Causes and Consequences of Variation in the Nutrition and Endemic Microflora of Food Stores in Managed Honey Bees (*Apis mellifera* L.)

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Declaration

I herewith declare that this work has been originally produced by myself for the present thesis, and that it has not previously been presented to obtain a degree in any form. Collaborations with other researchers, as well as publications or submissions for publications are properly acknowledged.

Philip Donkersley

Lancaster University, August 2014

"To make a prairie it takes a clover and one bee,

One clover, and a bee,

And revery."

—Emily Dickinson

Abstract

Honey bees are pollinators, accounting for around 90% of commercial pollination of animal-pollinated plants and approximately 35% of global food production. Global populations of honey bees have declined significantly recently with heavy losses attributed to Colony Collapse Disorder, pesticides, parasites and pathogens. One of the factors that may be contributing to an increase in susceptibility to these stresses is the quality of food available in a hive. This thesis focuses on the interactions between honey bee nutrition, microbial communities and fitness.

In Chapter 2 the nutritional composition of bee bread (pollen stored inside hives) was studied. The composition in terms of protein and reducing sugar was found to vary both spatially and temporally; lipid and starch content was found to vary temporally through the season. The spatial trends in protein content were found to be associated with changes in landscape composition, as estimated by the Countryside Survey database. The implications for these findings are that certain landscape types may produce higher quality diets for honey bees.

In Chapter 3, the link between nutritional composition of bee bread and the species of plant that comprise it was investigated. Previous research indicates that pollens vary in their nutritional content and using molecular tools, we investigated the impact of complex plant communities in this system. The number of plant species in bee bread was positively correlated with increasing protein levels, and specifically certain individual plant species were found to be driving this pattern. These results indicate that a more diverse diet of plants will benefit honey bees by increasing their dietary protein intake.

The conversion of pollen to bee bread requires the activity of certain microorganisms. In chapter 4, we again used molecular tools to study the microbial

community found associated with bee bread. We found a community that was not significantly different between hives located in different areas, but which varied significantly in is composition through the beekeeping season. This suggests that the environment does not determine the bacterial communities in honey bee hives; rather it is being determined by seasonal changes.

Finally, in chapter 5 the relationship between the nutritional composition of bee bread and the immunocompetence of larval and adult honey bees was examined. The results showed that dietary protein and carbohydrate is significantly correlated with the overall fitness of a hive in terms of expression a constituent immune response. The link between landscape composition and nutrition established in chapter 2 was used to predict honey bee nutrition across the UK, and then was used to predict immune response for all UK bees. These predictions were comparable to honey bee disease records maintained by UK government.

This thesis provides a detailed examination of the effects of landscape composition on honey bee nutrition and immunity. The results presented here have implications for understanding spatial patterns in bee fitness and bee disease epidemiology.

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Finally, I would like to thank my supervisors, Ken Wilson and Kevin Jones for all their support and guidance. To Glenn Rhodes and Roger Pickup thanks for their kind and tough words that were dearly needed (and deserved) and without which, I would not have made it this far.

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Abbreviations

BLAST	basic local alignment search tool
C	Celcius
CCD	colony collapse disorder
cDNA	complementary DNA
CFU	colony forming units
cm	centimetres
CS	countryside survey
DGGE	denaturing gradient gel electrophoresis
dH20	distilled water
DHI	5,6-dihydroxyindole
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EDTA	ethylene diaminetetraacetic acid
ESRI	Environmental systems research institute
FERA	Food and environment research agency
g	grams
GLMM	generalised linear mixed effects model
GOX	glucose oxidase
h	hours
ha	hectares
HPG	hypopharyngeal gland
HRB	high react buffer
IC	immunocompetence

IPTG	isopropyl β-D-1-thiogalactopyranoside
kg	kilogram
L	litres
L-DOPA	L-3,4-dihydroxyphenylalanine
LB	Luria Bertani
LCM07	land cover map 2007
mM	millimolar concentration
M	molar concentration
mg	milligrams
μg	micrograms
ml	millilitres
μl	microlitres
mm	millimetres
NA	nutrient agar
NaCac	sodium cacodylate
ng	nanograms
NGS	next generation sequencing
nm	nanometres
OTU	operational taxonomic unit
P:C	protein:carbohydrate
PCR	polymerase chain reaction
PO	phenoloxidase
proPO	prophenoloxidase
R2A	low nutrient agar
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SSCP	single strand conformation polymorphism
ssDNA	single stranded DNA
TBE	tris-borate-EDTA buffer
TGGE	temperature gradient gel electrophoresis
TE	tris-EDTA buffer
UV	ultra violet
V	volts
v/v	volume/volume
w/v	weight/volume
WBC	William Broughton Carr
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Species list

Common dandelion	*Taraxacum officinale*
Maize	*Zea mays*
Mugwort	*Tanacetum vulgare*
Oil seed rape	*Brassica napus*
Padre's shooting star	*Dodecatheon clevelandii*
Plantain	*Plantago major*
Tobacco	*Nicotiana tabacum*
White clover	*Trifolium repens*
American foulbrood	*Paenibacillus larvae*
Asian nosema	*Nosema cerenae*
Chalkbrood	*Ascosphaera apis*
European foulbrood	*Melissococcus plutonis*
Western nosema	*Nosema apis*
African armyworm	*Spodoptera littoralis*
Black garden ant	*Lasius niger*
Buff tailed bumblebee	*Bombus terrestris*
Common fruit fly	*Drosophila melanogaster*
Mealworm beetle	*Tenebrio molitor*
Neotropical sweat bees	*Halictidae sp.*
Varroa Mite	*Varroa Destructor*
Western honey bee	*Apis mellifera*

Chapter 1. General introduction

National hives located near Burnley.

1.1 Background

1.1.1 Insect pollination

Globally, over 3000 plants are grown as food, of which only 12 currently supply 90% of the world's food supply (McGregor, 1976). These twelve are rice, wheat, maize, sorghums, millet, rye, barley, potatoes, sweet potatoes, cassavas, bananas and coconuts (Harrar, 1961). As these crops are mostly wind or self pollinated it may seem that insect pollinated crops play a minimal role in feeding the world. 70% of the remaining crop plants, including most fruits and vegetables are in fact insect pollinated (Klein *et al.*, 2007). Although these twelve food plants provide the world with basic carbohydrates, the key aspects of nutrition – protein, fat, minerals and vitamins – come from insect pollinated fruits and vegetables. Insect pollinated crops are therefore central to healthy diets (Calderone, 2012).

Insect pollination is both an ecosystem service provided by wild and managed pollinators and a production practice used by farmers for crop production (Gallai *et al.*, 2009). Wild pollinators, including bees, wasps and flies, and managed honey bees contribute significantly to the pollination of a large array of crops (Winfree *et al.*, 2008; Greenleaf and Kremen, 2006). Pollination does not only enable production of these crops, it also increases productivity of crop land and market value of final product. In 2008, the production value of non-insect pollinated crops averaged £130 tonne^{-1}, whilst the same insect-pollinated crops averaged £658 ton^{-1} (Gallai *et al.*, 2009).

Recently, global populations of insect pollinators have been in decline (Winfree *et al.*, 2009; Potts *et al.*, 2012; Thomas *et al.*, 2004) and pollinator

abundance has become a major limiting factor in many agroecosystems (Klein *et al.*, 2007). The suggested drivers of pollinator population decline include: agricultural intensification and habitat destruction (Steffan-Dewenter *et al.*, 2005; Steffan-Dewenter and Tscharntke, 2000; Kremen *et al.*, 2002), poisoning as a result of the use of chemical pesticides (Brittain and Potts, 2011; Thompson and Thorbahn, 2008), introduction of foreign species (Ghazoul, 2004; De la Rua *et al.*, 2009), dissemination of foreign pathogens and parasites (Cameron *et al.*, 2011) and climate change (Bale *et al.*, 2002).

1.1.2 Importance of honey bees

Honey bees are responsible for 90% of the commercial pollination of animal pollinated plants (Steffan-Dewenter *et al.*, 2005), which accounts for ~35% of global food production (Klein *et al.*, 2007). The economic value of the pollination service provided by honey bees is hugely variable and has been estimated to range from £190 million per annum in the United Kingdom (Knight *et al.*, 2009) to $14.5 billion per annum globally (Morse and Calderone, 2000; Pimentel *et al.*, 1980).

Recently, concerns have been raised of a global decline in populations of pollinating insects, such as solitary bees, bumble bees and honey bees (Abrol, 2012; Tylianakis, 2013; van Dooremalen *et al.*, 2013; Vidau *et al.*, 2011). As a result, the implications of a decline in pollinator populations on the services they provide to agricultural systems have been scrutinised. A decline in populations of pollinators at a time of rapid agricultural intensification may lead to a shortfall in pollination and a food production crisis (Holden, 2006). Contrary to popular belief, however, honey bee populations have not declined in the past 50 years, in fact they have increased by

approximately 45% globally (Aizen and Harder, 2009). However, in the same time period global agricultural production dependent on pollination by honey bees has increased by 300% (Aizen and Harder, 2009). Insufficient increases in honey bee populations are combining with increased challenges to honey bee health, leading to growing concerns over the future of pollination services. These challenges include land-use change and reduction in available foraging grounds (Naug, 2009), compromised immune systems (Evans and Pettis, 2005), agrochemicals (Alaux *et al.*, 2010a) and parasite introductions (van Dooremalen *et al.*, 2013); each of these issues can be linked to nutritional deprivation (Alaux *et al.*, 2010b; Mattila and Otis, 2006; Wahl and Ulm, 1983).

Since 2007 there have been large-scale, unexplained losses of managed honey bee colonies in the United States. This phenomenon was named Colony Collapse Disorder (CCD) because the main trait was a rapid loss of adult worker bees (vanEngelsdorp *et al.*, 2009). Although no single cause has yet been identified (Becher *et al.*, 2013), many potential triggers have been suggested including parasites such as infection by varroa mite (Martin *et al.*, 2012), which may linked to increased viral infection (Bromenshenk *et al.*, 2010; Runckel *et al.*, 2011). Agrochemical poisoning incidents have also been blamed (Mullin *et al.*, 2010; Farooqui, 2013), although these incidents are often more acute in nature than those associated with CCD (Thompson, 2010), with the exception of neonicotinoid pesticides (Bernal *et al.*, 2010).

1.1.3 Honey bee biology

Honey bees (*Apis mellifera* L.) are hymenopteran invertebrates, from the same order as sawflies, wasps and ants. The order Hymenoptera includes approximately 100,000 described species. Hymenoptera can be divided into two orders, Symphyta including the sawflies and wood wasps and the Apocrita, including the wasps, ants and bees. Within the Apocrita, honey bees are members of the family Apidae, which has approximately 200 genera (Gullan, 2010).

The European honey bee and its various subspecies (Table 1.1, (De la Rua *et al.*, 2009) are the most commonly managed bees in the world (vanEngelsdorp and Meixner, 2010). *Apis mellifera* originated in Europe from the African A lineage (Cornuet and Garnery, 1991), which has since split into the M lineage in North-west Europe (Ruttner *et al.*, 1978) and C lineage in Central and South-East Europe (Figure 1.1, (Kandemir *et al.*, 2006). The black honey bee, *A. mellifera* subsp. *mellifera*, has been present in England for over 4000 years, is currently the most commonly managed in this country (De la Rua *et al.*, 2009) and is therefore the focus of this study.

Table 1.1. The geographical distribution of the European honey bee *Apis mellifera* and its various subspecies. Lineages represent the theoretical origin of each group of subspecies

Geographic distribution	Historical Lineage	Sub species of *Apis mellifera*
Central Mediterranean sea and south-west Europe:	A-M	*ligustica, carnica, macedonia, sicula, cecropia*
Western Mediterranean sea and North-west Europe:	M	***mellifera**, iberica, sahariensis, intermissa*
Middle East:	C	*meda, adami, cypria, caucasica, armeniaca, anatolica*
Africa:	A	*intermissa, major, sahariensis, adansonii, unicolor, capensis, monticola, scutellata, lamarkii, yementica, litorea*

Figure 1.1. Approximate distribution of the most common *Apis mellifera* lineages and subspecies in Europe. Figure originally printed in De la Rua *et al.* (2009).

Honey bees are highly eusocial invertebrates and form colonies with distinguishable castes. Colonies of honey bees comprise populations of female workers, male drones and a single female queen (Free, 1966). Although female workers are physically identical, they perform different tasks within the colony as they age – a phenomenon known as age polyethism (Gullan, 2010). The youngest female workers are involved in the raising of the larval brood; older workers are then primarily associated with maintenance of the homeostatic hive environment and guarding the hive entrance; the oldest workers collect pollens and nectar from the environment (Figure 1.2).

A single female queen is responsible for egg production and controls the behaviour of her colony through the secretion of pheromones (Free, 1966; Morse and Boch, 1971). A queen may live up to 15 years, however as she ages, her ability to lay eggs and produce pheromones that control the hive decreases. An old, unhealthy or absent queen may induce her colony into swarming behaviour. Swarming results in the worker bees leaving their queen and colony with a large proportion of the food stores to found a new colony and raise a new queen (Ferrari *et al.*, 2008; Zeng *et al.*, 2005). Swarming is colony reproduction by fission and is the main process by which a colony will naturally relocate (Morse and Boch, 1971; Seeley and Buhrman, 1999).
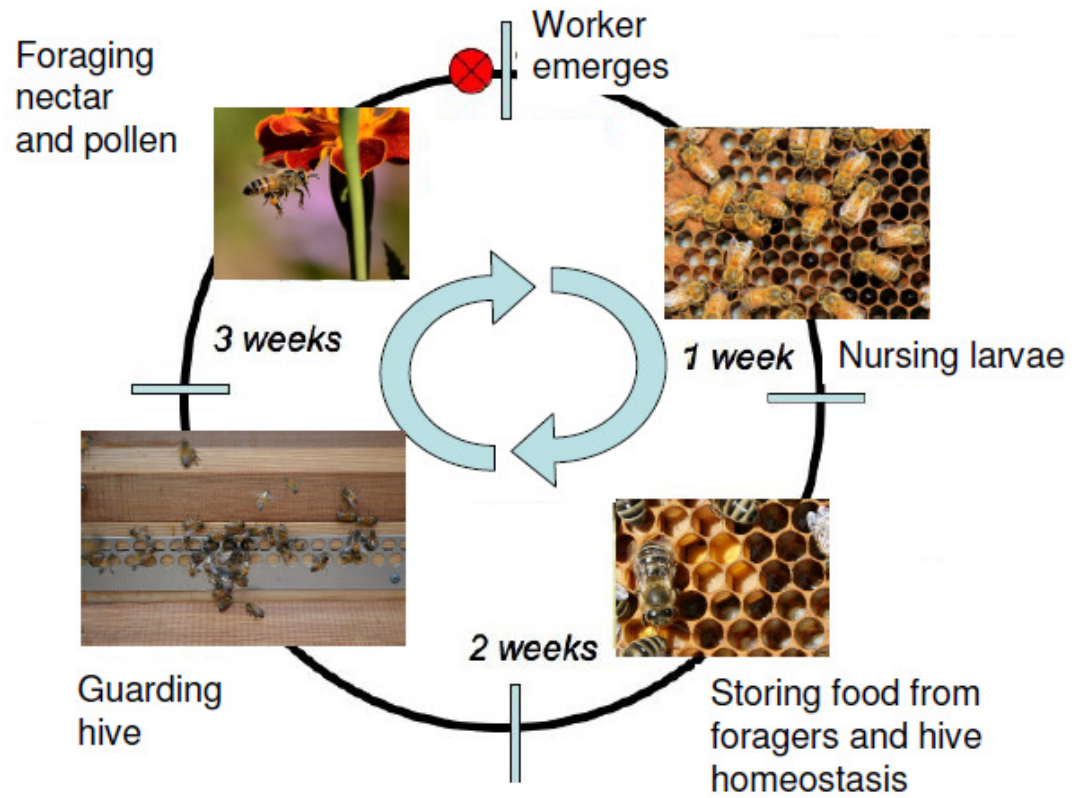
Figure 1.2. Schematic diagram of honey bee work task development. As worker bees age, the tasks they are assigned to within the hive change from nursing larvae, to hive maintenance and ending with foraging outside the hive.

1.1.4 Beekeeping

Honey bees have been domesticated and can be managed in hives by beekeepers. A hive may come in any of hundreds of variations in design and form. The most commonly used designs in the UK include National, WBC (named after its inventor William Broughton Carr), and Langstroth (Crane, 1999). These hives all follow the same basic design of frames of honey comb stored within multiple boxes which comprise the hive. Designs may differ in the size of the frame, boxes or outer walls, but maintain the same purpose of housing a single colony, governed by one queen.

The central tenet of beekeeping is the maintenance of a stable population of foragers to optimise both production of honey with year-to-year survival and minimal swarming behaviour. Beekeepers manipulate their hives through removal or introduction of extra space for a queen to lay eggs, changing positions of hives to optimise access to forage and removal of senescing queens to prevent swarming. The consequence of this is that managed honey bees differ greatly from wild colonies. However, both managed and wild colonies of honey bees are threatened by parasites, diseases and the other challenges mentioned previously.

Our understanding of honey bee nutrition, the extent of variation in nutrition and the processes that govern this variation is relatively limited. Nutrition has an important role in the health of honey bees and their survival (Brodschneider and Crailsheim, 2010). The potential future pollinator crisis requires us to investigate the roles nutrition may play in averting risks to food security (Woodcock *et al.*, 2013). By managing honey bees in a way that may promote better nutrition and therefore better

health, pollinator populations may be able to match the increasing agricultural requirements for pollination (Steffan-Dewenter *et al.*, 2005).

1.2 Honey bee nutrition

1.2.1 Nutritional requirements

Honey bee workers forage in their environment for pollen and nectar from plants they find. Both pollen and nectar are stored in cells on frames in hives and are sources of protein and carbohydrate (Camazine, 1991). The nutritional components vary in importance to members of the different castes within the hive. However, protein is an important factor to consider when discussing bee health (Oliver, 2007b). Protein is fed to the larval bees by nurse bees giving them protein derived from the pollen of the flowers upon which adult bees forage.

In addition to age polyethism in the tasks that worker bees are assigned to (Figure 1.2), their diets also change with age. Older adult worker bees abandon pollen consumption and subsist almost exclusively on carbohydrates sourced from nectar from flowers or honey from within the hive. Carbohydrates are necessary to generate heat to warm the hive and to fuel foraging flights (Oliver, 2007a). Forager bees do not require a high protein diet because they abandon brood rearing activities and are believed to survive on a high carbohydrate diet (Guzmannovoa *et al.*, 1994; Crailsheim and Stolberg, 1989; Moritz and Crailsheim, 1987).

As worker bees age and become foragers they lose the ability to digest protein, due to decreased protease activity in their guts (Grogan and Hunt, 1980). This may be linked to reduced replicative ability of intestinal stem cells, leading to reduction of

digestive ability in the intestinal tract (Ward *et al.*, 2008). Protease activity is limited in pupae and newly emerged bees, then increases rapidly in the first hours of the imago stage of development. The greatest levels of protease activity are found in larvae and in nurse bees (Moritz and Crailsheim, 1987). Enzymatic levels decrease as the workers begin foraging flights and remain low for the entirety of later adult life (Crailsheim, 1990). A healthy colony of honey bees comprises of members from all ages and the nutritional requirements of a colony are the sum of nutritional requirements of all the individuals within a hive. Whereas most of the carbohydrate comes from nectar (transformed and stored within the hive into honey), the proteins, fats, minerals, and vitamins come from pollen.

1.2.2 Sources of nutrition

A honey bee colony may need to collect up to 30 kg of pollen per year to meet its requirements for protein, lipids, micronutrients and other nutrients (Todd and Bishop, 1940, Free, 1966, Brodschneider and Crailsheim, 2010, McLellan, 1978). The amount collected by foragers often varies, depending on both worker foraging capacity and the availability of forage, ranging from 5.92 to 221.70 kg annum^{-1} hive^{-1} (Keller *et al.*, 2005b). Accurate estimates of the actual quantity of pollen collected by a colony are difficult to formulate as there are many factors that may bias the collection efficiency of pollen traps. Pollen traps are typically designed as boxes that cover the entrance of a hive with a mesh that allows foragers to enter, but which passively removes pollen sacs from their legs. Species composition of the collected pollen affects the average efficiency of pollen traps, varying between 33% and 60%, depending on the size of pollen grains collected from the forage (Levin and Loper,

1984). In addition, colonies may change their behavior in response to pollen trapping, by increasing their foraging effort (Levin and Loper, 1984, Webster *et al.*, 1985).

Honey bees differentially choose to collect certain pollen species over others and many studies have attempted to quantify which pollens are most regularly collected by honey bees (Free, 1963; Cook *et al.*, 2003; Solberg and Remedios, 1980). The most common pollen source found from studies within Europe was maize (*Zea mays*), with it occurring in the top 5 in 55 (48%) of 114 studies. Other pollens included white clover (*Trifolium repens*: 45%), common dandelion (*Taraxacum officinale:* 45%), plantain (*Plantago sp.* 41%) and oil seed rape (*Brassica napus* 40%) (Keller *et al.*, 2005b; Keller *et al.*, 2005a). These were far more abundant at the European scale of the studies, however at more local levels, some plants may be far more important to the colonies as food sources (Keller *et al.*, 2005a).

Bees preferentially forage for some pollen species over others, possibly because pollens vary in their nutritional composition (Roulston and Cane, 2000, Roulston *et al.*, 2000, Somerville, 2001, Somerville, 2005). Nutrients in pollen include protein, amino acids, starch, sterols, and lipids (Roulston and Cane, 2000). Some pollen species have been shown to contain very low levels of protein, with the lowest being 11.7% in the pollen of mugwort (*Tanacetum vulgare*: Asteraceae), whereas the greatest (61.7%) was found in padre's shooting star (*Dodecatheon clevelandii*: Primulaceae; (Roulston and Cane, 2000).

Although pollen is a primary part of their diet, honey bees do not possess the necessary enzymes to fully digest pollen grains. Many invertebrates that struggle to digest plant materials maintain a symbiotic relationship with specific microorganisms (Mueller *et al.*, 2001; Moran, 2007; Moran and Telang, 1998). Microbial communities associated with honey bee colonies may play a significant role in nutrition of a hive

(Kaltenpoth and Engl, 2014). Instead of consuming pollen in its raw state, honey bees mix pollen with nectar, enzymes from the hypopharyngeal gland and microbes from their guts, this mixture is then left to ferment for four to six weeks (Oliver, 2007ab). The resulting material has been called bee bread, and it forms the primary source of protein consumed by bees (Oliver, 2007a).

1.3 Bee bread

1.3.1 Nutritional composition

The nutritional composition of bee bread differs significantly from that of the pollens that comprise it. Pollen stores undergo a lactic acid fermentation to become bee bread (Herbert and Shimanuki, 1978): the fermentation of pollen results in a change in the pH value of bee bread – lowering from 6.8 to 4.2 as the fermentation occurs, which may provide a favourable environment for selective bacterial growth (Gilliam, 1979b). In addition to a change in pH, significant changes in the nutritional composition of stored pollen occur when they are converted to bee bread. The starch content is reduced and converted to less complex carbohydrates and water content is significantly reduced (Herbert and Shimanuki, 1978).

Since the nutritional value of pollens collected by foragers varies considerably, so it seems likely that the bee bread they become should also vary in nutritional composition (Somerville, 2005; Somerville, 2001; Roulston *et al.*, 2000; Roulston and Cane, 2000). Further examination of the differences in nutritional composition of bee bread produced from different mixtures of pollens is required. Honey bees foraging on different pollen sources should result in different diets being consumed in the hive.

There is evidence that different pollen spectra result in changes to fitness of bee colonies (Schmidt, 1984; Alaux *et al.*, 2010b). This further supports the need to establish the link between pollen source and bee bread chemical composition.

1.3.2 Importance of bee bread

Pollen abundance is not constant through the year (McLellan, 1978). In the same way that honey is used by bees as storage for carbohydrates, bee bread may be used as storage for protein. When fresh pollen supplies cease during winter, bee bread becomes the only source of protein for the hive. Protein is required to stimulate the queen to begin reproduction the following year and may be sourced primarily from bee bread. Year-on-year survival in honey bee colonies is traditionally thought to be determined by the volume of honey stores, which are required to maintain hive temperatures during winter. However, resumption of egg-laying by the queen is equally important in maintaining the hive population and foraging capacity after winter.

The conversion of pollen to bee bread may make pollen more easily digested by bees, by degrading the complex carbohydrate walls that surround pollen grains (Brodschneider and Crailsheim, 2010). The quality of diet insects consume has a direct effect on their fitness, most importantly their ability to survive infection by parasites or microbes. Honey bees that are infected with the microsporidian parasite *Nosema apis* and fed bee bread have a greater longevity than raw pollen-fed bees (Beutler and Opfinger, 1948). The immune system of honey bees has been shown to increase in efficacy as they are fed greater amounts of protein in their diet (DeGrandi-Hoffman *et al.*, 2010).

1.4 Microorganisms associated with honey bees

Honey bee colonies support a diverse assemblage of bacteria, fungi and protozoa (Figure 1.3, (Gilliam, 1979b, Gilliam, 1979a, Gilliam *et al.*, 1989) which are thought to be of significant importance to the functioning of the hive (Kaltenpoth and Engl, 2014). Specifically, these bacteria are theorised to be involved in the aforementioned process of converting pollen into bee bread through fermentation (Vásquez and Olofsson, 2009). The composition of microbial communities associated with bee bread production has been discussed extensively.

1.4.1 Culture based detection of microbial communities

Classically, research into the microbial communities of honey bees was based on culturing these organisms and estimating the community diversity based on these techniques. The earliest study on the microbial communities associated with honey bees was published in 1921. The study found the guts of honey bees to be dominated exclusively by "coli-like" bacteria using culture based techniques across England and Scotland (White, 1921). Further, Chevtchik (1950) used culture techniques to identify the progression of microbial communities in stored pollen to bee bread. Initially, the communities were found to be heterogeneous, made up of yeasts, bacteria and other assorted fungi. Lactic acid bacteria were detected after 12 hours, and were associated with increasing levels of lactic acid that lowered the pH of pollen and "killed off yeasts and other putrefactive bacteria". The study stated that after 1 week the

production of lactic acid was so great that it made bee bread "microbially sterile". Pain and Maugenet (1966) identified three microbial genera in bee bread: *Pseudomonas*, *Lactobacillus*, and *Saccharomyces*, supporting the results of previous studies (Chevtchik, 1950; White, 1921).

Further to this work on the diversity of microbial species, several studies were published based on samples collected in California regarding the diversity of yeast species and the bacterial genus *Bacillus* (Gilliam, 1979b; Gilliam, 1979a). The *Bacillus* species identified were *Bacillus megaterium*, *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. circulans* (Gilliam, 1979a). *Bacillus* organisms have the ability to produce lactic acid and acetic acid in silage (Woolford, 1977). However, they are less efficient than lactic acid bacteria in the production of lactic acid and may play a lesser role in lactic acid fermentation in bee bread (Gilliam, 1979a). The yeast species identified included *Torulopsis magnoliae*, *Cryptococcus flavus*, *C. laurentii*, *C. albidus* and *Rhodotorula glutinis* (Gilliam, 1979b).

