned randomly to pots before dosing and worker allocation. At the end of each exposure period, five replicate micro-colony pots per dosage were frozen at −80°C to euthanize all workers prior to gut dissection. Gut samples were only taken from workers that were alive at the end of their exposure period, to avoid reflecting a post-mortem microbiota.

Prior to dissections anatomical measurements were taken, including total weight and gut weights, along with head width, which can be used as a proxy for body size as it is largely unaffected by freezing (Hagen & Dupont 2013).

7.2.4 DNA isolation and sequencing

Prior to dissection, bees were surface sterilised with 70% ethanol then gently spun in phosphate buffered saline (1xPBS) to remove external contaminants, whilst maintaining cell integrity. Intact guts were placed into individual wells of a PowerSoil®-htp 96 Well Soil DNA isolation kit (Mobio Laboratories, Carlsbad, CA, USA) and worker gut bacterial DNA was extracted following the manufacturer's recommended protocol. Approximately 20 ng of extracted template DNA per sample was amplified and sequenced following the protocol outlined in Section 2.7.1.

7.2.5 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was used to estimate absolute copy numbers of 16S rRNA genes to complement the high-throughput sequencing data (Appendix 13). Bacterial DNA in a subset (n = 48) of samples was amplified using the Femto™ Bacterial DNA Quantification Kit (Zymo Research, USA). Samples were prepared in triplicate using approximately 2 µl of template DNA in reaction volumes of 10 µl. Duplicate standards (1 µl per reaction) and

controls were also added, and the whole plate was placed in a LightCycler® 480 (Roche Molecular Systems, Inc.) following the manufacturer's recommended thermocycling parameters. Absolute gene copy numbers were calculated from the thermocycler, based on the known input values of the standard curve DNA.

7.2.6 Sequence analysis

Resultant raw sequences were processed and analysed through a sequencing analysis workflow summarised in Section 2.8.3. Low abundance OTUs (operational taxonomic units) and probable kit contaminants (Salter *et al*. 2014; Glassing *et al*. 2016) were filtered from the sequence data, including sequences that appeared once, non-bacterial taxa, and potential false positives.

OTUs were assigned taxonomic identity against the Greengenes Release 13_5 (at 97%) (DeSantis *et al*. 2006), but some OTUs could not be resolved further than phylum classification. In these instances, the Basic Local Alignment Search Tool (Altschul *et al*. 1990) and EzBioCloud database (Yoon *et al*. 2017) were used to perform additional taxonomic classification. Identity was assigned to OTUs based on > 99% matches, however assigning bee microbiota taxonomy is highly dependent on training set used (Newton & Roeselers 2012), and with short sequence reads produced by sequencing (~300 bp), OTU identities should be considered putative.

From the 273 samples sequenced, 15 were removed from further analysis due to poor read counts (<1000), leaving 258 samples, including 222 experimental samples across the five treatments and negative sucrose control and 36 'parent' colony baseline samples. These were rarefied to an equal sequencing depth based on the lowest sequence count of 2,073 sequences in R using the VEGAN package (Dixon 2003). Following rarefication and filtering, a subset 525,916 sequence reads, representing 402 OTUs was used in subsequent analyses.

Before testing for the effects of clothianidin exposure on the worker gut community, any significant differences in worker gut microbiota between colonies prior to the experiment were assessed. To do this baseline samples collected from each 'parent' colony prior to the establishment of experimental micro-colonies. These samples showed that there

was no significant difference in OTU counts ($\chi^2 = 14.36$, $p = 0.19$, $n = 36$) or Fisher's alpha diversity between the colonies sampled ($\chi^2 = 13.42$, $p = 0.26$, $n = 36$).

7.2.7 Bacterial culturing and optical density measurements

In addition to the micro-colony exposure experiments, the direct effects of neonicotinoid on the growth of key bacterial species were also investigated. Freeze-dried cultures of *Bombus* gut bacterial isolates were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Snodgrassella alvi* (DSM 104735; Kwong & Moran 2013) was cultured in Trypticase Soy Broth (Thermo Fisher-Scientific), and three *Gilliamella* species characterised by Praet *et al.* (2017), including *Gilliamella bombi* (DSM 104030), *Gilliamella bombicola* (DSM 104085), and *Gilliamella intestini* (DSM 104029) were cultured in Tryptone Soya Broth (Oxoid). All isolates were cultured at 37°C for 2–3 days on a shaker.

Approximately 20 µl bacterial suspension was transferred in triplicate to a 96-well plate containing 180 µl of culture broth or 180 µl broth with 0.004–0.026 µg/ml clothianidin. Duplicate controls consisted of culture broth or broth with 0.004–0.026 µg/ml clothianidin. Plates were incubated at 37°C for 48 h, and optical density (OD) of bacterial cultures was measured using a BioTek Synergy HT plate reader at 600 nm every 12 h.

7.2.8 Statistical analysis

Rarefaction curves were used to confirm that MiSeq sequencing depth was sufficient to capture all diversity within the worker gut (Appendix 3), indicating that communities were completely, or near completely sampled and that read counts did not affect the number of OTUs detected.

Fisher's alpha (log series alpha) was calculated as a measure of bacterial diversity as it is independent of sample size (Magurran 2004). Normalised sequence data was converted to a Bray-Curtis dissimilarity matrix using PAST v.3.0.1 (Hammer *et al*. 2001) to investigate bacterial composition. In addition, univariate statistics and survival analyses were carried out in R (base version 3.3.2) with additional analyses from the R: Phyloseq package (McMurdie & Holmes 2013). Analysis of similarity (ANOSIM) and similarity of percentages (SIMPER) analysis were performed with PAST v3.08, with Bray-Curtis as the underpinning community similarity measure.

7.3 RESULTS

7.3.1 Worker mortality

Over the 10-day trial, 34 workers died before they could be sampled at their allocated time point (i.e. T_1, T_2, or T_3). The majority of these samples (41%) were from the highest dose treatment and occurred between 4–9 days of exposure. While by comparison, only the 0.004 µg/ml treatment had 100% survival rate over the whole trial, while survival was greatly reduced in the higher dose treatments. In particular, treatment 0.026 ug/ml saw survival decline to ~56% after 10 days, though these deaths occurred nearer the end of the experimental exposure period.

A Kaplan-Meier survival analysis estimated that the average survival times for each treatment group ranged from 80 hrs (0.026 ug/ml) to 116 hrs (0.004 ug/ml). Additionally, a log-rank test for between-group significance suggested that there were significant differences in survival between treatments over the 10-day exposure trial (χ^2 = 205, p < 0.0001, n_{events} = 2943).

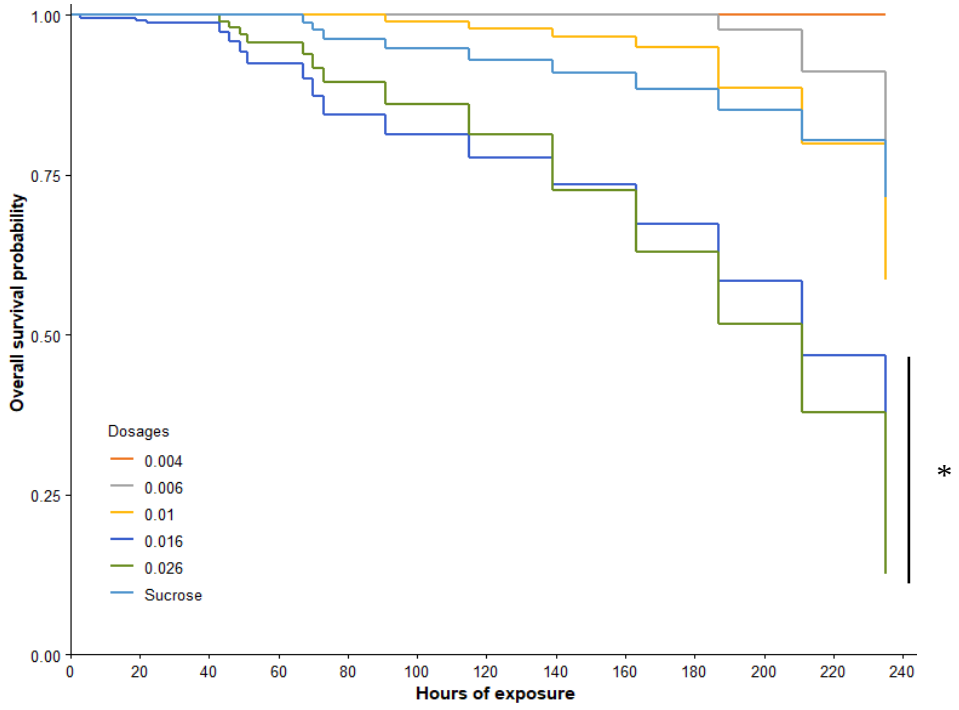

Figure 7.1 Kaplan-Meier survival analysis of worker *Bombus terrestris audax* fed differing pesticide doses over an exposure period of 240 hours. Asterisk highlights significantly differences at the p < 0.0001 level.

Worker traits, such as size, were measured to see if worker survival to clothianidin exposure was susceptible to physical attributes. There was a significant relationship between head width and survival ($F_{(2,269)} = 12.92$, $p < 0.001$, $n = 270$), where significantly smaller workers were more likely to expire before their T_1 ($p < 0.01$) and T_2 counterparts ($p = 0.02$). However, there was no significant difference in head width between workers at T_3.

7.3.2 Feeding rates

The amount of sucrose consumed per worker was estimated for each individual treatment pot with the assumption that bees in each treatment pot drank at the same rate. There was a significant difference in worker feeding rates between different doses ($F_{(1,5)} = 15.42$, $p < 0.001$, $n_{events} = 1562$), with workers in the negative control and low dose treatments (0.004 and 0.006 μg/ml) drinking more sucrose over the course of the experiment compared to workers in the higher clothianidin dose treatments (Appendix 14).

The total sucrose consumed at each time point was compared between treatments. There were no differences in sucrose consumption after 24 h of exposure ($\chi^2 = 10.48$, $p = 0.06$, $n = 90$), but an anti-feeding effect was observed after 96 h ($\chi^2 = 22.99$, $p < 0.001$, $n = 59$), with significantly higher consumption in the negative control ($p < 0.05$). After 240 h of exposure there was a significant difference in between the total sucrose consumed between treatments ($\chi^2 = 15.02$, $p = 0.01$, $n = 28$), with consumption significantly higher in the negative and low dose treatments ($p < 0.05$).

7.3.3 Common gut microbiota affected by clothianidin exposure

From all individuals sequenced ($n = 258$) 402 bacterial operational taxonomic units (OTUs) were identified. Baseline worker samples taken prior to dosing showed that eight common OTUs were present in >75% of samples (Table 7.1) and constituted approximately 95.14% of the total species richness of the gut community. On average 18.4 ± 4.5 OTUs (± 95% confidence interval) were detected per individual worker gut.

Across baseline samples and treatments, the most abundant OTU was a Betaproteobacterium (*Snodgrassella alvi*), while Gammmaproteobacteria were the most abundant class, which consisted of multiple *Gilliamella* OTUs. Relative abundance was consistent across treatment groups with little variation, except at the highest dose (0.026 ug/ml); both *Snodgrassella alvi* and *Gilliamella bombi* were significantly lower compared

to all other treatment groups (Fig 7.2; $\chi^2 = 21.63$, $p < 0.01$, $n = 258$, and $\chi^2 = 14.50$, $p = 0.02$, $n = 258$, respectively). In addition, baseline workers contained significantly less *Bombiscardovia coagulans* ($\chi^2 = 41.95$, $p < 0.0001$, $n = 258$) compared to negative control (sucrose) and low to medium dosages of clothianidin (0.004–0.016 ug/ml; $p < 0.05$). Baseline and sucrose-fed workers contained significantly more *Lactobacillus bombicola* than clothianidin-dosed bees ($\chi^2 = 45.39$, $p < 0.0001$, $n = 258$). Also, qPCR data indicated that overall there was no significant difference in community size between treatments, ($\chi^2 = 6.82$, $p = 0.34$, $n = 48$; Appendix 13).

Table 7.1 Common OTUs present in the worker gut with percentage confidence of taxa identification. OTU ID is used to distinguish taxa in further tables and graphs in this chapter.

Class	OTU ID	Family	Taxa	% Sample occupancy
β-proteobacteria	OTU7	Neisseriaceae	*Snodgrassella alvi* 97%	94.4%
γ-proteobacteria	OTU6	Orbaceae	*Gilliamella bombicola* 99%	93.6%
	OTU1899	Orbaceae	*Gilliamella bombi* 100%	93.6%
	OTU596	Orbaceae	*Gilliamella bombi* 97%	82.3%
	OTU954	Orbaceae	*Gilliamella bombi* 97%	78.2%
Bacilli	OTU376	Lactobacillaceae	*Lactobacillus apis* 99%	89.9%
	OTU379	Lactobacillaceae	*Lactobacillus bombicola* 100%	88.4%
Actinobacteria	OTU584	Bifidobacteriaceae	*Bombiscardovia coagulans* 98%	84.9%

7.3.4 Clothianidin doses and genus level dynamics

The gut communities present across replicates for each dosage were characterised to determine how gut microbiota responded to sublethal doses of clothianidin. As with the baseline samples, worker gut microbiota during the trial in both sucrose and clothianidin treatments was dominated by four main genera: *Gilliamella* (mean 45.75 ± 2.01%), *Snodgrassella* (mean 37.53 ± 1.77%), *Lactobacillus* (mean 10.72 ± 0.95%), and *Bombiscardovia* (mean 1.90 ± 0.29%). There was a significant difference in the numbers of OTUs detected between baseline, control, and dosed bees ($\chi^2 = 12.38$, $p < 0.01$, $n = 261$).

In addition, Fisher's alpha index of diversity highlighted significant differences in gut community between baseline, control, and dosed bees ($\chi^2 = 9.67$, $p < 0.01$ $n = 261$), with dosed bees containing more diverse OTUs. These differences in gut community diversity were present between the control and clothianidin dosages ($\chi^2 = 14.81$, $p < 0.05$, $n = 222$), as well as between clothianidin doses themselves ($\chi^2 = 10.56$, $p < 0.05$, $n = 187$).

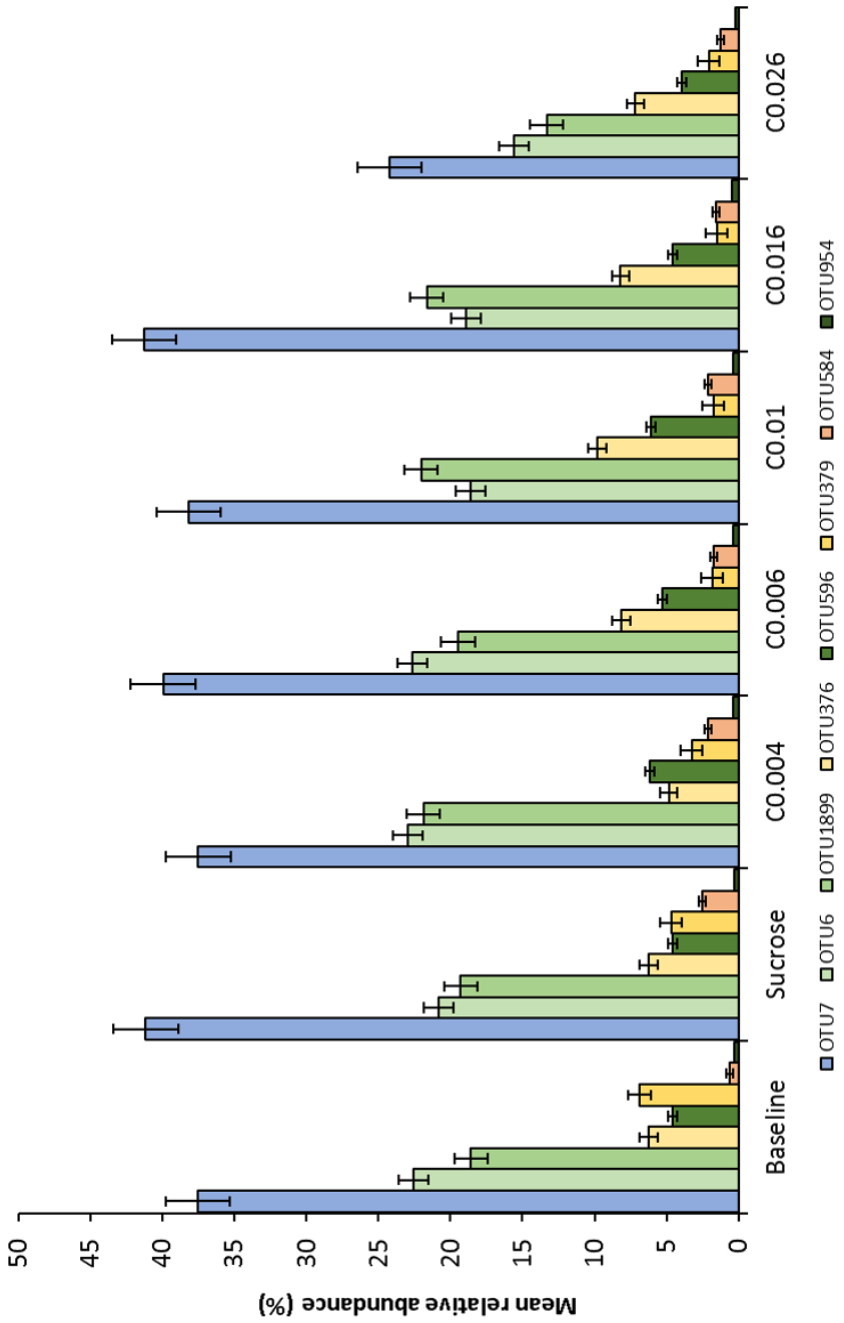

Figure 7.2 Mean relative abundance of the common bacterial OTUs present in workers across all treatments and time points. Bars shaded in blue belong to Betaproteobacteria, green are Gammaproteobacteria, yellow are Bacilli, and the OTU shaded in orange belongs to Actinobacteria. Error bars represent ± 1 standard deviation (SD).