Microbial community analysis that exclusively uses culture based techniques is limited in its ability to determine the entire community. Culture is selective by the nature of media used or growth conditions imposed (Stolp, 1988); these studies may have underestimated the diversity of microbial organisms associated with bees. From the culture based studies, the microbial communities associated with bees and bee bread are demonstrably variable between studies, but are primarily dominated by the Enterobacteriaceae, Bacillaceae and assorted Fungi, such as *Candida* or *Saccharomyces* (Figure 1.3a).

a

Enterobacteriaceae

Corynebacteriaceae

Bifidobacteriaceae

Bacillaceae

Apiosporaceae

Pseudomonadaceae

Pleosporaceae

Penecillium

Paenibacillaceae

Microbacteriaceae

Lactobacillaceae

Fungi
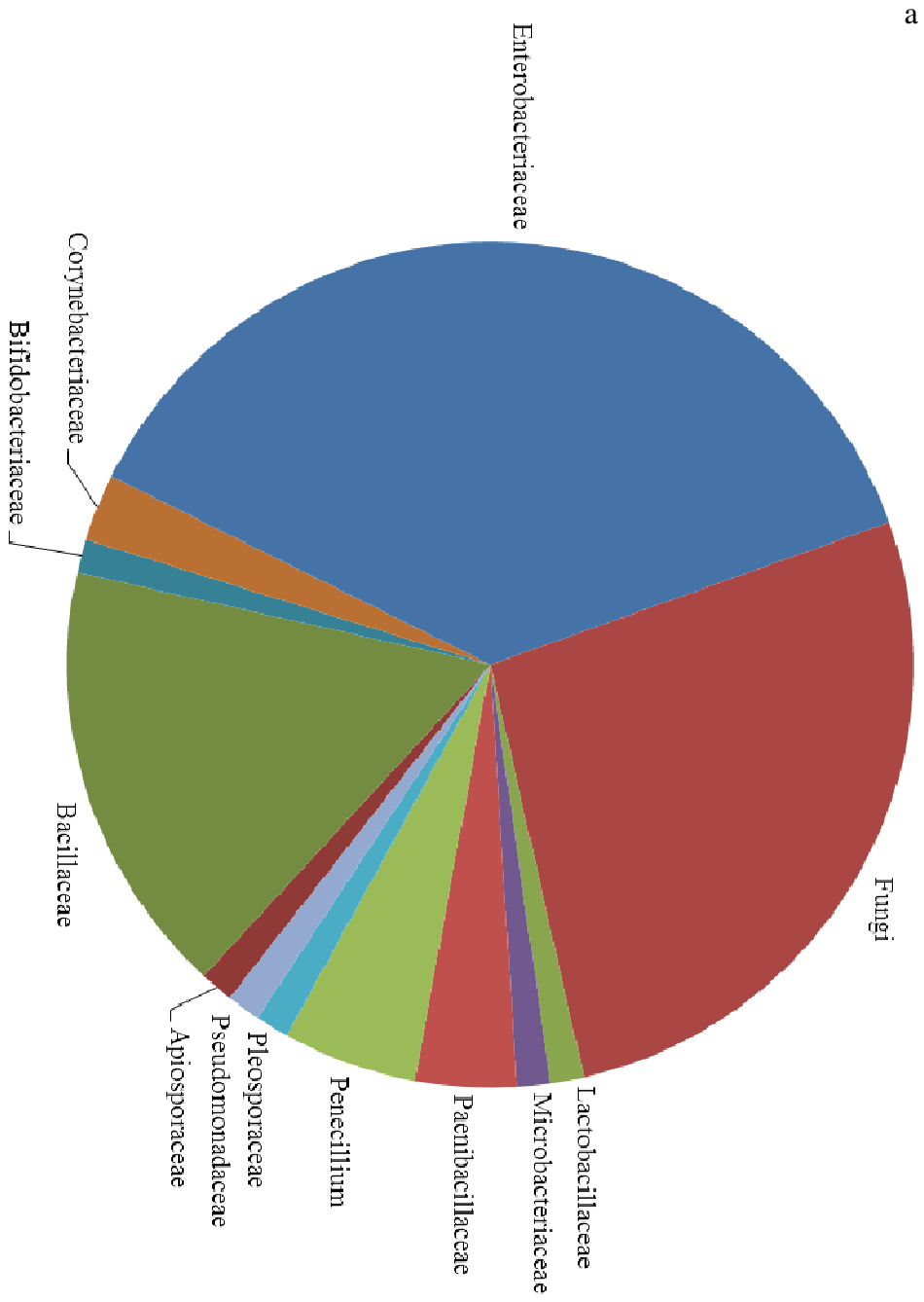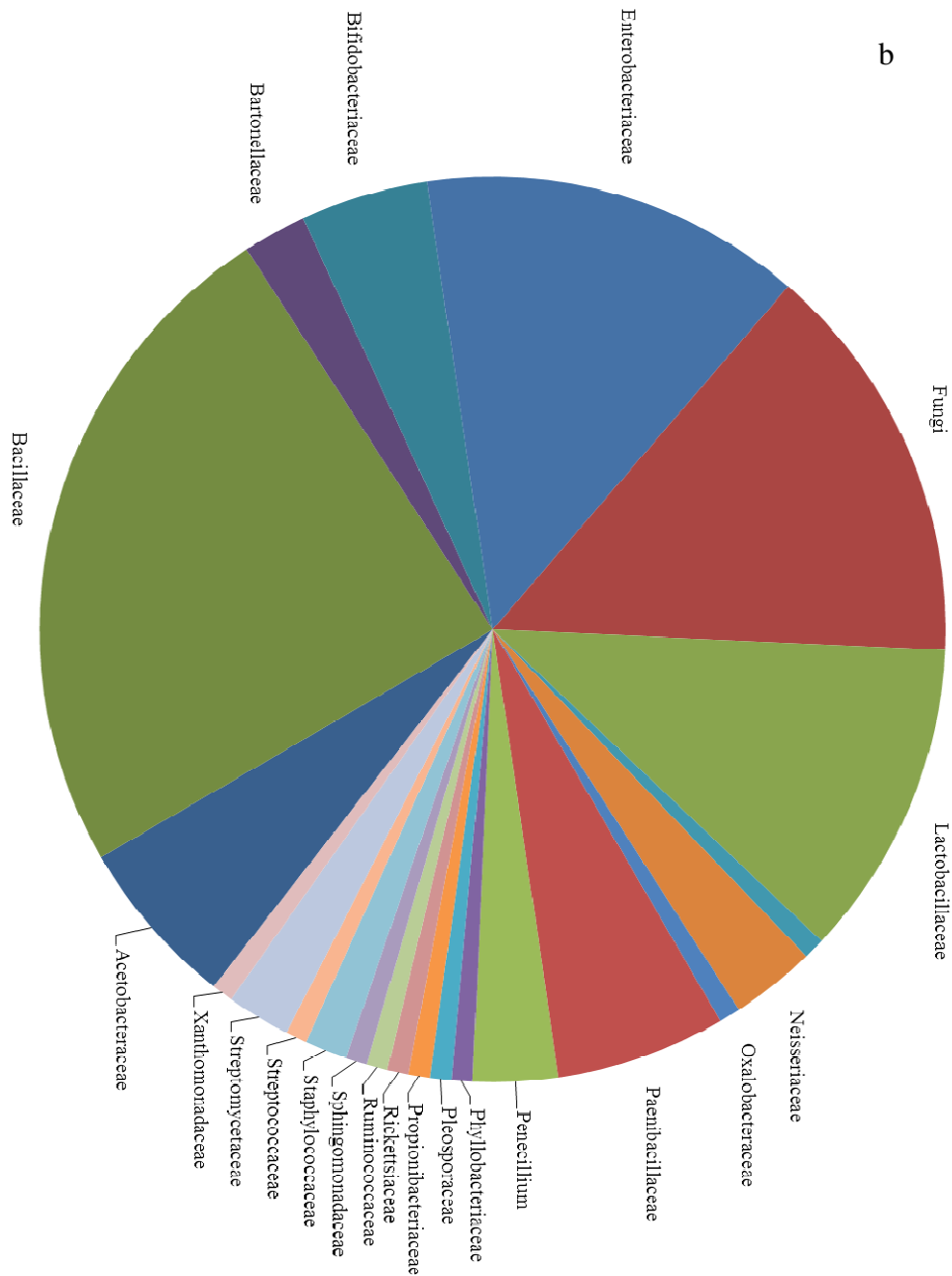
Figure 1.3. Results from a meta-analysis on records of microbial organisms found in studies of bee bread until 2012. (a). The frequency of organisms ordered to Family level from studies that used culture based techniques to examine microbial diversity (b) The frequency of organisms ordered to Family level from studies that used molecular techniques to examine microbial diversity. Fungal organisms, except *Penicillium*, were amalgamated into a single category, and viruses were excluded.

1.4.2 Molecular detection of microbial communities

Molecular techniques have become the primary method for analysis of microbial communities in associated with honey bees. . A comparison was made of the microbial communities detected using traditional culture-based methods (Figure 1.3a; (Gilliam, 1979a; Chevtchik, 1950; White, 1921) and those using more recent molecular techniques (Figure 1.3b; Forsgren *et al*., 2010, Kaznowski *et al*., 2005, Martinson *et al*., 2012, Mattila *et al*., 2012, Mohr and Tebbe, 2006, Olofsson and Vásquez, 2008, Runckel *et al*., 2011, Vasquez *et al*., 2012, Yoshiyama and Kimura, 2009, Alippi and Reynaldi, 2006, Vásquez and Olofsson, 2009). This revealed that using molecular techniques the diversity of microorganisms in association with bees was significantly higher than that detected using culture-based methods (Figure 1.3); although the majority of molecular technique based studies used single samples of bees to study the microbial community, which reduces the capacity to detect environmental variation. Communities detected using molecular techniques were shown to be dominated by *Lactobacillae*, *Enterobacteriaceae and Bacillae* (Figure 1.3b).

Vásquez and Olofsson (2008, 2009) examined the diversity of *Lactobacillus*, *Bifidobacteria* and *Pasteurelaceae* in bee bread using molecular techniques that selectively target these genera. Eight isolates in the *Lactobacillus* group were successfully detected, along with five isolates of *Bifidobacterium* and two of the *Pastueuralaceae*. By using selective techniques, this study was able to detect the sequences of 15 species in these three families. In comparison, across the rest of the studies included in the earlier comparison, 51 genera were identified across 22 families.

Despite the greater diversity of microorganisms found in bee bread using molecular methods compared with culture methods, the estimates presented here are likely underestimates of the true microbial diversity of bee bread (Dowd *et al.*, 2008). Next generation sequencing (Lee *et al.*, 2012, Schwartz *et al.*, 2011), has been used to study microbial communities of bee bread (Mattila *et al.*, 2012). This technique is capable revealing a wider diversity than culture or traditional PCR, and is particularly useful in mixed community DNA samples. (Matilla *et al.* (2012) identified organisms from 12 bacterial families in bee bread: Actinobacteria, Enterobacteriaceae, Enterococcaceae, Lactobacillaceae, Bacteroidetes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes, Staphylococcaceae, Ruminococcaceae and Rikenellaceae). The result of these studies is that we know a great deal about the nature of diversity in bee hives in general (Figure 1.3), but little about how this community develops or the temporal stability of it in the hive or about the community specifically associated with bee bread. Little research has been performed regarding the origin of these communities associated with honey bees; bee-specific lactobacilli have been identified and are believed to be largely maternally inherited within the hive (McFrederick *et al.*, 2012; McFrederick *et al.*, 2013). Other *Lactobacilli* are variable in their presence and may be regularly acquired from environmental sources such as floral nectaries or pollen (McFrederick *et al.*, 2012).

1.4.3 Potential interactions between microbial communities and host organism

Despite the relatively large number of studies that have focused on the diversity of the microbial community in honey bee colonies, the potential function of this diversity has received little attention. Limited studies have examined the potential of some species of bacteria isolated from bees to inhibit the growth of honey bee

pathogens, such as American foulbrood (*Paenibacillus larvae*) (Yoshiyama *et al*., 2013, Evans and Armstrong, 2005, Alippi and Reynaldi, 2006).

The impact of complex microbial communities on the biology of their hosts has been studied extensively in humans (Clayton *et al*., 2012, Flier and Mekalanos, 2009, Lozupone *et al*., 2012) and the importance of the gut microbiome has recently become an important field of study. In honey bees, the importance of the gut microbiome in modifying health is now beginning to be considered (Vasquez *et al*., 2012, McFrederick *et al*., 2012). When a colony of honey bees suffers colony collapse syndrome, it displays an increased susceptibility to disease and this may be due to a shift in microbial communities called dysbiosis (Hamdi *et al*., 2011, Mattila *et al*., 2012). The effects of dysbiosis and restoration to a stable microbiome through introduction of a complex community of microorganisms has been used in human medical trials as a potential treatment for *Clostridium difficile* infection (Lawley *et al*., 2012). By manipulating honey bee microbial communities in a similar manner, the effects of dysbiosis on honey bee colonies may also be alleviated.

Many organisms, in particular invertebrates, maintain bacterial symbioses when feeding on plant material. Most aphid species are associated with nitrogen-fixing bacteria which, in exchange for carbohydrates from their hosts, provide the aphids with the necessary amino acids to maintain growth and fitness (Mueller *et al*., 2001, Moran and Telang, 1998, Moran, 2001). Honey bees are not capable of effectively digesting pollen grains, and they are regularly found undigested in the gut of adult bees (Roulston and Cane, 2000). Pollen grains are surrounded by a complex wall of cellulose and sporopollenin. Honey bees possess the genes that encode cellulases (Kunieda *et al.*, 2006), but not the enzymes necessary to digest the (currently unknown) complex polysaccharides found in sporopollenin (Grienenberger

et al., 2010; Lallemand *et al.*, 2013). Upon storing pollen in cells on a frame, bees mix the pollen with nectar from their honey stomach, along with amylase, invertase and cellulose enzymes (Rinaudo *et al.*, 1973). The result of this reaction is the conversion of stored pollen into bee bread (Herbert and Shimanuki, 1978). The enzymes supplied by honey bees are not sufficient to digest the sporopollenin surrounding pollen grains. Some members of the microbial community associated with stored pollen may be responsible for providing the necessary biochemical pathways to digest sporopollenin. Specifically, these may be the organisms responsible for the lactic acid fermentation that occurs in cells of pollen (Pain and Maugenet, 1966, Oliver, 2007b, Brodschneider and Crailsheim, 2010, Ellis and Hayes, 2009, Lundgren and Lehman, 2010). The process of fermentation may be linked to the digestion of sporopollenin; microbial communities are believed to play a key role in production of bee bread, but digestive pathways and the identity of species responsible are currently unknown (Gilliam *et al*., 1988, Vásquez and Olofsson, 2009, Pain and Maugenet, 1966).

1.5 Immunology and fitness

1.5.1 The invertebrate immune system

Invertebrates are constantly being challenged by pathogens and parasites and have correspondingly developed anatomical, behavioural and humoral resistance mechanisms to protect themselves (Tanada and Kaya, 1993). Anatomical defences involve physical barriers to infection, such as the epithelia of respiratory system, digestive tract and body cuticle. In general, fungi, nematodes and arthropod parasites such as mites or parasitoids are capable of actively breaching these defences, although

bacterial, viral and protozoan infections can co-occur at the site of entry (Tanada and Kaya, 1993). Behavioural resistance mechanisms are most well studied for honey bees, they demonstrate hygienic behaviours, whereby infected larvae are uncapped and removed from the hive (Rothenbuhler, 1964; Evans and Spivak, 2010). Humoral immunity allows invertebrates to resist development of disease after successful colonisation by pathogenic or parasitic organisms. In studies of invertebrate immunology, the most regularly studied and most reliably quantified immune responses are typically humoral. The innate humoral immune system can be further distinguished into cellular and enzymatic responses (Gillespie *et al.*, 1997).

The cellular immune system includes cells known as haemocytes, which are cells that circulate in the invertebrate haemolymph, but are incapable of transporting oxygen. They have a variety of functions including blood coagulation, phagocytosis, encapsulation and detoxification (Rodríguez *et al.*, 1995). Encapsulation of invading material by haemocytes follows melanisation, and is used to exclude foreign material from the body (Siva-Jothy and Thompson, 2002). The enzymatic immune system response involves production of proteins or peptides that bind to invading material and produce toxic effects.

1.5.2 Enzymatic immune responses

Enzymatic responses include, but are not limited to the prophenoloxidase pathway, lysozyme and antimicrobial peptide production (Gupta *et al.*, 1985; Söderhäll and Cerenius, 1998; Alaux *et al.*, 2010b; Rheins and Karp, 1982). The prophenoloxidase system is constitutively expressed in invertebrates and is involved in phagocytosis, production of melanin and the encapsulation response (Asano and

Ashida, 2001). Phenoloxidase (PO) is a copper containing enzyme that catalyses oxygenation of mono-phenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones and ultimately to melanin (Ashida and Brey, 1995). The enzyme is highly reactive and is stored as an inactive precursor prophenoloxidase (pro-PO), the ratio of pro-PO to PO in haemolymph varies between different invertebrates (Ashida and Brey, 1995; Söderhäll and Cerenius, 1998). Pro-PO is activated by microbial cell wall components, such as peptidoglycan and zymosan, in the presence of Ca^{2+} via a serine protease cascade (González-Santoyo and Córdoba-Aguilar, 2012) Figure 1.4). The PO pathway is thought to be involved in recognition of non-self, as well as the encapsulation of larger organisms, it is central to an invertebrate immune response (Ashida and Brey, 1995; Söderhäll and Cerenius, 1998).

Lysozymes are glycoside hydrolase enzymes produced in haemocytes, pericardial cells and prothoracic glands (Gupta *et al.*, 1985). Lysozyme activity increases upon infection by a bacterial pathogen and continues to be high after the infection has subsided (Chadwick, 1970). Lysozyme on its own does not result in increased resistance to bacterial infection however, it is likely that complete destruction of bacterial cells requires the presence of antimicrobial peptides, such as cecropins, attacins and diptericins (Anderson and Cook, 1979; Tanada and Kaya, 1993). These immune-proteins are rapidly synthesised in response to infection, primarily in the fat body, and may persist for hours or days after infection (Boman and Hultmark, 1987).
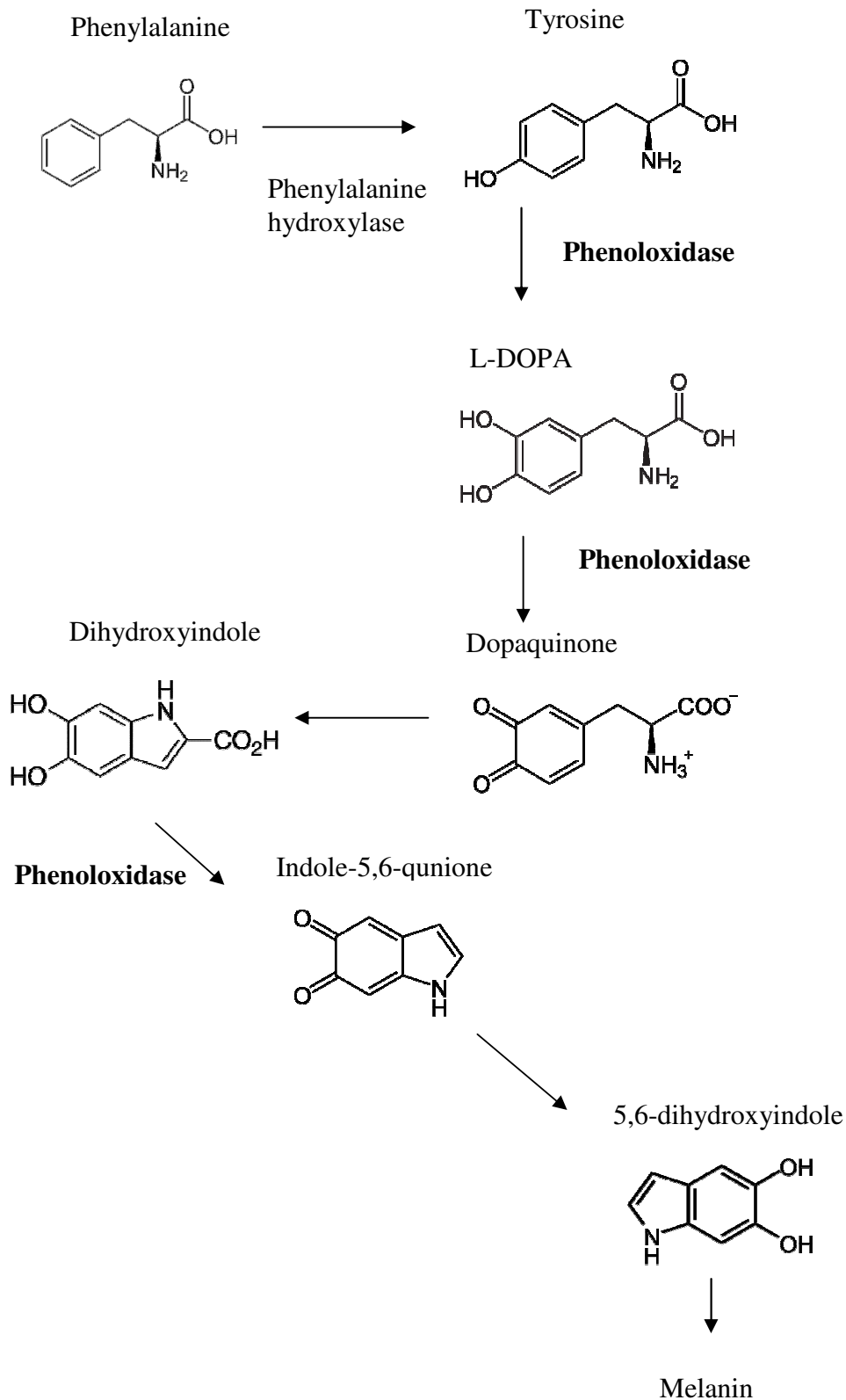
Figure 1.4. Phenoloxidase signalling pathway and PO role in melanin production.

PO participates in the formation of DOPA from Tyrosine (Tyr). PO also convert

DOPA to dopaquinone, and 5,6-dihydroxyindole (DHI) to indole-5,6-quinone.
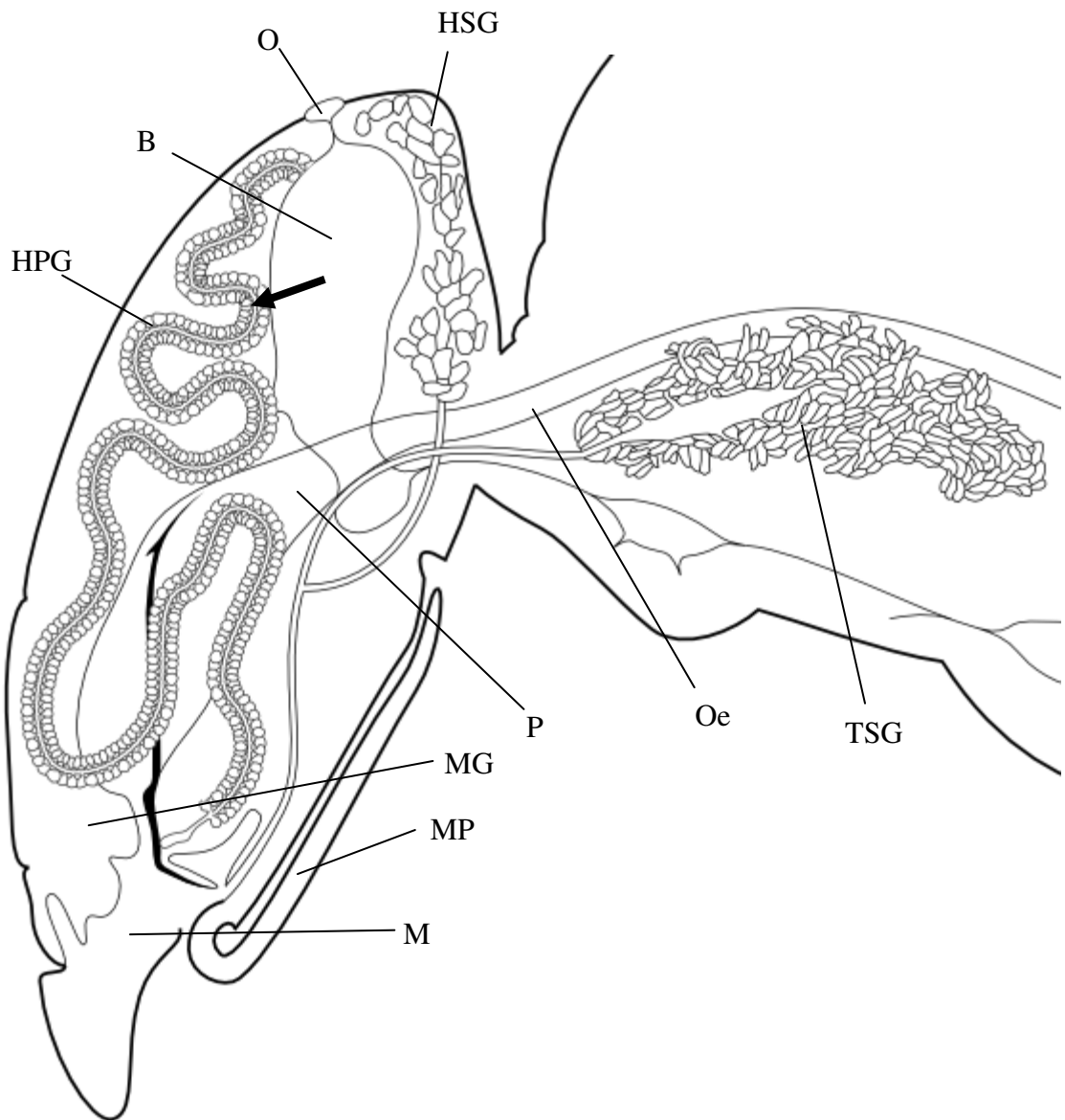
Figure 1.5. Schematic diagram of head of a honey bee worker; hypopharyngeal glands indicated by an arrow. B – brain; HPG – hypopharyngeal glands; HSG – head salivary glands; M – mouth; MG – mandibular glands; MP – mouthparts; O – ocellus; Oe – oesophagus; P – pharynx; TSG – thoracic salivary glands.

1.5.3 Honey bee immunity

In honey bees, the production of enzymatic immune responses is primarily focused in the hypopharyngeal gland (HPG), located in the head (Figure 1.5). The HPG is believed to be responsible for production of a large suite of enzymes, including glucose oxidase and phenoloxidase (DeGrandi-Hoffman *et al.*, 2010). As with other invertebrates, honey bees constitutively express phenoloxidase but they are unique in other aspects of their immunology. Honey bees possess a specialised immune pathway, known as the glucose oxidase pathway (GOX). GOX converts glucose to gluconic acid and hydrogen peroxide, which combined have antiseptic properties (Oliver, 2010). Like PO, this pathway is also believed to be expressed constitutively (Ohashi *et al.*, 1999). The products of GOX are applied to larvae directly through the mouthparts of nurse bees, where the antiseptic properties protect the larvae from opportunistic infection (Alaux *et al.*, 2010b; Oliver, 2010).

Immunosenescence is a systemic reduction of immune function as an organism ages, it occurs in vertebrates and invertebrates (Amdam *et al.*, 2005). In honey bees, immunosenescence drives a change in the suite of immune responses. As worker bees transition from nurse to forager stages, juvenile hormone titres increase (Amdam, 2011) and they lose the ability to produce haemocytes (Schmid *et al.*, 2008). The immune system of young nurse bees is more diverse and stronger than in older foragers (Wilson-Rich *et al.*, 2008; Amdam *et al.*, 2005). The immune gene pathways in adult forager bees are significantly up-regulated compared to adult nurse bees (Bull *et al.*, 2012).

1.5.4 Nutritional impacts on immunity

Invertebrates require sufficient nutritional input in order to maintain immune function, deficient nutrition can impair immune function and increase the susceptibility of individuals to disease (Alaux *et al.*, 2010b). Production of necessary enzymes or cells to elicit an immune response requires both protein and carbohydrate (Cotter *et al.*, 2010). A reduction in protein and carbohydrate in the diet has been shown to reduce the phenoloxidase response in mealworms (*Tenebrio molitor* L.), even under short term dietary restriction (Siva-Jothy and Thompson, 2002). Many studies have also examined the effects of infection on the nutritional intake of invertebrates. The protein requirements of immune responses are great, and it has been demonstrated in Lepidoptera that a host may actively alter its nutritional intake in response to infection by a parasite or pathogen (Povey *et al.*, 2009; Lee *et al.*, 2006; Povey *et al.*, 2014b). Depending on the extent of the infection, host species and quality of diet initially, protein may be more important than carbohydrate as a resource promoting host resistance against viral infection (Lee *et al.*, 2006). The success of an infection is also based on both the quantity and quality of amino acid content in the diet (Hoover *et al.*, 1998). Honey bees suffering from an infection by *Nosema cerenae* have an increased appetite compared to uninfected bees (Mayack and Naug, 2010). In order to actively compensate for the introduction of a pathogen, invertebrates should be able to detect the nutritional quality of a diet, not only in terms of protein content, but also amino acid composition (Cotter *et al.*, 2010; Paoli *et al.*, 2014). There is no consensus on the ability of honey bees to detect composition of their diets, with evidence existing both for and against (Bertazzini *et al.*, 2010; Kim and Smith, 2000).

Although the perception of diet quality is debated in honey bees, the effects of it on immunity have been studied. The effects of diet on the immune systems of honey bees have focused on the relationship between dietary protein, the hypopharyngeal gland (HPG, Figure 1.5) and the enzymes it produces. Increased dietary protein intake increases the development of the HPG in worker bees (DeGrandi-Hoffman *et al.*, 2010; Crailsheim and Stolberg, 1989), which in turn can lead to reduced viral load in the haemolymph (DeGrandi-Hoffman *et al.*, 2010). The expression of immune gene pathways is also up-regulated by *p*-coumaric acid intake, which may be one of the monomers of sporopollenin, and is found in both bee bread and honey stores (Mao *et al.*, 2013). Diets composed of several species of pollen may provide a more diverse source of amino acids. These diets have been shown to enhance some immune functions when compared with diets based on single species, in particular GOX activity (Alaux *et al.*, 2010b).

1.5.5 Interactions between non-pathogenic microbial communities and immune systems

A large proportion of microbial organisms associated with honey bees are not pathogenic (Cox-Foster *et al.*, 2007; Anderson *et al.*, 2013). Many of these organisms interact with honey bees on a regular basis without causing harm to their host. Recently, these interactions have been studied for their potential impact and benefit to the host immune system (Maynard *et al.*, 2012; Anderson *et al.*, 2011). Some microorganisms associated with honey bees have been studied for their antibiotic properties. Members of the *Lactobacillus* genus are regularly noted for their antibiotic activity, specifically against the pathogen, *Paenibacillus larvae*, causative organism of

American Foulbrood (AFB) in honey bees (Hammes and Hertel, 2006; Mudronova *et al.*, 2011; Vasquez *et al.*, 2012). The diversity of this genus, common occurrence across hives in different studies and the numerous accounts of their antibiotic activity (Mudronova *et al.*, 2011; Vasquez *et al.*, 2012; Forsgren *et al.*, 2010; Yoshiyama and Kimura, 2009; Yoshiyama *et al.*, 2013; Alippi and Reynaldi, 2006; Evans and Lopez, 2004) suggest that these organisms may play an important role in colony-level immunity.

Recently, the interactions of complex communities of microorganisms, rather than individual species within that community have begun to be considered. It has been suggested that, in humans in particular, the gut microbiome is involved in a protective layer of immunity against specific gut pathogens, such as *Clostridium difficile* (Robinson *et al.*, 2010; Walker *et al.*, 2011). Successful infections by this pathogen are associated with a shift in the composition of the microbiome in the gut. Methods to treat this disease by restoring the previous microbiome show promise (Borody and Khoruts, 2012); similarly, restoration of microbiota have been shown to be effective in treating chytridomycosis in amphibians (Bletz *et al.*, 2013). A healthy, stable microbiome in honey bees has been studied extensively, and it is theorised that shifts in this microbiome may be linked to increased susceptibility to disease, as with humans (Hamdi *et al.*, 2011). The trigger for such a shift in microbiome in honey bees has not been identified currently however.

1.6 Study site

Honey bee hives are maintained by beekeepers across the UK. The research presented in this thesis was focused on hives in the North-west (SD362272/SD926048/SD625210/SD486889) and West Midlands regions (SO463454/SO478444/SO469442/SO471452) of the UK. The hives in the North West UK were located across the counties of Lancashire and Cumbria and were maintained by either hobbyist beekeepers, breeding sites for maintaining local genetic lineages of bee or maintained as part of training suites for local beekeeping associations. The hives in West Midlands UK were located near the city of Hereford and were maintained as part of a commercial apiculture operation. These hives were selected because they were maintained in high density apiaries, with up to 90 hives located in a single apiary.

The North-west UK includes natural areas such as the Forest of Bowland and Morecambe Bay. The geography is primarily dominated by natural grasslands and improved grasslands (Carey *et al.*, 2008b), which are maintained for the purposes of pastoral farming. Hereford (West Midlands UK) includes natural areas such as the Wye Valley and Malvern Hills. The habitat used in this thesis was primarily apple orchards and other broadleaf woodlands (Carey *et al.*, 2008b).

1.7 Hypotheses

This thesis addresses the significant gap of knowledge linking the environment to bee nutrition to fitness. Here, the factors that influence the composition of diets honey bees are collecting are examined, with an aim to investigate the environmental composition of land surrounding hives, the plant species composition that bees are foraging from and the composition of the microbial community associated with their food stores. Based on previous research on the nutrition values of pollen, I predict that certain plant species with pollens high in protein content will be associated with particular landscape compositions and therefore, these landscapes will correlate with higher protein content food stores.