A two-way repeated measures ANOVA highlighted a significant effect of time*treatment ($F_{(2,222)}$ = 2.592, p = 0.016, n = 223), but this was only detected at T_2 (χ^2 = 26.69, p < 0.0001, n = 74), where worker gut microbiota was much more diverse at the highest clothianidin concentration (p < 0.05). These analyses together highlight that the high clothianidin dose (0.026 µg/ml) resulted in workers with much more diverse microbiota than observed in all other treatments, likely skewing these data.

Pairwise Bray-Curtis dissimilarity values were calculated to characterise the distribution of microbiota between treatments. Analysis of similarity (ANOSIM; Table 7.2) tests highlighted significant differences between the high clothianidin dose and the other treatments. Similarity percentage (SIMPER; Table 7.3) analyses revealed that the dissimilarity appeared to be a result of changes in abundance of the major OTUs in the system. As clothianidin dosage increased, decreases were observed in *Snodgrassella alvi* (χ^2 = 21.02, p < 0.001, n = 222), *Gilliamella bombi* (χ^2 = 13.59, p = 0.02, n = 222), *Lactobacillus bombicola* (χ^2 = 16.36, p < 0.01, n = 222) and *Bombiscardovia coagulans* (χ^2 = 14.30, p = 0.14, n = 222).

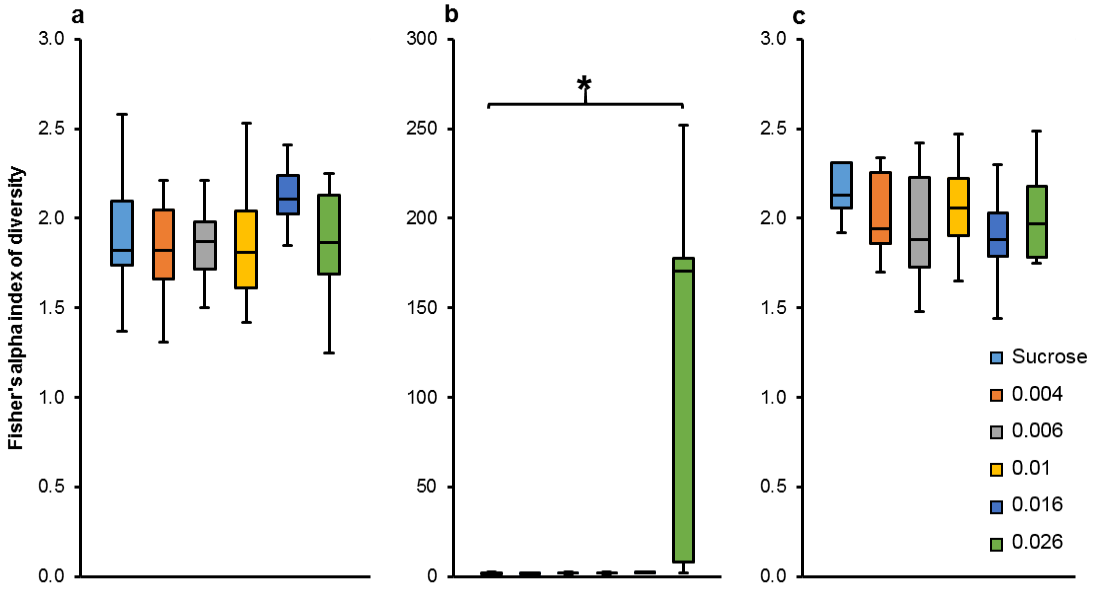

Figure 7.3 Alpha diversity of gut microbiota for each treatment (sucrose, 0.004, 0.006, 0.01, 0.016, and 0.026 µg/ml), between the three sample time points: (a) T_1 = 24 h, (b) T_2 = 96 h (*note scale*), (c) T_3 = 240 h. Asterisk denotes significance at the p < 0.05 level.

Table 7.2 Analysis of similarity (ANOSIM) of control and clothianidin-dosed workers. Values were generated using the Bray-Curtis measure of similarity, with the ANOSIM test statistic (R) shown below the diagonal with the probability (*p*) above. Those highlighted in bold font with an asterisk indicate significance ($p < 0.05$).

Table 7.3 Similarity of percentages (SIMPER) analysis of worker gut microbiota in control and clothianidin dosages. Given is the percentage mean abundance, with the overall average dissimilarity between samples =34.22%. N.B Only >1% contribution taxa are shown, and ASV identities are based on ~300 fragments and should be considered putative.

Table 7.2	Suc	C0.004	C0.006	C0.01	C0.016	C0.026
Suc	-	0.595	0.3822	**0.010***	0.0939	**0.000***
C0.004	-0.008	-	0.2842	0.0816	0.1641	**0.000***
C0.006	0.001	0.006	-	0.3039	0.417	**0.000***
C0.01	0.053	0.026	0.005	-	0.116	**0.000***
C0.016	0.021	0.019	0.000	0.020	-	**0.000***
C0.026	0.164	0.181	0.181	0.211	0.180	-

Table 7.3			Mean abundance %						Av. dis.	Cont. %	Cuml. %
Class	**Family**	**Taxon name**	**Suc**	**C0.004**	**C0.006**	**C0.01**	**C0.016**	**C0.026**			
Neisseriales	Neisseriaceae	*Snodgrassella alvi* 97%	41.20	37.50	40.00	38.20	41.30	24.30	7.39	21.59	21.59
Orbales	Orbaceae	*Gilliamella bombicola* 99%	20.80	23.00	22.60	18.60	18.90	15.60	7.22	21.10	42.70
Orbales	Orbaceae	*Gilliamella bombi* 100%	19.30	21.90	19.50	22.00	21.60	13.30	5.79	16.92	59.62
Lactobacillales	Lactobacillaceae	*Lactobacillus apis* 99%	6.24	4.84	8.15	9.78	8.19	7.16	3.37	9.86	69.48
Orbales	Orbaceae	*Gilliamella bombi* 97%	4.60	6.15	5.27	6.06	4.56	3.97	2.40	7.01	76.48
Lactobacillales	Lactobacillaceae	*Lactobacillus bombicola* 100%	4.67	3.23	1.80	1.72	1.51	2.05	1.67	4.86	81.34
Bifidobacteriales	Bifidobacteriaceae	*Bombiscardovia coagulans* 98%	2.50	2.14	1.74	2.10	1.53	1.26	1.07	3.12	84.47
Lactobacillales	Lactobacillaceae	*Lactobacillus bombi* 100%	0.18	0.43	0.29	0.78	1.40	1.48	0.57	1.65	86.12

7.3.5 Temporal dynamics of neonicotinoid exposure

Temporal diversity analyses indicated that the dissimilarity in gut communities was found in workers exposed to 0.026 µg/ml clothianidin for 96 h. Changes in relative abundance of the common taxa was tracked throughout the exposure at the high clothianidin dose (Fig. 7.4). After 96 h of exposure, there were declines in *Snodgrassella alvi*, *Gilliamella bombicola*, *Gilliamella bombi*, and *Lactobacillus apis*.

After 240 h of exposure, workers that had survived to be sampled had microbiota similar to that observed at the start of the experiment, albeit at lower relative abundances. Statistically significant declines were observed at T_2 in *Snodgrassella alvi* ($\chi^2 = 22.2$, $p < 0.001$, $n = 30$), *Gilliamella bombicola* ($\chi^2 = 10.19$, $p < 0.01$, $n = 30$), *Gilliamella bombi* (OTU1899; $\chi^2 = 9.69$, $p < 0.01$, $n = 30$), *Lactobacillus apis* ($\chi^2 = 11.07$, $p < 0.01$, $n = 30$) and *Lactobacillus bombicola* ($\chi^2 = 10.80$, $p < 0.01$, $n = 30$). In T_3 workers, by comparison, *Bombiscardovia coagulans* and *Gilliamella bombi* had higher relative abundances than T_1 or T_2 workers did ($\chi^2 = 8.96$, $p < 0.01$, $n = 30$; $\chi^2 = 7.97$, $p < 0.01$, $n = 30$, respectively).

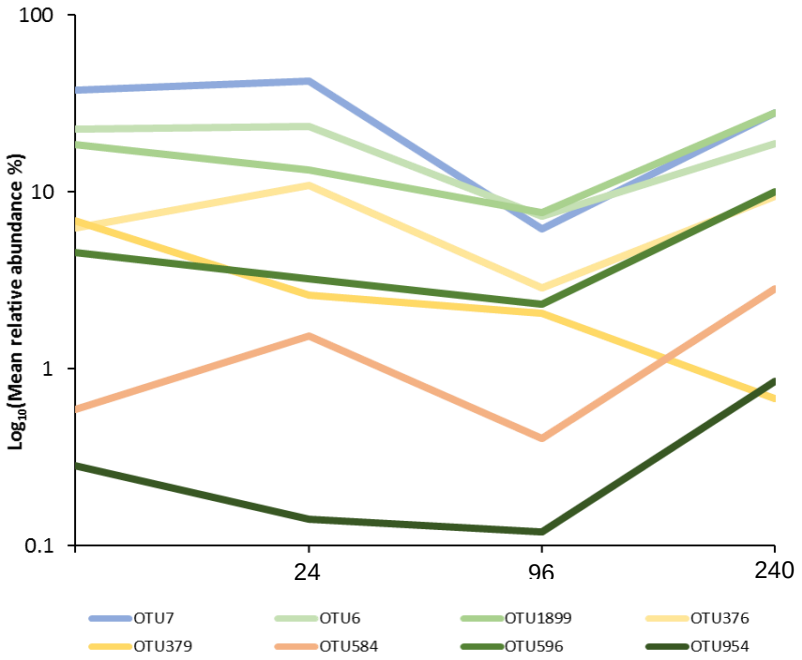

Figure 7.4 Average trends in relative abundance of the common OTUs present in workers during the 0.026 µg/ml clothianidin treatment.

7.3.6 Bacterial responses to clothianidin exposure

To explore how some of the most common gut taxa responded at the individual species level, strains of the core *Bombus terrestris* gut microbiota genera, *Gilliamella* and *Snodgrassella* were exposed to the same concentrations of clothianidin. Their optical density was measured to track growth rates over time (Fig. 7.5).

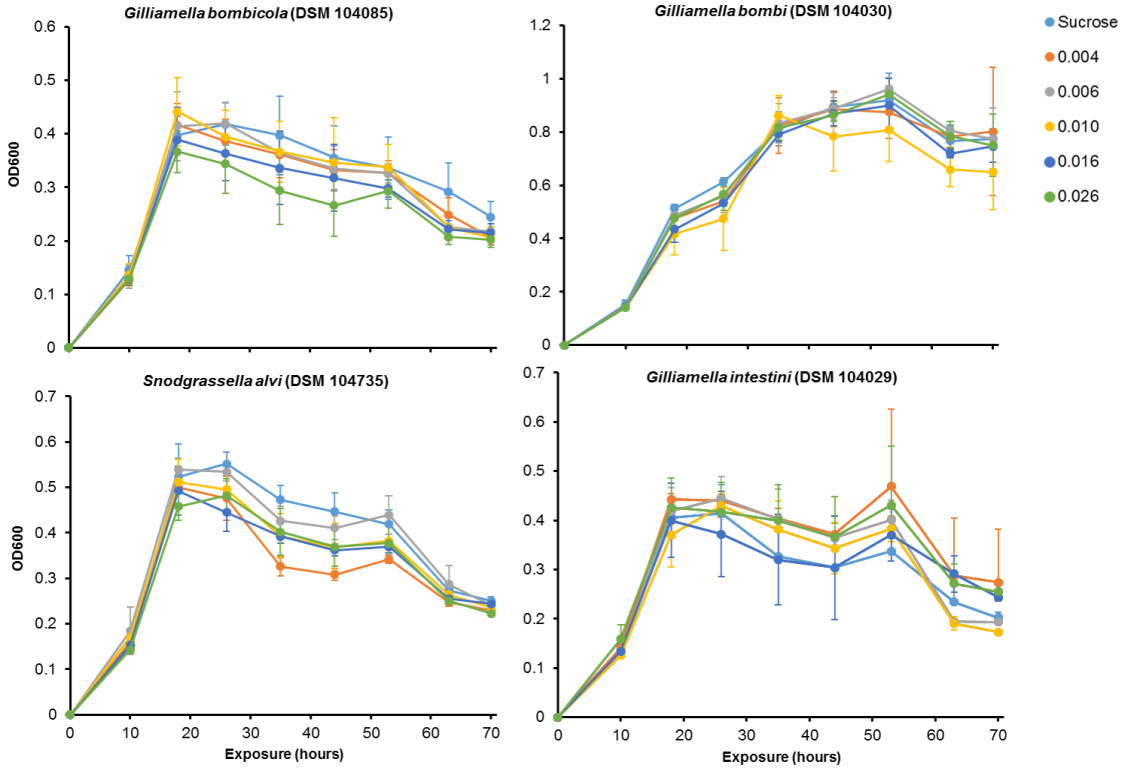

Figure 7.5 Bacterial growth (measured every 12 h) of key *B. terrestris* gut microbiota exposed to culture medium (sucrose), or culture medium spiked with doses of clothianidin.

Growth did not appear to be prevented in any the bacteria tested, though *Gilliamella bombicola* appeared to show a staggered effect between treatments, where higher dose treatments had lower optical densities. Overall, the highest optical density was observed in the negative culture broth control (Fig. 7.5a; $\chi^2 = 11.46$, $p = 0.04$, $n = 278$). The remaining bacteria show differing trends over time: *Snodgrassella alvi* reaches a peak and decreases, *Gilliamella bombi* still appears to be growing after 70 hours of exposure, and *Gilliamella intestini* grows to a peak, slowly declines then peaks again between 50–60 hours, and sharply declines again.

7.4 DISCUSSION

Throughout this 10-day trial, exposure to sub lethal concentrations of clothianidin had a significant effect on the worker gut microbiota, only at the highest dose (0.026 μg/ml = 26 ppb). This dose exceeds the upper limits of field estimates of clothianidin levels in nectar (0.17–20.8 ppb; Botías *et al.* 2015). In other studies, sub lethal exposure to neonicotinoids have been demonstrated to impact both the individual and colony level in *Bombus* species, including impairing reproduction, foraging efficiency, and colony health (Laycock *et al*. 2012; Whitehorn *et al*. 2012; Feltham *et al.* 2014; Gill & Raine 2014; Rundlöf *et al*. 2015; Baron *et al*. 2017). However, the resilience of the gut microbiota to all but the highest clothianidin concentration tends to suggest that in the field, typical exposure levels would have negligible impact on the worker gut community over similar exposure durations.

After 96 h of exposure to 0.026 μg/ml clothianidin, the relative abundance of common bacterial taxa decreased, opportunistic taxa colonised newly exposed niches, which resulted in more diverse gut communities. However, by the end of the trial, workers that survived the full 10-day exposure at the high clothianidin dose had a similar gut microbiota to that observed after 24 h of exposure. Although the common taxa were at lower relative abundances, they still constituted the majority of the community, suggesting partial 'recovery' of key taxa after the initial microbiota disturbance observed after 4 days.

Between days 4–10, there was also a significant decrease in the amount of dosed sucrose being consumed in the medium to high clothianidin treatments (0.01–0.026 μg/ml). This clear antifeedant effect (Thompson *et al*. 2014) may have resulted in a reduction in clothianidin exposure, thereby allowing it to be cleared by the bee, and without repeated consumption of the insecticide, the common taxa appeared to recover after a few days. This result could indicate a mechanistic interaction between feeding and exposure to clothianidin, which interferes with the regulation of the gut bacterial community. Previous studies have highlighted that exposure to neonicotinoids adversely impacts the immunocompetence of bumblebees with long lasting effects (Czerwinski & Sadd 2017), and challenges to the immune system can detrimentally alter the regulation of bacterial populations (Kwong *et al*. 2017). The disturbance in the gut community when exposed to

higher than typical clothianidin levels is likely to make *Bombus* workers more susceptible to pathogenic infection in non-sterile surroundings and warrants further investigation.