Further, this thesis will also provide evidence for the debate on whether monofloral and polyfloral diets benefit bees greater. Additionally, this thesis will examine the microbial community associated with bee bread food stores in a more detailed manner than has been attempted previously. With these data, I predict that this thesis will establish the core bacterial species present in the bee bread and will determine whether certain bacteria are constantly present in bee bread throughout the year.

Finally, these factors influencing honey bee nutrition will be collated and correlated with the immune function of honey bees. Based on previous research linking nutrition to immune function in bees, I predict that this thesis will find that even given the variable quality of environments that bees are exposed to; there will be a reliable response of immune function to bee bread nutritional composition.

1.8 Thesis aims and structure

The overall objective of this thesis is to examine the patterns in spatial and temporal variation in the biological and chemical properties of bee bread and how these variations may further influence the biology of honey bees.

In Chapter 2, the spatial and temporal variation in the nutritional content of bee bread is examined at a regional scale. The variation in nutrition is compared with variation in the landscape composition, which has been used as an estimate of environmental effects on honey bee nutrition.

Following the examination of the effects environmental composition may have on the nutritional content of bee bread, in Chapter 3 the floral resources available under these constraints are examined. The nutritional composition of bee bread produced from different species of pollen was examined and the effect of floral diversity on the nutritional content of pollen was determined.

In Chapter 4, the community of microorganisms associated with bee bread is examined. The spatial and temporal variation of the complexity of these communities was analysed and the species present were identified using molecular techniques.

In Chapter 5, the relationship between a honey bee immune response (phenoloxidase activity) and the nutritional composition of their diet in the hive was analysed. The work here expanded on previous laboratory based experiments in the area of honey bee nutritional immunology. These data were integrated with the data collected in Chapter 2 on the spatial variation in bee bread nutritional content in order to assess the effects of nutritional variation on variation in immune responses and therefore health at a regional scale.

By examining the effects of nutrition on the various levels of honey bee biology in a combined study, this thesis attempts to examine how each of the aspects studied here interact to determine the health of bees (Figure 1.6). Many of these aspects of nutritional constraints and effects have yet to be studied and may be provide new insights into our understanding honey bee biology.
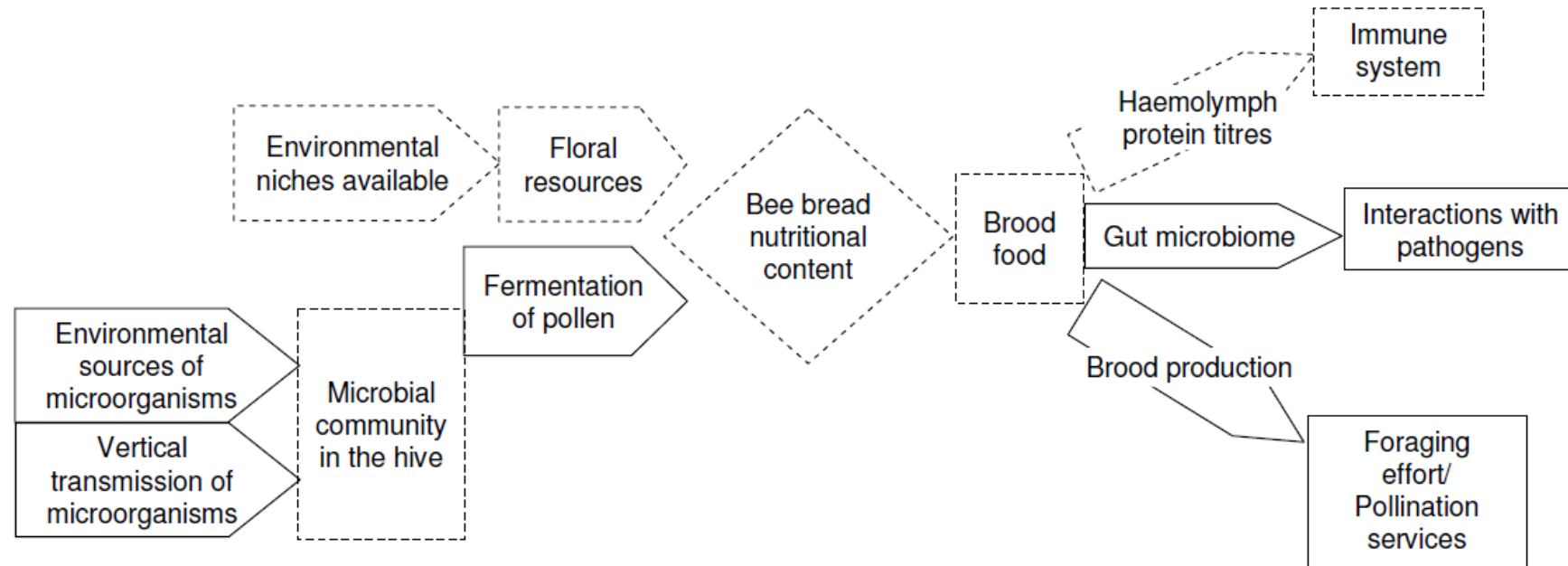
Figure 1.6. Aspects of honey bee biology that can be influenced by nutrition. The nature of bee nutrition can be constrained by abiotic factors derived from the environment around a hive and biotic factors derived from the bacterial assemblages transmitted vertically and from the environment. The dashed boxes are those which were analysed as part of this thesis.

Chapter 2: Honey bee nutrition is linked to landscape composition: implications for land use change

A view from Warton Crag, overlooking some of the sample sites in Lancashire.

2.1 Abstract

Honey bee nutrition is dependent on floral resources (i.e. nectar and pollen), which are linked to landscape composition. Therefore, land use surrounding hives may impact on bee nutrition. Pollen is fermented in honeycomb cells to become bee bread and is the primary source of protein to a honey bee colony. Here, I have presented a stratified analysis of the chemical composition of bee bread in managed hives with a view to examining potential sources of variation in its nutritional composition. Specifically, I tested the hypothesis that bee bread composition correlates with local land use and available floral resources.

The results demonstrated that the starch, lipid and moisture contents of bee bread are all highly conserved across hives, whereas levels of protein and reducing sugar vary significantly both temporally and spatially. Protein and non-reducing sugar increased as the year progressed, reducing sugars however, decreased during the first half of the year and then increased towards the end.

Local land use around hives was quantified using data from the Countryside Survey 2007 Land Cover Map. Bee bread protein content was negatively correlated with increasing levels of arable and horticultural farmland cover surrounding hives and positively correlated with the cover of natural grasslands. Reducing sugar content was positively correlated with the amount of broad-leaved woodland within a 3 km² radius of the hives.

Previous studies on a range of invertebrates, including honey bees, indicated that dietary protein intake may have a major impact on correlates of fitness, including longevity and immune function. The finding that bee bread protein content varies with land use suggests that landscape composition may impact on insect pollinator

wellbeing, and provides a link between landscape and the nutritional ecology of socially foraging insects in a way not previously considered.

2.2 Introduction

The honey bee, *Apis mellifera* L., is a eusocial insect with a global distribution. Honey bees provide one of the most important ecosystem services in agriculture, accounting for around 90% of commercial pollination of animal-pollinated plants, translating to approximately 35% of global food production (Steffan-Dewenter *et al.*, 2005; Klein *et al.*, 2007). Global populations of honey bees have been subject to heavy losses attributed to Colony Collapse Disorder, pesticides, parasites and pathogens (Cox-Foster, 2007; Behrens *et al.*, 2010; Mullin *et al.*, 2010; Martin *et al.*, 2012). It is therefore essential that the causes of global pollinator declines are investigated.

One of the factors that may be contributing to an increase in susceptibility to these stresses is the quality of food available in a hive (Naug, 2009). Different worker castes within the hive have distinct nutritional requirements, with honey bee larvae primarily requiring protein (Ward *et al.*, 2008) and adult honey bees mostly needing carbohydrate and a lesser amount of protein (Mayack & Naug 2010). Most of the carbohydrate required by a colony comes from nectar (transformed into and stored as honey), whilst the proteins, fats, minerals, and vitamins are all derived from pollen. Protein is used by nurse bees to produce brood food, which they use to nurture larvae within the hive (Oliver, 2007b). In the same way that nectar is converted to honey, pollen is converted to "bee bread". Workers return to the hive and store pollen pellets in cells along with honey, nectar and glandular secretions, which then undergoes lactic acid fermentation to become bee bread. The nutritional content of bee bread has rarely

been examined and previous studies have been limited in sample size and so fail to capture the potential variation in bee bread nutritional value (e.g. Herbert and Shimanuki (1978).

Although protein is the primary nutritional constituent of bee bread, the nutritional composition of bee bread is multifaceted and variable. Bee bread contains protein, reducing sugars (e.g. monosaccharides, glucose), non-reducing sugars (e.g. polysaccharides, sucrose), starches, lipids and fibre content (Herbert and Shimanuki 1978). Bee bread also contains numerous micronutrients (e.g. Fe, Mn, Mg, Na etc.) and vitamins necessary for larval development (Mattila and Smith, 2008; Morgano *et al.*, 2012).

The nutritional content of pollen varies depending on the species of plant from which it derives. Previous studies suggest that pollen protein content varies from around 2.5% (*Solanum sp.*: Solanaceae) to 62% (*Dodecatheon clevelandii*: Primulaceae; Buchmann 1986; Roulston & Cane 2000). The availability and diversity of forage varies with the local landscape composition, the nutritional content of pollen and the foraging preferences of the insects (Keller *et al.*, 2005a). Pollen provides an essential raw material for bee bread, and pollen species vary in their nutritional compositions. Therefore, I predicted that bee bread would vary in nutritional composition depending on the local and seasonal availability of pollens from different plant species.

The nutritional composition of diets is far more important to consider than the volume of food consumed; although an animal could consume more food to acquire a nutrient in short supply, this means they consume an excess of other nutrients, and this may be harmful (Simpson & Raubenheimer 2012). Total consumption of a diet may

be misleading; therefore this study considers the relative proportions of various components of the honey bee diet in addition to overall quantity of key nutrients.

In the present study, I used a stratified sampling approach to examine the nutritional composition of bread samples collected from hives from across the North-west of England. By collecting multiple samples of bee bread within and among hives throughout the honey bee foraging season, we were able to partition variation in bee bread nutritional composition both spatially and temporally. Specifically, I tested the following hypotheses: (i) bee bread nutritional composition will vary both within and between hives due to spatial and temporal variation in the availability of floral resources and/or the changing needs of the colony; and (ii) geographical variation in bee bread composition will correlate with local land use surrounding the hives, as this is a key determinant of the flowering species available.

2.3 Materials and Methods

2.3.1 Bee bread sampling

Individual cells of bee bread were obtained from 35 hives from within 20 apiaries (a site of several hives) distributed across 3000 km^2 of the North-west England (Figure 2.1). Individual hives were sampled once every 8 weeks from 7th April to 2nd September 2012. All of the hives were owned by either hobbyist beekeepers, a commercial beekeeper, or maintained as part of the training suites for local beekeeping associations. All beekeepers maintained colonies of *A. m. mellifera*.

Stratified sampling within-hives (internal variation) and between-hives (external variation) was used to partition variation in bee bread composition at different spatial scales. The hives in this study were structured in a nested fashion

whereby honeycomb cells covered space on frames (Figure 2.1). These frames were stored in connected boxes (usually two) which comprise a single hive. The number of hives sampled from each apiary is shown in Figure 2.1. Cells were extracted from each of two frames within a brood chamber, from multiple brood chambers within a hive (if present), and from each of up to two hives within an apiary (Figure 2.1). Bee bread was recovered from cells aseptically into sterile 1.5 mL microfuge tubes from three individual cells (with minimal disturbance to neighbouring cells). Samples were transferred to the laboratory on ice and processed within two hours.
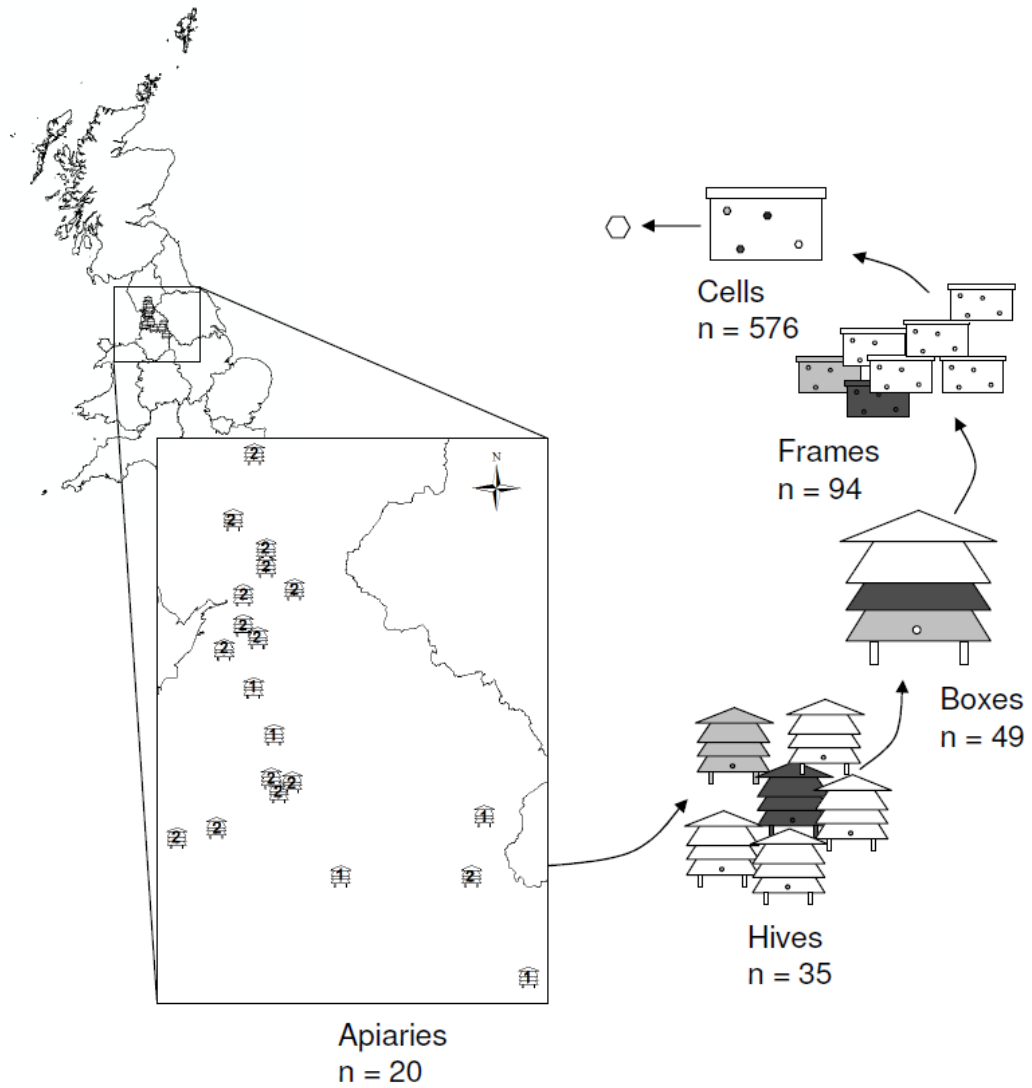
Figure 2.1. Schematic diagram of the stratified sampling technique used to sample apiaries in the North-west of England. The location of apiaries (n = 20) is highlighted by the hive drawings which in turn have number of hives sampled at each (either 1 or 2) inside. In total 576 cells were sampled for bee bread, which were obtained from 94 frames, held in 49 boxes from 35 hives across the 20 apiaries.

2.3.2 Nutritional analysis

Nutritional content of plant material may be analysed through biochemical assays. The results of these assays produced a repeatable colourimetric change that was measured using spectrophotometry. The nutritional content of bee bread was estimated by a series of spectrophotometric chemical analyses using a VERSAmax™ Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA) using Softmax® Pro v4.7 software for Windows®. The following bee bread constituents were analysed for each sample: protein, reducing sugars (e.g. glucose), non-reducing sugars (e.g. sucrose), starch, lipid and moisture. Both reducing and non-reducing sugars were considered because previous studies have shown that they vary consistently independently (Herbert and Shimanuki, 1978). Negative controls were maintained using each of the reaction buffers. Methods for the chemical analysis for each of the constituents are briefly described below:

Proteins: The free protein content of bee bread was estimated using the Biuret reaction (Gornall *et al.*, 1949; Sapan *et al.*, 1999). The Biuret reaction involves introducing Cu^{2+} ions from a solution of hydrous copper sulphate and potassium sodium tartrate in potassium hydroxide ($KOH \cdot CuSO_4 \ 6H_2O \cdot \ KNaC_4H_4O_6 \cdot 4H_2O$) to protein solution resulting in a violet-purplish colour when the cupric ions (Cu^{2+}) interact with peptide bonds under alkaline conditions. The major advantage of this technique is that there is no interference from materials that absorb at lower wavelengths, and it is less sensitive to protein type because it utilizes absorption involving peptide bonds that are common to all proteins, rather than specific side groups as in the Bradford-Lowry method. It is also much faster and safer, and therefore more repeatable than the Kjeldahl method (Sapan *et al.*, 1999; Vanderplanck *et al.*, 2013). Here, 10 µg (wet weight) of bee bread was incubated in 200 µL Biuret

solution for 30 min at room temperature. Absorbance was read at a wavelength of 550 nm, using bovine serum albumen as a standard.

Reducing & non-reducing sugars: Reducing and non-reducing sugar contents were estimated using the dinitrosalicylic acid (DNS) reaction (Lees, 1971). This method tests for the presence of free carbonyl group (C=O) in reducing sugars. This involves the oxidation of the aldehyde functional group (R-CHO) present in glucose and the ketone functional group (RC(=O)R') in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions (Lees, 1971; Miller, 1959). Here, 20 µg (wet weight) of bee bread was incubated in 200 µL DNS for 15 minutes at 95 ℃. Non-reducing sugar content was also estimated using the DNS reaction, with an additional digestion step using 100 µL 1M invertase enzyme solution in sodium acetate buffer (pH 5.0) for 5 minutes at 55 ℃. For both reducing and non-reducing sugar analysis, absorbance was read at a wavelength of 575 nm.

Lipids: Lipid content was estimated using phosphoric acid-vanillin analysis colorimetry (Cheng *et al.*, 2011). Samples were prepared in a chloroform-methanol solvent, which was then evaporated. Samples were then reacted with sulphuric acid, which causes oxidation of the lipids to produce carbonium ions (C+-C=C-R'). This solution is then reacted with a vanillin-phosphoric acid solution ($C_8H_8O_3 \cdot H_3PO_4$) to produce 1,1-di(4-hydroxy-3-methoxyphenyl)ethylene ions (Johnson *et al.*, 1977). Here, 5 µg (wet weight) of bee bread underwent lipid extraction using 500 µL 2:1 chloroform:methanol solution. The lipid layer was removed and added to 100 µL 20M sulphuric acid at 80 ℃ for 15 minutes, followed by 2 minutes on ice. Finally, 100 µL vanillin–phosphoric acid reagent (400µg vanillin per ml 34% phosphoric acid) was

added and left for color development for 10 min. Absorbance was read at a wavelength of 540 nm.

Starch: Although starch has been reported in a previous study as absent in bee bread (Herbert and Shimanuki, 1978), advances in sensitivity in starch detection methods (McCleary *et al.*, 1994) warranted its inclusion in nutritional analysis. The method used to determine starch was the Association of Official Analytical Chemists (AOAC) method 996.11 (McCleary *et al.*, 1994), which uses multi-stage starch hydrolysis to determine the total content of a given sample. In phase 1, starch is partially hydrolysed and totally solubilised. In phase 2, the starch dextrins are quantitatively hydrolysed to glucose by amyloglucosidase. Here, 50 µg (wet weight) bee bread was analysed using the AOAC method 996.11 starch analysis kit (Megazyme, Ireland), following manufacturers specifications (McCleary *et al.*, 1994; Megazyme, 2006). Absorbance was read at 510 nm.

Moisture: Moisture content of bee bread samples was determined by placing 100µg homogenized bee bread in a drying oven at 100 ºC for 24 hr to a constant mass. Moisture content was estimated as the difference in mass between wet and dried samples.

2.3.3 Land cover composition estimation

In order to estimate the correlation between land cover and honey bee nutrition, data were sourced from the Countryside Survey Land Cover Map 2007 (Morton *et al.*, 2011). The composition and configuration of different land cover classes (Steffan-Dewenter *et al.*, 2002; Kleijn and van Langevelde, 2006) within three radial buffer zones (defined as circular areas comprising the landscape surround each hive in the study) around each hive was used. The primary buffer zone for analysis

was 3 km in radius. Honey bee foraging is most efficient at 3 km (Visscher *et al.*, 1985; Visscher and Seeley, 1982; Steffan-Dewenter and Tscharntke, 2000), but they are capable of foraging up to 10 km from the hive (Seeley, 1986). Only 10% of the bees forage within 0.5 km of the hive, 50% forage at more than 6 km, 25% more than 7.5 km and 10% more than 9.5 km from the hive (Beekman and Ratnieks, 2000). To test for potential localised effects around each hive, we included an inner buffer zone of 0.5 km and a 10 km buffer zone to test beyond the scale of the study described by Steffan-Dewenter *et al.* (2002). Land cover classes that accounted for >0.5% of total cover within a buffer zone were excluded from analysis, at 0.5 km 8 classes were included, at 3 km 14 classes and 14 at 10 km.

2.3.4 Statistical analysis

The effects of the spatial and temporal variation on the nutritional constituents of bee bread were assessed using a series of generalised linear mixed-effects models (GLMMs) using "*lme4*" package (Bates *et al.*, 2012) in the *R* statistical software v3.0.0 (R Core Team, 2013). The extent of internal variation at a nested hierarchy of spatial scales was analysed. The scales included were within-frame, within-hive box, within-hive, within-apiary and between-beekeepers. The variation was analysed by including a series of random effects in the model (*1|Apiary/Hive/Box/Frame*) to account for hierarchal variation in sampling, and (*1|Block*) for the sampling triplicate through the season (Bolker *et al.*, 2009; Bates *et al.*, 2012). Confidence intervals for random effects were generated using Chi squared tests on residual maximum likelihood estimates (Zuur, 2009).

Each of the nutritional constituents, protein, non-reducing sugars, reducing sugars, lipid, starch and moisture were analysed as dependent variables in separate

models. Total carbohydrate (the sum of non-reducing sugars, reducing sugars and starch values) was also considered as a dependent variable, but explained less variation and were not presented in the final analysis. To analyse the spatial and temporal variation in nutritional content, potential fixed effects tested included *Eastings*, *Northings* and *Day* (Julian date). Markov chain Monte Carlo simulation was used to estimate *p*-values for the fixed effects using "*languageR*" package (Baayen 2007). The results presented represent the output of the most parsimonious models, determined using stepwise deletion based on residual deviance contrasts (Crawley, 2007; Zuur, 2009).

For those nutritional constituents that were found to vary spatially, the spatial variables *Eastings* and *Northings* were replaced with variables describing the landscape composition, as estimated by the Countryside Survey 2007 Land Cover Map (Morton *et al.*, 2011). Countryside Survey land cover data divides total land cover (km²) between different landscape types (Table 2.3 and (Morton *et al.*, 2011). Buffer zones (Steffan-Dewenter *et al.*, 2002) with radii of 500 m, 3 km and 10 km around each hive had values for total land cover in raw area (km²) converted to relative land cover (%) and arcsine transformed to normalise the residuals for statistical analysis. The landscape composition variables were included in linear mixed effects models (LMER) as independent variables tested against the nutritional constituents as dependent variables, with the hierarchal sampling structure included in the random effects (*1|Apiary/Hive/Box/Frame*). The fixed effects included in the most parsimonious models at each of the buffer zone sizes are shown in Table 2.3.

2.4 Results

2.4.1 General observations on the nutritional content of bee bread

Analysis of the nutritional composition of the 576 bee bread samples showed that the major nutritional constituent was protein (mean concentration = 65.92 mg g^{-1} ± 22.25), followed by reducing sugars (28.68 mg g^{-1} ± 14.28) and non-reducing sugars (22.21 mg g^{-1} ± 17.33). Lipids, moisture and starch were present in low concentrations (1.99 ± 1.69, 0.23 ± 0.25 and 0.08 mg g^{-1} ± 0.06, respectively). The mean protein to carbohydrate ratio (P:C) was 1.53:1 (± 0.83). The mean mass of bee bread sampled from hives in this study was 165.89 mg ± 73.40.

Table 2.1. Variance components analysis of random effects on the variance of inter- and intra-hive of the two most significant nutritional constituents. Variances and standard deviations (S.D.) indicate how variable nutritional constituents are at different spatial scales, standard deviations. Random effects were tested using Chi squared on residual maximum likelihood estimates using ML error structure and analysis of variance between models including random effects.

		Protein (mg per g bee bread)			
Between	df	Variance	S.D.	Chi²	*P*
Cells	575	15.24	3.90	10.40	0.015
Frames	93	19.18	4.38	5.91	0.054
Boxes	48	27.94	5.29	10.48	0.001
Hives	34	35.37	5.95	24.11	<0.001
Blocks	2	730.95	27.04	36.86	<0.001
Residual	-	315.17	17.75	-	-

		Reducing sugar (mg per g bee bread)			
Between	df	Variance	S.D.	Chi	P
Cells	575	0.00	0.00	0.00	1.000
Frames	93	5.38	2.32	8.14	0.004
Boxes	48	8.21	2.86	7.70	0.005
Hives	34	5.43	2.33	20.43	<0.001
Blocks	2	164.48	12.83	49.04	<0.001
Residual	-	120.62	10.98	-	-

		Non-reducing sugar (mg per g bee bread)			
Between	df	Variance	S.D.	Chi²	*P*
Cells	575	0.00	0.00	0.00	1.000
Frames	93	0.29	0.54	0.19	0.617
Boxes	48	0.15	0.38	0.71	0.403
Hives	34	0.00	0.00	0.00	1.000
Blocks	2	0.00	0.00	0.00	1.000
Residual	-	3.72	1.93	-	-

		Lipid (mg per g bee bread)			
Between	df	Variance	S.D.	Chi	P
Cells	575	0.00	0.06	0.00	1.000
Frames	93	0.00	0.07	0.00	1.000
Boxes	48	0.05	0.22	2.11	0.135
Hives	34	0.02	0.15	1.31	0.252
Blocks	2	0.00	0.00	0.00	1.000
Residual	-	0.18	0.42	-	-

		Starch (mg per g bee bread)			
Between	df	Variance	S.D.	Chi²	*P*
Cells	575	0.00	0.00	0.00	1.000
Frames	93	0.00	0.02	0.00	1.000
Boxes	48	0.00	0.03	0.00	1.000
Hives	34	0.00	0.05	0.00	1.000
Blocks	2	0.00	0.00	0.00	1.000
Residual	-	0.00	0.07	-	-

		Moisture (mg per g bee bread)			
Between	df	Variance	S.D.	Chi	P
Cells	575	0.00	0.03	0.00	1.000
Frames	93	0.00	0.06	0.00	1.000
Boxes	48	0.00	0.00	0.00	1.000
Hives	34	0.01	0.10	1.76	0.444
Blocks	2	0.00	0.00	0.00	1.000
Residual	-	0.05	0.23	-	-

Table 2.2. Summary statistics for significant spatio-temporal variation in nutritional composition of bee bread. Nutritional constituents presented here were included in GLMMs as independent variables, and Effects included as dependent variables. b is the effect size estimate, S.E. is the standard error of the effect. P values were generated for fixed effects using Markov chain Monte Carlo simulation and p values produced.

Nutritional constituent	Effect	b	S.E.	F	df	P
	Eastings (E)	0.966	0.010	8.099	1, 572	0.003
	Northings (N)	0.948	0.018	1.430	1, 572	0.011
Protein	Day (D)	1.006	0.003	5.769	1, 572	0.009
	D^2	1.000	0.000	63.890	1, 572	< 0.001
	E*N	1.000	0.000	1.44	1, 572	0.002
Non-reducing sugar	Day (D)	0.015	0.002	46.775	1, 576	0.020
Reducing sugar	Day (D)	-0.303	0.083	5.869	1, 574	<0.001
	D^2	0.001	0.001	7.823	1, 574	0.004
	E*N	0.001	0.001	4.857	1, 574	0.032
Lipid	Day (D)	-0.011	0.002	29.560	1, 575	< 0.001
	D^2	0.001	0.001	13.090	1, 575	< 0.001
Starch	Day (D)	0.002	0.000	46.570	1, 576	< 0.001

2.4.2 Variation in bee bread nutritional content at different spatial scales

Variance components for each of the nutritional constituents are shown in Table 2.1. Unless otherwise stated, variance components were not significantly different from zero. The greatest level of variance was found in protein and reducing sugars components. Variance components were not statistically significant for non-reducing sugar, lipid, starch and moisture, indicating that levels of these four components of bee bread was relatively invariant between bee bread samples. Protein concentration varied significantly between cells on the same frame, but other nutritional constituents did not. Both reducing sugars and proteins varied significantly within-box; i.e. cells located on different frames within the same box had significantly different protein and reducing sugar contents. Both of these nutritional components also varied significantly within-hives as cells of bee bread located within different boxes had significantly different concentrations of protein and reducing sugars. The highest variances for proteins and reducing sugars were at the *Block* level, indicating significant variation at the different sampling stages in both of these nutritional constituents.

2.4.3 Geographical and temporal variation in nutritional content

The protein content of bee bread varied significantly through the season (*Day* and *Day^2* in Table 2.2, Figure 2.2a) and also varied significantly geographically, as reflected in the significant Eastings*Northings interaction (Table 2.2). These results suggest that there may be areas where bee bread has significantly higher or lower protein content (Figure 2.2a) and that overall protein content varies non-linearly across the season, peaking in late-July (Figure 2.3a). Non-reducing sugars did not vary

spatially, but did increase through the season (*Day* in Table 2.2, Figure 2.3b). Reducing sugars varied significantly with the interaction between *Eastings* and *Northings* (Table 2.2), indicating that, like protein content, reducing sugar content has areas of both significantly higher and lower values (Figure 2.2b). The reducing sugar content of bee bread also varied non-linearly through the season (*Day* + *Day*^{2} in Table 2.2), appearing to decline from spring to mid-summer, before then increasing to a peak in August-September (Figure 2.3c). Although lipid content did not vary spatially, it did increase non-linearly through the season (Table 2.2). Starch also increased through the season (Table 2.2), whereas the moisture content of bee bread varied neither temporally nor geographically

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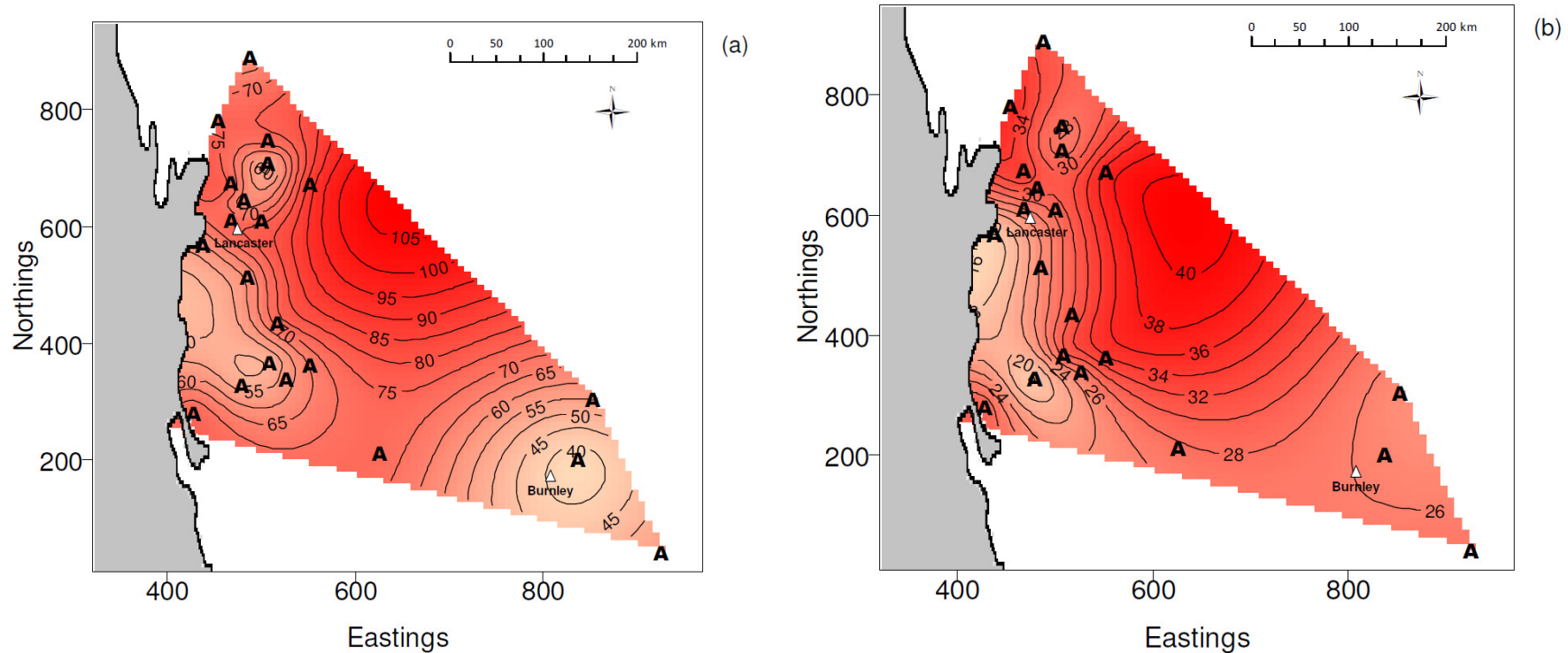
Figure 2.2. Spatial variation in bee bread nutritional composition. Geographical variation in (a) protein content and (b) reducing sugar content of bee-bread visualised using thin plate spline (TPS) surface plots based on data collected in 2012, with smoothing factors based on generalized cross validation. Contour lines and colour scaling represents the mass of protein detected in the samples (mg); the darker the colour, the higher the protein content; "A" indicates apiaries, white triangles indicate local cities and a surface raster of the coastline has been included.

(a)

(b)
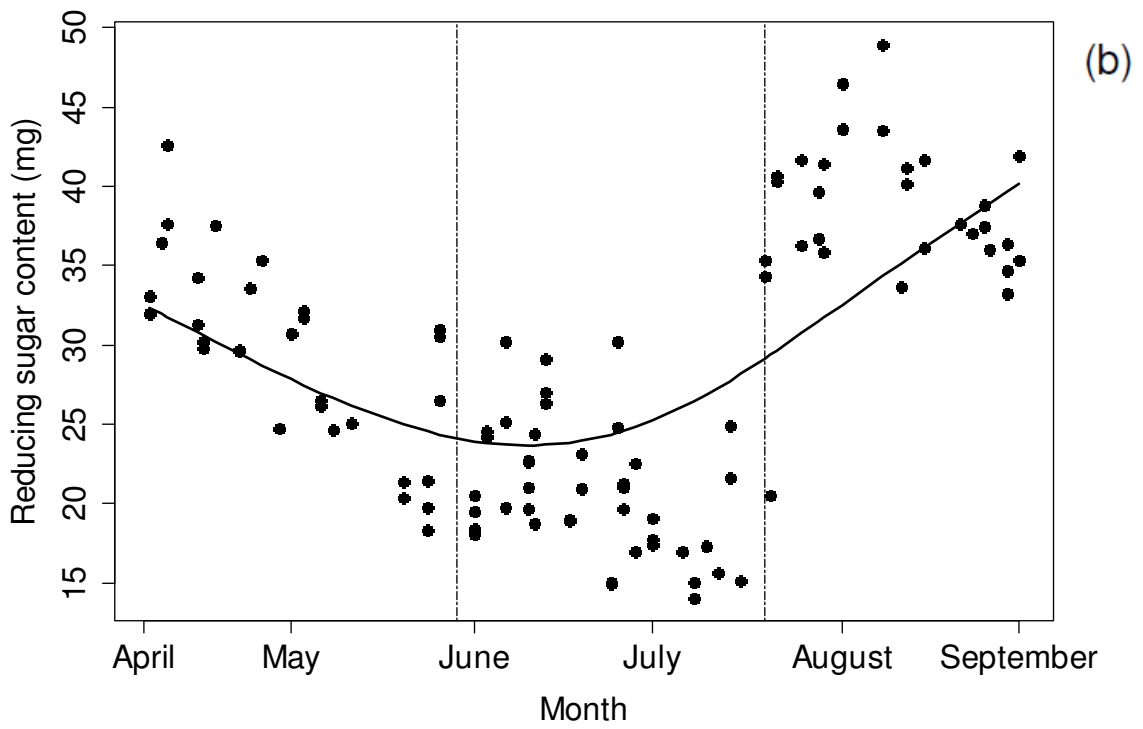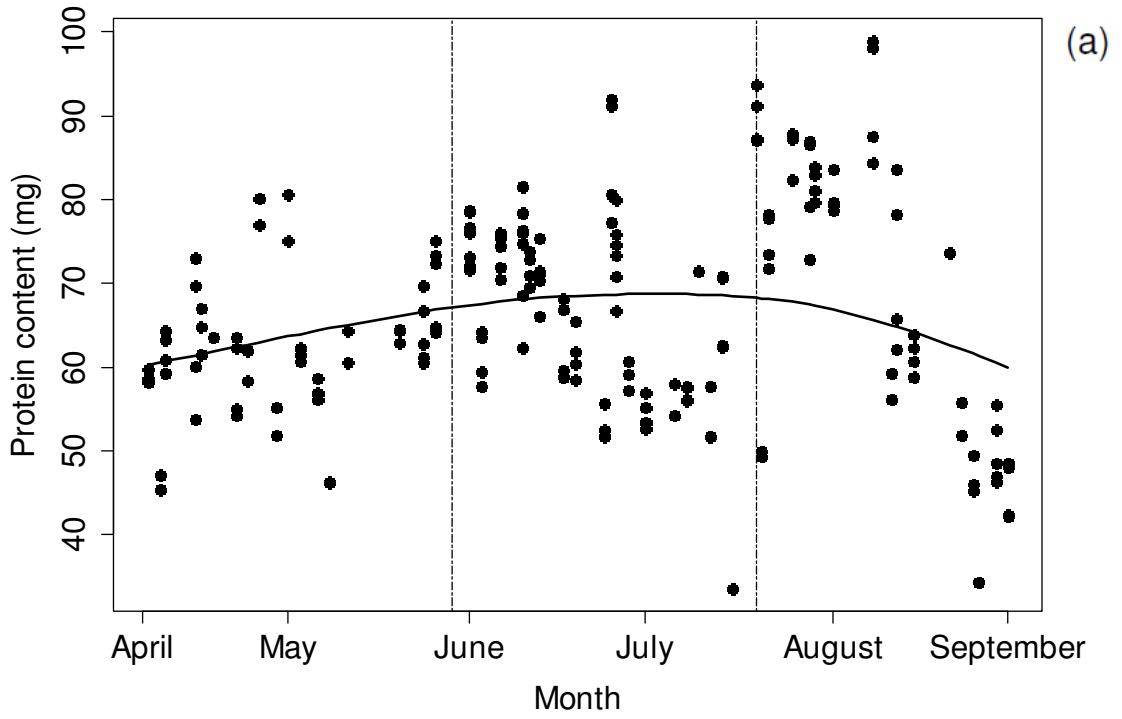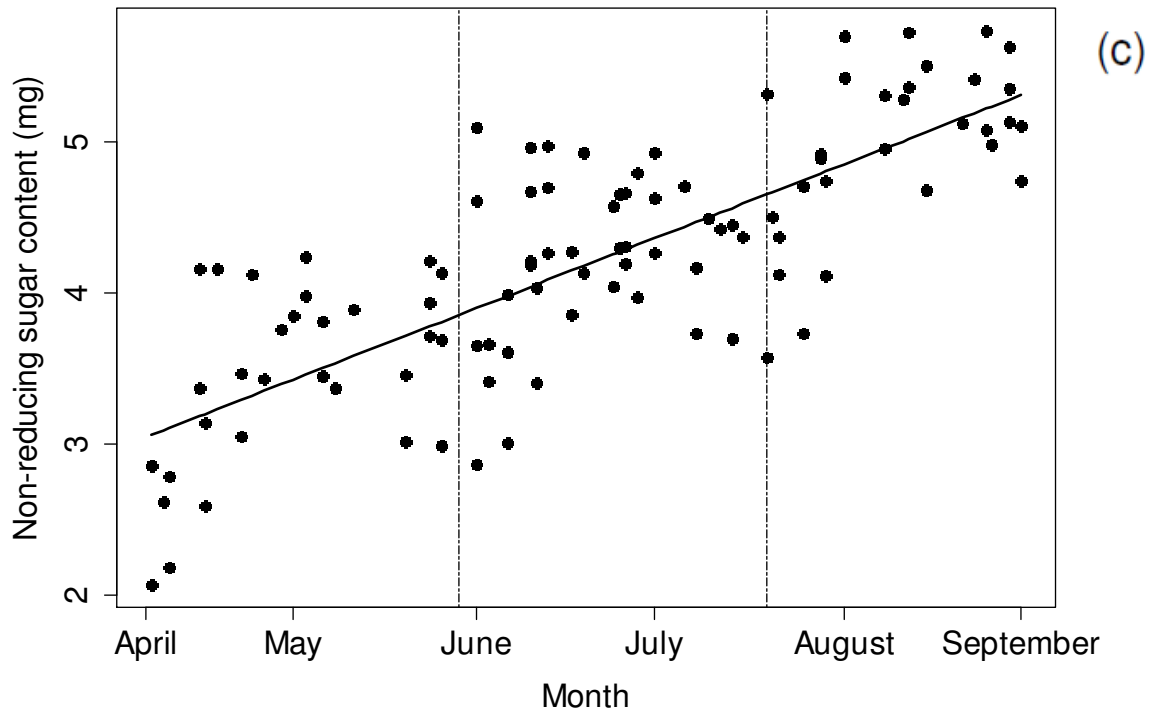
Figure 2.3. Temporal variation in bee bread nutritional composition. Time plot of relative (a) protein content, (b) reducing sugar content and (c) non-reducing sugar content of bee bread sampled over the 2012 field season. Fitted data are plotted and have been divided into each of the three sampling repeat occasions, representing data taken in April – June, June – July and July – September.

2.4.4 Landscape composition and bee bread composition

Analysis of bee bread nutrition composition in relation to landscape composition variation was restricted to the two nutrients that varied most at a geographic scale: protein and reducing sugars. Correlations between the protein content of bee bread and landscape cover composition were strongest for cover estimates made within a 3 km radius of the hive (Table 2.3; n = 6/14 significant correlations), and were weakest at the 0.5 km buffer zone (n = 2/8 significant correlations). Bee bread protein content was consistently negatively correlated with the percentage of local arable and horticultural land across all of the buffer zone sizes and significantly positively correlated with the percentage of acid grassland and improved grasslands; there was also a marginally significant negative correlation between bee bread protein content and the percentage neutral grasslands, but only at the 3 km buffer zone. Protein content was also positively correlated with increasing littoral sand cover at both 3 km and 10 km buffer zones, and with increasing percentage built-up areas and gardens at the 10 km buffer zone.

In contrast, for reducing-sugars there were no consistently significant landscape types across the different buffer zone sizes (Table 2.4, 6 out of 8 classes at 0.5 km, 14 at 3 km and 14 at 10 km).

Table 2.3. Summary statistics of effects of different landscape types, area of the types and buffer zones on protein content of bee bread; only statistically significant results (P<0.05) are included, and landscape types that did not vary significantly at any buffer zone size were omitted. (*df* = 1, 576).

	Buffer zone sizes								
	500 m			3 km			10 km		
Landscape type	Estimate	S.E.	*P*	Estimate	S.E	*P*	Estimate	S.E.	*P*
Acid grassland				127.77	33.07	<0.001	346.98	114.86	0.003
Arable and horticulture	-113.52	58.44	0.048	-805.81	220.40	<0.001	-261.28	82.24	0.002
Broad leaved, mixed and yew woodland				93.53	26.52	<0.001			
Built up areas and gardens							681.11	281.59	0.016
Freshwater	175.85	67.97	0.001				-650.94	226.48	0.004
Improved grassland				32.72	15.03	0.050			
Littoral sands				175.81	61.28	0.004	106.36	32.98	0.001
Neutral grassland				-76.69	37.60	0.042			

Table 2.4. Summary statistics of effects of different landscape types, area of the types and buffer zones on reducing sugar content of bee bread; non significant results have been omitted, and landscape types that were not found to be significant at any buffer zone size were also omitted. ($df = 1, 576$).

	Buffer zone sizes								
	500 m			3 km			10 km		
Landscape type	Estimate	S.E.	*P*	Estimate	S.E.	*P*	Estimate	S.E.	*P*
Broad leaved, mixed and yew woodland				151.89	75.47	0.045			
Freshwater	83.38	24.53	0.001						
Littoral rock				-305.93	123.03	0.013			
Littoral sediment							59.17	20.79	0.005
Rough grassland	59.26	22.39	0.008						
Salt water							-75.97	28.083	0.007

2.5 Discussion

This is the first study to quantitatively assess variation in the nutritional composition of bee bread. I used stratified-sampling of hives in the North-west of England to show that there is significant internal (within-hive) and external (between-hive) variation in the nutritional composition of bee bread, and that the external variation is significantly associated with environmental factors such as landscape composition. Bee bread is an essential component of the honey bee hive, providing nutrition to develop the brood as well as stimulating egg laying by the queen after winter (Oliver, 2007b). Bee bread contains both protein and amino acids, both of which are highly variable between pollens (Vanderplanck *et al.*, 2013); however the assays in this study were not able to detect amino acid quantities. There is also growing evidence that the protein content and amino acid composition may play a role in determining the amount of pollen bees consume (Nicolson, 2011; Nicolson and Human, 2013).

2.5.1 Internal (within-hive) variation

The high degree of variation in bee bread protein and reducing sugar levels within each frame noted in this study is considered likely due to constraints on the number of pollen grains (and therefore species) that can be stored in each cell within a frame, the composition of which is determined by worker bee foraging effort. The protein content of bee bread is primarily driven by the plant species that bees have collected pollen from, which vary in their protein content (Somerville, 2001). Although pollen does contain some reducing sugar (Roulston and Cane, 2000), the sugars in bee bread are likely come to from floral nectar (Vásquez *et al.*, 2009). The

nutritional value of floral nectars also varies in different plant species (Waddington, 1983; Pacini *et al.*, 2003). The combination of different plant species available to bees, with pollens of different nutritional values and nectars with different sugar contents may result in the observed variation in bee bread nutritional composition.

The internal variation shown here suggests that pollen may still be sourced from several different flower species from foraging areas targeted by bees. They may preferentially forage pollen from different plant species based on amino acid content (Cook *et al.*, 2003) or based on certain phagostimulatory lipids (Schmidt and Hanna, 2006). Foraging bees use the "waggle dance" (Riley *et al.*, 2005) to describe the location of forage to others, allowing for repeated foraging efforts on a single patch of flowers. Accordingly, neighbouring cells on a single frame may contain very different pollen combinations, leading to the observed variation in protein content.

The variation in nutritional composition of bee bread distributed between different boxes (see Table 2.1) may be attributable to bees working in one box only at a given time. There is substantial anecdotal evidence from beekeepers that a colony of bees will work one box and then progress to another as the colony expands in size. It is unknown whether bees deposit pollen species to specific loci within the hive. However if this was shown to be the case it could explain the high level of within-hive and within-box variation observed in the present study.

The variation in nutritional composition of bee bread observed within-hive makes multiple food sources of different nutritional content accessible to the bees which could be important to their overall fitness as previous studies have suggested that protein content of honey bee diet may influence some aspects of fitness (Alaux *et al.* 2010; Brodschneider & Crailsheim 2010). Additionally, most insects, such as *Drosophila melanogaster*, Meigen, *Spodoptera littoralis*, Boisduval, and honey bees

have an optimal diet composition that maximises fitness (Lee *et al.* 2006; 2008; Altaye *et al.* 2010), designated the "intake target" (Simpson & Raubenheimer 2012). By having access to multiple variable sources of nutrition within the hive, this may be the method by which honey bees can achieve their optimum "intake target".

2.5.2 External (between-hive) variation

Sampling of the 20 geographically distinct apiaries three times through the beekeeping season allowed both the temporal and geographical variation in bee bread composition to be quantified. The protein content of bee bread displayed the most significant geographical variation (see Figure 2.2a and Table 2.2), suggesting that it is determined by environmental factors that vary around the hives. Species areas were also identified that are significantly associated with higher protein content of bee bread and observed geographical variation in the amount of reducing sugars. Spatial variation in the floral resources that supply bees with both dietary protein and sugar may explain this variation in nutritional content of bee bread across the study area. Consistent with the findings of previous research, bee bread is comprised of both reducing and non-reducing sugars (Herbert and Shimanuki, 1978). The former occurs at greater levels than the latter and this may be because although nectar contains both types of sugar, honey bees are more attracted to nectar high in reducing sugars (Nicolson, 2011). Previous studies have indicated that pollen can have protein content ranging from 12 to 62% (Roulston and Cane, 2000). Here, a mean concentration in bee bread below 7% (65.92 mg g^{-1}) was found; this may be because pollen and nectar are mixed to produce bee bread, effectively reducing the maximum concentration of protein possible.

The combined effects of protein and sugar on invertebrate fitness are well documented (Lee *et al.*, 2006; Cotter *et al.*, 2010). High protein diets of protein:carbohydrate (P:C) >5:1, have been shown to reduce lifespan in *Drosophila melanogaster* (Lee *et al.*, 2008) and even lead to colony collapse in the aphid-tending ant, *Lasius niger* L. (Dussutour and Simpson, 2012). Here, we observed a mean P:C ratio in bee bread of 1.53:1, although neither the effects bee bread composition nor the consumption of nectar and honey on bee fitness are presently understood.

Temporal analysis of bee bread nutritional composition suggests that the 'preferred' foraging locations for bees may change through the season, as different plant species come into flower. The Himalayan balsam (*Impatiens glandulifera*, Royle) blooms from July to October and dominates the study area in North-west England. It is well known amongst the local beekeeping community to be foraged almost exclusively by their colonies upon its appearance. The emergence of balsam is correlated here with an increase in the variability of protein content of bee breads (see Figure 2.3a) which may be a reflection of the variable access of bees from different apiaries to this plant.

Protein is also known to directly influence some aspects of immunocompetence in individual honey bees (Crailsheim and Stolberg, 1989; Alaux *et al.*, 2010a; Brodschneider and Crailsheim, 2010; DeGrandi-Hoffman *et al.*, 2010) and lifespan in ant colonies (Dussutour and Simpson, 2008; Dussutour and Simpson, 2012). Decreased immunity and memory impairment of individual foragers could reduce the foraging capacity of a colony, potentially leading to further fitness costs.

2.5.3 Landscape composition and nutritional composition

In demonstrating that bee nutrition is significantly linked with changing properties of the environment, this study utilised data from the Countryside Survey 2007 Land Cover Map (Morton *et al.*, 2011) as a proxy for the floral resources available to each hive (Kleijn and van Langevelde, 2006). This approach elucidated that the observed spatial variation in the nutritional composition of bee bread was significantly correlated with several landscape types. Most landscape cover types, excluding arable and horticulture land and freshwater, were not correlated with nutritional composition at 0.5 km, but were found to be significant at larger spatial scales, most likely due to honey bee foraging being most efficient at 3 km (Visscher *et al.*, 1985; Visscher and Seeley, 1982; Steffan-Dewenter and Tscharntke, 2000) and capable of up to 10 km from the hive (Seeley, 1986).

The association of high protein bee breads with areas of high acid grassland and broadleaf woodland cover may be because these environments are dominated by plant species with high protein content in their pollens, whereas arable farmland may be associated with plants with low protein content pollen. The 'selectivity' of different land use types on the availability of different forage flowers may be the main mechanism by which honey bee nutrition is being determined (Ricketts *et al.*, 2008).

Arable and horticulture land was consistently negatively correlated with bee bread protein content at all buffer zone sizes (see Table 2.3). It is well established that the monoculture of crop species in arable lands has a negative impact on insect diversity (Tscharntke *et al.*, 2005; Tscharntke *et al.*, 2002), and certain crop plants such as sunflower and rape, have been shown to reduce longevity in honey bees (Schmidt *et al.*, 1995). Evidence suggests that agri-environmental schemes may have benefits to invertebrate diversity (Kleijn *et al.*, 2006). However, the results presented

here suggest these benefits may not be in the form of pollens with higher protein content.

At the 10 km buffer zone, it was found that built-up areas and gardens are associated with an increase in the protein content of bee bread. Numerous factors have been shown to influence floral and arthropod diversity, such as green corridors (Vergnes *et al.*, 2012), roundabouts (Jones and Leather, 2012) and cemeteries (Lussenhop, 1977). These areas have been shown to provide small, but significant refuges in habitat or resources. The high diversity of exotic introduced garden species associated with high income urban environments (Hope *et al.*, 2003) may present a possible source of high-protein pollen that is driving this interaction. Bates *et al.* (2011) also suggested that bee diversity is strongly affected by local diversity in urban environments, particularly in high diversity pockets such as garden centres.

Increases in bee bread protein content were also significantly associated with coastal (littoral) sands at both the 3 km and 10 km buffer zones. This may suggest that certain plants, particularly sea aster (*Aster tripolium* L.), which are exclusively available in these areas, are particularly high in protein and thus may be the primary drivers of this trend, although relevant data are currently lacking. Recently, Naug (2009) attempted to further explain honey bee population declines due to loss of forage leading to nutritional stress. The results of our study show that built up urban environments and gardens are associated with an increase in the protein content of bee bread, which may be alleviating nutritional stress in terms of protein.

The extent and depth of this study was made possible by the association of beekeepers with this study, and has encouraged communication and interaction between this important group of stakeholders and the research team, making the

results more relevant to the key stakeholder group involved in honey bee management.

This study has presented a unique examination of how bee nutrition may be influenced by localised land use utilizing data from the Countryside Survey. By using stratified-sampling, I show how the likely nutritional value of honey bee bread varies both within and between hives. Nutrition plays a key role in how animals can resist physiological stresses; poor nutrition may contribute to the widespread and on-going population decline by increasing vulnerability to various stresses. There is currently however, a lack of understanding of how a variable environment can influence the nutrition of social insects; the findings here have provided a link that will inform future studies of social foraging. Finally, these results suggest that local land use practises and bee health may be linked, which has important implications for honey bee management, by allowing optimisation of hive location according to land use.

Chapter 3: Comparisons of pollen from bee bread in Honey bee (*Apis mellifera mellifera* L.) colonies determined by molecular methods

Honey bee (A. mellifera) assisting in sterile sampling of bee bread.

3.1 Abstract

Honey bees collect pollen from their environment and use it as a source of dietary protein for raising their offspring. The quality of pollen varies according to the plant community in the environment. Although much is known about the nutritional quality of pollens, there is a lack of information on the net effects of complex plant communities on honey bee nutrition.

Bee bread stored within bee hives was sampled from locations in the North-west of England. The nutritional content of these pollen stores was estimated using a series of biochemical analyses designed to determine the relative amounts of protein, reducing sugar, non-reducing sugar, lipid, starch and moisture. The amino acid composition of bee bread was assessed using high-performance liquid chromatography (HPLC). The diversity of plant species within the bee bread samples was estimated using PCR to select for the *trn*L gene on the chloroplast Group I intron; this gene is present in all plant genomes. Species diversity was estimated using denaturing gradient gel electrophoresis (DGGE).

Between one and ten different pollen operational taxonomic units (OTU) were detected within single cells of bee bread. Increasing protein content of bee bread was positively correlated with increasing number of pollen OTUs and with the prevalence of specific OTUs. In addition, the proportion of the amino acids proline and histidine in bee bread increased with the increasing number of OTUs.

These results suggest that increasing plant species diversity available for honey bees to forage on may have a significant and quantifiable benefit to honey bee nutrition, and that certain plant species have a greater impact on the nutritional composition of the bee bread. Previous studies have established that the protein content of a diet has a significant impact on the fitness of many invertebrates. The

results of this study indicate that increasing diversity of forage available to bees may lead to improved fitness. Future studies could expand on these findings by revealing species identity and the effects they have in a complex plant community.

3.2 Introduction

The honey bee *Apis mellifera* L. is an important pollinator of major agricultural systems around the world, with estimated annual values varying between £190 million per annum in the United Kingdom (Knight *et al.*, 2009) and $220 million (Pimentel *et al.*, 1980) to $14,560 million (Morse and Calderone, 2000) per annum in the United States. Honey bees collect pollen and nectar from the forage plants as their primary source of nutrition. Pollen collected from the environment is stored on cells within the hive as "bee bread". Honey bees are capable of collecting pollen from a diverse selection of forage plants (Keller *et al.*, 2005b; Keller *et al.*, 2005a) and hives located in different areas can accumulate pollen from significantly different selections of forage plants (Koppler *et al.*, 2007).

Honey bees have been shown to preferentially visit some flowers over others. In a European study, Keller *et al.* (2005b) found, across studies that use pollen traps, that maize (*Zea mays*) was the most common pollen source for honey bees; in 48% of cases (55/114), maize was in the top 5 most common pollen sources. Other pollens that occur in the top 5 included white clover (*Trifolium repens* - 45%), common dandelion (*Taraxacum officinale* – 45%), plantain (*Plantago* spp. – 41%) and oil seed rape (*Brassica napus* – 40%). Conversely, Kirk and Howes (2012) note that honey bees will avoid foraging on certain common flower species, including buttercups (*Ranunculus* spp.), foxgloves (*Digitalis* spp.) and vetch (*Vicia* spp.). Therefore, the

composition of forage collected by bees will depend on the relative abundances of these attractive and unattractive plant species.

Pollens vary not only in relative attractiveness to honey bees, but also in nutritional content (Roulston and Cane, 2000; Roulston *et al.*, 2000; Somerville, 2001; Somerville, 2005). Some species have been shown to contain very low levels of protein, with the lowest being 11.7% in the pollen of mugwort (*Tanacetum vulgare*: Asteraceae), whereas the greatest (61.7%) was found in Padre's Shooting Star (*Dodecatheon clevelandii*: Primulaceae). The species believed to be most commonly visited by honey bees in England (Kirk and Howes, 2012) is white clover, which is reported to contain only 25.3% protein content (Somerville, 2001).

Pollens also differ in amino acid composition. The studies of DeGroot (1953) indicated that arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were essential amino acids for honey bees (and indeed most insects (Chapman *et al.*, 2013), while alanine, cysteine, glycine, hydroxyproline, proline, serine and tyrosine were nonessential. Pollens that contain a high proportion of these essential amino acids are assumed to be of greater nutritional value than pollens with lower proportions of essential amino acids (Day *et al.*, 1990).

Agricultural intensification has led to an increase in monoculture, and a reduction in the diversity of potential forage collected by honey bees around agricultural land (Batra, 1995; Schmidt *et al.*, 1987). Several studies have attempted to address the effects of reduced floral diversity on various bee species. Results of these studies have demonstrated both an increase in fitness associated with polyfloral (multiple species) diets (Alaux *et al.*, 2010b; Tasei and Aupinel, 2008) and increasing fitness associated with monofloral (single species) diets when certain plants were used, e.g. mustard (*Brassica capestris* L.; (Singh and Singh, 1996).

There have been no previous studies to determine the correlation between the floral composition of bee bread and its nutritional composition. To understand these associations, the species of each of the pollens present in bee bread must be identified. Previously, approaches involving microscopic identification of pollen species have been used to assess the species composition of pollens before bees enter a hive (Ohe *et al.*, 2004). Bee bread is however, found only inside hives and once pollen has been placed onto frames inside the hive, the grains become damaged due to the action of both the bees and the bacteria present in bee bread (see Chapter 4) and this makes them difficult to identify by these traditional methods. By using molecular fingerprinting that targets plant DNA that is not damaged during the storage of pollen in the hive (Olivieri *et al.*, 2012), it may be possible to detect and identify the species assemblage of pollens in bee bread.

A highly conserved region of the plant chloroplast Group I intron, the *trn*L (UAA) locus (Taberlet *et al.*, 2007), was selected as a target for amplification and analysis. This region was selected for its conserved nature within species (Bakker *et al.*, 2000), which allows differentiation between species, whilst limiting the amplification of fungal species (Chen *et al.*, 2010). The *trn*L locus was selected for the number of records on GenBank (over 100,000 entries). In addition, previous studies have shown that DNA amplification can be performed even on highly degraded DNA, such as from processed food or from permafrost samples (Taberlet *et al.*, 2007), which is particularly relevant for analysing processed pollen found in bee bread.