The results of this study suggest that neonicotinoid exposure can impact bumblebee microbiota, despite no such effect being found in honey bees (Raymann *et al*. 2018). One reason for this discrepancy may be due to differences in experimental design between this study and Raymann *et al.* (2018). Here, *B. terrestris* micro-colonies were exposed to clothianidin-dosed sucrose for a total of 10 days, in which a significant change in gut community was apparent by day 4 of the trial. By comparison, the total exposure period in Raymann *et al.* (2018) was just 3 days, with post-exposure samples taken 3 and 5 days after returning to their hive. It is possible that changes to microbiota only occur after extended periods of insecticide exposure, and that future long-term experiments are needed.

Additionally, there is extensive literature on the difference in response to neonicotinoids between honey bees and bumblebees, likely corresponding to differences in metabolism and dietary sensitivity. Individually, due to their size, bumblebee workers consume much more nectar than honey bees resulting in higher pesticide doses (Kessler et al., 2015). Honey bees are therefore less sensitive to neonicotinoids in their diet and appear to detoxify quicker than bumblebees (Cresswell *et al*. 2012, 2014). While, at the colony level, neonicotinoid exposure also appears more detrimental to bumblebees than honey bees Rundlöf *et al.* (2015). These results suggest that honey bees are an inadequate model for pollinator microbiota, and that pesticide research should incorporate more long-term studies on different wild bee species (Baron *et al*. 2017).

The adult bumblebee gut microbiota appears to show some resilience in response to field-realistic pesticide exposure and was only disturbed at the highest clothianidin dose. Moreover, the effect of neonicotinoid exposure was more apparent in the whole bacterial gut community than on core taxa grown in isolation. Raymann *et al.* (2018) detected metabolites of imidacloprid (nitrosoguanidine and guanidine) in whole *A. mellifera* bee gut cultures exposed to the pesticide. Potentially, there is some mechanistic interaction in the whole community in which neonicotinoids are metabolised into toxic compounds and detrimentally impacts the resident gut microbiota and warrants further study.

The findings in the worker bumblebee however do not mean that all members of the colony will respond in the same way (Chapters 3, 4). In *Drosophila melanogaster*, the

composition of larval microbiota was significantly affected by exposure to imidacloprid, while no change was detected in adults (Daisley *et al*. 2017). In *Bombus*, there are distinct differences in adult/larval *Bombus* gut microbiota, with larval microbiota less stable and more prone to opportunistic colonisation than mature adults (Chapters 3, 4). Additionally, larvae are more susceptible to pathogenic infections (Wilson-Rich *et al*. 2009; Forsgren 2010), so any potential disturbance (e.g. from insecticide exposure) is likely to be more damaging, which makes the stability and resilience of the larval gut microbiota highly important to host health (Osborne 2012).

The findings of this study suggest that more individual level, long-term data is required if we are to understand the impact of pesticides on bee gut microbiota and the associated health impacts. While there is some evidence of gut community disturbance at high concentrations, more work is needed to tease apart the mechanistic interactions between feeding, exposure, and the impacts on the bacterial taxa themselves.

CHAPTER 8:

GENERAL DISCUSSION

The overall objective of this research project was to investigate the ecology of bumblebee gut microbiota in the model organism *Bombus terrestris* (ssp. *audax*). I used manipulative experiments in the field and lab combined with molecular screening to explore how the specialised bacterial communities varied throughout the host's lifetime, between castes (Chapter 3), and in response to changes in the foraging environment (Chapter 4). A key phase of the *Bombus terrestris* lifecycle was explored in detail and revealed that key gut taxa are retained by the queen during diapause (Chapter 5) with potentially important impacts for the next colony cycle. The next chapter studied how colonies and their associated gut communities respond and develop when the host is fed different pollen diets (Chapter 6), before exploring how pesticide exposure could impact the worker gut microbiota (Chapter 7).

8.1 KEY FINDINGS

8.1.1 Gut microbiota differs between castes and during development

Life history traits differ greatly between members of the same bumblebee colony. Workers, the most abundant caste, perform numerous tasks, such as caring for the larvae and foraging, and rarely reproduce. This contrasts with males who spend their early life at rest, performing some brood care (Cameron 1985) or consuming food before permanently leaving the nest and attempting to mate. Future queens receive additional nutrients compared to workers and, after mating and establishing a colony, do not leave the nest to forage, instead devoting themselves to egg production. Despite these differences, research investigating the gut microbial diversity in social bees has largely ignored how caste, phenotypic and behavioural differences affect the structure of host microbiota (Kapheim *et al*. 2015; Tarpy *et al*. 2015).

My findings show overwhelmingly that caste and developmental stage strongly influence the composition of the gut microbiota. During worker development, gut microbiota exhibited subtle changes in diversity and composition from eggs to larval instars, through pupation and to adult emergence. No research prior to this has investigated bacterial communities from all developmental stages. The proposed model of microbial assembly in Chapter 3 is the first of its kind to provide a holistic view throughout the complete *B. terrestris* lifecycle. It characterised that through worker development, the gut microbiota gradually shifts from a diverse community to a more conserved set of common taxa after adult worker emergence.

Typically, diversity and composition did not change drastically between larval instars, contrary to what has been published for the honey bee (Vojvodic *et al*. 2013). This is likely a result of dietary and behavioural differences between honey bees and bumblebees. *Apis mellifera* larvae receive nutrition from nurse bees via trophallaxis, which in early instars is comprised of royal jelly. Later in development this diet is supplemented with honey, nectar and pollen (Beetsma 1985). The antiseptic properties of royal jelly appear to result in a depauperate early instar gut (García *et al*. 2010), while other food stores (particularly pollen) can act as a source of bacterial inoculum (Vojvodic *et al*. 2013). In bumblebees, however, all larvae are fed a liquid mixture of pollen and honey regardless of age (Sladen 1912; Michener 1974), so there is no 'microbial filtering' of the larval food. As a result, this specific bacterial colonisation pattern is not exhibited in bumblebees and could suggest that microbial succession is less important in bumblebee larvae compared to honey bees.

Bumblebee larval instars tended to retain the same core taxa through development, including some that were rarely found in adults. These taxa may be uniquely adapted to the morphology of the larval gut and its diet, and have important functional roles not required in adults (Parmentier *et al*. 2018). In addition, worker and male gut microbiota were also less diverse and more stable than those of larvae (Chapter 3). Temporal sampling showed that environmental perturbation affected adult and larval gut microbiota differently, demonstrating greater variability of larval microbiota compared to those of mature castes (Chapter 4). This adult/larval distinction was only recently suggested by Parmentier *et al.* (2018) after a survey of a single wild *B. pascuorum* nest. Chapters 3 and 4 have more

robustly demonstrated this dichotomous effect through repeated destructive sampling of multiple *B. terrestris* colonies using a more rigorous sampling regime and tested colonies as they developed in different environments.

Clearly my work demonstrates that testing the worker gut microbiota is not sufficient for modelling impacts of the gut microbiota on the whole colony. Differences in life history traits between individuals within the same colony or between species can influence the gut microbiota and are not representative of all bees. Further research, therefore, is needed into the gut microbiota of less studied constituents such as the larvae and queens. For instance, it would be interesting to look at how pesticide exposure impacts the larval microbiota and whether they become more susceptible to opportunistic colonisation from pathogens.

8.1.2 Maternal influence on gut microbiota

Queens are responsible for overall colony health and productivity (Rangel *et al*. 2016), so understanding the composition and effectiveness of their gut microbiota is critical to their health, and ensuring the productivity of the colony. In honey bees, the queen is not thought to influence the gut microbiota of her daughters, chiefly because the core worker microbiota is much more diverse than that of the queen (Tarpy *et al*. 2015). My work on bumblebee queens and colony development, however, challenges whether this viewpoint holds across all bee species. In bumblebees, the queen gut community is much more diverse than that of her daughter workers and contains the same characteristic core species (Chapters 3–5).

When bumblebee queens emerge in spring, the microbiota they retain through diapause, in addition to what is gained from foraging as she establishes her new colony, will likely be the primary source of microbiota for her colony (Chapter 5; Koch *et al.* 2013). It is likely that gut microbiota from the founding queen is present within the nest materials, pollen and nectar stores (Anderson *et al*. 2013). Here they would act as a source of inoculum for the first-generation workers, although the potential role of vertical transmission should not be discounted (Section 8.3.2).

By comparison to honey bees, the annual colony cycle of bumblebees puts the population and gut microbiota through a bottleneck (Kwong *et al*. 2014), suggesting that

maternal transmission of beneficial symbionts is highly important for successfully inoculating the first generation of workers. If they do not possess all the necessary bacteria (e.g. protective *Gilliamella*, *Snodgrassella* and *Lactobacillus* spp.) then the colony may be weaker when out foraging (Koch & Schmid-Hempel 2011b; Kwong *et al*. 2017).

In laboratory-reared colonies on sterile diets, a subset of wild gut microbiota are present despite restrictions to horizontal transmission from the environment (Meeus *et al*. 2015). Similarly, indoor-reared bees had the same typical microbiota composition regardless of the irradiated diet they were fed (Chapter 6). Colonies were in near-sterile surroundings, and the main route for transmission and acquisition of bacteria would have been from shared food resources and/or faeces from the queen as well as the unrelated helper worker (newly emerged worker with immature gut microbiota; Chapter 3). Nevertheless, 100% of workers sampled in the trial contained the three core bacteria, *Gilliamella*, *Snodgrassella*, and *Lactobacillus*, despite the reduced opportunities for horizontal transmission and sterile diets.

The presence of certain bacterial taxa within the queen gut also appears to play a role in colony health. *Lactobacillus* spp. were only detected in post-diapause queens that were producing healthy colonies, while queens that did not lay eggs appeared to have very species-poor microbiota dominated by just a handful of genera (Chapter 5).

Clearly these results indicate that in a closed setting the bumblebee queen gut microbiota is likely to be a large bacterial source to inoculate her daughter workers. Therefore, both her health (and microbial health) is important to sustain the colony. However, the mechanisms by which the queen microbiota influences her offspring remain unclear, and further investigation would be valuable. Isolation experiments in which isolated larvae are reared in a clean environment without contact with the nest could provide important information for what is potentially passed on from the maternal queen to her offspring.

8.1.3 Persistence of core bacterial species in response to perturbation

It is well established that like honey bees, bumblebees have a consistent, simple gut microbiota, largely comprised of *Gilliamella*, *Snodgrassella*, *Lactobacillus*, and often *Bombiscardovia* and *Schmidhempelia* species (Mohr & Tebbe 2006; Cox-Foster *et al*.

2007; Martinson *et al*.2011; Engel *et al*. 2012; Moran *et al*. 2012; Engel & Moran 2013a). The establishment and persistence of these beneficial taxa is highly important for health and survival (Koch & Schmid-Hempel 2011b), yet few studies have investigated bacterial populations over time or in response to perturbation from changes in habitat, nutrition, or exposure to pesticides.

Common gut bacterial taxa are detected throughout bumblebee worker development, including in pupal stages when the bee goes through metamorphosis (Chapter 3). Initially pupation was thought to severely disrupt or even eliminate the bacterial population (Moll *et al*. 2001; Hakim *et al*. 2010) due to the production of antimicrobial peptides (Russell & Dunn 1996). However, both common genera, *Gilliamella* and *Snodgrassella* were found at low levels in pupae and survived metamorphosis to fully dominate the adult worker gut. These taxa remain relatively stable throughout the foraging season in workers, but gut microbiota appears to fluctuate more in larvae (Chapter 4).

All core bacterial species were present in bumblebee queens and workers, regardless of dietary changes. In queens undergoing extreme starvation during diapause, the common microbiota species were also able to survive, though the bacterial population itself declined (Chapter 5). In workers on different sterile diets common taxa were dominant, though it also appeared that nutritional aspects could affect the relative abundance of certain genera (Chapter 6).

In a system such as the bee gut, there is also a great need to investigate the functional response diversity to perturbation, i.e. how species that perform a similar function respond to the same stimuli. During diapause in the queen gut, fermentative species appeared to outcompete each other for nutrients, with certain species tending to be outcompeted by others (Chapter 5). Similarly, in workers exposed to clothianidin, *Gilliamella* species differed in response, with some declining dramatically, and others seemed relatively unaffected (Chapter 7). Here, the gut community appeared severely restricted by pesticide exposure, though once workers stopped feeding the sequenced gut community resembled that of near-normal levels. This suggests a mechanism of bacterial recovery when conditions in the gut are more favourable.

The presence of core taxa that are able to persist in the face of challenges such as starvation during queen diapause, gut remodelling during pupation, and exposure to field-

realistic pesticide concentrations suggests that they are highly adapted to the ecology of their hosts and resilient to extreme perturbation. Understanding the mechanisms that confer this bacterial resilience may help to inform effective strategies (dietary, probiotic, drug-based, etc.) for returning microbial systems back to their healthy states. Positive or negative feedback loops that respond to alterations within the gut population could induce changes to the gut environment/the host making it more tolerable. For instance, when faced with starvation challenges, microbial metabolites produced by the microbiota could induce changes to host pathways controlling gut retention, thereby keeping food in the gut for longer (Lozupone *et al*. 2012).

8.1.4 Environmental influences on the gut microbiota

Existing studies on honey bees and bumblebees have identified stable, core populations of microbiota, generally unaffected by geographic location (Kwong & Moran 2016). Exposure to the environment however, has been shown to affect the non-core gut community of workers (Chapters 3, 4; Newbold *et al.* 2015). This rare, non-core community represents only a small portion of the worker microbiota and though changes to it do not alter overall diversity they could still potentially bring about functional changes.

Other colony members however, such as queens and larvae, may (at certain times in development) be more susceptible to environmental bacteria than has been observed in workers. Larval microbiota, for instance, was found to be more responsive to environmental cues than in adults (Chapters 3, 4). This again reiterates that not only do gut communities differ between adults and larvae but that they also respond differently to environmental stimuli (Chapter 4).

It is likely that the larval diet as well as their gut structure makes the microbiota more susceptible to seasonal trends in floral resources. They feed on pollen, which is stored in their simplistic gut until after pupation. Compared to the carbohydrate-rich worker diet largely consisting of acidophilic nectar, pollen is a more suitable growth medium for bacterial colonisation (Manirajan *et al*. 2016). As workers forage in the environment, potential habitat changes, either increasing or decreasing floral resources, could impact the spread, diversity, or survival of bacteria at flowers and thereby impact the feeding larvae back in the colony (Anderson *et al*. 2013). Bacterial diversity in foraged food however,

does not appear to influence the core gut microbiota of workers. It is likely that the established adult gut microbiota buffers against colonisation by new taxa (Koch & Schmid-Hempel 2011b), making them less prone to perturbation than developing larvae.

The gut microbiota of wild post-diapause bumblebee queens also appeared to show strong environmental influence, with wild post-diapause queen microbiota separating based on the area in which they were foraging/searching for nesting sites (Chapter 5). It is likely that the decrease in the bacterial population during diapause predisposed queen guts to environmental bacterial colonisation. With what is known about the stability of the adult gut (Chapter 4), the core gut microbial community of the queen established after diapause may be largely unchanged for the remainder of her life. This implies the importance of environmental quality encountered by the queen during this crucial stage.

Flowers can be a source for this 'new' bacterial community, acting as bacterial reservoirs for insect visitors (Moran *et al*. 2012). Along with the diversity of plant species, the environment that bumblebees interact with also contains a diversity of nutritional sources. Any changes to this habitat can severely disrupt or influence the foraging environment and in turn impact the nutritional quality of pollen brought back to the nest (Vaudo *et al*. 2015; 2016). The interaction between nutritional sources and floral bacterial reservoirs may also be influencing the bumblebee gut. Sterilised pollen diet treatments can impact the gut community and upregulate certain taxa (Chapter 6). While core bacteria were present in all diet treatments, those containing Brassicaceae, Fabaceae and Rosaceae plant families had more productive colonies on average, and upregulation of *Lactobacillus* species.

Not only are foragers exposed to potentially pathogenic or beneficial bacteria when foraging, but also pesticides, which are increasingly found as environmental residues (Woodcock *et al*. 2018). Key microbiota in bumblebee workers were again found to be resilient to this perturbation in all but the highest dose, suggesting that field-realistic exposure to neonicotinoids when foraging is unlikely to impact the worker gut microbiota (Chapter 7). However, there is still an abundance of literature to show that exposure to pesticides causes detrimental damage to honey bees and bumblebees regardless of whether this is via changes to the gut microbiota or not (Goulson 2013b).

Still as previously explored, studies on workers may not be representative of the whole colony. Queens foraging after diapause with a depauperate gut community may be adversely affected as well as larvae, which have a much more responsive gut community to external stimuli and reduced immune function (Wilson-Rich *et al*. 2009; Forsgren *et al*. 2010) so may be highly impacted by pesticide exposure.

8.2 LIMITATIONS

Though every effort was made to optimise my experimental procedure where possible, there are certain aspects of my methodology that, although I believe were justified, should be discussed for their potential to impact the conclusions drawn. In particular, the bioinformatic and data analysis approaches that I used highlight the many issues faced by researchers when dealing with sequencing data – simply, there is no one size fits all method. For this reason, I explored different methods to separate my data, for instance with taxonomic grouping (see Sections 2.8.3 and 2.8.4).

In Chapters 3, 4 and 7 sequences were grouped into OTUs by a fixed similarity threshold (≥97%), which helps to avoid over-estimating biodiversity when there are few accurate reference sequences available (Kunin *et al*. 2010). This is often the case in *Bombus* microbiota research, but can result in a lack of sensitivity, and increases the likelihood of omitting functional diversity (Patin *et al*. 2013).

When using this grouping method, I found multiple OTUs that were identified to the same taxa, particularly within the *Gilliamella* genus. These are known to exhibit functional strain diversity, suggesting that the OTU method did identify some functional diversity. I compared this with an alternative method in Chapters 5 and 6 (ASV method), but whether it uncovered more functional diversity, or indeed more bacterial diversity is difficult to determine. The sequencing data generated in these chapters was of lower quality and during processing taxa could only be determined to genus level. These datasets were also smaller, but this is likely due to being mostly adult castes, or individuals reared in restrictive indoor environments, resulting in a lack of microbial diversity. However, both methods provided similar snap shots of the conserved bumblebee gut community, suggesting that they portray an accurate representation of the host gut that is in agreement with the wider literature.

Another potential issue arising from the methodology concerns rarefaction of sequencing data. In microbial ecology it is standard in most major data analysis toolkits, but is often criticized for eliminating usable data, as well as reducing sensitivity and statistical power (McMurdie and Holmes 2014; Weiss *et al.* 2017).

I rarefied my data to the lowest read count to normalize library sizes an allow for fair comparisons between samples, while enough redundancy was built into the experimental design to compensate for the data removed. There is, however, difficulty arising when rarefying differing sample types, for instance for comparisons between larvae, workers, and reproductive castes. It is possible that to compare between groups, rarefying obscured some differences between samples by removing data from larger libraries – considering that absolute abundance of 16S rRNA copies may differ between castes/stages.

To verify this, the analysis in Chapter 3 was conducted with and without rarefaction initially before inclusion in this thesis. Without rarefaction OTU counts were much higher (2,140 compared to 789) and more "common" taxa were identified. However, the community dynamics and pathway of microbial succession throughout development over time was concurrent – regardless of the method used. This is possibly because of the restricted microbial community in the bumblebee gut – as most bacterial taxa are rare; the most abundant taxa are present regardless of any potential data loss through rarefaction.

While I am confident that these limitations did not significantly alter my conclusions, future analyses should look more closely at the processing of post-sequencing data. In this instance, and in the wider current literature, no single approach has proven more reliable than others in 16S rRNA metagenomics. Each have their advantages and disadvantages based on the dataset being used, sequencing quality and the region targeted for amplification.

8.3 DIRECTIONS FOR FUTURE RESEARCH

8.3.1 Probiotic potential of Lactobacillus species

The composition and effectiveness of the microbiota is critical to host health, and relevant to both wild and managed bumblebee populations. The microbiota of commercially-reared bumblebees is a subset of that found in wild colonies, consisting chiefly of core taxa

(Meeus *et al*. 2015). Though these taxa are able to adapt to new environmental challenges (Chapters 3, 4; Newbold *et al.* 2015), it is unclear if this community alone is sufficient to provide protection against viral or parasite infections when pollinating indoor/outdoor crops.

As highlighted by work in Chapters 5 and 6, the detection of *Lactobacillus* spp. was positively correlated with nesting and egg-laying behaviours in post-diapause queens and high colony yields. *Lactobacillus* spp. are already known for probiotic benefits to health and colony performance in *A. mellifera* (Evans & Lopez 2004; Audisio & Benítez-Ahrendts 2011; Pătruică & Mot 2012), but diet supplementation is not currently the standard practice for bumblebee rearing. From a commercial perspective, supplementing the diet of reared bumblebees with *Lactobacillus* is affordable and practical, but still the implications of this for colony productivity is unknown. It would be useful to design and test assays using different potential probiotics, and different modes of delivery (in pollen or nectar, or topical exposure, etc.) that may be used by bee breeders and researchers to select for species and strains that benefit the host.

8.3.2 Vertical transmission of gut microbiota

In many animals, symbionts are often transmitted to offspring vertically through the female germ line (Bright & Bulgheresi 2010), but in honey bees and bumblebees, the potential contribution of vertical transmission is often overlooked.

In Chapter 3, I detected key bacterial taxa in surface sterilised eggs, which remained in the larval and pupal gut at low levels throughout development. The presence of these core bacteria at such an early developmental stage, and prior to feeding, could suggest that they are maternally inherited from the queen through egg laying. Both *Gilliamella* and *Snodgrassella* spp. have been detected in queen ovaries (Billiet 2016), making the transfer of symbionts to the developing egg likely. However, further research will be required to verify this and determine the underlying mechanisms of transmission. It would be useful to apply techniques such as real time quantitative PCR (RT-qPCR) to measure transcription rates in bacterial cells and combine this with fluorescence microscopy and transmission electron microscopy to detect and visualise live bacterial cells and track movement between queen ovaries and the developing eggs.

8.3.3 Studies on bumblebees and other wild bees

The worker honey bee is often used as an emerging model organism for the study of host gut microbiota. Its microbiota is simple and well documented, colonies can be easily managed in the lab, and they exhibit social behaviours, allowing comparisons to more complex organisms (Koch *et al*. 2013; Kwong *et al*. 2014; Pernice *et al*. 2014). However, the value of a model organism lies in its representation of a wide range of species (Leonelli & Ankeny 2013). Yet, between honey bees and bumblebees, there are already distinct life history differences that appear to influence the host and its gut microbiota. My results clearly indicate the importance of researching gut microbiota in other bee species, and future work should consider expanding this field to wild bees, to monitor how environmental changes can impact gut communities of natural populations and influence host health. This could be achieved through gut microbiota surveys of wild bee populations in combination with land use and habitat change data to model how gut microbiota of species may be shaped by environmental quality.

8.3.4 Determining functions of gut microbiota

The next step in understanding the gut microbiome and its influence on the host is to determine the functions of key taxa. In the honey bee we already have a lot of data on core bacterial functions (Section 1.6), and while it is assumed that these roles are the same for bumblebees, this has yet to be determined. Even strain levels differences in bacterial species can change function (Engel *et al*. 2012; Sabree *et al*. 2012; Kwong *et al*. 2014) and with host-specific taxa, such as the *Gilliamella* spp. we have yet to determine their function in the bumblebee gut. In Chapter 7, there were sufficient differences in my findings of the impacts of neonicotinoid insecticides on bumblebee gut microbiota species, suggesting that even bacteria of the same genera may be performing divergent functions, or metabolising different compounds.

Metagenomic analysis is vital to further our understanding on this communities. Diversity itself does not provide complete information on the changes in physiological functions with the host, though functional changes can be inferred from changes to the overall gut microbial community. What is required is a hologenomic approach, examining the host genome and its gut metagenome to decipher functional changes from changes to

the gut microbial community. The hologenome theory proposed by Zilber-Rosenberg & Rosenberg (2008) argues for incorporating both the host genome and microbiome (i.e. the holobiont) into evolutionary analyses, since natural selection will act upon both. Investigations of the bumblebee holobiont, therefore, could use comparative genomic analyses to explore of the role of host-associated microbiome in the evolution of specialised diets, or response to pathogens (Schwarz *et al*. 2015).

8.3.5 Pesticides and the gut microbiota

Further work on pesticides and the gut microbiota of important pollinator species is needed. Neonicotinoids were shown to have no effect on the gut microbiota of honey bees, and while there are methodological considerations between my study and that of Raymann *et al.* (2018), which may suggest that the two species are not comparable (Section 7.4). Reaching a consensus in this area of research is crucial to understanding the implications of land management practices, such as pesticide use. While they as yet do not appear to significantly impact the gut microbiota of honey bees, this result in *B. terrestris* indicates that there are many other wild pollinator species that could respond differently, and therefore using honey bees as a model for all pollinators is inaccurate.

The effect of neonicotinoid exposure was more pronounced in the bumblebee worker microbiota than on growth rates of core bumblebee taxa grown in isolation. Future work should investigate whether this result is caused by a mechanism in which bacteria are directly impacted by the pesticide (i.e. they metabolise it into toxic products), or if impacts on the host compound and then impact the gut community. It would be beneficial to investigate the functional roles of the bumblebee bacterial taxa to help elucidate if exposure to the pesticide is directly affecting the bacteria, or if it affects the host and the result impacts the gut community.

8.3 CONCLUSIONS AND RECOMMENDATIONS

Bumblebees, along with other insect pollinators, are critically important for the maintenance of plant reproduction in natural and managed systems, so maintaining host health is of great importance. An essential part of harnessing the beneficial capability of the gut microbiota, lies in understanding how the core community develops with the host and

adapts to new environments and challenges. My research has highlighted methodological considerations for future research, including demonstrating that honey bees are not a good model for all bee species, as they can differ considerably in key areas of life history, larval development, and responses to perturbation. More work should be conducted on other pollinators providing this key environmental service, especially in wild populations, to monitor the impacts of multiple stressors such as land use change and pesticide exposure.

In addition, I have emphasised that studies solely based on workers may not be a sufficient indicator for the 'microbial health' of the whole colony. Other castes and developmental stages play highly integral roles in the life of a colony, so more work should focus on identifying the diversity and functions of their gut community.

As a key agricultural and commercial pollinator, bumblebee health is a major concern both ecologically and economically. Future avenues of research should target improving our understanding of the factors that shape and influence host gut microbiota, which may offer solutions for improving pollinator health. Researchers and commercial bumblebee suppliers would also benefit from exploring the potential probiotic applications of *Lactobacillus* species as food supplements to promote healthy, sustainable bumblebee populations in the future.

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APPENDIX 1

IDENTIFYING CASTE AND DEVELOPMENTAL STAGES (CHAPTERS 3, 4)

Descriptions for identifying the caste and developmental stage categories examined in Chapters 3 and 4 based on (Alford, 1975). Images provided courtesy of William Harvey©.

Developing stage			Description
Egg			Pearly white in colour and sausage-shaped.
Larva	*1st Instar*		Larger than eggs. Clumped within brood cell.
	2nd Instar		Slight larger. Still clumped together within brood cells.
	3rd Instar		Mandibles start to become visible.
	4th Instar		Larvae upright in individual brood cell.
Pupa			Soft and adult-like in form, although wings are not yet expanded. Tissues may be coloured white to dark grey.

Caste stage	Description
Newly Emerged Worker	Adult female fully emerged from its brood cell. Hair is pale/silvery in colour and wings are crumpled and soft.
Worker	Fully formed adult female. Hair has darkened with wings hardened in position.
Newly Emerged Male	Adult male fully emerged from its brood cell. Hair is pale/silvery in colour and wings are crumpled and soft.
Male	Fully formed adult male. Hair has darkened with wings hardened in position.
New Queen	Very large adult female. Hair colouration and wings in much better condition than original colony queen.
Queen	Very large adult female. Distinguished from new queen by wing wear and hair colour.

APPENDIX 2

OPTIMISED BEE GUT DNA EXTRACTION PROTOCOL (CHAPTERS 3, 4)

This protocol uses items from both PowerMag® Microbiome and PowerSoil®-htp kits from Mobio Laboratories, USA (now Qiagen).

Before beginning, warm the PowerMag® Microbiome Lysis Solution at 60°C for 15–20 minutes before starting to dissolve any precipitates. Then, add β-mercaptoethanol (β-ME) at a ratio of 25 µl per 1 ml of the PowerMag® Microbiome Lysis Solution.

1. Remove the Square Well Mat from the PowerSoil®-htp Bead Plate and add samples. Then add 650 µl of warmed PowerMag® Microbiome Lysis Solution/β-ME, followed by 2.5 µl of proteinase-K (20 mg/ml) to each well of the PowerSoil®-htp Bead Plate.
2. Secure a new Bead Plate Sealing Mat tightly to the PowerSoil®-htp Bead Plate and vortex horizontally for 5 seconds to ensure that sample/solutions are mixed.
3. Add 60 µl of Solution C1. Secure the Square Well Mat tightly to the PowerSoil®-htp Bead Plate and place on a 96 Well Plate Shaker. Shake at speed 20 for 10 minutes, remove plates and re-orient them so that the side closest to the machine body is now furthest from the machine body and shake again at speed 20 for 10 minutes.
4. Centrifuge at room temperature for 6 minutes at 4500 x *g*, then remove the Bead Plate from the centrifuge. Carefully and without splashing remove and discard the Square Well Mat and transfer the supernatant to a clean 1 ml Collection Plate.
5. Add 250 µl of Solution C2 to each well and apply Sealing Tape to 1 ml Collection Plate. Vortex for 5 seconds and incubate at 4°C for 10 minutes. Centrifuge the 1 ml Collection Plate at room temperature for 6 minutes at 4500 x *g*. Remove and discard Sealing Tape.
6. Avoiding the pellet, transfer entire volume of supernatant to a new 1 ml Collection Plate. Apply new Sealing Tape to the 1 ml Collection Plate and centrifuge at room temperature for 6 minutes at 4500 x *g*. Transfer entire volume of supernatant to a new 1 ml Collection Plate.

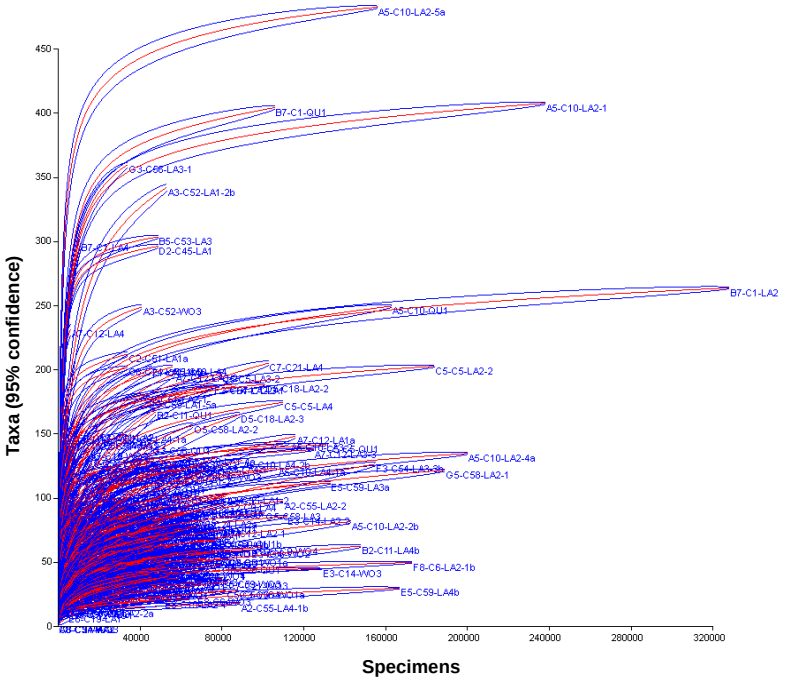
7. Add 200 µl of Solution C3 and apply Sealing Tape to the 1 ml Collection Plate. Vortex for 5 seconds and incubate at 4°C for 10 minutes. Centrifuge at room temperature for 6 minutes at 4500 x *g*. Remove and discard Sealing Tape.
8. Avoiding the pellet, transfer entire volume of supernatant to a new 1 ml Collection Plate. Apply Sealing Tape to 1 ml Collection Plate. Centrifuge the 1 ml Collection Plate at room temperature for 6 minutes at 4500 x *g*.
9. Transfer no more than 650 µl of supernatant to a 2 ml Collection Plate avoiding any residual pellet, then add 1300 µl of Solution C4 to each well of the 2 ml Collection Plate and pipet samples "up and down" to mix.
10. Load approximately 650 µl into each well of the Spin Plate and apply Centrifuge Tape then centrifuge at room temperature for 3 minutes at 4500 x *g*. Discard the flow through and place the Spin Plate back on the same 0.5 ml Collection Plate. Discard the Centrifuge Tape. Repeat until all the supernatant has been processed and then discard the final flow through.
11. Place the Spin Plate back on the same 0.5 ml Collection Plate. Add 500 µl of Solution C5-D (containing ethanol) to each well of the Spin Plate. Apply Centrifuge Tape to the Spin Plate then centrifuge at room temperature for 3 minutes at 4500 x *g*. Discard the flow through and place the same 0.5 ml Collection Plate beneath the Spin Plate. Centrifuge again at room temperature for 5 minutes at 4500 x *g*. Discard the flow through to remove residual ethanol.
12. Carefully place the Spin Plate onto a Microplate. Remove Centrifuge Tape and discard and allow to air dry for 10–20 minutes at room temperature. (Check that plate in dry by inverting and tapping on a paper towel) then add 100 µl of Solution C6 to the centre of each well of the Spin Plate. Apply Centrifuge Tape.
13. Centrifuge at room temperature for 3 minutes at 4500 x *g*. Remove Centrifuge Tape and discard. Cover wells of the Microplate with the Elution Sealing Mar provided. DNA is now ready for any downstream application.