One method of analysis of plant genes is to use denaturing gradient gel electrophoresis (DGGE; (Liu and Shyu, 2006), which is based on separation of intact amplicons, which are known to be of similar or identical lengths on a polyacrylamide

gel according to variability in sequence composition. Separation of these amplicons is based on the changing mobility of DNA fragments as they melt from double stranded (dsDNA) to single stranded (ssDNA) form on a chemical gradient (Etscheid and Riesner, 1998, Wen-Tso and Stahl, 2002). This gradient comprises formamide (CH_3NO) and urea (CH_4N_2O), which destabilises dsDNA at different rates according to variable mobility in amplicons due to sequence content. They therefore stop in replicable positions on a denaturing gradient, and can be used to compare community composition between different environment samples. Although previous studies have used DGGE to analyse amplicons of plant DNA (Liu and Shyu, 2006), these have not used the *trnL* (UAA) locus as a target region.

By estimating the floral diversity of bee diets using these molecular methods, the following main questions will be addressed: (i) Are there some pollen OTUs that always occur in cells of bee bread? (ii) Do monofloral and polyfloral bee bread diets differ in their nutritional compositions? (iii) Is the nutritional composition of bee bread, in terms of macronutritents (i.e. protein and carbohydrate) and amino acid composition, correlated with the prevalence of particular pollen species in the diet? An overarching objective of this study is to establish whether the complexities of the local plant community are reflected in bee bread nutritional composition and to use these findings to provide a potential management tool to improve bee nutrition.

3.3 Materials and methods

3.3.1 Bee bread sampling

39 samples of bee bread were collected from 14 honey bee (*A. m. mellifera*) hives within 13 apiary sites in North-west England between 7th April and 23rd August

2012 (Figure 3.1). The hives were owned by either hobbyist beekeepers, a commercial beekeeper, or maintained as part of the training suites for local beekeeping associations. To minimize contamination of the samples, each cell was extracted using a separate, sterile sampling tool and placed into sterile 1.5 mL microfuge tubes for transport to the laboratory. Samples were returned to the lab and processed within 2 hours of collection. The mean mass of bee bread samples from 2012 was 165.9 mg ± 73.4; these samples were divided for nutritional and molecular analysis, with 85% of the sample (143.5 mg ± 65.3) being used for nutritional analysis and the remaining 15% for DNA extraction and amplification.
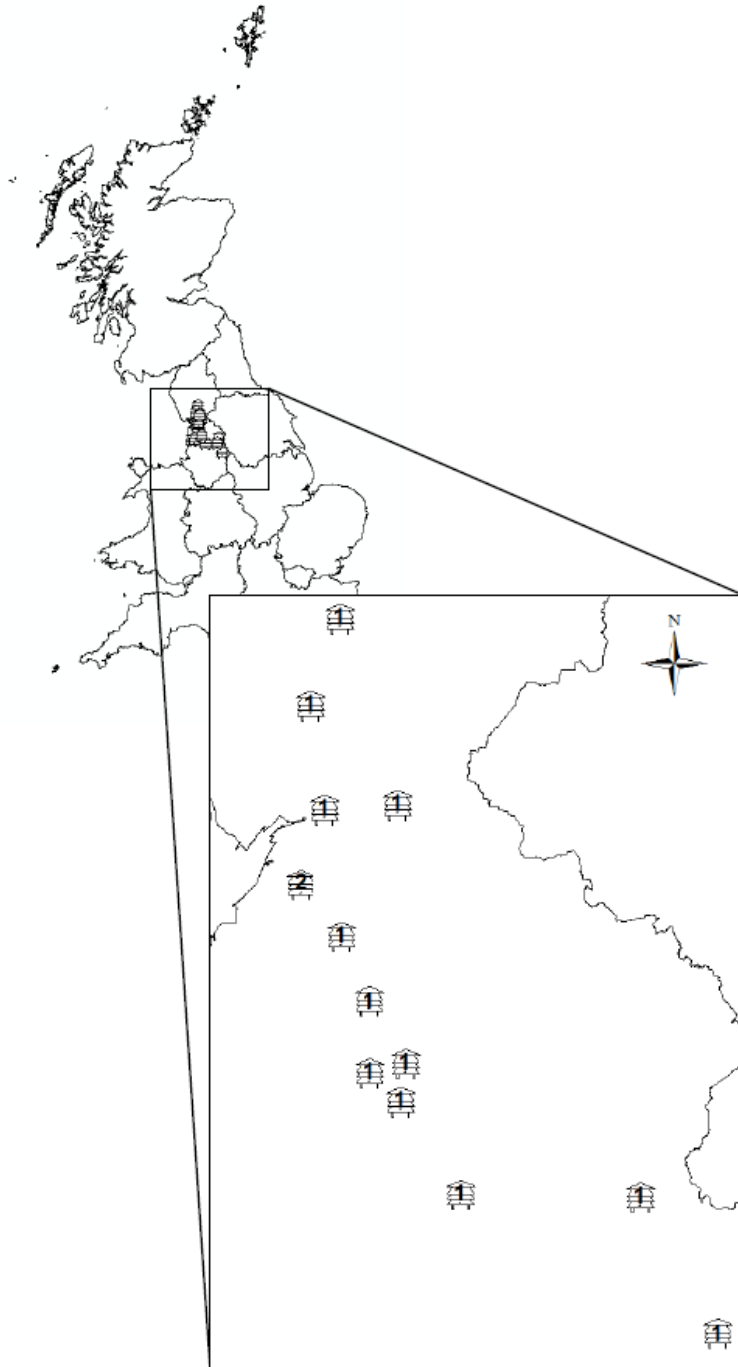
Figure 3.1. Map of sampling locations for pollen identification from sample apiaries in the North-west of England. The location of apiaries (n = 13) is highlighted by the hive drawings which in turn have number of hives sampled at each apiary (either 1 or 2) inside.

3.3.2 Nutritional analysis

The nutritional content of bee bread was estimated using a series of chemical analyses designed to determine the relative amounts of protein, reducing sugars, non-reducing sugars, starch, lipid and moisture (see Chapter 2, section 2.3.2.).

In brief, for protein, carbohydrate, starch and lipid assays, absorbance was measured using a VERSAmax™ Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA) set to 550, 575, 510 and 540 nm respectively using Softmax® Pro v4.7 software for Windows®. Protein content of bee bread was estimated using the Biuret reaction (Sapan *et al.*, 1999). Carbohydrate content was estimated using the dinitrosalicylic acid (DNS) reaction (Lees, 1971). Lipid content was estimated using Phosphoric acid-vanillin analysis colorimetry (Cheng *et al.*, 2011). Starch content was measured by multi-stage starch hydrolysis using the AOAC method 996.11 starch analysis kit (Megazyme, Ireland), following manufacturers' specifications (McCleary *et al.*, 1994). Moisture content of bee bread samples was determined by placing bee bread in a drying oven at 100 ºC for 24 hr and calculating the difference in mass between wet and dried samples.

3.3.3 Amino acid analysis

Amino acids composition in bee bread was analysed through high-performance liquid chromatography (HPLC) performed at the Institute of Neuroscience and School of Biology, Newcastle University by Dr E. Power. The mass of bee bread used for extraction was 3.31 mg ± 2.51. Each sample was placed in 200 µl HPLC-grade methanol (Sigma-Aldrich, Dorset, UK.) and mixed for 60 sec in an electrical vortex to extract free amino acids, followed by centrifugation at 13,000 rpm

for 5 min. The supernatant was filtered through a 0.2 μm syringe-tip filter (Phenomenex, Torrance, California) to remove particulates.

HPLC was carried out using a solvent delivery programme modified from Jones *et al*. (1981): 0% solvent B on initiation, linear step to 14% B over 4 min, isocratic step for 5 min, linear step to 50% B over 3 min, isocratic step for 8 min, linear step to 100% B over 10 min, isocratic step for 2 min, linear step to 0% B over 4 min. The HPLC system comprised a Rheodyne 20 μl injector column with a 150 x 4.6 μm Kingsorb C18 guarded column (Phenomenex, Torrance, California), a Gilson computerised solvent delivery system, and a Roslagsvägen CMA/280 fluorescence detector, set at 490 nm.

Free amino acids present in 10 μl of a 1:500 dilution of the bee bread extracts were identified and quantified by comparison with Sigma-Aldrich AA-S-18 amino acid calibration standards supplemented with asparagine, glutamine tryptophan, and γ-aminobutyric acid (GABA), diluted to 2.5 μM using HPLC-grade water.

3.3.4 Nucleic acid extraction and amplification

Total community DNA was extracted from bee bread using the QIAamp DNA Plant Mini kit (Qiagen Ltd, Crawley, UK). DNA extractions were performed according to manufacturers' specifications. Universal PCR primers targeted at Group I intron chloroplast DNA (Shinozaki *et al.*, 1986) *trn*L (UAA) locus were selected (Taberlet *et al.*, 2007) to estimate plant diversity in bee pollen forage within the hive using molecular methods. All PCR amplifications were carried out in an ABI Veriti™ Thermal Cycler (Applied Biosystems, UK).

Table 3.1. Primers used in PCR reactions.

Primer	Sequence (5`-3`)	Position*	Primer Target	Reference
*trn*LF	CGAAATCGGTAGACGCTACG	49330	Plant chloroplast *trn*L (UAA) intron	(Taberlet *et al.*, 2007)
*trn*LR	GGGGATAGAGGGACTTGAAC	49833	Plant chloroplast *trn*L (UAA) intron	(Taberlet *et al.*, 2007)
*trn*LF-GC ‡	*CGCCCGCCGCGCCCCGCGCCCG TCCCGCCGCCCCCGCCCGG* - CGAAATCGGTAGACGCTACG	49330	Plant chloroplast *trn*L (UAA) intron	Present study

Nicotiana tabacum chloroplast DNA numbering—corresponds to the positions in *N. tabacum* 18S chloroplast gene

‡ Italicised sequence to identify GC-clamp sequence

Plant target DNA was amplified using universal primers *trn*LF/*trn*LR (Table 3.1) on DNA extracted from bee bread (Taberlet *et al.*, 2007). PCR amplifications were performed in 20 µL volumes, such that each reaction contained the following: 2 µL (20 pmol) of each primer, 4 µL sterile PCR grade water, 4 µL sample extracted DNA and 10 µL Amplitaq Gold® 360 Master Mix (Applied Biosystems, UK). Positive controls contained genomic DNA from *Taraxacum officinale* Wigg. in place of sample DNA, while negative controls contained sterile PCR grade water for a PCR negative control and a DNA extraction on sterile PCR grade water for an extraction negative control. Initial denaturation was carried out for 10 min at 95 ℃, and cycling was performed as follows: 95 ℃ for 30 seconds, 50 ℃ for 30 seconds and 72 ℃ for 60 seconds for 35 cycles, with a final elongation at 72 ℃ for 7 min.

3.3.5 Denaturing gradient gel electrophoresis (DGGE)

Extracted DNA was amplified with the above conditions, but with alternative primers incorporating a GC clamp to enable analysis by DGGE (Table 3.1). PCR products were separated by DGGE using the Scie-Plas TV400 vertical electrophoresis system (Scie-Plas, Cambridge, UK). Denaturing gels consisted of 1 mm thick 6% polyacrylamide with a denaturing gradient of 0-50%, whereby 100% denaturing corresponds to 7M urea and 40% v/v deionized formamide, 1x TAE buffer (40mM Tris–acetate (pH 8.0) and 2mM EDTA) and 2% v/v glycerol. Electrophoresis was performed at 60 ℃ and limited to 20V-30mA for 10 minutes and then at 100V-30mA for 1250 V·Hrs (approximately 16 h) in 1x TAE buffer. Gels were stained in 1x SYBR Gold nucleic acid stain (Invitrogen) and visualised on a UV-transilluminator block, gel images were obtained and documented with Kodak MI software

(Carestream Health, Inc., Rochester, NY). Bands were extracted according to Chapter 4 (section 4.3.6) and sequenced commercially by GATC Biotech (Konstanz, Germany); however the results of the sequencing were of a very poor quality, and produced short sequences in most of the products. Therefore, it was not possible to generate species identities from the sequences. Floral species richness in each sample was measured by counting the number of bands found in each lane (Figure 3.2), with the assumption that each band represents a unique chloroplast gene sequence, and therefore are classed as operational taxonomic unit (OTUs; Atlas and Bartha, 1998). A binary matrix was produced for each sample by denoting OTU presence∕absence.
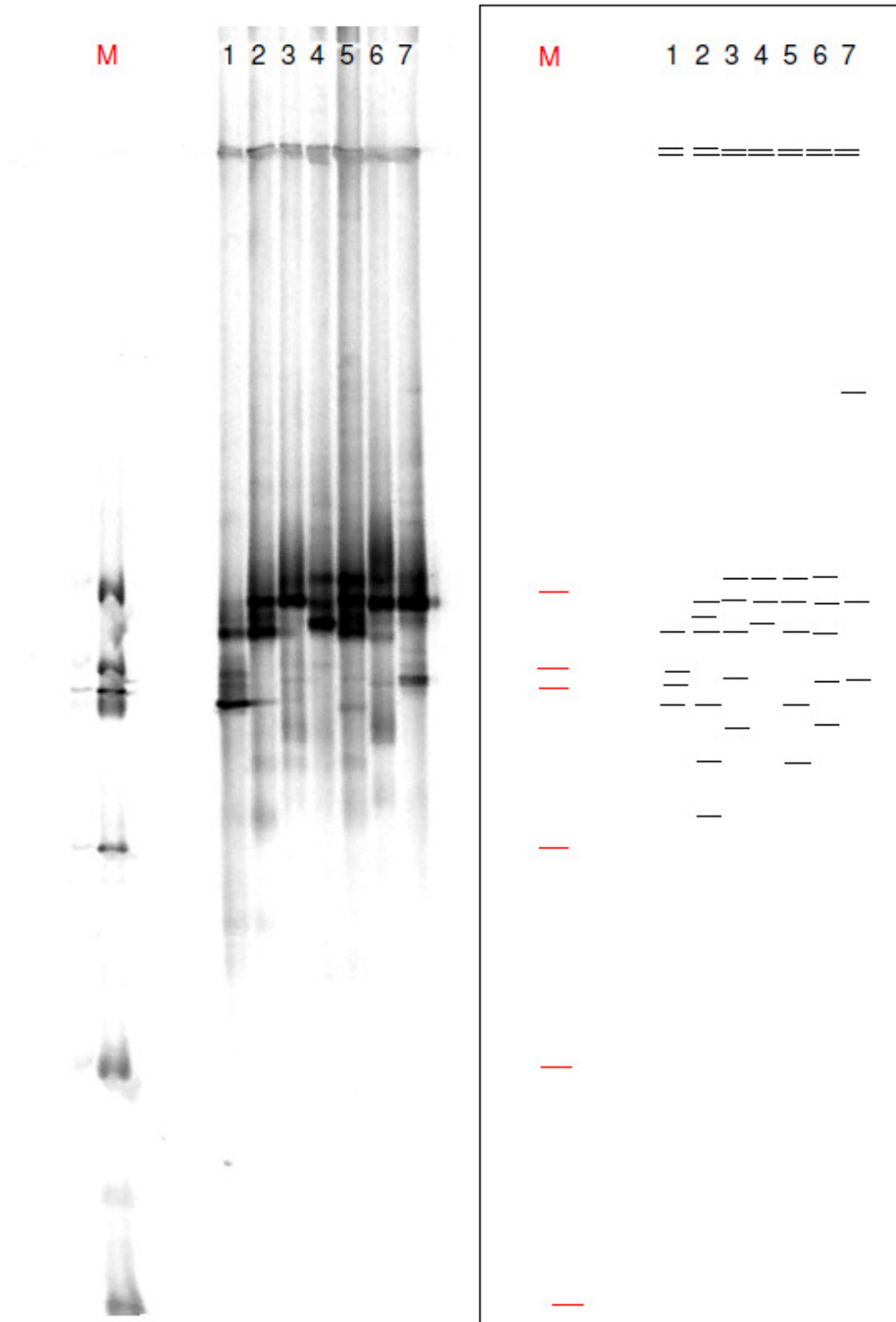
Figure 3.2. Section of a DGGE profile with a schematic mark-up to indicate how bands on gel images were determined to be DNA bands. Numbers 1-7 indicate samples of bee bread; M: internal DGGE marker.

3.3.6 Statistical analysis

Dependent variables in generalised linear models (GLMs) were analysed in two stages: first, through changes in the community composition, which were analysed using α-diversity as a dependent variable. These metrics provide different information on the trends to be observed; α-diversity, represents the number of OTUs within each sample and was included because it most closely represents the data recorded from DGGE gels. Second, a series of logistic regressions on the most common OTUs from across the dataset were used to determine variation in the prevalence of certain OTUs, which the previous indices cannot achieve. These OTUs were analysed as a binomial dependent variable in a logistic regression in GLMs.

The independent variables tested in each of the models were the results from the nutritional analyses (i.e. sample *moisture*, *lipid*, *carbohydrate*, s*tarch* and *protein*) and the relative percentage of protein to carbohydrate (*PC*). The concentration of free amino acids in bee bread (*Asp*, *Glu*, *Asn*, *Ser*, *Gln*, *His*, *Gly*, *Thr*, *Arg*, *Ala*, *GABA*, *Tyr1*, *Cys*, *Val*, *Met*, *Trp*, *Phe*, *Ile*, *Leu*, *Lys*, *Pro*) were also used as an independent variable in a multivariate analysis of variance (MANOVA). The results shown here represent the output of the most parsimonious models, determined using stepwise deletion, with residual deviance contrasts.

3.4 Results

3.4.1 Bee bread nutritional composition

For each of the samples of bee bread, a nutritional profile was generated consisting of mg per gram (wet weight) bee bread concentrations of moisture, lipid, reducing sugar, non-reducing sugar, starch and protein. These samples were from a

subset of those reported in Chapter 2, the major nutritional constituent of the bee bread subset was protein (mean concentration ± S.D. = 81.03 mg g^{-1} ± 31.43), followed by non-reducing sugars (36.45 mg g^{-1} ± 22.84) and reducing sugars (32.76 mg g^{-1} ± 15.17). Lipids, moisture and starch were present in low concentrations (1.85 ± 1.87, 0.18 ± 0.22 and 0.08 mg g^{-1} ± 0.05, respectively). The mean relative proportion of protein in bee bread was 0.537 ± 0.138 and the protein to carbohydrate ratio was 1.43 ± 1.04.

3.4.2 Bee bread amino acid composition

Seventeen amino acids were found through HPLC analysis of the 39 bee bread samples in variable amounts (Figure 3.3). Three amino acids (tryptophan, glutamine and asparagine) and one non-protein amino acid (GABA) were consistently below the limit of detection and were eliminated from subsequent analysis. Among those amino acids detected, proline accounted for the greatest proportion of total free amino acid (mean ± S.D. = 0.22 ± 0.09); other dominant amino acids included lysine (0.15 ± 0.05) and leucine (0.10 ± 0.04).
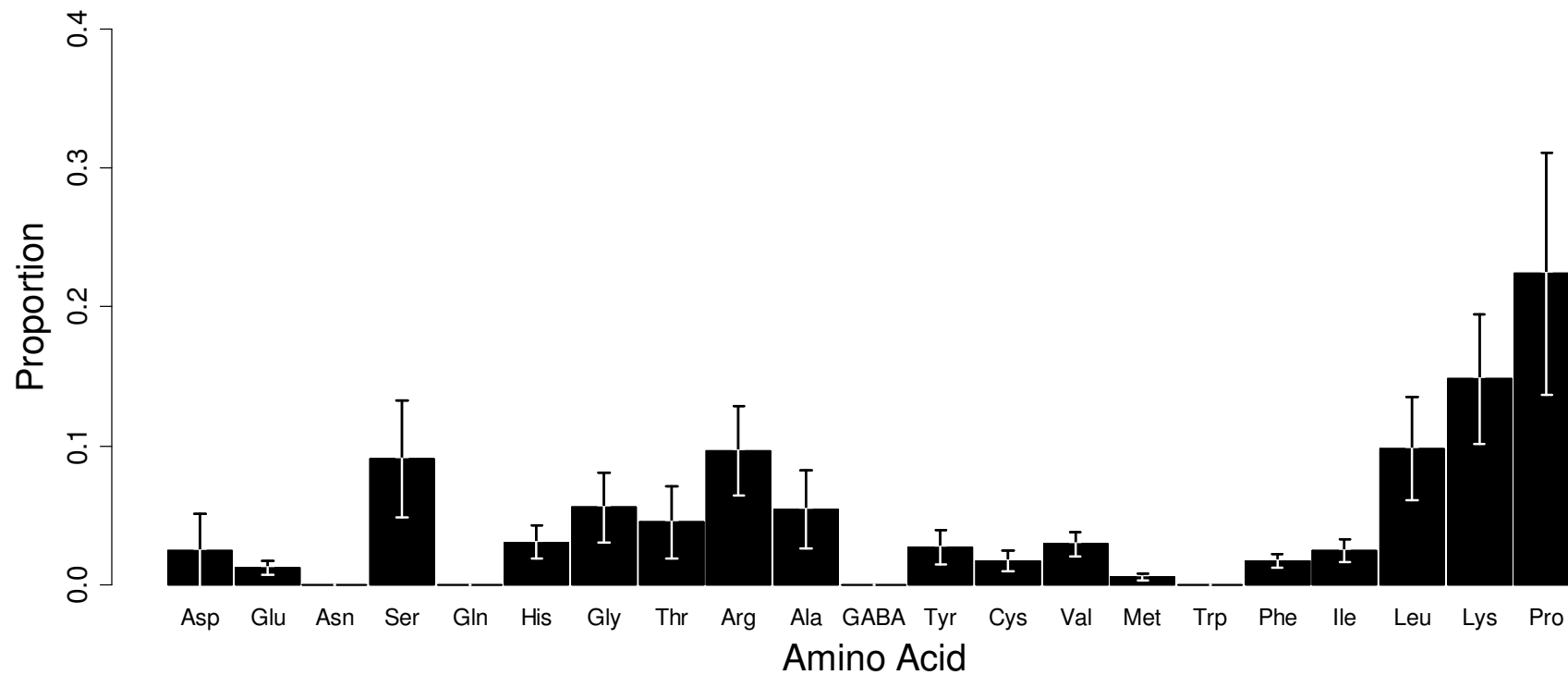
Figure 3.3. Mean proportions of amino acids of the total free amino acid content. Asp = aspartic acid/aspartate, Glu = glutamic acid/glutamate, Asn = asparagine, Ser = serine, Gln = glutamine, His = histidine, Gly = glycine, Thr = threonine, Arg = arginine, Ala = alanine, GABA = γ-aminobutyric acid, Tyr = tyrosine, Cys = cysteine, Val = valine, Met = methionine, Trp = tryptophan, Phe = phenylalanine, Ile = isoleucine, Leu = leucine, Lys = lysine, Pro = proline. Error bars represent one standard deviations of the mean proportion.

3.4.3 Bee bread pollen diversity

DGGE profiles were generated and analysed for 39 samples of bee bread. From these profiles, 16 distinct OTU fingerprints were identified, comprising 24 distinct bands, which were designated BP01-BP24 (Figure 3.2). Each sample of bee bread displayed on average 6 ± 2 OTUs (mean ± SD.: 6.18 ± 2.27, range: 2 – 11, Figure 3.4). The most common OTUs were BP01, BP03 and BP11; in a logistic regression with α-diversity, BP01 and BP03 were found to occur more frequently in more OTU-diverse samples (Table 3.2).

3.4.4 Correlations between pollen diversity and nutritional composition

The relative percentage of protein to carbohydrate in bee bread was significantly positively correlated with pollen α-diversity (b ± S.E. = 0.056 ± 0.023, $F_{1,38}$ = 5.919, P = 0.019, Figure 3.5); a quadratic relationship for this variable was considered, but the quadratic term was non-significant (b ± S.E. = -0.017 ± 0.015, $F_{1,36}$ = 1.241, P = 0.273). The relationship between pollen diversity and the protein and carbohydrate contents of bee bread were marginally non-significant (0.05<P<0.10); and none of the other nutritional contents were significant. In other words, as the number of pollen OTUs in the bee bread sample increased, so too did the relative amount of protein. There was no significant correlation between floral alpha diversity, as determined using DGGE, and any of the other nutritional components estimated here.
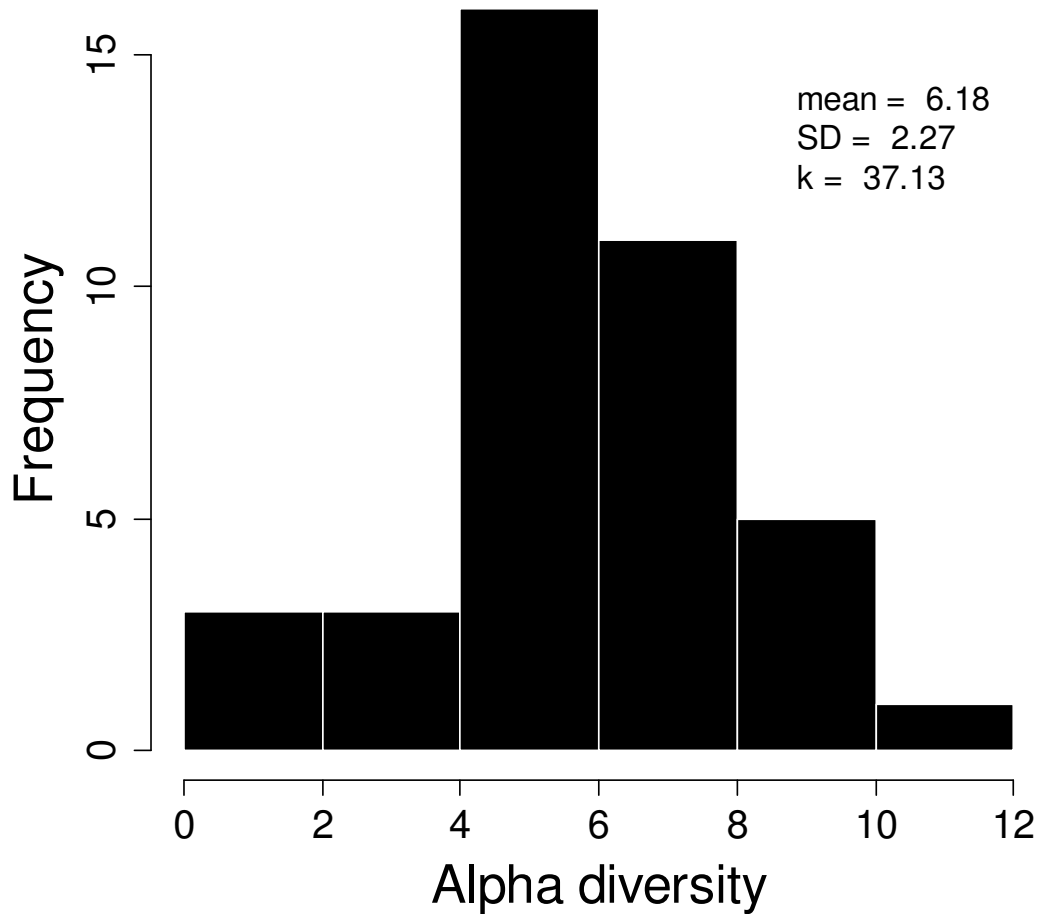
Figure 3.4. The prevalence of pollen operational taxonomic units (OTUs) detected by denaturing gradient gel electrophoresis, in bee bread samples (n = 39) from 30 honey bee hives. Mean, one standard deviation (SD) and degree of aggregation from the negative binomial distribution (k) are indicated.

Table 3.2. DGGE OTUs detected from amplification and analysis of plant trnL gene. The top five most frequently detected OTUs (present in >15 samples) were analysed in a logistic regression with α-diversity.

DGGE OTU	Frequency (n = 39)	Relationship with α-diversity		
		$b \pm$ S.E.	F	P
BP01	**23**	**0.423 ± 0.190**	**6.495**	**0.011**
BP02	**16**	**0.603 ± 0.234**	**10.178**	**0.001**
BP03	**24**	**0.722 ± 0.266**	**13.140**	**<0.001**
BP04	3			
BP05	9			
BP06	7			
BP07	5			
BP08	15			
BP09	13			
BP10	7			
BP11	29	-0.074 ± 0.168	0.195	0.659
BP12	6			
BP13	17	0.441 ± 0.535	0.759	0.384
BP14	12			
BP15	6			
BP16	3			
BP17	9			
BP18	7			
BP19	1			
BP20	4			
BP21	5			
BP22	10			
BP23	8			
BP24	2			
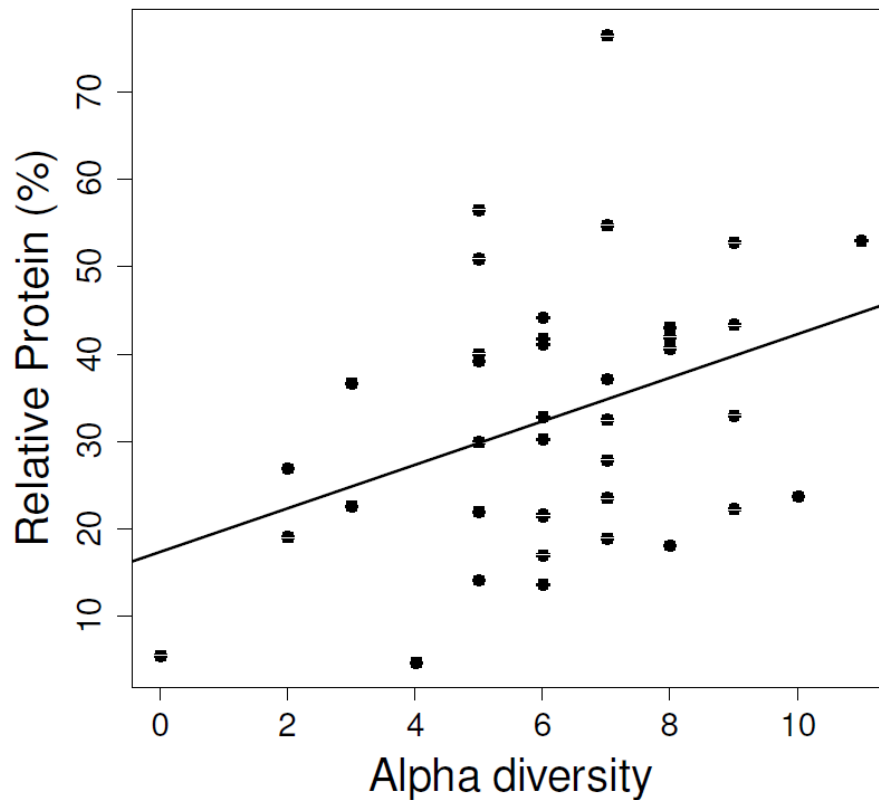
Figure 3.5. Variation in pollen diversity in bee bread determined by denaturing gradient gel electrophoresis. Relative percentage of protein to carbohydrate (*P/P+C*) content of bee bread increases with increasing diversity of pollens.