APPENDIX 3

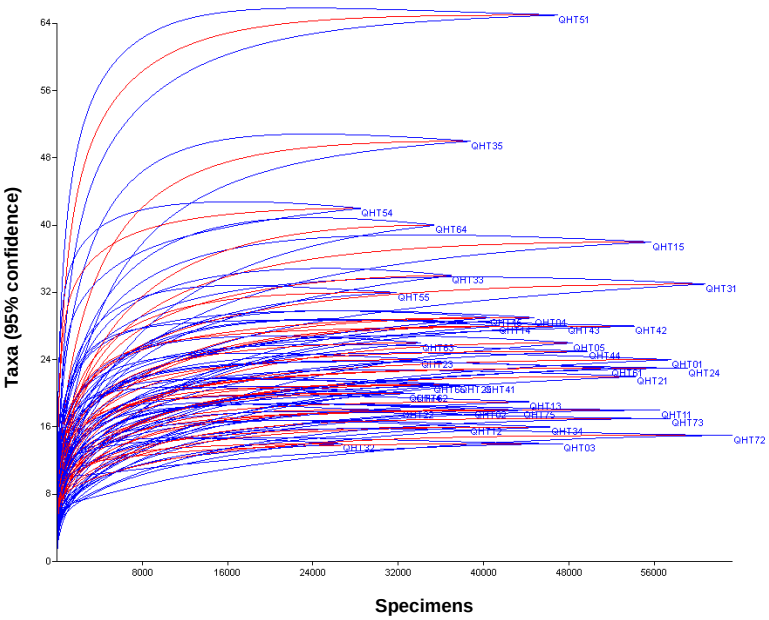
SAMPLE RAREFACTION CURVES (CHAPTERS 3–7)

Presented below are overlaid rarefaction curves (red line) of all samples processed (±95% confidence, blue line) demonstrating that sequencing depth was sufficient for the majority of samples.

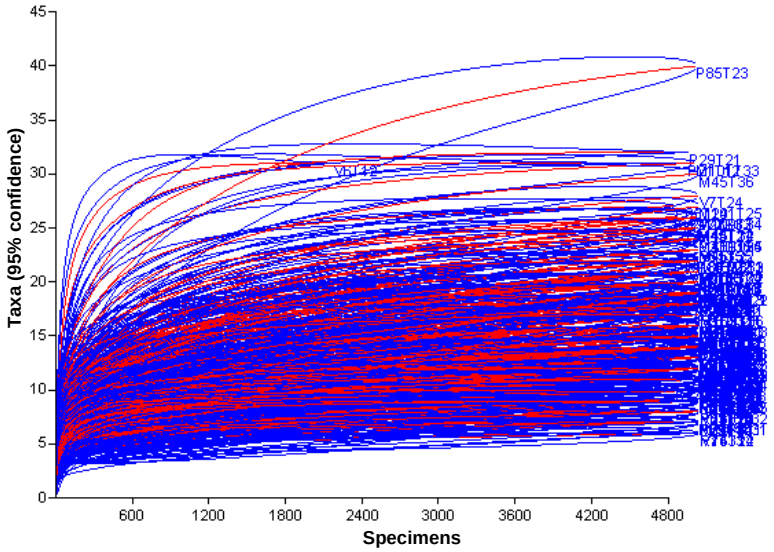
Chapters 3, 4



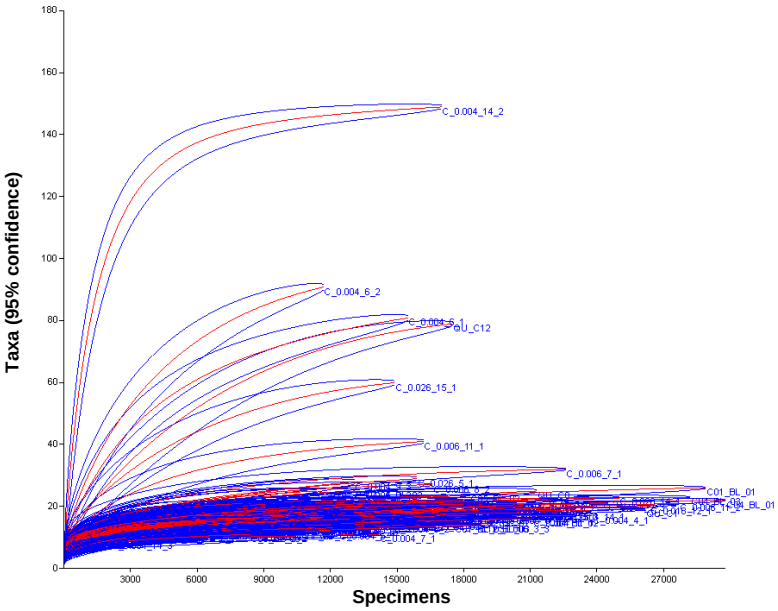
Chapter 5



Chapter 6



Chapter 7



APPENDIX 4

ACCESSION NUMBERS FOR SAMPLES (CHAPTERS 3, 4)

The sequence data reported in Chapters 3 and 4 were deposited in the European Nucleotide Archive under study accession number PRJEB21869, and sample accession numbers ERS1935907 - ERS2131105. Dual-indexes barcode primer combination based on Kozich *et al.* (2013).

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1935907	Pupa	5 weeks	Control	V3.F.SA507 V4.R.SB703
ERS1935908	Pupa	5 weeks	Control	V3.F.SA507 V4.R.SB704
ERS1935909	Pupa	5 weeks	Control	V3.F.SA501 V4.R.SA708
ERS1935910	1st instar larva	5 weeks	Control	V3.F.SA501 V4.R.SB701
ERS1935911	1st instar larva	5 weeks	Control	V3.F.SA501 V4.R.SB702
ERS1935912	2nd instar larva	5 weeks	Control	V3.F.SA501 V4.R.SB703
ERS1935913	2nd instar larva	5 weeks	Control	V3.F.SA501 V4.R.SB704
ERS1935914	3rd instar larva	5 weeks	Control	V3.F.SA501 V4.R.SB705
ERS1935915	4th instar larva	5 weeks	Control	V3.F.SA501 V4.R.SB706
ERS1935916	Male	5 weeks	Control	V3.F.SB501 V4.R.SB707
ERS1935917	New worker	5 weeks	Control	V3.F.SB501 V4.R.SB701
ERS1935918	Queen	5 weeks	Control	V3.F.SA501 V4.R.SB708
ERS1935919	Worker	5 weeks	Control	V3.F.SB501 V4.R.SB702
ERS1935920	Worker	5 weeks	Control	V3.F.SB501 V4.R.SB703
ERS1935921	Worker	5 weeks	Control	V3.F.SB501 V4.R.SB704
ERS1935922	Worker	5 weeks	Control	V3.F.SB501 V4.R.SB705
ERS1935923	Worker	5 weeks	Control	V3.F.SB501 V4.R.SB706
ERS1935924	Pupa	6 weeks	Control	V3.F.SA508 V4.R.SB707
ERS1935925	1st instar larva	6 weeks	Control	V3.F.SA504 V4.R.SB712
ERS1935926	1st instar larva	6 weeks	Control	V3.F.SA503 V4.R.SA705
ERS1935927	2nd instar larva	6 weeks	Control	V3.F.SA505 V4.R.SB702
ERS1935928	2nd instar larva	6 weeks	Control	V3.F.SA505 V4.R.SB703
ERS1935929	2nd instar larva	6 weeks	Control	V3.F.SA505 V4.R.SB704
ERS1935930	3rd instar larva	6 weeks	Control	V3.F.SA505 V4.R.SB705
ERS1935931	New worker	6 weeks	Control	V3.F.SB503 V4.R.SB706
ERS1935932	Worker	6 weeks	Control	V3.F.SB503 V4.R.SB707
ERS1935933	Worker	6 weeks	Control	V3.F.SB503 V4.R.SB708
ERS1935934	Worker	6 weeks	Control	V3.F.SB503 V4.R.SB709
ERS1935935	Worker	6 weeks	Control	V3.F.SB503 V4.R.SB710
ERS1935936	Worker	6 weeks	Control	V3.F.SB503 V4.R.SB711

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1935937	Worker	6 weeks	Control	V3.F.SB503 V4.R.SB712
ERS1935938	4th instar larva	6 weeks	Control	V3.F.SA505 V4.R.SB706
ERS1935939	Egg	8 weeks	Control	V3.F.SA508 V4.R.SB709
ERS1935940	Pupa	8 weeks	Control	V3.F.SA504 V4.R.SA711
ERS1935941	Pupa	8 weeks	Control	V3.F.SB502 V4.R.SB702
ERS1935942	Pupa	8 weeks	Control	V3.F.SB502 V4.R.SB703
ERS1935943	Pupa	8 weeks	Control	V3.F.SA505 V4.R.SA701
ERS1935944	1st instar larva	8 weeks	Control	V3.F.SA508 V4.R.SB710
ERS1935945	1st instar larva	8 weeks	Control	V3.F.SA508 V4.R.SB711
ERS1935946	2nd instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB701
ERS1935947	2nd instar larva	8 weeks	Control	V3.F.SA504 V4.R.SA703
ERS1935948	2nd instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB703
ERS1935949	2nd instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB704
ERS1935950	2nd instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB705
ERS1935951	2nd instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB706
ERS1935952	3rd instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB707
ERS1935953	3rd instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB708
ERS1935954	3rd instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB709
ERS1935955	4th instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB710
ERS1935956	4th instar larva	8 weeks	Control	V3.F.SA504 V4.R.SA708
ERS1935957	4th instar larva	8 weeks	Control	V3.F.SB501 V4.R.SB712
ERS1935958	Male	8 weeks	Control	V3.F.SB506 V4.R.SB711
ERS1935959	New worker	8 weeks	Control	V3.F.SB505 V4.R.SB712
ERS1935960	Queen	8 weeks	Control	V3.F.SB502 V4.R.SB705
ERS1935961	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB701
ERS1935962	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB710
ERS1935963	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB702
ERS1935964	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB703
ERS1935965	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB704
ERS1935966	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB705
ERS1935967	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB706
ERS1935968	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB707
ERS1935969	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB708
ERS1935970	Worker	8 weeks	Control	V3.F.SB506 V4.R.SB709
ERS1935971	Egg	10 weeks	Control	V3.F.SB506 V4.R.SB710
ERS1935972	Pupa	10 weeks	Control	V3.F.SB507 V4.R.SB706
ERS1935973	Pupa	10 weeks	Control	V3.F.SA506 V4.R.SA707
ERS1935974	Pupa	10 weeks	Control	V3.F.SB507 V4.R.SB708
ERS1935975	Pupa	10 weeks	Control	V3.F.SB507 V4.R.SB709
ERS1935976	1st instar larva	10 weeks	Control	V3.F.SB506 V4.R.SB711
ERS1935977	2nd instar larva	10 weeks	Control	V3.F.SB506 V4.R.SB712

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1935978	2nd instar larva	10 weeks	Control	V3.F.SB507 V4.R.SB701
ERS1935979	3rd instar larva	10 weeks	Control	V3.F.SB507 V4.R.SB702
ERS1935980	3rd instar larva	10 weeks	Control	V3.F.SB507 V4.R.SB703
ERS1935981	3rd instar larva	10 weeks	Control	V3.F.SB507 V4.R.SB704
ERS1935982	4th instar larva	10 weeks	Control	V3.F.SB507 V4.R.SB705
ERS1935983	Male	10 weeks	Control	V3.F.SA501 V4.R.SB711
ERS1935984	Male	10 weeks	Control	V3.F.SA502 V4.R.SB708
ERS1935985	Male	10 weeks	Control	V3.F.SA501 V4.R.SB712
ERS1935986	Male	10 weeks	Control	V3.F.SA502 V4.R.SB701
ERS1935987	Male	10 weeks	Control	V3.F.SA502 V4.R.SB702
ERS1935988	Male	10 weeks	Control	V3.F.SA502 V4.R.SB703
ERS1935989	Male	10 weeks	Control	V3.F.SA502 V4.R.SB704
ERS1935990	Male	10 weeks	Control	V3.F.SA502 V4.R.SB705
ERS1935991	Male	10 weeks	Control	V3.F.SA502 V4.R.SB706
ERS1935992	Male	10 weeks	Control	V3.F.SA502 V4.R.SB707
ERS1935993	New worker	10 weeks	Control	V3.F.SA501 V4.R.SB706
ERS1935994	Queen	10 weeks	Control	V3.F.SB507 V4.R.SB710
ERS1935995	Worker	10 weeks	Control	V3.F.SA501 V4.R.SB707
ERS1935996	Worker	10 weeks	Control	V3.F.SA501 V4.R.SB708
ERS1935997	Worker	10 weeks	Control	V3.F.SA501 V4.R.SB709
ERS1935998	Worker	10 weeks	Control	V3.F.SA501 V4.R.SB710
ERS1935999	Egg	11 weeks	Control	V3.F.SB502 V4.R.SA708
ERS1936000	Pupa	11 weeks	Control	V3.F.SB502 V4.R.SA711
ERS1936001	Pupa	11 weeks	Control	V3.F.SB502 V4.R.SA712
ERS1936002	4th instar larva	11 weeks	Control	V3.F.SB502 V4.R.SA710
ERS1936003	Male	11 weeks	Control	V3.F.SA504 V4.R.SB710
ERS1936004	Male	11 weeks	Control	V3.F.SA501 V4.R.SA706
ERS1936005	Male	11 weeks	Control	V3.F.SA504 V4.R.SB711
ERS1936006	Male	11 weeks	Control	V3.F.SA505 V4.R.SB701
ERS1936007	Male	11 weeks	Control	V3.F.SA505 V4.R.SB702
ERS1936008	Male	11 weeks	Control	V3.F.SA505 V4.R.SB703
ERS1936009	Male	11 weeks	Control	V3.F.SA505 V4.R.SB704
ERS1936010	Male	11 weeks	Control	V3.F.SA505 V4.R.SB705
ERS1936011	Male	11 weeks	Control	V3.F.SA505 V4.R.SB706
ERS1936012	New male	11 weeks	Control	V3.F.SA504 V4.R.SB709
ERS1936013	Queen	11 weeks	Control	V3.F.SB503 V4.R.SA701
ERS1936014	New queen	11 weeks	Control	V3.F.SB503 V4.R.SA702
ERS1936015	Worker	11 weeks	Control	V3.F.SA503 V4.R.SB712
ERS1936016	Worker	11 weeks	Control	V3.F.SA504 V4.R.SB701
ERS1936017	Worker	11 weeks	Control	V3.F.SA504 V4.R.SB702
ERS1936018	Worker	11 weeks	Control	V3.F.SA504 V4.R.SB703

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1936019	Worker	11 weeks	Control	V3.F.SA504 V4.R.SB704
ERS1936020	Worker	11 weeks	Control	V3.F.SA504 V4.R.SB705
ERS1936021	Worker	11 weeks	Control	V3.F.SA504 V4.R.SB706
ERS1936022	Worker	11 weeks	Control	V3.F.SA504 V4.R.SB707
ERS1936023	Worker	11 weeks	Control	V3.F.SA504 V4.R.SB708
ERS1936024	Pupa	5 weeks	Foraging	V3.F.SA507 V4.R.SB706
ERS1936025	Pupa	5 weeks	Foraging	V3.F.SA501 V4.R.SA710
ERS1936026	3rd instar larva	5 weeks	Foraging	V3.F.SA501 V4.R.SB709
ERS1936027	4th instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SA701
ERS1936028	New worker	5 weeks	Foraging	V3.F.SB501 V4.R.SB708
ERS1936029	Queen	5 weeks	Foraging	V3.F.SA501 V4.R.SB711
ERS1936030	Worker	5 weeks	Foraging	V3.F.SB501 V4.R.SB709
ERS1936031	4th instar larva	6 weeks	Foraging	V3.F.SA505 V4.R.SB708
ERS1936032	Pupa	6 weeks	Foraging	V3.F.SA502 V4.R.SA708
ERS1936033	3rd instar larva	6 weeks	Foraging	V3.F.SA505 V4.R.SB707
ERS1936034	Male	6 weeks	Foraging	V3.F.SB504 V4.R.SB703
ERS1936035	Worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB701
ERS1936036	Worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB702
ERS1936037	Egg	8 weeks	Foraging	V3.F.SB502 V4.R.SB706
ERS1936038	Pupa	8 weeks	Foraging	V3.F.SB502 V4.R.SB709
ERS1936039	3rd instar larva	8 weeks	Foraging	V3.F.SB502 V4.R.SB707
ERS1936040	4th instar larva	8 weeks	Foraging	V3.F.SB502 V4.R.SB708
ERS1936041	New worker	8 weeks	Foraging	V3.F.SB506 V4.R.SB712
ERS1936042	Queen	8 weeks	Foraging	V3.F.SB502 V4.R.SB710
ERS1936043	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB702
ERS1936044	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB703
ERS1936045	Egg	10 weeks	Foraging	V3.F.SB507 V4.R.SB711
ERS1936046	Pupa	10 weeks	Foraging	V3.F.SB508 V4.R.SB703
ERS1936047	Pupa	10 weeks	Foraging	V3.F.SB508 V4.R.SB704
ERS1936048	1st instar larva	10 weeks	Foraging	V3.F.SB507 V4.R.SB712
ERS1936049	2nd instar larva	10 weeks	Foraging	V3.F.SB508 V4.R.SB701
ERS1936050	4th instar larva	10 weeks	Foraging	V3.F.SB508 V4.R.SB702
ERS1936051	Male	10 weeks	Foraging	V3.F.SA502 V4.R.SB710
ERS1936052	Queen	10 weeks	Foraging	V3.F.SB508 V4.R.SB705
ERS1936053	Worker	10 weeks	Foraging	V3.F.SA502 V4.R.SB709
ERS1936054	Pupa	5 weeks	Foraging	V3.F.SA507 V4.R.SB708
ERS1936055	Pupa	5 weeks	Foraging	V3.F.SA501 V4.R.SA711
ERS1936056	1st instar larva	5 weeks	Foraging	V3.F.SA501 V4.R.SB712
ERS1936057	2nd instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB701
ERS1936058	3rd instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB703
ERS1936059	3rd instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB704