Logistic regression of the five most common OTUs revealed that as the prevalence of BP01 increased, so the relative protein content of the bee bread decreased linearly ($b \pm$ S.E. = -0.024 $\pm$ 0.012, $F_{1,38}$ = 4.444, P = 0.049, Figure 3.6a), but the opposite was true for BP11 ($b \pm$ S.E. = 0.035 $\pm$ 0.017, $F_{1,38}$ = 5.633, P = 0.039, Figure 3.6b). BP03 did not vary significantly with bee bread protein ($b \pm$ S.E. = 0.001 $\pm$ 0.010, $F_{1,38}$ = 0.018, P = 0.893), nor did BP02 ($b \pm$ S.E. = -0.014 $\pm$ 0.011, $F_{1,38}$ = 1.678, P = 0.195) or BP13 ($b \pm$ S.E. = -0.041 $\pm$ 0.039, $F_{1,38}$ = 1.419, P = 0.234).

3.4.5 Correlations between pollen diversity and amino acid composition

The overall composition of free amino acids did not vary significantly with pollen α-diversity (MANOVA: $F_{1,18}$ = 1.405, P = 0.495). However, individual amino acids were subsequently shown in post-hoc analysis to vary significantly with pollen α-diversity: both proline ($F_{1,18}$ = 9.757, P = 0.006, Figure 3.7a) and histidine ($F_{1,18}$ = 6.803, P = 0.018, Figure 3.7b) occurred in higher proportions in samples with greater pollen α-diversity. None of the amino acids were found to decline significantly in proportion with increasing α-diversity. In logistic regression, none of the amino acids varied significantly with any of the OTUs, possibly due to a lack of statistical power.
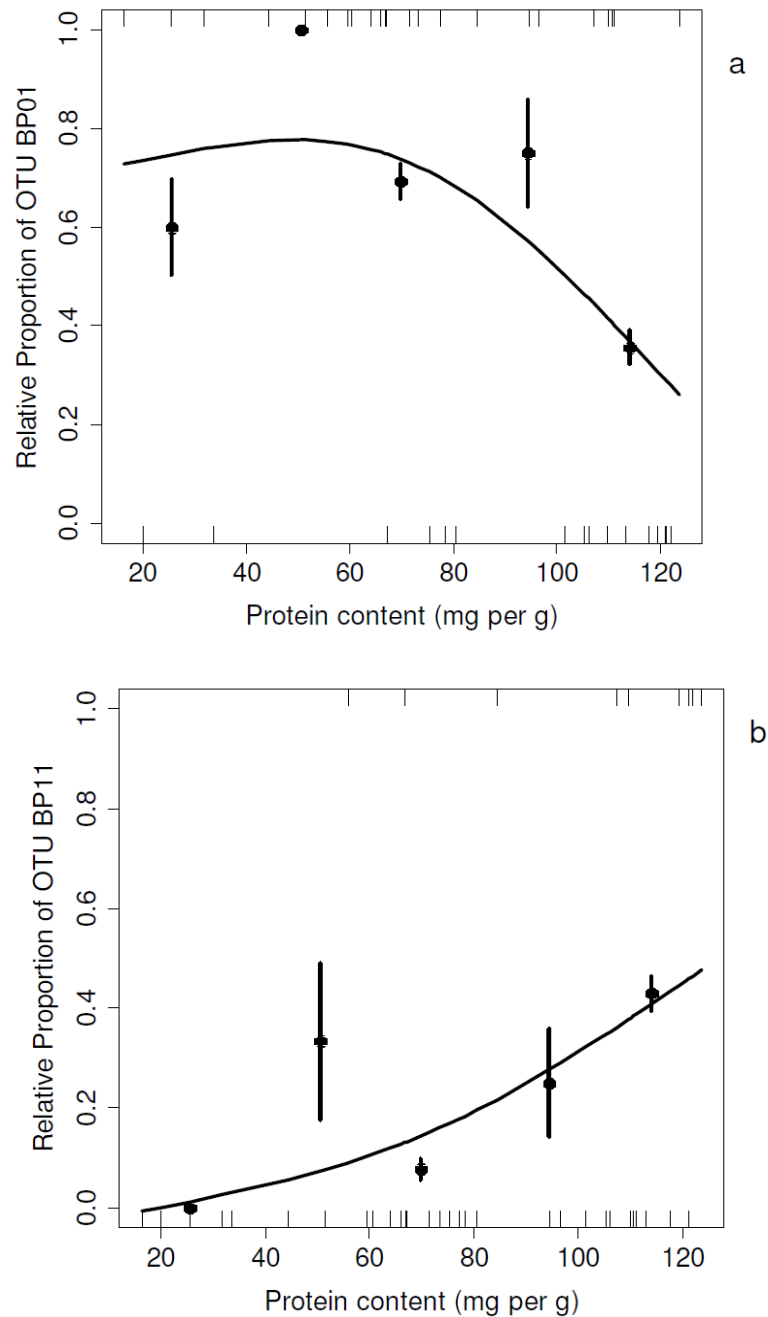
Figure 3.6. Relative proportional abundance of OTUs amplified from bee bread against protein content of samples. (a) OTU BP01 from DGGE shows lower protein contents as it increases in presence and (b) as OTU BP11 from DGGE increases in presence higher protein contents occur. Error bars represent one standard error of the mean abundance for an OTU within each of five bins of protein content (>37.7 mg, 37.7 - 59.3 mg, 59.3- 80.8 mg, 80.8 – 102.0 mg, <102.0 mg).
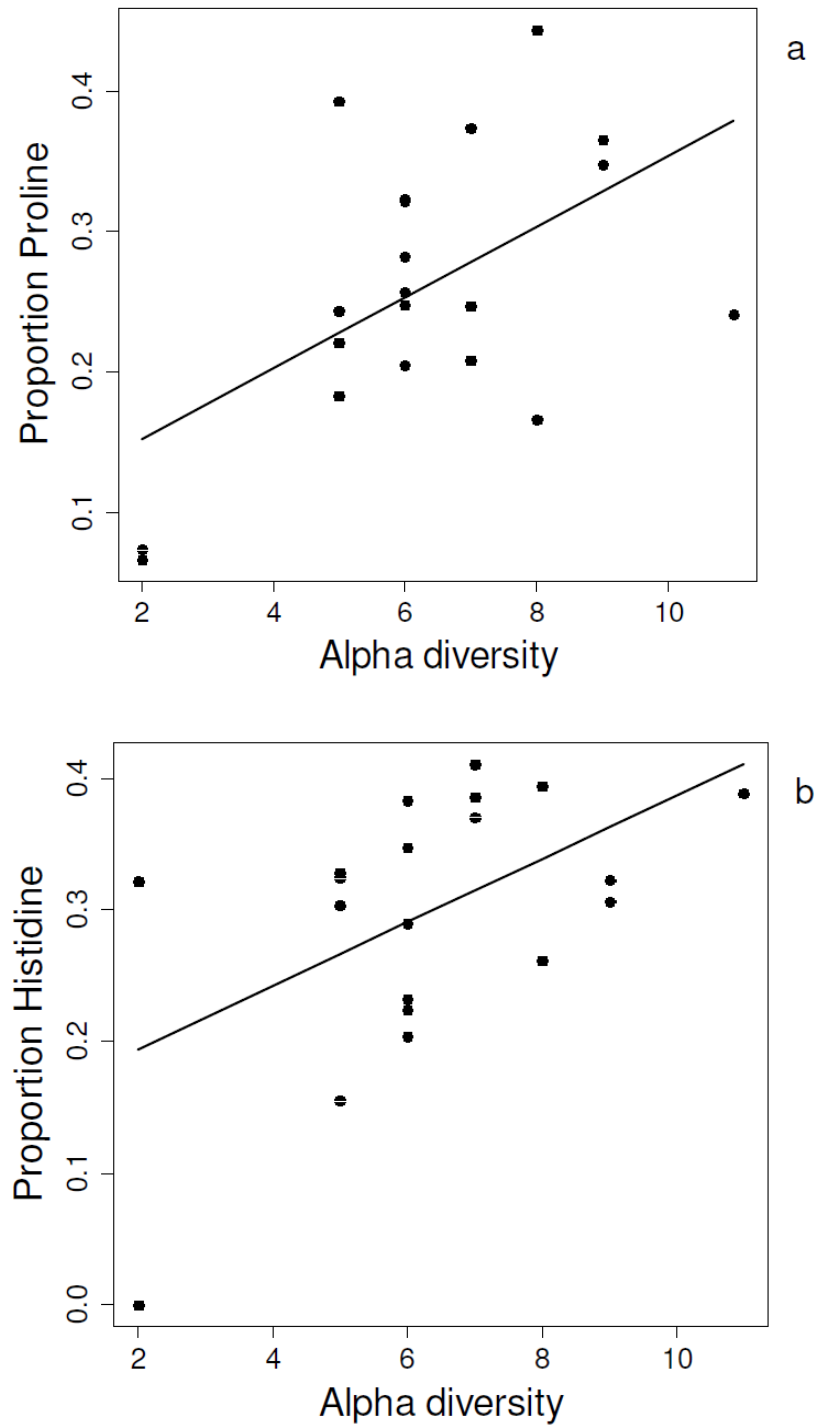
Figure 3.7. Proportions of free amino acids bread against the diversity of pollens amplified from bee bread. Proportion of (a) proline and (b) histidine relative to the total free amino acids in samples detected by high-performance liquid chromatography with lines showing significant correlations of fitted values from generalised linear models.

3.5 Discussion

A molecular approach based on analysis of *trn*L (UAA) intron sequence (Taberlet *et al.*, 2007) variability using denaturing gradient gel electrophoresis (DGGE) was used to determine the diversity of pollen species in stores of pollen within bee hives and to correlate this with honey bee nutrition. Traditionally, methods for estimating the diversity of pollens in studies associated with honey bees have involved the use of pollen traps, which are devices designed to remove grains of pollen from the legs of forager bees entering the hive (Todd and Bishop, 1940; Koppler *et al.*, 2007). There is disparity between pollen brought into the hive by bees and those actually stored on frame within the hive, caused by a loss of pollen grains during transport through the hive (Dimou *et al.*, 2006). Therefore, this study has focused on pollen stored on the frame, as this is the most representative of the diet of the bees in a hive. Identification of pollens has also typically been achieved through visual identification through light microscopy (Ohe *et al.*, 2004). During the process of storing pollen on frame, honey bees partially digest the exine (outer wall) of pollen grains (Crailsheim *et al.*, 1992). As the exine is used to identify pollen grains, this makes visual identification of pollen from the frame unreliable. The molecular approach may become a more reliable alternative, as the DNA in pollen remains intact on the frame (Eady *et al.*, 1995), but a method for successfully identifying pollens to species level requires further study.

When making assessments from data derived from the molecular approaches used here (PCR-DGGE), certain constraints must be acknowledged. PCR-based techniques are subject to the limitations of PCR itself: the most significant of these include that amplification can be inhibited by contaminants that co-extract with DNA; that there can be preferential or selective amplification of DNA from mixed

communities; and there can be formation of chimeric or heteroduplex DNA molecules (Nannipieri *et al.*, 2003; Takada Hoshino and Morimoto, 2010). Due to the limitations of PCR-DGGE therefore, it must be noted that the interpretations presented here are likely based on an underestimation of the "true" diversity of pollen species within bee bread.

Analysis of the DGGE profiles showed a significant relationship between the diversity and composition of pollen species in cells of bee bread and the nutritional composition of these cells, specifically relating to the percentage protein overall and the protein:carbohydrate ratio. Previous studies have indicated that a higher concentration of protein in the diet can be beneficial for honey bees, improving longevity, pesticide resistance (Altaye *et al.*, 2010; Wahl and Ulm, 1983). These benefits may be balanced by the amount of carbohydrate in the diet, with some studies showing that diets with an extreme protein to carbohydrate ratio (P:C < 5:1) may have reductive effects on life history traits, such as lifespan (Lee *et al.*, 2008) and development rates (Cotter *et al.*, 2008a). High protein content diets may also have fitness costs in terms of reproductive ability and survival (Pirk *et al.*, 2010), these effects have been further demonstrated in ant colonies (Dussutour and Simpson, 2012).

Protein has also been shown in many previous studies to enhance immune functions in honey bees (DeGrandi-Hoffman *et al.*, 2010; Alaux *et al.*, 2010b), which in turn will make bees more robust against infection. It must be noted however, that recommendations for planting regimes based on enhancing protein content alone may have detrimental effects on honey bees. In the aphid-tending ant, *Lasius niger* L., a high-protein diet can increase mortality of adult workers and lead to colony collapse (Dussutour and Simpson, 2012). Therefore dietary carbohydrate may also play an

important role in maintaining or modulating invertebrate fitness; the combined intake of protein and carbohydrate has been shown to significantly alter honey bee fitness (Altaye *et al.*, 2010).

Of the nutritional constituents considered, only protein content varied significantly with the diversity of pollens in this study. Lipids, starch and moisture were not correlated with these floral estimates. Although lipid content has been shown to modify the longevity of worker bees (Kunert and Crailsheim, 1988) and there are significant differences in the fatty acid contents of different pollen species (Loidl and Crailsheim, 2001), it is likely that the assay used in this study was not sensitive enough to detect these differences in terms of total lipid (Cheng *et al.*, 2011). Consistent with previous findings (see Chapter 2, section 2.4.3), the moisture and starch contents of bee bread have been shown previously to not vary significantly between samples.

Diets derived from a mixed assemblage of pollens (polyfloral) have been shown to significantly increase the immune response of larval bees (Alaux *et al.*, 2010b). Bumble bee (*Bombus terrestris*) larvae fed with a polyfloral blend were found to have greater mass than larvae fed with monofloral diets of higher protein content (Tasei and Aupinel, 2008). In contrast, monofloral diets have also been shown to benefit honey bees. A monofloral diet of mustard flower pollen was found to increase brood production and larval weight, whereas a polyfloral diet of mixed populations of non mustard plants did not shown a significant effect (Singh and Singh, 1996). Although the pollen-diversity of honey bee diets may have a direct link to health benefits for the bee, previous research has suggested that this may be due to the benefits of a few key pollen species such as sweet chestnut (*Castanea* spp.) and blackberry (*Rubus* spp.; (Di Pasquale *et al.*, 2013). Although the identity of the OTUs

in the present study is not known, BP11 (see Figure 3.6b) may play one of these key pollen species within bee bread here, whereas BP01 could potentially be "diluting" the protein content of bee bread (see Figure 3.6a).

The results of this study potentially have implications for honey bee management, not only in terms of optimising locations of hives but also for floral resource planting schemes. Honey bees have demonstrated preferences to forage certain flowers over others (Kirk and Howes, 2012; Keller *et al.*, 2005b; Keller *et al.*, 2005a), and current recommendations for planting are based on this (Thompson *et al.*, 2003; Royal Horticultural Society, 2014). Pollen species also vary in their nutritional qualities (Somerville, 2001), which does not correspond to feeding preference (Schmidt and Hanna, 2006), meaning that bees may preferentially forage on poor quality pollens if they are made available in their environment.

In Chapter 2, it was noted that honey bees are capable of foraging in areas up to 10 km² around their hives, but they are most efficient at 3 km² (Steffan-Dewenter *et al.*, 2002); this factor is not considered in the design of planting schemes, however this is likely due to the assumed near-cosmopolitan nature of honey bees in UK (House of Commons Public Accounts Committee, 2009). By identifying which plant species have the greatest benefit to bees in terms of their nutrition within the hives; this information would contribute significantly to the decision-making process for municipal and public planting of these species.

Here, the occurrence of individual pollen species was shown to have potential impacts on the nutritional content of food stores. The appearance and disappearance of certain plant species as the beekeeping season progresses has been suggested previously to be driving significant changes in honey bee nutrition (see Chapter 2.5). The single OTU found in this study to appear to have the greatest influence on the

nutritional composition of bee bread was the as yet unidentified BP11, which was most commonly found in high protein bee bread samples. The single OTU found most commonly in low protein content bee bread was the unidentified BP01. Having indicated that these possible plant species may play an important role in determining honey bee nutrition across multiple hives, a further study using advanced sequencing techniques, such as next generation sequencing (Margulies *et al.*, 2005; Morey *et al.*, 2013) based around the same target sequence would allow the identity of these organisms to be revealed. Next generation sequencing is capable of detecting a much higher degree of diversity in microbial systems due to the ability to amplify extremely low copy-number target DNAs. It generates up to several hundred thousand individual sequencing reads simultaneously, which are used to estimate the composition and relative abundances of organisms in a given community (Lee *et al*., 2012).

The results presented here have also attempted to take into account the variable amino acid contents of different pollens (Bertazzini *et al.*, 2010; Cook *et al.*, 2003; Kim and Smith, 2000), of which honey bees have specific requirements for maximal survival (see Table 3.3). All of the essential amino acids suggested by deGroot (1953), except for tryptophan, were detected in all of the bee bread samples (see Figure 3.3); indicating that the current mixtures of pollens that honey bees are collecting within the study site are sufficient to meet their amino acid requirements. Previous research has indicated that relative concentrations of amino acids to dietary carbohydrate have significant impacts on honey bee feeding habits and increasing amino acid content having a putative effect on lifespan (Paoli *et al.*, 2014).

Ideally, a further study that can account for the changes in amino acid composition in bee bread and how this is affected by the individual pollen species

would be valuable in constructing a recommended planting regime that would benefit honey bee nutrition.

From the results of DGGE, bee breads sampled in this study were all found to be polyfloral – meaning that they likely all contained at least two or more pollen species (see Table 3.2). Individual pollens vary significantly in their amino acid composition; many are deficient in some of the essential amino acids required by honey bees (Somerville, 2001). Therefore, as a result of the polyfloral nature of bee bread, this food store does not suffer from the deficiencies of individual pollens. There is evidence that honey bees are able to detect amino acid composition in their diets and are able to optimise their intake of essential amino acids (Cook *et al.*, 2003). Honey bees may therefore be mixing pollens in the production of bee bread in order to optimise their intake of essential amino acids.

Although the results here have shown the potential effects of pollen community composition on the nutritional composition of bee bread, species level identification of these pollen species was not successful, limiting the conclusions that could be drawn from the data currently. Consistent with previous research, the results here suggest that more pollen-diverse diets may lead to higher protein content diets for honey bees (Di Pasquale *et al.*, 2013), which may have potential healthy benefits (see Chapter 5); and that individual species may play a particularly important role in driving this potential benefit (Di Pasquale *et al.*, 2013).

Furthermore, there has also been some research that suggests that plant genotype may play an important role in determining nutritional quality of plant derived diets (Rowntree *et al.*, 2010). A further study on the nutritional compositions of pollens and how they differ at the subspecies level would inform planting recommendations to benefit honey bees.

Table 3.3. Pollen amino acids grouped according to their importance for honey bee nutrition (deGroot, 1953).

Requirement class			
Non-essential	Least essential	Intermediate essential	Most essential
Alanine	Histidine†	Arginine*	Isoleucine
Asparagine‡	Methionine	Lysine*	Leucine*
Aspartate	Tryptophan‡	Phenylalanine	Valine
Cysteine	Threonine		
γ-Aminobutyric acid (GABA) ‡			
Glutamate			
Glutamine‡			
Glycine			
Hydroxyproline			
Proline*†			
Serine*			
Tyrosine			

*Amino acids that accounted for the greatest proportions of free amino acids in bee bread samples.

‡ Amino acids that occurred below the limit of detection.

† Amino acids shown to increase in proportion with increasing pollen diversity.

Chapter 4: Diversity and dynamics of microbial communities in bee bread stores of the honey bee (*Apis mellifera mellifera* L.)
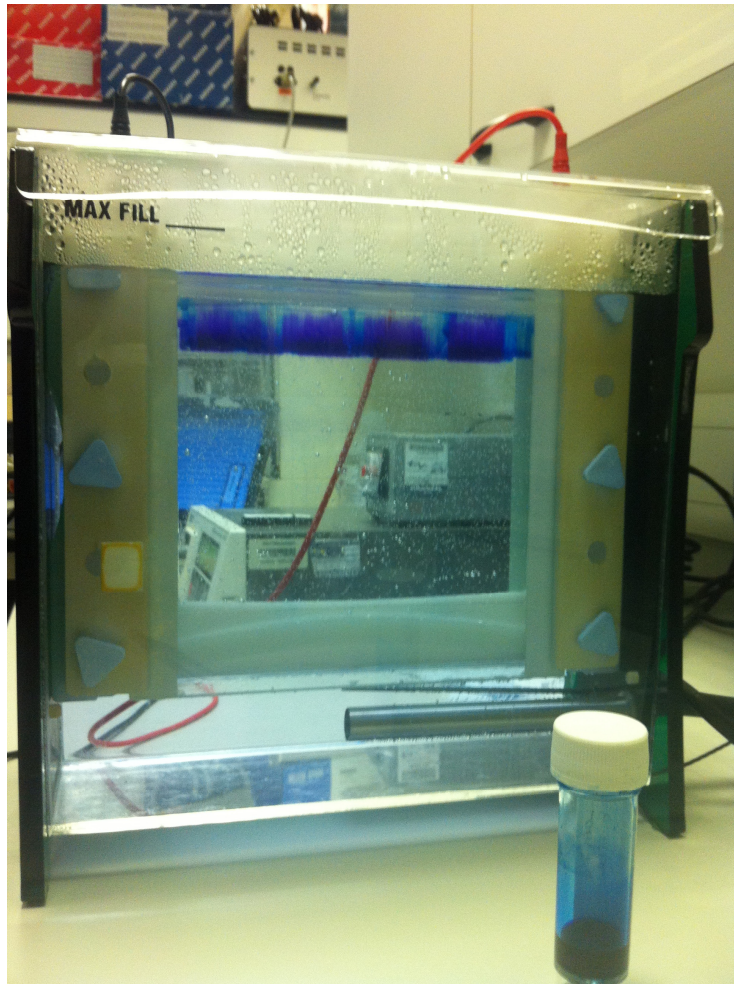
Denaturing gradient gel electrophoresis machine used in this chapter to analyse bacterial communities found in bee bread.

4.1 Abstract

Honey bees (*Apis mellifera* L.) are associated with a community of microorganisms. Members of this community may play a key role in the production of fermented pollen (bee bread), which is the main source of protein used to raise larvae. Previous studies have examined the diversity of microbial communities of the gut and honey stores, but few have considered bee bread and only at limited spatial and temporal scales.

Here, the composition and dynamics of the microbial communities associated with bee bread of honey bees across broad spatial and temporal scales are described. This was achieved through culture and molecular analysis using PCR, denaturing gradient gel electrophoresis (DGGE) and partial sequencing of PCR-amplified eubacterial 16S rRNA gene products, from bee breads sampled once every eight weeks over six months from 30 hives across North-west England.

Eighteen distinct bacterial genera from 73 operational taxonomic units (OTUs) were detected across all samples (n = 472), with each sample of bee bread revealing on average six OTUs. Within hives, bacterial communities maintained remarkable stability between frames in the same box at each sampling time point, but there was significant variation between boxes within the same hive, possibly because different cohorts of bees work in different parts of the hive. Bacterial communities differed significantly between hives found in different locations and diversity changed within hives as the beekeeping season progresses.

Though the bacterial community on which the bee bread is dependent varies significantly between hives in this way, bee bread production is maintained throughout the season. Honey bees may maintain these changing bacterial communities as an adaptation to the variable sources of pollen that they collect

through the season, although there is currently no evidence for this. Future studies could usefully explore the mechanisms driving the temporal changes in the microbial community as the beekeeping season progresses and its consequences for honey bee fitness.

4.2 Introduction

Insects maintain both internal and external associations with diverse bacterial communities for many functions, including nutritional support (Engel and Moran, 2013), one such insect is the honey bee (*Apis mellifera*, L.). Honey bees consume pollen as a source of protein and amino acids, lipids, vitamins and minerals (Roulston and Cane, 2000; Herbert and Shimanuki, 1978). In addition they collect nectar from plants, dehydrate and store it within their hives as honey. Nectar and honey are the primary source of carbohydrate, consumed to fuel the activity of adult workers. Within the hive, pollen is converted to bee bread via fermentation, through microbial community fermentation (Pain and Maugenet, 1966; Gilliam, 1979b; Gilliam, 1979a; Vásquez and Olofsson, 2009). Bee bread is an essential component of the honey bee diet, providing the protein and carbohydrate necessary for the production of brood food used in raising larval honey bees (Oliver, 2007b). Bee bread also plays a key role in determining the microbial community of the honey bee gastrointestinal tract (McFrederick *et al.* 2012), which itself may be important in providing protection against honey bee pathogens (Alippi and Reynaldi, 2006). Previous studies have shown that increasing the protein content of bee bread may result in increased immune function, larval production and adult longevity in honey bees (Alaux *et al.*, 2010b; DeGrandi-Hoffman *et al.*, 2010), but have not attempted to link these effects to the dynamics of the microbial community.

The disruption of microbial communities, called dysbiosis, can lead to increased susceptibility to disease in honey bees (Hamdi *et al.* 2011; Mattila *et al.* 2012). Although a trigger for dysbiosis in bees has not been determined, it has been suggested there may be a link between this and colony collapse disorder (CCD; (Johnson *et al.*, 2009). CCD is a major driver of global pollinator decline (vanEngelsdorp and Meixner, 2010); see Chapter 1, section 1.1.2) and the direct cause of this phenomenon has not yet been identified (Ellis *et al.*, 2010; vanEngelsdorp *et al.*, 2009) as it is likely related to a number of interacting factors including nutrition and microbial communities (Becher *et al.*, 2013).

Currently, little research has been undertaken on the diversity and composition of the microbial community found in bee bread, with the exception of some early research based on culturable bacteria (White, 1921; Gilliam, 1979b; Gilliam, 1979a; Chevtchik, 1950), which may not be representative of the whole microbial community (e.g. it has been estimated that 60-80 per cent of human colonic bacteria are not culturable; (Langendijk *et al.*, 1995; Suau *et al.*, 1999). These studies indicated that the microbial community of bee bread is primarily dominated by *Bacillus*, *Lactobacillus* and several species of yeast.

A recent study used molecular techniques to analyse the lactic acid bacteria (Pasteurallaceae, *Acinetobacter* spp. and *Lactobacillus* spp.) found in bee bread (Vásquez and Olofsson, 2009). It found that these bacteria were likely sourced exclusively from the honey stomach of honey bees when they add honey to pollen via regurgitated nectar from the honey stomach. Another study that combined cultural and molecular methods found that bee bread was dominated by *Lactobacillus kunkeei* (Anderson *et al.*, 2013) and from this it was suggested that the production of bee bread

from pollen may be due to a process similar to that observed in silage preservation (Loper *et al.*, 1980).

The microbial community of bee bread originates, therefore, from a combination of the microbes found on the pollen grains that comprise the main ingredient of bee bread and the gut microbiota of the honey bees themselves (McFrederick *et al.*, 2012). Molecular techniques will advance the understanding of spatio-temporal dynamics of bee bread microbial communities, which is particularly important given the variation in food plants and hive fitness that has been demonstrated elsewhere in this thesis (see Chapter 3 and Chapter 5).

Therefore, the aim of this study was to address the following questions: (i) Is there any spatial structuring of bacterial communities within the hive or are they homogeneously distributed? (ii) Are there some 'core' bacterial species that are common across most hives? (iii) Is the bacterial community stable across the year? (hereafter, "the season", as the beekeeping season does not last for a whole year). The answers to these questions may elucidate understanding of the influence worker bees have over the microbial communities associated with bee bread.

4.3 Materials and methods

4.3.1 Sample collection

Bee bread was sampled from 30 hives sited within 25 apiaries, each of which comprises several hives, distributed across 3000 km^2 of North-west England in two years. In 2011, each hive was sampled once from 7th August to 27th September. In 2012, individual hives were sampled once every eight weeks from 7th April to 2nd September 2012. Bee bread lasts approximately six to eight weeks in the hive before

being eaten by brood (Oliver, 2007b; van der Steen, 2007), meaning that each sample taken from a hive represents an independent bee bread sample from each hive through the season. All hives sampled housed *A. m. mellifera*.

For each sampling occasion, stratified sampling was adopted in accordance with the nested nature of honey bee hives (see Chapter 1, section 1.1.5 and Chapter 2, section 2.3.1). Bee bread is stored within hives in separate honeycomb cells on frames, such that each cell on a frame contains a unique "unit" of bee bread. In the present study, bee bread was extracted from three cells on two frames within a box, from multiple boxes within a hive (if present), and from each of up to two hives within an apiary (Figure 4.1). Bee bread was recovered from cells aseptically into sterile 1.5 mL microfuge tubes. Samples were transferred to the laboratory on ice and processed within two hours. These samples were then divided such that 50% was used in culture-dependent analysis (section 4.3.2), 10% for culture-independent analysis (section 4.3.3) and the remaining mass was set aside for nutritional analysis (see Chapter 2) and long term storage.

4.3.2 Culture-dependent analysis

The mean mass for cells of bee bread was 299.4 mg ± 64.0, of which 50% was selected for culture, for 2011 samples only. These bee bread samples were suspended in 1 mL sterile phosphate buffer solution and both anaerobic bacteria and the community of aerobic heterotrophic organisms were cultured.

Aerobic heterotrophs were cultured on R2A agar (Oxoid, UK) under aerobic conditions at 35ºC for 14 days. Anaerobic lactic acid bacteria were selectively cultured on Rogosa agar (Oxoid, UK) under anaerobic conditions at 35ºC for 14 days (De Man *et al.*, 1960). Conditions were chosen to mimic as closely as possible those

within a beehive (Bujok *et al.*, 2002). Counts of colony forming units (CFU) were converted to total CFU per unit weight of bee bread based on the mass of bee bread that was used.

4.3.3 Culture-independent analysis

Microbial communities were analysed using 16S rRNA gene PCR that amplified the hypervariable region between positions 515 – 806 (Table 4.1). The 16S rRNA gene consists of several sequence domains that have evolved at different rates, resulting in regions that are universally highly conserved across species, interspersed with regions that are more variable (García-Martínez *et al.*, 1999).