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1936060	4th instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB705
ERS1936061	Male	5 weeks	Foraging	V3.F.SB501 V4.R.SB712
ERS1936062	Queen	5 weeks	Foraging	V3.F.SA502 V4.R.SB706
ERS1936063	Worker	5 weeks	Foraging	V3.F.SB501 V4.R.SB710
ERS1936064	Worker	5 weeks	Foraging	V3.F.SB501 V4.R.SB711
ERS1936065	Egg	6 weeks	Foraging	V3.F.SA505 V4.R.SB709
ERS1936066	Pupa	6 weeks	Foraging	V3.F.SA502 V4.R.SA701
ERS1936067	Male	6 weeks	Foraging	V3.F.SB504 V4.R.SB707
ERS1936068	New worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB704
ERS1936069	Queen	6 weeks	Foraging	V3.F.SA503 V4.R.SA708
ERS1936070	Worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB705
ERS1936071	Worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB706
ERS1936072	Pupa	8 weeks	Foraging	V3.F.SB503 V4.R.SB705
ERS1936073	Pupa	8 weeks	Foraging	V3.F.SB503 V4.R.SB706
ERS1936074	Pupa	8 weeks	Foraging	V3.F.SB503 V4.R.SB707
ERS1936075	1st instar larva	8 weeks	Foraging	V3.F.SA505 V4.R.SA703
ERS1936076	2nd instar larva	8 weeks	Foraging	V3.F.SB502 V4.R.SB712
ERS1936077	2nd instar larva	8 weeks	Foraging	V3.F.SB503 V4.R.SB701
ERS1936078	3rd instar larva	8 weeks	Foraging	V3.F.SB503 V4.R.SB702
ERS1936079	3rd instar larva	8 weeks	Foraging	V3.F.SB503 V4.R.SB703
ERS1936080	4th instar larva	8 weeks	Foraging	V3.F.SB503 V4.R.SB704
ERS1936081	New worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB704
ERS1936082	Queen	8 weeks	Foraging	V3.F.SB503 V4.R.SB708
ERS1936083	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB705
ERS1936084	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB706
ERS1936085	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB707
ERS1936086	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB708
ERS1936087	Pupa	10 weeks	Foraging	V3.F.SB508 V4.R.SB709
ERS1936088	Pupa	10 weeks	Foraging	V3.F.SB508 V4.R.SB710
ERS1936089	Pupa	10 weeks	Foraging	V3.F.SB508 V4.R.SB711
ERS1936090	Pupa	10 weeks	Foraging	V3.F.SB501 V4.R.SA701
ERS1936091	2nd instar larva	10 weeks	Foraging	V3.F.SB508 V4.R.SB706
ERS1936092	3rd instar larva	10 weeks	Foraging	V3.F.SB508 V4.R.SB707
ERS1936093	4th instar larva	10 weeks	Foraging	V3.F.SB508 V4.R.SB708
ERS1936094	Male	10 weeks	Foraging	V3.F.SA503 V4.R.SB703
ERS1936095	New worker	10 weeks	Foraging	V3.F.SA502 V4.R.SB712
ERS1936096	Queen	10 weeks	Foraging	V3.F.SB501 V4.R.SA703
ERS1936097	New queen	10 weeks	Foraging	V3.F.SB501 V4.R.SA704
ERS1936098	Worker	10 weeks	Foraging	V3.F.SA502 V4.R.SB711
ERS1936099	Worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB701
ERS1936100	Worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB702

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1936101	Pupa	11 weeks	Foraging	V3.F.SB503 V4.R.SA707
ERS1936102	1st instar larva	11 weeks	Foraging	V3.F.SA507 V4.R.SA705
ERS1936103	1st instar larva	11 weeks	Foraging	V3.F.SA507 V4.R.SA706
ERS1936104	2nd instar larva	11 weeks	Foraging	V3.F.SB503 V4.R.SA705
ERS1936105	3rd instar larva	11 weeks	Foraging	V3.F.SB503 V4.R.SA706
ERS1936106	New worker	11 weeks	Foraging	V3.F.SA501 V4.R.SA707
ERS1936107	Worker	11 weeks	Foraging	V3.F.SA505 V4.R.SB709
ERS1936108	Worker	11 weeks	Foraging	V3.F.SA505 V4.R.SB710
ERS1936109	Worker	11 weeks	Foraging	V3.F.SA505 V4.R.SB711
ERS1936110	Queen	11 weeks	Foraging	V3.F.SA507 V4.R.SA710
ERS1936111	Pupa	5 weeks	Foraging	V3.F.SA501 V4.R.SA712
ERS1936112	Pupa	5 weeks	Foraging	V3.F.SA507 V4.R.SB711
ERS1936113	3rd instar larva	5 weeks	Foraging	V3.F.SA508 V4.R.SB701
ERS1936114	1st instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB707
ERS1936115	2nd instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB708
ERS1936116	3rd instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB709
ERS1936117	3rd instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB710
ERS1936118	4th instar larva	5 weeks	Foraging	V3.F.SA502 V4.R.SB711
ERS1936119	Worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB701
ERS1936120	Worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB703
ERS1936121	Pupa	6 weeks	Foraging	V3.F.SA508 V4.R.SB710
ERS1936122	3rd instar larva	6 weeks	Foraging	V3.F.SA505 V4.R.SB711
ERS1936123	4th instar larva	6 weeks	Foraging	V3.F.SA505 V4.R.SB712
ERS1936124	Male	6 weeks	Foraging	V3.F.SB504 V4.R.SB709
ERS1936125	Queen	6 weeks	Foraging	V3.F.SA506 V4.R.SB701
ERS1936126	Worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB708
ERS1936127	Egg	8 weeks	Foraging	V3.F.SB503 V4.R.SB709
ERS1936128	Pupa	8 weeks	Foraging	V3.F.SB504 V4.R.SB704
ERS1936129	Pupa	8 weeks	Foraging	V3.F.SB504 V4.R.SB705
ERS1936130	Pupa	8 weeks	Foraging	V3.F.SB504 V4.R.SB706
ERS1936131	2nd instar larva	8 weeks	Foraging	V3.F.SB503 V4.R.SB710
ERS1936132	2nd instar larva	8 weeks	Foraging	V3.F.SB503 V4.R.SB711
ERS1936133	2nd instar larva	8 weeks	Foraging	V3.F.SB503 V4.R.SB712
ERS1936134	3rd instar larva	8 weeks	Foraging	V3.F.SB504 V4.R.SB701
ERS1936135	4th instar larva	8 weeks	Foraging	V3.F.SB504 V4.R.SB702
ERS1936136	4th instar larva	8 weeks	Foraging	V3.F.SB504 V4.R.SB703
ERS1936137	Queen	8 weeks	Foraging	V3.F.SB504 V4.R.SB707
ERS1936138	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB710
ERS1936139	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB711
ERS1936140	Worker	8 weeks	Foraging	V3.F.SB507 V4.R.SB712
ERS1936141	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB701

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1936142	Pupa	10 weeks	Foraging	V3.F.SB501 V4.R.SA707
ERS1936143	3rd instar larva	10 weeks	Foraging	V3.F.SB501 V4.R.SA705
ERS1936144	4th instar larva	10 weeks	Foraging	V3.F.SB501 V4.R.SA706
ERS1936145	Male	10 weeks	Foraging	V3.F.SA503 V4.R.SB705
ERS1936146	Queen	10 weeks	Foraging	V3.F.SB501 V4.R.SA708
ERS1936147	Worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB704
ERS1936148	Egg	5 weeks	Foraging	V3.F.SA502 V4.R.SB712
ERS1936149	Pupa	5 weeks	Foraging	V3.F.SA507 V4.R.SB712
ERS1936150	Pupa	5 weeks	Foraging	V3.F.SA508 V4.R.SB702
ERS1936151	1st instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB702
ERS1936152	1st instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB703
ERS1936153	2nd instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB704
ERS1936154	2nd instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB705
ERS1936155	3rd instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB706
ERS1936156	4th instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB707
ERS1936157	4th instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB708
ERS1936158	New worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB704
ERS1936159	Queen	5 weeks	Foraging	V3.F.SA503 V4.R.SB709
ERS1936160	Worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB705
ERS1936161	Worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB706
ERS1936162	Worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB707
ERS1936163	Worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB708
ERS1936164	Egg	6 weeks	Foraging	V3.F.SA506 V4.R.SB702
ERS1936165	Pupa	6 weeks	Foraging	V3.F.SA502 V4.R.SA711
ERS1936166	2nd instar larva	6 weeks	Foraging	V3.F.SA506 V4.R.SB703
ERS1936167	2nd instar larva	6 weeks	Foraging	V3.F.SA506 V4.R.SB704
ERS1936168	2nd instar larva	6 weeks	Foraging	V3.F.SA506 V4.R.SB705
ERS1936169	2nd instar larva	6 weeks	Foraging	V3.F.SA506 V4.R.SB706
ERS1936170	2nd instar larva	6 weeks	Foraging	V3.F.SA506 V4.R.SB707
ERS1936171	3rd instar larva	6 weeks	Foraging	V3.F.SA506 V4.R.SB708
ERS1936172	3rd instar larva	6 weeks	Foraging	V3.F.SA506 V4.R.SB709
ERS1936173	Male	6 weeks	Foraging	V3.F.SB505 V4.R.SB704
ERS1936174	New worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB710
ERS1936175	Queen	6 weeks	Foraging	V3.F.SA506 V4.R.SB710
ERS1936176	Worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB711
ERS1936177	Worker	6 weeks	Foraging	V3.F.SB504 V4.R.SB712
ERS1936178	Worker	6 weeks	Foraging	V3.F.SB505 V4.R.SB701
ERS1936179	Worker	6 weeks	Foraging	V3.F.SB505 V4.R.SB702
ERS1936180	Pupa	8 weeks	Foraging	V3.F.SB505 V4.R.SB706
ERS1936181	1st instar larva	8 weeks	Foraging	V3.F.SB504 V4.R.SB708
ERS1936182	1st instar larva	8 weeks	Foraging	V3.F.SB504 V4.R.SB709

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1936183	1st instar larva	8 weeks	Foraging	V3.F.SB504 V4.R.SB710
ERS1936184	1st instar larva	8 weeks	Foraging	V3.F.SB504 V4.R.SB711
ERS1936185	1st instar larva	8 weeks	Foraging	V3.F.SB504 V4.R.SB712
ERS1936186	2nd instar larva	8 weeks	Foraging	V3.F.SB505 V4.R.SB701
ERS1936187	2nd instar larva	8 weeks	Foraging	V3.F.SA505 V4.R.SA711
ERS1936188	2nd instar larva	8 weeks	Foraging	V3.F.SB505 V4.R.SB703
ERS1936189	3rd instar larva	8 weeks	Foraging	V3.F.SB505 V4.R.SB704
ERS1936190	4th instar larva	8 weeks	Foraging	V3.F.SA506 V4.R.SA701
ERS1936191	Queen	8 weeks	Foraging	V3.F.SA506 V4.R.SA703
ERS1936192	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB702
ERS1936193	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB703
ERS1936194	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB704
ERS1936195	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB705
ERS1936196	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB706
ERS1936197	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB707
ERS1936198	Pupa	10 weeks	Foraging	V3.F.SB501 V4.R.SA711
ERS1936199	3rd instar larva	10 weeks	Foraging	V3.F.SB501 V4.R.SA710
ERS1936200	New worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB706
ERS1936201	Queen	10 weeks	Foraging	V3.F.SB501 V4.R.SA712
ERS1936202	Worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB707
ERS1936203	Worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB708
ERS1936204	Worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB709
ERS1936205	Pupa	11 weeks	Foraging	V3.F.SB504 V4.R.SA701
ERS1936206	1st instar larva	11 weeks	Foraging	V3.F.SB503 V4.R.SA709
ERS1936207	2nd instar larva	11 weeks	Foraging	V3.F.SB503 V4.R.SA710
ERS1936208	2nd instar larva	11 weeks	Foraging	V3.F.SB503 V4.R.SA711
ERS1936209	4th instar larva	11 weeks	Foraging	V3.F.SB503 V4.R.SA712
ERS1936210	Male	11 weeks	Foraging	V3.F.SA506 V4.R.SB703
ERS1936211	New male	11 weeks	Foraging	V3.F.SA506 V4.R.SB702
ERS1936212	Queen	11 weeks	Foraging	V3.F.SB504 V4.R.SA702
ERS1936213	Worker	11 weeks	Foraging	V3.F.SA505 V4.R.SB712
ERS1936214	Worker	11 weeks	Foraging	V3.F.SA506 V4.R.SB701
ERS1936215	Egg	5 weeks	Foraging	V3.F.SA503 V4.R.SB710
ERS1936216	Pupa	5 weeks	Foraging	V3.F.SA502 V4.R.SA705
ERS1936217	Pupa	5 weeks	Foraging	V3.F.SA508 V4.R.SB704
ERS1936218	1st instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB711
ERS1936219	2nd instar larva	5 weeks	Foraging	V3.F.SA503 V4.R.SB712
ERS1936220	3rd instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB701
ERS1936221	3rd instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB702
ERS1936222	4th instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB703
ERS1936223	New worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB709

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1936224	Queen	5 weeks	Foraging	V3.F.SA504 V4.R.SB704
ERS1936225	Worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB710
ERS1936226	Worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB711
ERS1936227	Egg	6 weeks	Foraging	V3.F.SA506 V4.R.SB711
ERS1936228	Pupa	6 weeks	Foraging	V3.F.SA503 V4.R.SA712
ERS1936229	2nd instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB701
ERS1936230	2nd instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB702
ERS1936231	2nd instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB703
ERS1936232	2nd instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB704
ERS1936233	2nd instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB705
ERS1936234	3rd instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB706
ERS1936235	3rd instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB707
ERS1936236	3rd instar larva	6 weeks	Foraging	V3.F.SA503 V4.R.SA711
ERS1936237	4th instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB709
ERS1936238	New worker	6 weeks	Foraging	V3.F.SB505 V4.R.SB705
ERS1936239	Worker	6 weeks	Foraging	V3.F.SB505 V4.R.SB706
ERS1936240	Worker	6 weeks	Foraging	V3.F.SB505 V4.R.SB707
ERS1936241	Pupa	8 weeks	Foraging	V3.F.SB505 V4.R.SB711
ERS1936242	1st instar larva	8 weeks	Foraging	V3.F.SB505 V4.R.SB708
ERS1936243	2nd instar larva	8 weeks	Foraging	V3.F.SB505 V4.R.SB709
ERS1936244	4th instar larva	8 weeks	Foraging	V3.F.SB505 V4.R.SB710
ERS1936245	New queen	8 weeks	Foraging	V3.F.SB506 V4.R.SB701
ERS1936246	New worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB708
ERS1936247	Queen	8 weeks	Foraging	V3.F.SB505 V4.R.SB712
ERS1936248	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB710
ERS1936249	Worker	8 weeks	Foraging	V3.F.SB508 V4.R.SB711
ERS1936250	Egg	10 weeks	Foraging	V3.F.SA506 V4.R.SA711
ERS1936251	Pupa	10 weeks	Foraging	V3.F.SB502 V4.R.SA705
ERS1936252	2nd instar larva	10 weeks	Foraging	V3.F.SB502 V4.R.SA702
ERS1936253	3rd instar larva	10 weeks	Foraging	V3.F.SB502 V4.R.SA703
ERS1936254	4th instar larva	10 weeks	Foraging	V3.F.SA507 V4.R.SA701
ERS1936255	Queen	10 weeks	Foraging	V3.F.SB502 V4.R.SA706
ERS1936256	New queen	10 weeks	Foraging	V3.F.SB502 V4.R.SA707
ERS1936257	Worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB710
ERS1936258	Worker	10 weeks	Foraging	V3.F.SA503 V4.R.SB711
ERS1936259	Egg	11 weeks	Foraging	V3.F.SB504 V4.R.SA703
ERS1936260	Pupa	11 weeks	Foraging	V3.F.SB504 V4.R.SA710
ERS1936261	Pupa	11 weeks	Foraging	V3.F.SB504 V4.R.SA711
ERS1936262	2nd instar larva	11 weeks	Foraging	V3.F.SA507 V4.R.SA711
ERS1936263	2nd instar larva	11 weeks	Foraging	V3.F.SB504 V4.R.SA705
ERS1936264	2nd instar larva	11 weeks	Foraging	V3.F.SB504 V4.R.SA706