Table 4.1. Primers used in PCR reactions.

Primer	Sequence (5`-3`)	Position*	Primer Target	Reference
515F	CCAGCAGCCGCGGTAA	515-533	Eubacterial 16S rRNA gene	(Relman *et al.*, 1992)
806R	GGACTACCACGGTATCTAAT	806-786	Eubacterial 16S rRNA gene	(Relman *et al.*, 1992; Wilson *et al.*, 1990)
515F-GC ‡	*CGCCCGCCGCGCCCCGC GCCCGTCCCGCCGCCCC CGCCCGG* - CCAGCAGCCGCGGTAA	515-533	Eubacterial 16S rRNA gene	(Relman *et al.*, 1992)

*Escherichia coli numbering—corresponds to the positions in *E. coli* 16S rRNA gene

‡ Italicised sequence to identify GC-clamp sequence

4.3.4 Nucleic acid extraction and amplification

Total DNA was extracted from bee bread samples from both sample years using the QIAamp DNA Plant Mini kit (Qiagen Ltd, Crawley, UK). DNA extractions were performed according to manufacturer's specifications. The mass of bee bread used for extraction was 21.3 mg ± 1.9. Bacterial 16S rRNA genes were amplified using the universal primers 515F/806R (Wilson *et al.*, 1990; Relman *et al.*, 1992), Table 4.1) with the forward primer (515F) incorporating a 34-bp GC clamp (Sheffield *et al.*, 1989) for electrophoresis analysis. All PCR amplifications were carried out in an ABI Veriti™ Thermal Cycler (Applied Biosystems, UK).

PCR amplifications were performed in 20 µL volumes; each reaction contained the following: 2 µL (20 pmol) of each primer, 4 µL sterile PCR grade water, 4 µL extracted DNA and 10 µL Amplitaq Gold® 360 Master Mix (Applied Biosystems, UK). Positive controls contained genomic DNA from *Pseudomonas* sp. (Accession: HF536517) in place of sample DNA. The negative control for the PCR reaction contained sterile water. The negative control from the DNA extraction was included as an additional negative control reaction. Initial denaturation was carried out for 5 min at 94 ℃, and cycling was performed as follows: 94 ℃ for 20 seconds, 52 ℃ for 20 seconds and 72 ℃ for 45 seconds for 30 cycles, with a final elongation at 72 ℃ for 5 min.

4.3.5 Denaturing gradient gel electrophoresis (DGGE)

PCR products were separated by DGGE using the Scie-Plas TV400 vertical electrophoresis system (Scie-Plas, Cambridge, UK). Denaturing gels consisted of

1mm thick 6% polyacrylamide with a denaturing gradient of 40-65%, whereby 100% denaturing corresponds to 7M urea and 40% v/v deionized formamide, 1x TAE buffer (40mM Tris–acetate (pH 8.0) and 2mM EDTA) and 2% v/v glycerol. Electrophoresis was performed at 60 ºC and current was limited to 20V-30mA for 10 minutes and then at 100V-30mA for 1250 V·Hrs (approximately 16 h) in 1x TAE buffer. Gels were stained in 1x SYBR Gold nucleic acid stain (Invitrogen, UK) and visualised on a UV-transilluminator block, gel images were obtained and documented with Kodak MI software (Carestream Health, Inc., Rochester, NY).

4.3.6 DNA sequencing

Following staining and visualisation, identified bands were excised from DGGE gels with a sterile scalpel blade and placed in 30 µL sterile PCR grade water in a sterile 1.5 mL microfuge tube. DNA was extracted by centrifugation at 13, 300 rpm for 30 min in Corning® Costar® Spin-X® centrifuge tubes according to manufacturers instructions. SYBR Gold stain was removed from extracted DNA by ethanol precipitation with 300mM sodium acetate. Purified DNA (in 50 µL PCR grade water) was then re-amplified with primers 515F/806R (Table 4.1), using the PCR conditions described above. DNA sequences of excised bands were determined commercially by Beckman-Coulter Sequencing (Essex, UK) by a single read on an ABI 3730XL Sanger sequencer, using the original primer 515F. Following elimination of chimeric or heteroduplex sequences using QIIME (http://qiime.org/index.html) via ChimeraSlayer (Caporaso *et al.*, 2010); sequences were then aligned with those in the GenBank (ncbi.nlm.nih/genbank) database with the megaBLAST program (blast.ncbi.nlm.nih.gov; (Zhang *et al.*, 2000).

4.3.7 Estimation of microbial richness

Bacterial richness in each sample was measured by counting the number of bands found in each lane of the DGGE gel, with the assumption that each band represents a unique 16S rRNA gene sequence, and therefore operational taxonomic unit (OTUs; (Atlas and Bartha, 1998). Assigned positions were confirmed by comparing between different gels using sequences from the same position across multiple gels. The sequences of bands adjacent within a lane were compared and combined when found to be identical. A binary matrix was produced for each bee bread sample by denoting OTU presence ⁄ absence (Figure 4.1)
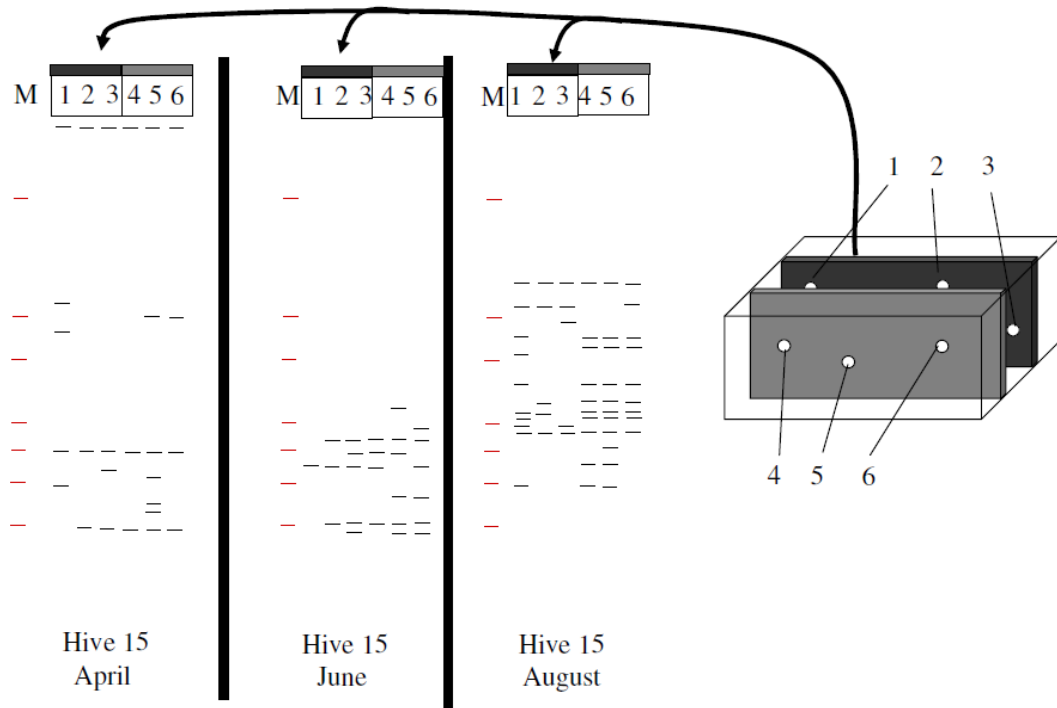
Figure 4.1. Section of a DGGE profile of PCR-amplified partial 16S rRNA bacterial genes targeted in six samples of bee bread taken from the same hive in three repeats across the season. Numbers 1-6 indicate samples of bee bread from two frames; M: internal DGGE marker.

4.3.8 Statistical analysis

Analyses of the intra- and inter-hive variation in the microbial community structure were performed using a series of generalised linear mixed-effects models (GLMMs) using the *lme4* package (Bates *et al.*, 2012) in the *R* statistical software (R Core Team, 2013). Variation in community structure was examined over/across a nested hierarchy of spatial scales. The scales included were within-frame, within-box, within-hive and between-beekeepers, as such random effects included in the model were (*1|Hive/Box/Frame*) to account for hierarchal variation in sampling. Variance components were extracted from these random effects according to Chapter 2, section 2.3.4. Chi-square statistics for these random effects were generated and tested using likelihood ratio estimates (Zuur, 2009).

The independent terms tested included the geographical variables Eastings and Northings (*Eastings* & *Northings*), a temporal variable, Julian date (*Julian*: days since 1st January), hive design (*Htype*: National, WBC or Langstroth, see Chapter 1, section 1.1.4) and beekeeping style (*Btype*: the style of beekeeping used on the beehive, segregated into Training, Breeding and Hobbyist). Comparisons across two sampling years were made only between the third sample period of 2012 (2nd July – 2nd September) and the 2011 samples (7th August to 27th September), i.e. when the timings of the two sampling periods aligned.

Dependent variables were analysed in two stages: first, through changes in the bacterial community composition, which were analysed using α-diversity (i.e. frequency of OTUs) in GLMMs, and a cluster identity in linear mixed effects models (LMMs) determined by Hartigan-Wong clustering algorithm as dependent variables (Hartigan and Wong, 1979).

Cluster patterns represent the composition of OTUs in samples and were included because they account for the similarity of samples through the presence and absence of taxa when generating similar clusters. For the cluster analysis, the appropriate number of clusters in the DGGE-derived OTU data was determined using k means-clustering using the Hartigan-Wong algorithm from the *R* package *cluster* (Maechler *et al.*, 2012; Hartigan and Wong, 1979) to assign each sample to a cluster based on its banding pattern. This algorithm calculates the Euclidean distance to assign cluster identity for each bee bread sample and the group of each sample was assigned to a new variable "*group*" (Hartigan and Wong, 1979), which was then used as a dependent variable in an LMM (as in Benskin *et al.*, (2010).

Second, a series of logistic regressions on the most common OTUs were used to determine variation in the prevalence of specific bacteria. These OTUs were analysed as a binomial dependent variable (presence/absence) in mixed effects logistic regression in GLMMs with the same hierarchical random terms as above. The random effects and fixed effects included in these models were the same as in previous GLMMs. The results presented are the output of the most parsimonious models, determined using stepwise-deletion of fixed effects, based on residual deviance contrasts (Bolker *et al.*, 2009). Markov chain Monte Carlo simulation was used to estimate *p*-values of the minimum models using *R* package *languageR* (Baayen 2007).

4.4 Results

4.4.1 Bacterial community analysis using DGGE

DGGE profiles were generated and analysed for 472 samples of bee bread, from 30 hives over the 2 years, generating 117 distinct fingerprints with 73 unique

bands. Each sample of bee bread produced, on average 6.16 ± 4.14 OTUs (mean $\pm$ SD; range = 1 – 20, k = 3.42, Figure 4.2).

All of the 73 bands excised from DGGE gels were successfully re-amplified and sequenced. Following analysis of the sequencing results (see section 4.3.7), 48 distinct OTUs were identified, designated BB1 - BB53 (OTUs BB2, BB26, BB32, BB36 and BB41 were omitted because of poor sequence quality), which were assigned GenBank accession numbers (KF881801- KF881848) and showed 95 - 99% identity with a known sequence in the GenBank database (Table 4.2). A total of 18 distinct bacterial genera were identified from sequence alignment (Table 4.2). Four OTUs (BB12, BB13, BB18 and BB21) shared the closest homology with the genus *Acinetobacter*, two with *Lactobacilli* (BB7 and BB16) and five with *Enterobacter* (BB5, BB38, BB40, BB49 and BB51); all three bacterial genera are believed to be capable of fermenting bee bread (Vásquez and Olofsson, 2009; Crotti *et al.*, 2010) and occurred in up to 16.5% (*Acinetobacter*), 18.2% (*Lactobacilli*) and 32.6% (*Enterobacter*) of samples. *Actinobacter* are capable of cellulose digestion, and are generally considered protective mutualists in insects, generating secondary metabolites that deter opportunistic food spoilage organisms (Kaltenpoth, 2009; Seipke *et al.*, 2011).
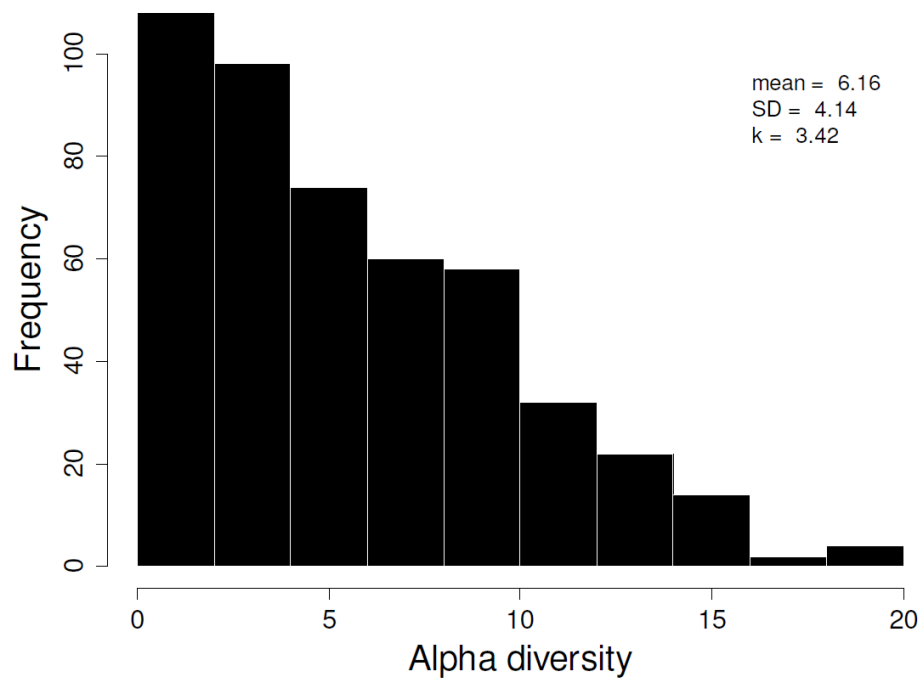
Figure 4.2. The α-diversity (frequency) of operational taxonomic units (OTUs) detected by denaturing gradient gel electrophoresis; in bee bread samples (n = 472) from 30 honey bee hives. Mean, standard deviation (SD) and degree of aggregation from the negative binomial distribution (k) are indicated.

Eight OTUs (BB8, BB19, BB22, BB26, BB28, BB30, BB46 and BB52) shared closest homologues with the *Pseudomonas* genus, although these were amongst the rarest, occurring in less than 6.8% of samples. The most common OTUs were those designated BB42, BB43, BB29, and BB16 (Table 4.2). There were no OTUs that occurred in all samples; however the most common was BB42 (KF881837), most closely aligned in GenBank to an uncultured proteobacterium, which occurred in 58.0% of all samples. BB28 (KF881826), a *Pseudomonas* sp., was the rarest, occurring in just 1.3% of samples.

Sequencing of DGGE bands revealed that several bacteria genera were represented by more than one band (Table 4.2), consistent with the findings of previous studies (Dahllöf, 2002; Fogel *et al.*, 1999). Consequently, the subsequent statistical analysis of banding profiles below relates to 16S rRNA gene sequence diversity rather than species richness within the bacterial community.

Table 4.2. Identification of bands excised from DGGE gels, as identified by partial 16S rRNA gene sequencing.

OTU	DGGE Gel Position	Closest 16S Homologue on GenBank	Identity score	Frequency in present population (%; n=472)	Accession number
BB18	c3	*Acinetobacter* sp. 18N3	87	8.69	KF881817
BB12	b5 , b7	*Acinetobacter* sp. APG8	95, 94	14.62	KF881811
BB13	b6	*Acinetobacter* sp. p95_A06	84	16.53	KF881812
BB21	c6	*Acinetobacter* sp. SAP 971.1	97	3.18	KF881820
BB2	a2	Arsenophonus endosymbiont of *Dermacentor variabilis*	86	4.45	-
BB34	f1	Arsenophonus endosymbiont of *Philaenarcys bilineata*	94	21.19	KF881831
BB45	h2	*Cedecea* sp. strain PB61	98	18.22	KF881840
BB15	b9	*Clostridium* sp. SL29	88	3.39	KF881814
BB40	f7	*Enterobacter* sp. p62_B05	98	12.29	KF881836
BB51	h9	*Enterobacter* sp. p62_B05	98	4.24	KF881846
BB49	h6, h7	*Enterobacter* sp. p95_C06	98, 98	7.63	KF881844
BB5	a8	*Enterobacter* sp. p96_C03	99	2.54	KF881804
BB38	f5	*Enterobacter* sp. p96_C03	99	18.22	KF881834
BB48	h5	*Erwinia* sp. CF03	99	3.18	KF881843
BB37	f4	*Erwinia* sp. KCB 19	68	22.46	KF881833
BB24	d1 , d2 , d3	*Frischella* sp. PEB0191	99, 99, 99	20.76	KF881823
BB29	d9	*Frischella* sp. PEB0191	88	35.17	KF881827
BB17	c1 , c2	*Gilliamella* sp. wkB11	85	5.72	KF881816

BB7	a10	*Lactobacillus* sp. 80-30	99	1.69	KF881806
BB16	b10 , b11	*Lactobacillus* sp. 80-30	99, 98	32.63	KF881815
BB35	f2	*Massilia* sp. FP2-21-4	96	22.67	KF881832
BB44	h1	*Massilia* sp. GI3-S-1-E04	80	9.75	KF881839
BB47	h4	*Pasteurellaceae* sp. BHMR4	99	9.96	KF881842
BB52	h10	*Pseudomonas* sp. C1 ecto 19	87	5.72	KF881847
BB30	e1	*Pseudomonas* sp. cc 1451	92	6.36	KF881828
BB8	b1	*Pseudomonas* sp. HUK21	82	1.69	KF881807
BB32	e3	*Pseudomonas* sp. M1/32	67	3.81	-
BB19	c4	*Pseudomonas* sp. MFS3	99	6.78	KF881818
BB22	c7	*Pseudomonas* sp. MFS3	99	2.54	KF881821
BB28	d8	*Pseudomonas* sp. OF38	97	1.27	KF881826
BB26	d5 , d6	*Pseudomonas* sp. p53_D01	76, 81	4.66	-
BB46	h3	*Pseudomonas* sp. Q1-S11	74	6.22	KF881841
BB1	a1	Uncultured bacterium clone BIGH1473	95	19.07	KF881801
BB4	a5 , a6 , a7	Uncultured bacterium clone BIGH1473	96, 98, 97	7.42	KF881803
BB9	b2	Uncultured bacterium clone BIGH1473	95	1.48	KF881808
BB31	e2	Uncultured bacterium clone BIGH1473	81	2.54	KF881829
BB39	f6	Uncultured bacterium clone BIGH1473	99	5.3	KF881835
BB42	g1 , g2 , g3, g4 , g5 , g6, g7	Uncultured bacterium clone BIGH1473	99, 99, 93, 99, 97, 98, 92	58.05	KF881837
BB36	f3	Uncultured bacterium clone FL5Ad11_3665	80	17.58	-
BB3	a3 , a4	Uncultured bacterium clone FL5Ad11_3665	97, 98	11.86	KF881802
BB20	c5	Uncultured bacterium clone FL5Ad11_3665	96	4.66	KF881819
BB23	c8	Uncultured bacterium clone FL5Ad11_3665	99	16.31	KF881822

BB27	d7	Uncultured bacterium clone FL5Ad11_3665	98	3.39	KF881825
BB50	h8	Uncultured bacterium clone FL5Ad11_3665	99	8.47	KF881845
BB53	h11	Uncultured bacterium clone FL5Ad11_3665	98	2.97	KF881848
BB14	b8	Uncultured bacterium clone Ontario1283	99	5.08	KF881813
BB11	b4	Uncultured bacterium clone Ontario1287	99	3.6	KF881810
BB25	d4	Uncultured bacterium clone Ontario1287	89	8.9	KF881824
BB33	e4 , e5	Uncultured bacterium clone Ontario1287	99, 98	26.06	KF881830
BB6	a9	Uncultured bacterium clone Phil_e14	93	3.18	KF881805
BB41	f8	Uncultured proteobacterium clone DDOUFD08	70	18.64	-
BB10	b3	Uncultured proteobacterium clone DDOUFD08	98	1.48	KF881809
BB43	g8 , g9 , g10 , g11	Uncultured proteobacterium clone GASP-MB1W1_C05	97, 97, 97, 98	39.62	KF881838

4.4.2 Culturable heterotrophs and anaerobes from bee bread

Culture analysis showed that in addition to the presence of OTUs detected using DGGE, culturable heterotrophic bacteria could be found within bee bread using the R2A agar culture conditions, and culturable lactic acid bacteria could be found using the Rogosa agar culture conditions (see section 4.3.2). CFU analyses were performed in triplicate for samples of bee bread from 10 hives, totalling 150 analyses. The mean CFU counts of aerobic heterotrophs was 54.44 ± 8.99 mg^{-1} wet mass bee bread and for anaerobes was 2.99 ± 0.37 mg^{-1} wet mass bee bread. CFU counts and number of OTUs detected through DGGE were not significantly correlated (Pearson's correlation: $r = -0.083$, $t = -0.712$, $df = 73$, $P = 0.479$).

4.4.3 Variation in microbial communities at different spatial scales

Variance components for the culture-independent data based on α-diversity and cluster grouping of the gene sequences are shown in Table 4.3. Unless otherwise stated, variance components were not significantly different from zero. Explained variance was greater in α-diversity than in cluster grouping analysis: α-diversity varied significantly between boxes within the same hive, but cluster grouping did not; i.e. cells of bee bread located within different boxes had significantly different OTU counts (α-diversity), but did not vary significantly in which cluster group they were assigned to (community composition). Neither α-diversity nor cluster grouping varied between cells on the same frame or between frames in the same box, though in the former instance this variation was only marginally non-significant ($0.05 < P < 0.01$).

Table 4.3. Variance components for each of the diversity indices determined from the DGGE profiles in the samples of bee bread, for each of the hierarchal sampling levels in the program. Variances and standard deviations (SD) indicate how variable diversity index constituents are at different spatial scales. Significance of random effects was tested using Chi-square tests on residual maximum likelihood estimates.

Alpha diversity					
Between	n	Variance	S.D.	χ^2	*P*
Cells	472	0.972	0.986	3.238	0.072
Frames	83	0.847	0.920	0.405	0.524
Boxes	**43**	**8.896**	**2.983**	**8.829**	**0.003**
Residual	-	10.743	3.278	-	-

Cluster analysis					
Between	n	Variance	S.D.	χ^2	*P*
Cells	472	0.353	0.594	2.809	0.094
Frames	83	0.287	0.536	0.854	0.356
Boxes	43	0.588	0.767	0.750	0.386
Residual	-	5.013	2.239	-	-

4.4.4 Diversity and community composition from DGGE

Hereafter, because the absolute effect sizes (b) and standard errors (S.E.) were small, they have been increased by a factor of 1000 for clarity.

Diversity: α-diversity significantly varied in a quadratic relationship through the season (GLMM: *Day + Day²*: *b1* ± S.E. = -27.749 ± 15.211, $F_{1,464}$ = 4.262, P = 0.042; *b2* ± S.E. = 0.300 ± 0.100, $F_{1,464}$ = 7.750, P = 0.011, Figure 4.3a). It did not vary significantly with hive location by either Eastings or Northings, and did not differ between the two sample years (GLMM: *Year*, *b* ± S.E. = 2525.0 ± 5262.0, $F_{1,469}$ = 0.230, P = 0.373).

Community composition: Using the Hartigan-Wong clustering algorithm, nine clusters were identified. The composition of the microbial community, as determined by the cluster group assigned to each sample, also varied in a quadratic relationship through the season (LMM: Day + Day², *b1* ± S.E. = -64.010 ± 21.820, $F_{1,469}$ = 8.602, P = 0.004; *b2* ± S.E. = 0.146 ± 0.065, $F_{1,469}$ = 5.038, P = 0.027, Figure 4.3b). Assigned cluster group also varied significantly with geographical hive position by Eastings coordinates (LMM: *Eastings*, *b* ± S.E. = -4.899 ± 2.005, $F_{1,469}$ = 5.972, P = 0.022, Figure 4.3c), but not across the two years (*Year*, *b* ± S.E. = -469.301 ± 354.202, $F_{1,468}$ = 1.755, P = 0.214).

Specific OTUs: The summary statistics for logistic regression with each of the OTUs (Table 4.4) show that five of the OTUs that were analysed changed in a quadratic relationship with prevalence through the season (*Day + Day²*), consistent with the seasonal change found in microbial diversity and community composition. These OTUs have periods during the season when they are more abundant and periods when they may be absent (Figure 4.4). When the abundance of OTUs BB16, BB34 and BB42 were combined, the absence noted in individual OTUs does not occur

(Figure 4.4). OTUs BB29 and BB35 both varied in prevalence with latitude (*Northings*), the former in interaction with longitude (*Eastings*), suggesting that there may be a possible hot-spot for these particular OTUs (Table 4.4).

Table 4.4. Summary statistics for logistic regression performed on the ten most common OTUs. OTU presence/absence presented here were included as dependent variables in binomial error GLMMs with a logit-link function. The maximal model included as independent variables: Day, Day², Eastings, Northings and Eastings: Northings. *b* is the effect size estimate, S.E. is its standard error. Significant effects included in the minimal model have been highlighted for emphasis, non-significant effects are exclusion probabilities generated during model simplification. The effect sizes (b) and error (S.E.) have been increased by a factor of 1000 for clarity..

Parameters				Observational statistics			
OTU	Accession number	*n*	Effect	*b*	S.E.	z	*P*
BB1	KF881801	90	**Day**	**804.460**	**168.837**	**4.765**	**<0.001**
	Uncultured		**Day²**	**-11.267**	**2.389**	**-4.716**	**<0.001**
	bacterium		Eastings	-42.118	39.102	-1.077	0.281
	clone		Northings	-26.889	30.232	-0.889	0.374
BB16	KF881815	154	**Day**	**-84.208**	**15.153**	**-5.557**	**<0.001**
			Day²	**0.857**	**0.122**	**7.004**	**<0.001**
	Lactobacillus		Eastings	4.032	4.391	0.918	0.358
	spp.		Northings	4.336	2.723	1.592	0.111
BB29	KF881827	166	Day	6.547	10.760	0.608	0.543
			Day²	-0.048	0.081	-0.596	0.551
			Eastings	-1.027	4.272	-0.240	0.810
	Frischella spp.		Northings	-6.963	4.048	-1.720	0.085
			Northings:Eastings	**0.018**	**0.009**	**2.039**	**0.041**