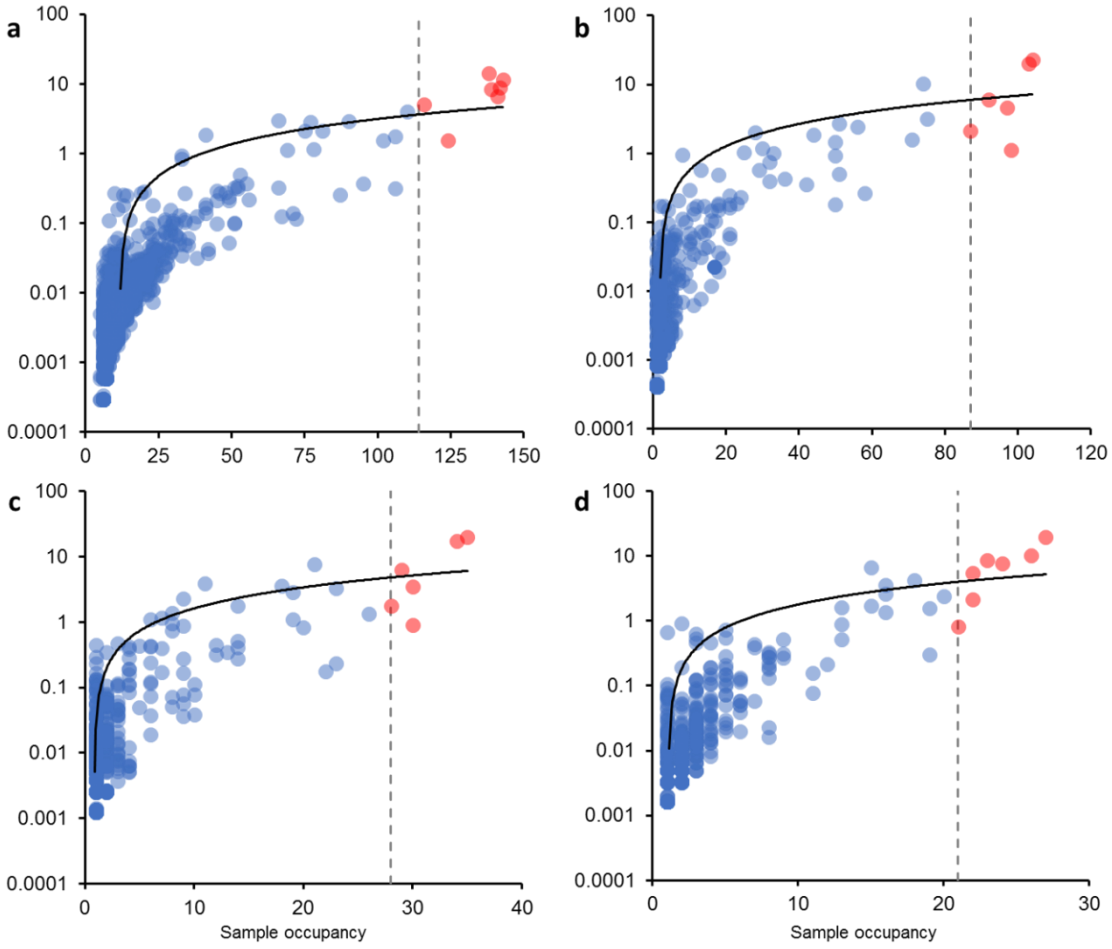
Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1936265	3rd instar larva	11 weeks	Foraging	V3.F.SB504 V4.R.SA707
ERS1936266	4th instar larva	11 weeks	Foraging	V3.F.SB504 V4.R.SA708
ERS1936267	Male	11 weeks	Foraging	V3.F.SA506 V4.R.SB710
ERS1936268	New male	11 weeks	Foraging	V3.F.SA506 V4.R.SB709
ERS1936269	Queen	11 weeks	Foraging	V3.F.SB504 V4.R.SA712
ERS1936270	Worker	11 weeks	Foraging	V3.F.SA506 V4.R.SB704
ERS1936271	Worker	11 weeks	Foraging	V3.F.SA506 V4.R.SB705
ERS1936272	Worker	11 weeks	Foraging	V3.F.SA506 V4.R.SB706
ERS1936273	Worker	11 weeks	Foraging	V3.F.SA506 V4.R.SB707
ERS1936274	Worker	11 weeks	Foraging	V3.F.SA506 V4.R.SB708
ERS1936275	Pupa	5 weeks	Foraging	V3.F.SA502 V4.R.SA706
ERS1936276	Pupa	5 weeks	Foraging	V3.F.SA508 V4.R.SB706
ERS1936277	1st instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB705
ERS1936278	2nd instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB706
ERS1936279	2nd instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB707
ERS1936280	3rd instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB708
ERS1936281	3rd instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB709
ERS1936282	4th instar larva	5 weeks	Foraging	V3.F.SA504 V4.R.SB710
ERS1936283	Male	5 weeks	Foraging	V3.F.SB503 V4.R.SB705
ERS1936284	New worker	5 weeks	Foraging	V3.F.SB502 V4.R.SB712
ERS1936285	Queen	5 weeks	Foraging	V3.F.SA504 V4.R.SB711
ERS1936286	Worker	5 weeks	Foraging	V3.F.SB503 V4.R.SB701
ERS1936287	Worker	5 weeks	Foraging	V3.F.SB503 V4.R.SB702
ERS1936288	Worker	5 weeks	Foraging	V3.F.SB503 V4.R.SB703
ERS1936289	Worker	5 weeks	Foraging	V3.F.SB503 V4.R.SB704
ERS1936290	Egg	6 weeks	Foraging	V3.F.SA504 V4.R.SA701
ERS1936291	2nd instar larva	6 weeks	Foraging	V3.F.SA507 V4.R.SB712
ERS1936292	3rd instar larva	6 weeks	Foraging	V3.F.SA508 V4.R.SB702
ERS1936293	3rd instar larva	6 weeks	Foraging	V3.F.SA508 V4.R.SB703
ERS1936294	4th instar larva	6 weeks	Foraging	V3.F.SA508 V4.R.SB704
ERS1936295	4th instar larva	6 weeks	Foraging	V3.F.SA508 V4.R.SB706
ERS1936296	Queen	6 weeks	Foraging	V3.F.SA508 V4.R.SB708
ERS1936297	Worker	6 weeks	Foraging	V3.F.SB505 V4.R.SB708
ERS1936298	1st instar larva	6 weeks	Foraging	V3.F.SA506 V4.R.SB712
ERS1936299	Worker	6 weeks	Foraging	V3.F.SB505 V4.R.SB709
ERS1936300	Worker	6 weeks	Foraging	V3.F.SB505 V4.R.SB710
ERS1936301	Male	8 weeks	Foraging	V3.F.SA501 V4.R.SB705
ERS1936302	Worker	8 weeks	Foraging	V3.F.SA501 V4.R.SB701
ERS1936303	Worker	8 weeks	Foraging	V3.F.SA501 V4.R.SB702
ERS1936304	Worker	8 weeks	Foraging	V3.F.SA501 V4.R.SB703
ERS1936305	Worker	8 weeks	Foraging	V3.F.SA501 V4.R.SB704

Accession	Caste/Stage	Colony age	Treatment	Primer combination
ERS1936306	Pupa	8 weeks	Foraging	V3.F.SB506 V4.R.SB708
ERS1936307	1st instar larva	8 weeks	Foraging	V3.F.SB506 V4.R.SB702
ERS1936308	1st instar larva	8 weeks	Foraging	V3.F.SB506 V4.R.SB703
ERS1936309	2nd instar larva	8 weeks	Foraging	V3.F.SB506 V4.R.SB704
ERS1936310	2nd instar larva	8 weeks	Foraging	V3.F.SB506 V4.R.SB705
ERS1936311	3rd instar larva	8 weeks	Foraging	V3.F.SB506 V4.R.SB706
ERS1936312	4th instar larva	8 weeks	Foraging	V3.F.SB506 V4.R.SB707
ERS1936313	Queen	8 weeks	Foraging	V3.F.SB506 V4.R.SB709
ERS1936314	Egg	11 weeks	Foraging	V3.F.SB505 V4.R.SA701
ERS1936315	Pupa	11 weeks	Foraging	V3.F.SA508 V4.R.SA701
ERS1936316	Pupa	11 weeks	Foraging	V3.F.SB505 V4.R.SA705
ERS1936317	4th instar larva	11 weeks	Foraging	V3.F.SA507 V4.R.SA712
ERS1936318	Male	11 weeks	Foraging	V3.F.SA507 V4.R.SB702
ERS1936319	Queen	11 weeks	Foraging	V3.F.SB505 V4.R.SA706
ERS1936320	Worker	11 weeks	Foraging	V3.F.SA506 V4.R.SB711
ERS1936321	Worker	11 weeks	Foraging	V3.F.SA507 V4.R.SB701

APPENDIX 5

COMMON AND RARE GROUPINGS OF OTUS (CHAPTERS 3, 4)

Distribution and abundance of OTUs from bee gut microbiota samples. Displayed is the number of samples for which each taxa was observed to occupy, plotted against the mean abundance across all samples for (a) Larva (n = 150, r^2 = 0.47, $F_{(1,\ 1883)}$ = 1686.28, p < 0.0001); (b) Worker (n = 107, r^2 = 0.44, $F_{(1,\ 983)}$ = 777.39, p < 0.0001); (c) Male (n = 34, r^2 = 0.38, $F_{(1,\ 583)}$ = 353.53, p < 0.0001); and (d) Queen (n = 28, r^2 = 0.51, $F_{(1,\ 1271)}$ = 1325.27, p < 0.0001). Blue dots are rare taxa, while those in red, past the grey 75% sample occupancy threshold are common taxa.



APPENDIX 6

SIMPER ANALYSIS (CHAPTER 3)

Similarity of percentages (SIMPER) analysis of *B. terrestris* gut microbiota dissimilarity (Bray-Curtis) between taxa in each caste and developmental stage, with 'N' corresponding to newly emerged individuals of a caste. Given is the % mean abundance, with the average dissimilarity between samples =79.96%. Percentage contribution is the mean contribution divided by mean dissimilarity across samples. The list of species is not exhaustive, so cumulative % does not add up to 100%. Only taxa above 1% contribution were used so as to identify major constituents within the community. OTU identities are based on ~300 bp sequence fragments and should be considered putative.

Class	Family	Taxon name	% Mean abundance								Av. dis.	Cont. %	Cuml. %
			Egg	Larva	Pupa	NWorker	Worker	NMale	Male	NQueen	Queen			
Gammaproteobacteria	Orbaceae	*Gilliamella bombicola* 99%	8.51	11.60	11.40	29.60	23.10	26.50	20.40	16.80	9.69	9.21	11.52	11.52
Betaproteobacteria	Neisseriaceae	*Snodgrasella alvi* 99%	3.29	6.57	6.46	20.10	19.90	40.20	17.60	5.44	5.38	7.99	10.00	21.52
Gammaproteobacteria	Enterobacteriaceae	*Enterobacter aerogenes* 100%	22.80	14.30	16.50	0.66	1.56	0.97	1.17	21.10	7.45	7.64	9.56	31.07
Bacilli	Lactobacillaceae	*Lactobacillus apis* 99%	5.51	8.71	5.00	4.64	6.06	8.07	5.98	9.63	19.20	5.53	6.91	37.99
Gammaproteobacteria	Pseudomonadaceae	*Ventosimonas* 100%	7.30	3.93	5.00	10.80	10.30	0.01	7.86	0.48	1.34	5.31	6.64	44.63
Bacilli	Lactobacillaceae	*Lactobacillus bombicola* 100%	5.71	8.43	4.39	5.89	4.53	2.27	3.44	5.24	8.36	4.47	5.59	50.21
Gammaproteobacteria	Enterobacteriaceae	*Pseudocitrobacter anthropi* 98%	5.73	5.12	3.01	1.75	1.44	0.65	3.49	3.12	6.50	3.32	4.15	54.36
Gammaproteobacteria	Enterobacteriaceae	*Providencia vermicola* 100%	5.71	2.85	2.82	0.06	2.45	0.01	3.30	4.00	4.02	2.69	3.36	57.72
Gammaproteobacteria	Enterobacteriaceae	*Arsenophonus arthropodicus* 99%	0.50	2.92	1.11	0.60	1.83	1.21	3.75	0.61	2.24	1.97	2.46	60.19
Bacilli	Lactobacillaceae	*Lactobacillus kimchicus* 99%	3.01	1.13	2.13	0.87	2.67	0.00	1.87	1.00	3.48	1.79	2.24	62.42
Bacilli	Lactobacillaceae	*Lactobacillus bombi* 98%	2.04	1.75	1.18	1.20	3.19	0.08	2.93	1.13	2.04	1.71	2.14	64.56
Bacilli	Lactobacillaceae	*Lactobacillus kunkeei* 97%	0.09	3.02	0.70	0.18	1.14	2.10	0.49	0.19	0.48	1.37	1.71	66.27
Gammaproteobacteria	Enterobacteriaceae	*Hafnia alvei* 96%	8.35	1.48	0.91	0.30	0.99	0.04	1.14	1.00	1.57	1.33	1.66	67.93
Alphaproteobacteria	Acetobacteraceae	*Saccharibacter floricola* 99%	1.37	0.28	2.50	1.82	2.01	4.92	0.33	0.03	0.02	1.16	1.46	69.38
Actinobacteria	Bifidobacteriaceae	*Bombiscardovia coagulans* 98%	0.39	1.51	1.10	2.67	2.09	1.79	1.87	1.00	0.80	1.14	1.43	70.81
Sphingobacteria	Saprospiraceae	Unknown	1.77	2.05	2.01	0.87	0.01	0.00	0.10	0.16	1.92	1.12	1.40	72.21
Bacilli	Lactobacillaceae	*Pediococcus acidilactici* 97%	0.28	1.86	1.99	0.05	0.02	0.00	0.00	16.10	0.31	1.08	1.35	73.56
Gammaproteobacteria	Enterobacteriaceae	*Rahnella woolbedingensis* 100%	0.35	2.06	0.54	0.08	0.94	0.06	0.83	0.11	1.38	1.07	1.33	74.89
Bacilli	Lactobacillaceae	*Lactobacillus bombicola* 96%	3.75	1.14	0.93	0.19	0.74	0.02	0.53	1.10	3.01	1.05	1.32	76.21

APPENDIX 7

SIMPER ANALYSIS – LARVAE/TREATMENT (CHAPTER 4)

Similarity of percentages (SIMPER) analysis of *B. terrestris* larvae gut microbiota dissimilarity (Bray-Curtis) between indoor control (C), resource-rich (RR), and resource-poor (RP) treatments. Given is the % mean abundance, with the overall average dissimilarity between samples = 79.98%. Percentage contribution is the mean contribution divided by mean dissimilarity across samples, and a cut off of >1% contribution was used so as to identify major taxa within the larval gut community. OTU identities are based on ~300 bp sequence fragments and should be considered putative.

			% Mean abundance					
Class	**Family**	**Taxon name**	**C**	**RR**	**RP**	**Av. Dis.**	**Cont. %**	**Cuml. %**
Gammaproteobacteria	Enterobacteriaceae	*Enterobacter aerogenes* 100%	11.70	14.90	15.90	10.34	12.93	12.93
Gammaproteobacteria	Orbaceae	*Gilliamella bombicola* 99%	9.98	13.70	9.22	7.17	8.96	21.89
Bacilli	Lactobacillaceae	*Lactobacillus bombicola* 100%	7.73	7.53	10.90	6.14	7.68	29.57
Bacilli	Lactobacillaceae	*Lactobacillus apis* 99%	6.93	9.64	8.82	6.04	7.55	37.12
Gammaproteobacteria	Enterobacteriaceae	*Pseudocitrobacter anthropi* 98%	9.65	3.34	4.53	4.87	6.08	43.20
Betaproteobacteria	Neisseriaceae	*Snodgrassella alvi* 99%	6.69	6.87	6.28	4.44	5.56	48.76
Gammaproteobacteria	Pseudomonadaceae	*Ventosimonas* sp. 100%	2.88	4.79	3.46	3.30	4.12	52.88
Bacilli	Lactobacillaceae	*Lactobacillus kunkeei* 97%	3.28	3.87	1.26	2.73	3.41	56.30
Gammaproteobacteria	Enterobacteriaceae	*Arsenophonus arthropodicus* 99%	4.69	2.91	1.50	2.64	3.30	59.60
Gammaproteobacteria	Enterobacteriaceae	*Providencia vermicola* 100%	0.11	4.73	1.76	2.40	3.00	62.60
Gammaproteobacteria	Enterobacteriaceae	*Rahnella woolbedingensis* 100%	1.60	1.40	3.83	2.09	2.61	65.21
Bacilli	Lactobacillaceae	*Pediococcus acidilactici* 97%	3.18	0.97	2.37	1.91	2.38	67.59
Sphingobacteria	Saprospiraceae	*Unknown*	2.04	1.36	3.58	1.90	2.38	69.97
Gammaproteobacteria	Enterobacteriaceae	*Hafnia alvei* 96%	0.66	1.23	2.94	1.42	1.77	71.74
Bacilli	Lactobacillaceae	*Lactobacillus bombi 98%*	1.42	2.36	0.94	1.29	1.62	73.36
Actinobacteria	Bifidobacteriaceae	*Bombiscardovia coagulans* 98%	1.92	1.42	1.42	1.15	1.43	74.79
Bacilli	Lactobacillaceae	*Lactobacillus bombicola* 96%	0.79	1.45	0.97	1.01	1.27	76.06
Bacilli	Lactobacillaceae	*Lactobacillus kimchicus* 99%	1.18	1.53	0.39	0.99	1.23	77.29
Bacilli	Enterococcaceae	*Enterococcus faecalis* 99%	1.92	0.03	1.39	0.98	1.23	78.52
Bacilli	Planococcaceae	*Sporosarcina sp.* 99%	1.17	0.80	1.01	0.95	1.19	79.70

APPENDIX 8

SIMPER ANALYSIS – LARVAE/TIME POINT (CHAPTER 4)

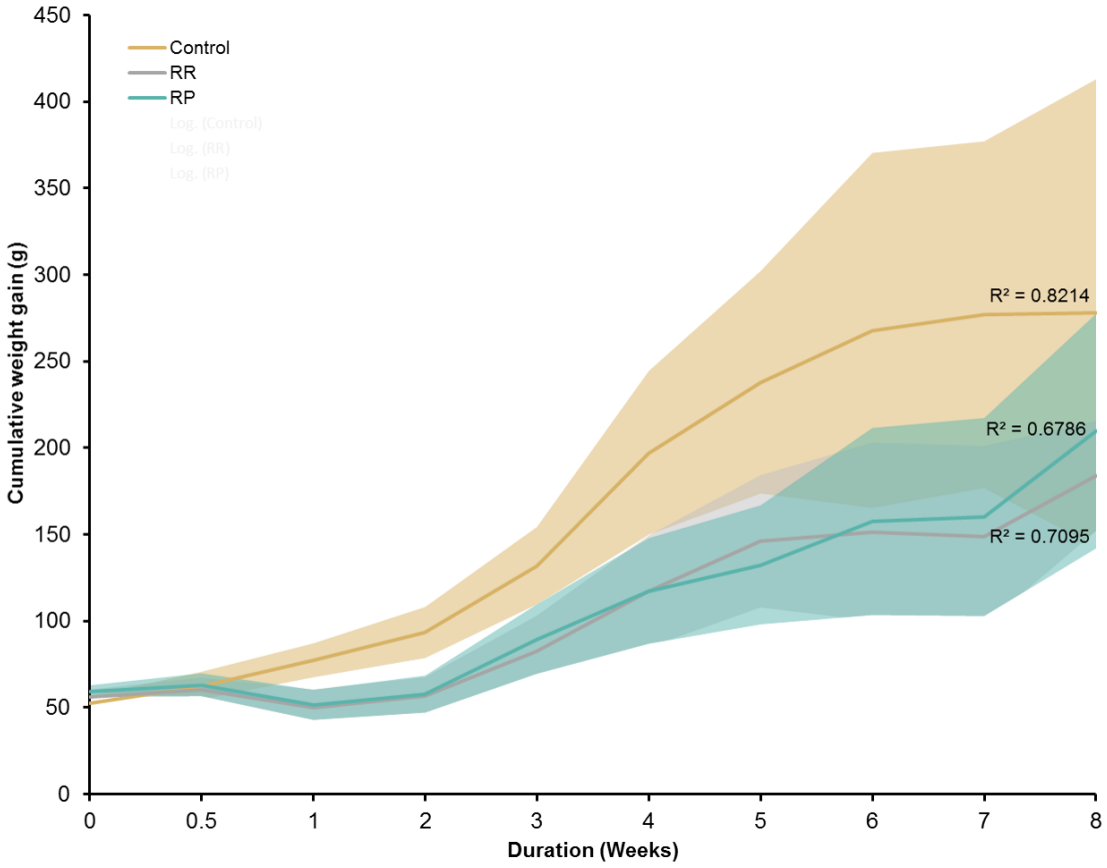
Similarity of percentages (SIMPER) analysis of *B. terrestris* larvae gut microbiota dissimilarity (Bray-Curtis) between time points during the resource-rich (RR) treatment. Given is the % mean abundance, with the overall average dissimilarity between samples = 80.92%. Percentage contribution is the mean contribution divided by mean dissimilarity across samples, and a cut off of >1% contribution was used so as to identify major taxa within the larval gut community. OTU identities are based on 400–440 bp sequence fragments and should be considered putative.

			% Mean abundancce					Av. dis.	Cont. %	Cuml. %
Class	Family	Taxon name	T_2	T_3	T_5	T_7	T_8			
Gammaproteobacteria	Enterobacteriaceae	*Enterobacter aerogenes* 100%	7.99	12.80	21.40	39.00	0.52	10.29	12.71	12.71
Gammaproteobacteria	Orbaceae	*Gilliamella bombicola* 99%	15.00	14.40	8.26	8.32	22.50	8.49	10.49	23.21
Bacilli	Lactobacillaceae	*Lactobacillus bombicola* 100%	14.60	11.50	4.56	0.99	1.20	6.32	7.81	31.01
Gammaproteobacteria	Enterobacteriaceae	*Providencia vermicola* 100%	0.13	1.36	1.63	0.02	33.20	6.22	7.69	38.70
Betaproteobacteria	Neisseriaceae	*Snodgrassella alvi* 99%	9.37	4.26	3.35	3.33	18.00	5.44	6.73	45.43
Gammaproteobacteria	Pseudomonadaceae	*Ventosimonas sp.* 100%	5.45	7.65	6.79	5.51	0.02	4.58	5.66	51.09
Bacilli	Lactobacillaceae	*Lactobacillus apis* 99%	8.05	10.10	5.75	0.88	2.66	4.50	5.56	56.65
Bacilli	Lactobacillaceae	*Lactobacillus sp.* 97%	5.82	4.19	3.42	8.51	0.00	3.81	4.71	61.36
Gammaproteobacteria	Enterobacteriaceae	*Pseudocitrobacter anthropi* 98%	9.63	2.10	0.55	0.01	0.15	2.80	3.46	64.82
Gammaproteobacteria	Enterobacteriaceae	*Arsenophonus arthropodicus* 99%	0.46	4.48	3.04	4.92	0.12	2.31	2.85	67.67
Bacilli	Lactobacillaceae	*Lactobacillus bombi* 98%	3.04	1.87	1.94	5.42	3.86	2.14	2.65	70.32
Bacilli	Lactobacillaceae	*Lactobacillus kimchicus* 99%	1.44	1.11	2.14	2.58	3.33	1.69	2.09	72.41
Bacilli	Lactobacillaceae	*Lactobacillus bombicola* 96%	1.14	1.94	1.02	0.48	3.98	1.66	2.05	74.46
Gammaproteobacteria	Enterobacteriaceae	*Rahnella woolbedingensis* 100%	0.66	1.29	3.95	0.02	0.05	1.35	1.67	76.12
Actinobacteria	Bifidobacteriaceae	*Bombiscardovia coagulans* 98%	1.63	1.24	1.29	1.20	3.08	1.20	1.48	77.60
Sphingobacteria	Saprospiraceae	Unknown	1.41	1.82	1.25	0.00	0.01	1.04	1.29	78.89
Bacilli	Planococcaceae	*Sporosarcina sp.* 99%	0.01	0.00	4.50	0.00	0.01	1.02	1.25	80.14

APPENDIX 9

GROWTH RATES OF LAB AND FIELD COLONIES (CHAPTERS 3, 4)

The average cumulative weight gain of colonies in each treatment over the duration of the experiment. Area around the average line represents ± 95% confidence interval.



APPENDIX 10

DADA2 PIPELINE SCRIPT (CHAPTERS 5, 6)

Sequence reads from Chapters 5 and 6 were processed using an amended version of the DADA2 pipeline to only look at forward reads (Callahan *et al.* 2016).

```
#Load DADA2 in R
source("https://bioconductor.org/biocLite.R")
biocLite("devtools"); biocLite("dada2")
library(dada2); packageVersion("dada2")
setwd("/raid1/scratch/sarwal/PollenQueen/FASTQ_all_files/")
path <- "/raid1/scratch/sarwal/PollenQueen/FASTQ_all_files"
list.files(path); list.files("filtered")

#Filter and Trim
fnFs <- sort(list.files(path, pattern="_R1_001.fastq"))
fnRs <- sort(list.files(path, pattern="_R2_001.fastq"))
#Extract sample names, assuming filenames have format: SAMPLENAME_XXX.fastq
sample.names <- sapply(strsplit(fnFs, "_"), `[`, 1)
# Specify the full path to the fnFs and fnRs
fnFs <- file.path(path, fnFs); fnRs <- file.path(path, fnRs)
#Examine quality profiles of forward and reverse reads
plotQualityProfile(fnFs[1:20], aggregate = TRUE)
plotQualityProfile(fnRs[1:20], aggregate = TRUE)

#Quality filtering, start by defining the file names where filtered fastq files will end up
filt_path <- file.path(path, "filtered") #Place filtered files in filtered/subdirectory
filtFs <- file.path(filt_path, paste0(sample.names, "_F_filt.fastq.gz"))
filtRs <- file.path(filt_path, paste0(sample.names, "_R_filt.fastq.gz"))
show(filtFs) #Only forward reads were processed after this point.

#Filter the forward reads using parameters.
out <- filterAndTrim(fnFs, filtFs, truncLen=c(290),
        trimLeft=c(0,0), maxN=0, maxEE=c(25), truncQ=2, rm.phix=TRUE,
```

```
        compress=TRUE, multithread=TRUE)
head(out); show(out)

#Learn and Visualize the error rates
errF <- learnErrors(filtFs, multithread=TRUE) #this will take about an hour
plotErrors(errF, nominalQ=TRUE) #Check that the data fits the predictions OK

#Dereplication, this combines all the identical sequences into unique sequences with
abundance values, along with a consensus quality profile of the unique sequence.
derepFs <- derepFastq(filtFs, verbose=TRUE) #takes approx 10min
names(derepFs) <- sample.names

#Apply the core sequence-variant inference algorithm to the dereplicated data.
dadaFs <- dada(derepFs, err=errF, multithread=TRUE)

#Construct sequence table and remove chimeric sequences
seqtab <- makeSequenceTable(dadaFs)
dim(seqtab) #Check table dimensions
seqtab.nochim <- removeBimeraDenovo(seqtab, method="consensus", multithread=TRUE,
verbose=TRUE); dim(seqtab.nochim)

#Identification
getN <- function(x) sum(getUniques(x))
track <- cbind(out, sapply(dadaFs, getN), sapply(mergers, getN), rowSums(seqtab),
rowSums(seqtab.nochim))
colnames(track) <- c("input", "filtered", "denoised", "tabled", "nonchim")
rownames(track) <- sample.names; head(track)

#Transpose, then write CSV and save with amplicon and/or run details
seqtab16S = t(seqtab.nochim)
write.csv(seqtab16S, file = "/raid2/scratch/sarwal/FASTQ/SeqTab16s.csv")

#Assign taxonomy using Greengenes database to assign 16S taxonomy
taxa16S <- assignTaxonomy(seqtab.nochim,
"/raid2/scratch/sarwal/RefDatabases/gg_13_8_train_set_97.fa.gz", multithread=TRUE)
unname(head(taxa16S)); write.csv(taxa16S, file = "/raid2/scratch/sarwal/FASTQ/16STaxa.csv")
```

APPENDIX 11

MEASUREMENTS TAKEN FROM POST-DIAPAUSE QUEENS (CHAPTER 5)

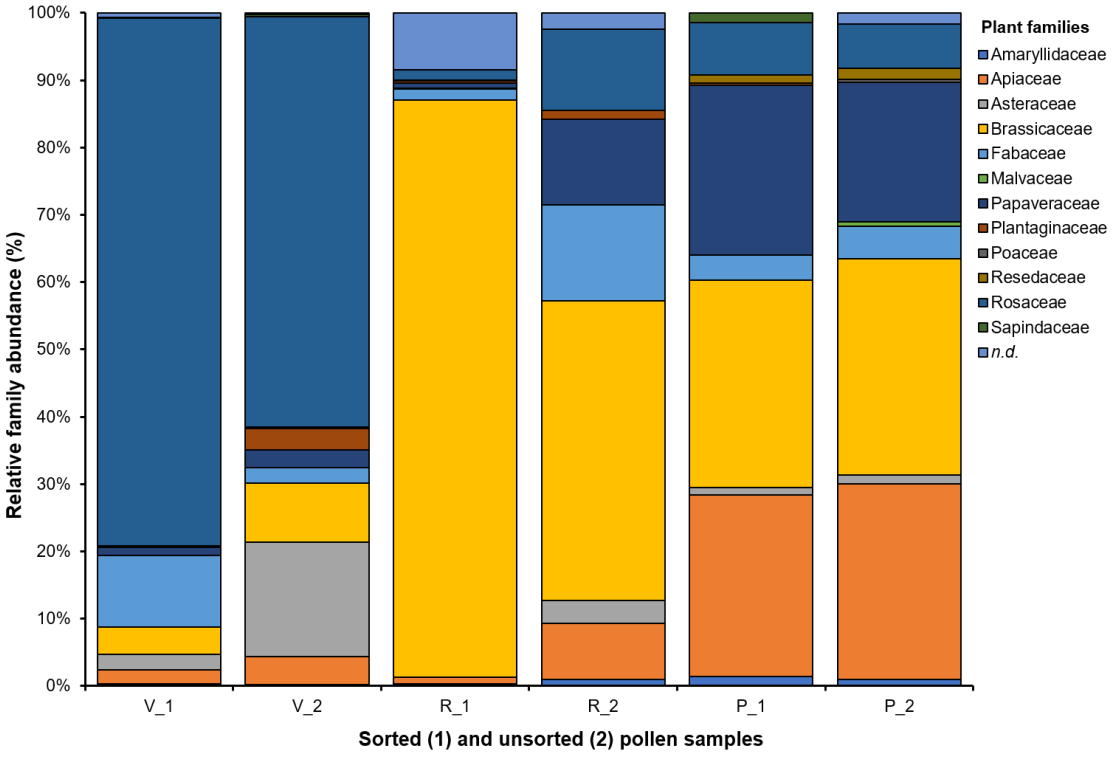
Post-diapause queen anatomical measurements indicated that there were no significant differences in wet weight, head width, or intertegular distance between 'healthy' and 'poor' condition queens.

	Treatment		Statistics	
Variable	**Healthy**	**Poor**	***t* value**	***p* value**
Head width (mm)	5.75	5.48	2.11	0.07
IT span (mm)	6.30	6.73	-1.66	0.15
Wet weight (g)	0.8947	0.7581	1.58	0.20
Gut weight (g)	0.1534	0.1672	-0.42	0.71

APPENDIX 12

POLLEN COMPOSITION BEFORE AND AFTER SORTING (CHAPTER 6)

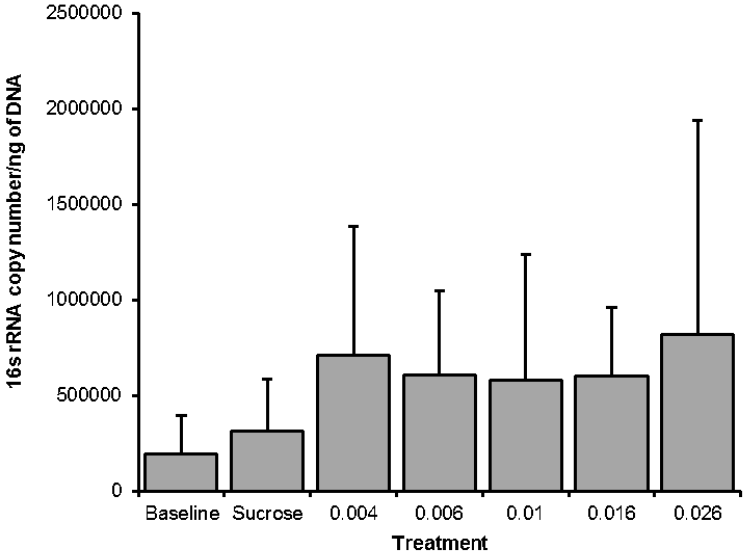
Commercial pollen blends were sorted by hand to reduce plant species diversity in each diet, which was then confirmed using high throughput sequencing. Diets with _1 were hand-sorted, while those with _2 were not.



APPENDIX 13

BACTERIAL 16S RRNA COUNT DATA (CHAPTER 7)

Bacterial copy number based on 16S rRNA copies averaged across all treatments in Chapter 7, showing no significant difference in bacterial copy number between treatments tested. Data generated was based on a subset (n = 48) of samples, using n = 7 samples per treatment, except in the sucrose control where only n = 6 were available for qPCR analysis.



APPENDIX 14

ESTIMATED WORKER FEEDING RATES (CHAPTER 7)

Estimated amount of sucrose consumed (in g) per bee for each treatment. Negative control and low dose treatments drank more than workers on the higher doses, suggesting an anti-feeding effect.