BB33	KF881830	123	**Day**	**-79.559**	**14.225**	**-5.593**	**<0.001**
	Uncultured		**Day²**	**0.571**	**0.104**	**5.518**	**<0.001**
	bacterium		**Eastings**	**5.668**	**2.389**	**2.373**	**0.018**
	clone		Northings	0.038	1.482	0.026	0.980
BB34	KF881831	100	**Day**	**-46.103**	**13.124**	**-3.513**	**<0.001**
	Arsenophonus		**Day²**	**0.248**	**0.103**	**2.411**	**0.016**
	endosymbiont		Eastings	-1.152	3.263	-0.353	0.724
	of *P. bilineata*		Northings	-0.811	1.974	-0.411	0.681
BB35	KF881832	107	Day	-19.163	14.058	-1.363	0.173
			Day²	0.231	0.155	1.490	0.149
	Massilia spp.		Eastings	2.328	4.916	0.474	0.636
			Northings	**5.927**	**2.627**	**2.257**	**0.024**
BB42	KF881837	274	**Day**	**60.850**	**9.635**	**6.315**	**<0.001**
	Uncultured		**Day²**	**-0.555**	**0.078**	**-7.148**	**<0.001**
	bacterium		Eastings	1.783	1.285	1.387	0.165
	clone		Northings	-0.673	0.701	-0.961	0.337
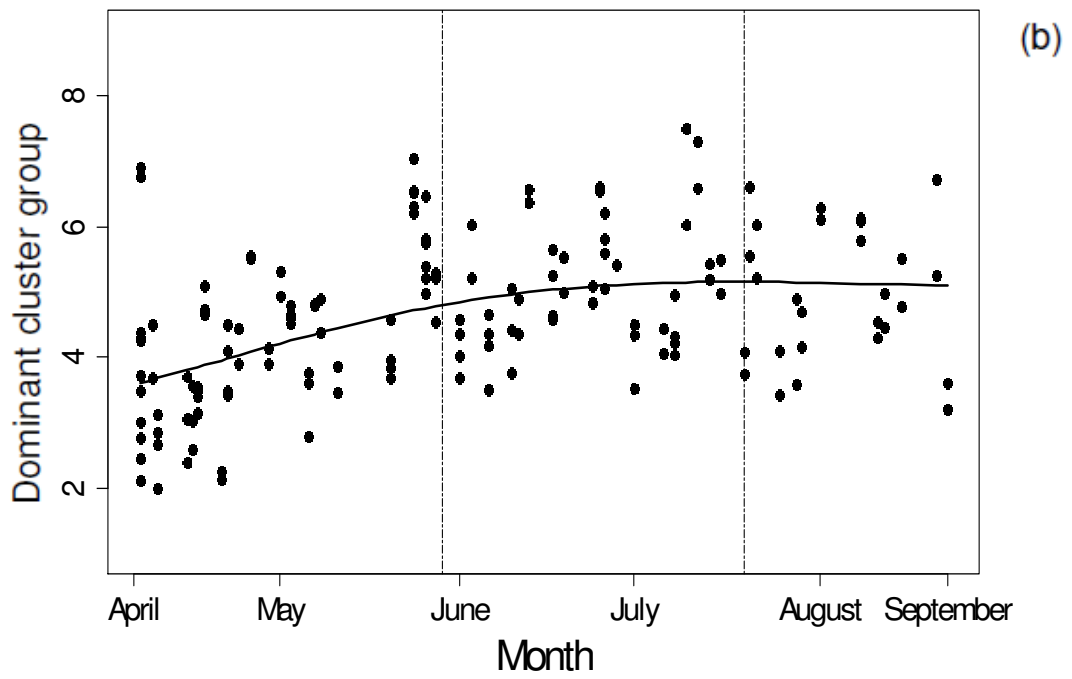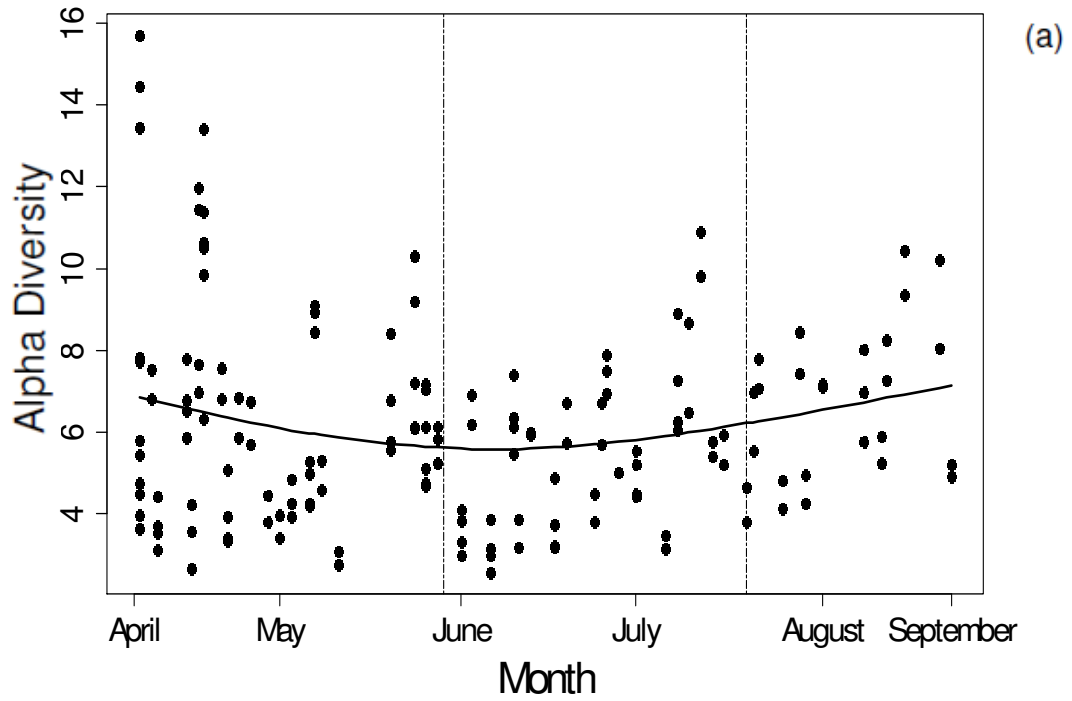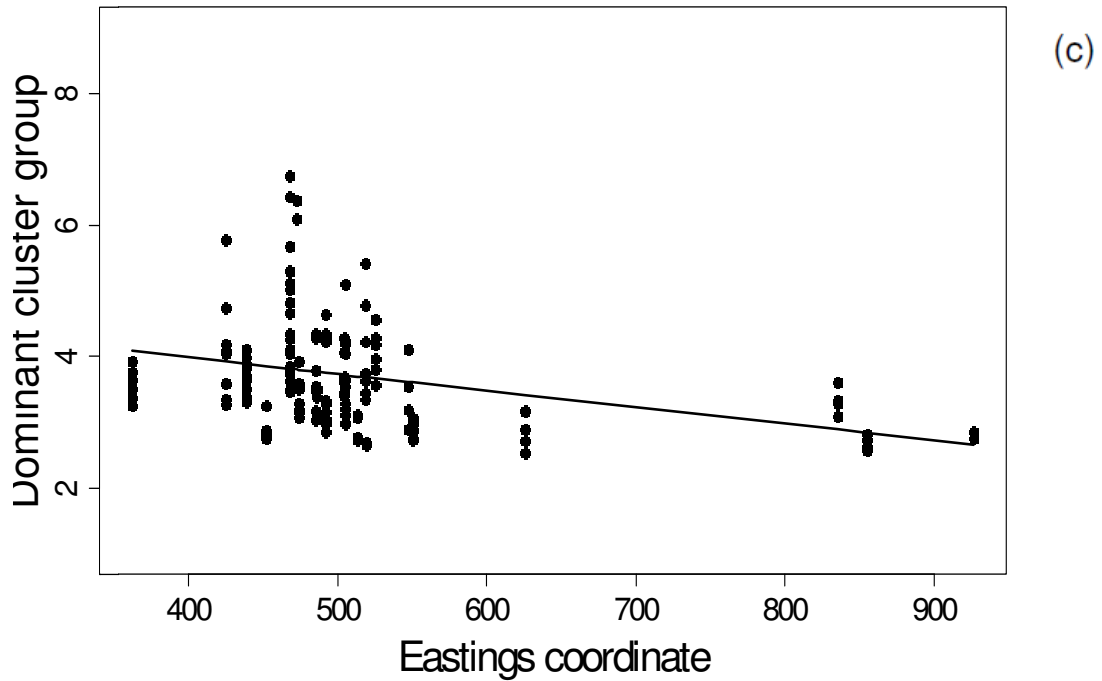
Figure 4.3. Spatiotemporal variation in bacterial community composition of bee bread determined by 16S rRNA gene PCR-DGGE. Fitted data from minimal models showing temporal variation in (a) alpha diversity, (b) assigned cluster over the 2012 field season; data points have been divided into each of the three sampling repeat locations, representing data taken in April – June, June – July and July – September. (c) Spatial variation in assigned cluster on the Eastings axis.
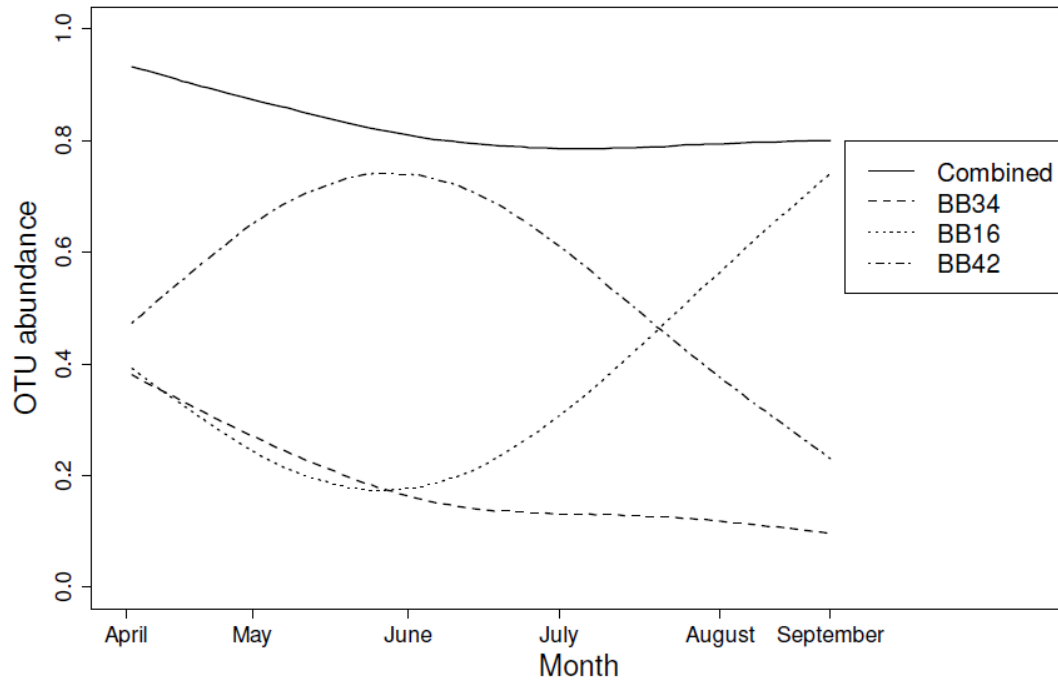
Figure 4.4. Changes in prevelance of OTUs BB42, BB16 and BB34 as the season progresses indicating changes in bacterial community composition. Dash-dot lines are OTU BB42, dotted lines are BB16, dashed lines are BB34, and the solid line is their combined presence in bee breads across the season – lines were based on logistic regression on individual OTUs with results shown in Table 4.4.

4.5 Discussion

This is the first study to statistically characterise variation in the microbial communities in the bee bread of honey bees both spatially (within- and between-hives) and temporally (within- and between-seasons). Bacterial communities were assessed using culture and PCR-DGGE and the composition of communities estimated using CFU and OTU respectively. Using PCR-DGGE 48 distinct OTUs were observed. This was a greater diversity than the 31 *Lactobacilli* and *Pasteurelaceae* isolates identified by Vásquez and Olofsson (2009), the 26 species identified by culture from three studies by Gilliam (1979a; 1997; 1989), and with greater detail than the 13 families identified by 454-pyrosequencing (Mattila *et al.*, 2012). This is probably a consequence of a more extensive sampling program employed in this study relative to previous ones (472 samples here, compared to 3, 5 and 20 samples, respectively, in previous studies). The key findings of this study were that there was significant temporal variation in bacterial community composition within the season and that this variation occurs in a similar manner between seasons. The study also found significant spatial variation in the diversity of microbial communities between different locations within the same hive.

In making assessments of PCR-DGGE-derived data, however, certain constraints have to be acknowledged. The first limitation in assessing microbial communities comes at the sampling stage; however, the extent and depth of sampling in this study has greatly reduced the potential of this limitation. PCR-based fingerprinting techniques are subject to the drawbacks and limitations of PCR itself, such as preferential or selective amplification of DNA from mixed communities, meaning that a subset of organisms in bee bread may remain undetected (Muyzer *et al.*, 1993); or the formation of chimeric or heteroduplex DNA molecules, which may

bias estimates of diversity (Nannipieri *et al.*, 2003). It is therefore important to stress that interpretations presented here are subject to these limitations. Despite this, it is equally important to emphasise that DGGE currently remains a very useful tool for assessments of microbial diversity (Shimano *et al.*, 2012; Machtelinckx *et al.*, 2012; Joossens *et al.*, 2011). Another option for studying microbial communities is 454-pyrosequencing, which can allow for a far greater depth of detail in determining microbial communities and has been used previously in studies involving honey bees (McFrederick *et al.*, 2012); however, this technology may be similarly limited by the formation of chimeric DNA, the relatively short length of sequences generated may limit the potential for species identification and applying these methods to large-scale studies often makes them prohibitively expensive (Morey *et al.*, 2013). Even with the limitations of PCR-DGGE, the extent of the community found here in bee bread is greater than previous studies and the analyses of these data are novel.

4.5.1 Variation in bacterial communities at different spatial scales

A high degree of variation in the bacterial community diversity of bee bread was found between cells located in different boxes within a hive, but not between cells or frames within the same box (see Table 4.1). As the composition of the microbial community in bee bread is dependent on its multiple plant and bee origins (McFrederick *et al.*, 2012), this suggests that bee bread communities have different origins in different boxes, but the same origins within them, though it should be recognised that variation in bacterial diversity and community composition between cells on the same frame came close to being statistically significant ($0.05 < P < 0.1$ in each case).

The microbial communities associated with bee bread are thought to be a combination of microbes derived from pollen, nectar and the gut bacteria of the bees themselves (McFrederick *et al.* 2012). The similarity noted here (within boxes) may therefore be due to similarity in any combination of these elements. Nectar and pollen are mixed by bees when making bee bread; however, it is currently unknown whether nectar and pollen from the same plants are used to make bee bread, or if different nectar and pollen sources are combined. Both the foraging bees that collect the pollen and the nurse bees that store it in cells are likely to expose bee bread to their gut bacteria (Fewell and Bertram, 1999). The significant similarity in communities found in bee bread within boxes, combined with differences in communities between boxes (see Table 4.1), could potentially suggest segregation of worker bees to different boxes. In other words, it is possible that a group of workers may process bee bread for a specific box within a hive, whereas an alternative group or cohort may work within another box within the same hive. This may have implications for our understanding of bee behaviour inside the hive, as currently our knowledge of the movement of honey bee cohorts inside the hive is relatively limited. Infared imaging can been used to visualise clustering of bee populations within hives (Shaw *et al.*, 2011) and radioentomology utilising medical CT-scanners can differentiate these populations into eggs, larvae, pupae, adult workers and queens (Greco and Sadd, 2012). However, these techniques have not been used to differentiate cohorts of workers clustered in specific areas of honey bee hives. It has been suggested there may be some spatially clustered behaviours, such as clustering of nurse bees around brood to maintain brood temperature and organisation of bee orientation within the hive to control ventilation (Bujok *et al.*, 2002; Southwick and Moritz, 1987).

4.5.2 Spatio-temporal dynamics of bacterial communities

Sampling of the 25 geographically distinct apiaries at three time points during the beekeeping season allowed both the temporal and geographical variation in bee bread composition to be quantified. This is the first study to consider inter-hive variation in microbial communities. Viable aerobic heterotrophs and fermentative anaerobic organisms were consistently found using selective culture across all hives, but the composition of microbial communities determined by DGGE showed significant variation between hives in different locations (see Figure 4.3c). This variation may be in part be determined by environmental factors that vary around the hives not just through the season, but also in specific areas. Spatial variation in the biomes that determine plant species assemblages around different hives may explain the variation across the study area. Previously, this study demonstrated that honey bee nutrition varies spatially and that this may be linked to changes in landscape composition and floral assemblages (see Chapter 2 and Chapter 3). Different flowers are host to different bacterial communities and this variation may influence the community present across an entire honey bee hive (Anderson *et al.*, 2013). Therefore, it may be that the spatial variation in bacterial community composition noted here (see Figure 4.3c) may be due to variation in floral composition in these areas.

The OTUs found here are similar to those found in previous studies, and were dominated by *Psuedomonas*, *Enterobacter*, *Acinetobacter* and *Lactobacillus* (see Table 4.2) (Pain and Maugenet, 1966; Brodschneider and Crailsheim, 2010; Ellis and Hayes, 2009; Lundgren and Lehman, 2010). The similarity of bacterial genera identified here with previous studies, and the consistency between all of the hives in the current study confirms the findings of previous studies that there is a defined selection of organisms from which honey bees can inoculate bee bread (Cox-Foster *et*

al., 2007; Mattila *et al.*, 2012). Alternatively, it may be because honey bees are selecting for a specific community to meet certain requirements in processing bee bread in a system similar to leaf cutter ants (*Acromyrmex* and *Atta*), which select for specific fungal isolates to digest plant material for food (Pinto-Tomas *et al.*, 2009). There is evidence that honey bees and bumble bees associate with host-specific *Lactobacilli* and that this may be due to regular horizontal transmission of gut contents and microbiota within the hive (McFrederick *et al.*, 2013). Or finally, it could be that the anaerobic-acidic environment of bee bread (Vásquez and Olofsson, 2009) is a highly selective environment that only specific organisms can colonise; this may play a role in preventing opportunistic decomposing fungi or yeasts from growing on bee bread stored in the hive (Anderson *et al.*, 2011). It is likely that all of these factors play a role in creating the specific community observed; the relative importance of each of these factors has not been explored.

Given that bee bread typically lasts within the hive during their active season for two months at most (McLellan, 1978; Chevtchik, 1950), the present study was deliberately scheduled to sample bee bread every eight weeks to ensure independent bee bread samples were taken from the same frames throughout the season (i.e. not the same selection of bee breads at different time points). Therefore, the significant temporal variation noted here cannot reflect changes in bacterial community composition that occur as bee bread matures (Chevtchik, 1950), but rather is reflecting changes in the bacterial community that bee bread is inoculated with. The hives in this study each had similar communities, but these change through the season (see Figure 4.3). Previous research has indicated that the activity of at least some bacterial genera (*Bifidobacteria* and *Lactobacilli*) is important for maintaining production of bee bread (Vásquez and Olofsson, 2009). The production of bee bread, however, is maintained

throughout the season despite the variation in bacterial community composition observed in this thesis. The association of distinct bacterial community compositions to different times of the beekeeping season suggests that these changes in bacterial community may be linked to changes in floral resources as the season progresses.

None of the bacteria defined by the OTUs in this study were present in all of the samples of bee bread (see Table 4.2) and yet bee bread production was consistently maintained through the season. This suggests that several of the OTUs found must be capable of the necessary functions to produce bee bread and that there may be redundancy in their function enhancing robustness. The bacterial communities maintained by other invertebrate species that digest plant materials, such as aphids, exhibit similar properties whereby there appear to be multiple species of bacteria that are capable of digesting plant material that their hosts feed on (Jousselin *et al.*, 2013; Najar-Rodriguez *et al.*, 2009). For example: in *Aphis gossypii*, the redundancy of bacterial symbionts has been suggested to be a mechanism by which their hosts can become generalist herbivores (Najar-Rodriguez *et al.*, 2009). By having multiple variable symbionts to assist in the digestion of plants, these organisms are able to survive on a greater variety of plants than their competitors (Najar-Rodriguez *et al.*, 2009). Therefore, having a diverse and redundant community of bacterial symbionts provides fitness benefits to the host; it may be the case that the diversity of bacterial communities associated with bee bread provides a similar benefit, by increasing the diversity of pollens that can be used in the production of bee bread for honey bees.

Through analysis of the 10 most common OTUs (see Figure 4.4 and Table 4.4), it was demonstrated that several individual OTUs varied non-linearly through the season, indicating that as the year progresses some of these OTUs appear and go below detection limits from the bee bread bacterial community . For example, BB42

was abundant in samples at the start of the year (April – May, see Figure 4.4), but as the year progressed it became less abundant and BB16 concurrently increased in abundance, peaking towards the end of the year (August – September, see Figure 4.4). The dynamics of OTUs BB16, BB42 and BB34, demonstrate that despite these individual 'species' being in low abundance at certain times of the year, when considered in combination they are consistently found in bee bread throughout the whole season. The assemblage of plants available for bees to forage from changes regularly through the season, and this results in changing pollen species composition present in bee bread (see Chapter 3). The redundancy in function may play an important role in maintaining the production of bee bread in the face of constantly changing composition.

4.5.3 Relevance of bacteria identified in bee bread

The predominant bacterial genera identified by sequence analysis of DGGE-derived bands were *Acinetobacter*, *Lactobacillus*, *Enterobacter* and *Pseudomonas* (see Table 4.2). *Acinetobacter, Lactobacillus* and *Enterobacter* species are rarely considered pathogenic (Crotti *et al.*, 2010) and have previously been detected in the digestive systems of honey bees (Vásquez and Olofsson, 2009; Vasquez *et al.*, 2012; Kaznowski *et al.*, 2005). Matilla *et al.* (2012) measured RNA abundances using 454-pyrosequencing and demonstrated that both Lactobacillae and Bifidobacteriaceae dominate the production of RNA from the bacterial community in bee bread. This means that they have the function capacity to be integral to the conversion of pollen into bee bread. Several of these species demonstrate antibiotic activity against honey bee pathogens (Alippi and Reynaldi, 2006; Evans and Armstrong, 2005; Mudronova *et al.*, 2011) and it has recently been suggested that the total microbial community

associated with honey bee guts may be protecting their hosts from infection (Hamdi *et al.*, 2011; Anderson *et al.*, 2011). Limited research on probiotic supplementation of honey bee colonies using *Lactobacillus* and *Enterobacter* suggests that these organisms may be used to reduce the colonization of bees' gastrointestinal tracts by pathogenic bacteria (Vasquez *et al.*, 2012; Kaznowski *et al.*, 2005).

These studies also showed the consistent occurrence of *Lactobacilli* across bee bread samples in all hives. However, this was not the case in the present study, probably because the larger sample size (n = 472) allowed for a more exhaustive examination of inter-hive microbial diversity. *Pseudomonas* spp. have been successfully cultured from the gut of the honey bee (Rada *et al.*, 1997), but as yet, not from bee bread. Studies have shown that *Pseudomonas* spp. associated with honey bees may have antimicrobial properties (Kwakman *et al.*, 2011; Uzel *et al.*, 2005). Several other genera were determined from sequence data here, including *Gilliamella*, *Erwinia* and *Frischella*, which have been previously found associated with honey bees (Kwong and Moran, 2013; Engel *et al.*, 2013; Alexandrova *et al.*, 2002).

In addition to potentially enhancing resistance to pathogens and opportunistic decomposers, some members of the bacterial community identified in the present study may play an important role in the digestion of pollen to produce bee bread. To access the nutritious core of pollen grains, honey bees must first digest a complex wall of cellulose and sporopollenin, a complex biopolymer whose chemical composition is not fully understood as yet. The bee genome possesses the necessary genes that encode for cellulases, but cannot digest sporopollenin (Kunieda *et al.*, 2006), hence large numbers of undigested pollen grains are found in their digestive tract (Crailsheim *et al.*, 1992). The microbial communities associated with bee bread could be responsible for the digestion of the sporopollenin, allowing access to the nutritious

core of pollen grains, although no direct evidence for this has been established (Vásquez and Olofsson, 2009; Gilliam, 1997; Gilliam *et al.*, 1988). Previous studies have suggested that these organisms are primarily dominated by *Lactobacillus* and *Acetobacter* (Olofsson and Vásquez, 2008; McFrederick *et al.*, 2012; Crotti *et al.*, 2010), which are further supported by the identities of OTUs demonstrated in this study.

Through application of DGGE, this study has described the temporal variability of the bacterial community that occurs in bee bread both within and between bee hives and has provided evidence that bee bread exhibits a consistent bacterial community. By demonstrating that even with environmental variation, the composition of bacterial assemblages do not vary significantly between hives, bacterial community patterns in bee bread stores within honey bee hives may be evaluated with greater confidence. The present study also provides an extensive characterization of the bacterial community of bee bread stores of the honey bee, a pollinator of global importance (Klein *et al.*, 2007). This study has also highlighted again the importance of bacterial associations with invertebrates and reiterates their importance in nutritional support for their hosts. Bee bread is an important food source to bees (Oliver, 2007b) and bacterial communities in bee bread may be important in maintaining its production (Vásquez and Olofsson, 2009). By revealing the sources of these bacteria, future studies can aim to better understand variation in pollinator nutrition and health.

Chapter 5: Variable diet quality within a hive affects a constitutive honey bee immune response

Sampling of bee bread and larvae in Hereford with the well appreciated assistance of E. Adams

5.1 Abstract

Honey bees require protein in their diet to raise the brood and maintain many of their physiological processes, including the immune system. The primary source of dietary protein for honey bees is bee bread, a product of pollen collected by bees and stored within hives. Previously, it has been demonstrated that hives in different locations collect pollen that has a variable protein content and that this may be due to such environmental factors as landscape composition and floral diversity (see Chapters 2 and 3). Here, the intra- and inter-hive variation in the nutritional content of bee bread has been correlated with variation in a key constitutive immune response of honey bees, the phenoloxidase enzymatic reaction.

Bee bread and bees were sampled from a commercial apiary in Herefordshire, England. Protein and carbohydrate contents of bee bread were estimated using spectrophotometric methods and immune function was estimated for individual bees using standard colorimetric analyses. The results revealed a significant correlation between dietary protein and carbohydrate levels in bee bread and phenoloxidase activity in both adult and larval honey bees. These results were then used in conjunction with landscape composition data from the Countryside Survey (see Chapter 2, section 2.3.3) to predict bee bread nutritional composition and hence immune function across the UK and the association with national levels of honey bee disease, as reported by the National Bee Inspectors database and FERA. Consequently, a link was proposed between landscape composition and honey bee fitness, based on the potential nutritional consequences of certain landscape types, such as improved grassland (negative effect) and broadleaf woodland (positive effect). While accounting for the variability in the nutritional content of bee bread, the results

here suggest that its nutritional composition may have a significant impact on the immune function of larval honey bees in terms of phenoloxidase activity.

5.2 Introduction

Global populations of honey bees have been challenged recently with heavy losses attributed to Colony Collapse Disorder, pesticides and introductions of pests and pathogens (Cox-Foster, 2007; Mullin *et al.*, 2010; Martin *et al.*, 2012; Behrens *et al.*, 2010). Honey bees contribute significantly to the pollination of a large array of crops (Winfree *et al.*, 2008), which account for approximately 35% of global food production (Klein *et al.*, 2007). Declines in honey bee populations may lead to reduced pollination services in agricultural systems, leading to shortfalls in pollination and global food insecurity (Steffan-Dewenter *et al.*, 2005; Holden, 2006). One of the drivers of pollinator decline is dissemination of foreign pathogens and parasites, such as European Foulbrood (*Melissococcus plutonius*, Bailey and Collins 1983) and Nosema (*Nosema apis*, Nägeli, 1857). Recent invasions of disease vectors like the Varroa mite (*Varroa destructor*, Anderson & Trueman, 2000) and increased stresses due to intensification of land use (Biesmeijer *et al.*, 2006; De la Rua *et al.*, 2009; Genersch, 2010; Amdam *et al.*, 2004) have resulted in an increased susceptibility to these organisms, pathogens and parasites.

Increased susceptibility to pathogens in honey bees could be due to a reduction in immune function, which is used to refer to "attributes of the innate or adaptive immune responses" (Wilson and Cotter, 2013). Many of the processes required to mount an immune response require dietary protein (Schmid-Hempel, 2003; Siva-Jothy and Thompson, 2002; Lee *et al.*, 2006; Povey *et al.*, 2014a), which honey bees collect

from their environment in the form of pollen (Roulston and Cane, 2000). Bees ferment pollen to make "bee bread" inside the hive, which they later consume (Vásquez and Olofsson, 2009). Nurse bees consume bee bread from multiple sources within the hive and then convert the protein from this diet into brood food in their hypopharyngeal glands (Hrassnigg and Crailsheim, 1998). By using multiple bee bread sources to produce bee bread, honey bees may reduce the variability of the nutritional content of brood food compared to bee bread (Altaye *et al.*, 2010; Basualdo *et al.*, 2013) because the variable protein contents are essentially 'averaged' together.

Invertebrate immunity comprises anatomical, behavioural and humoral immune systems. Humoral immune responses are regularly analysed when studying invertebrate immunology. Repeatable assays have been developed to study variation in the density of haemocytes, phenoloxidase activity, prophenoloxidase activity, lysozyme-like (lytic) activity, antimicrobial (growth inhibition) activity and others (Pauwels *et al.*, 2011; Arce *et al.*, 2012; Cerenius *et al.*, 2008; Miller and Simpson, 2010). The phenoloxidase system was chosen for this study because adult honey bees immunosenesce other aspects of their immune pathways, meaning that adult bees still maintain the phenoloxidase immune pathway, but do not produce haemocytes or antimicrobial peptides (Schmid *et al.*, 2008).

Phenoloxidase produces indole groups, which are subsequently polymerized to melanin (see Chapter 1, Figure 1.6), which plays a key role in encapsulating foreign bodies. These reactions also produce intermediate products, such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates, which are important during defence against bacterial, fungal, and viral agents (González-Santoyo and Córdoba-Aguilar, 2012).

Products of the phenoloxidase enzymatic reaction (see Chapter 1, Figure 1.4) rapidly become detectable upon adding sufficient substrate; the resultant melanisation has been used as an indicator of the levels of phenoloxidase in the haemolymph (Decker and Jaenicke, 2004). Phenoloxidase is expressed even in the absence of infection, but it can also be up-regulated following an immune challenge (Brown *et al.*, 2003) and is expressed by honey bees at both larval and adult life stages (Schmid *et al.*, 2008).

Laboratory diet manipulation studies indicate that increased dietary protein in the form of a limited selection of pollens (*Acer*, *Castanea*, *Cistus*, *Erica*, *Quercus*, *Salix* and *Taraxacum*) may enhance honey bee immune function, including phenoloxidase (Alaux *et al.*, 2010b; DeGrandi-Hoffman *et al.*, 2010). As these studies have used only a single (DeGrandi-Hoffman *et al.*, 2010) or two mixes of pollen (Alaux *et al.*, 2010b), they have not accounted for variation in the sources of pollen within and between hives (see Chapter 3). Previously, it has been suggested that the nutritional value of bee bread may vary depending on the diversity and composition of plant species within (see Chapter 3), meaning that these diets are far more variable than those used in previous experiments. By sampling pollen stores from within hives, this study aims to account for this variation when considering nutritional effects on honey bee immune function. The effects of this naturally occurring variation upon honey bee fitness are relatively unexplored.

This study also aims to establish a functional relationship between the nutritional content of pollen stores in honey bee hives and bee immune function, as measured by phenoloxidase activity in both adults and larvae. To this end, the following questions were addressed: (i) Is the nutritional content of pollen stores reflected in the nutritional composition of bees consuming the stores? (ii) Is there a

correlation between the composition of pollen stores and the immune function of the bees consuming them? (iii) Does the land use composition around honey bee colonies, and hence the community of plants available for foraging, correlate with immune function across honey bee hives?

5.3 Materials and methods

5.3.1 Sampling protocol

Sampling was performed in Herefordshire (England) on 28 commercially managed honey bee colonies (*Apis mellifera* L.) from seven apiaries on 3rd June 2013. Bee bread cells were sampled from 6 cells per hive, taken from a single frame. Bee bread samples were stored at -20ºC until nutritional analysis was performed. 15 final instar larval honey bees were sampled from each hive from the same frame as bee bread samples (mean ± S.D., body length 9.48 mm ± 0.99). Honey bee larvae removed from their cells were immediately washed in 200 µL sterile distilled water to sample brood food, which they are bathed in. 15 newly hatched adult honey bees were sampled from each hive from the patches of brood on the frame that was also sampled for larval bees. Bee samples were also stored at -20ºC until thawed for haemolymph extraction and immune response measurement in the lab.

5.3.2 Nutritional analyses

The protein and carbohydrate content of bee bread and larval brood food was estimated by spectrophotometric chemical analyses using a VERSAmax™ Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA) using Softmax® Pro v4.7

software for Windows®. Protein content of bee bread was estimated using the Biuret reaction (Gornall *et al.*, 1949; Sapan *et al.*, 1999): 10 µg of bee bread and 100 µL brood food were reacted in 200 µL Biuret solution for 30 min and absorbance was read at wavelength 550 nm. Carbohydrate content of bee bread was estimated using the dinitrosalicylic acid (DNS) reaction (Lees, 1971): 20 µg of bee bread and 100 µL were digested in 100 µL 1M invertase enzyme solution in sodium acetate buffer (pH 5.0) for 5 min at 55ºC, then reacted in an additional 100 µL DNS for 15 min at 95ºC. Absorbance was read at 575 nm (See Chapter 2.3.2).

5.3.3 Immune response analyses

Haemolymph phenoloxidase and prophenoloxidase activity was assayed spectrophotometrically with dopamine as a substrate (Cotter *et al.*, 2008b). Haemolymph was extracted by removing the head at the base of the neck, heads were homogenised in 100 µL ice cold 10mM sodium cacodylate (NaCac) buffer; then incubated at room temperature for 1 h, followed by centrifugation at 13,000 rpm for 10 min (Schmid *et al.*, 2008). After centrifugation, 75 µL of supernatant were mixed with 335 µL NaCac buffer. Larvae were macerated on ice and centrifuged at 13,000 rpm for 10 min, the haemolymph layer was extracted from below the lipid layer and the volume of haemolymph was made up to 410 µL using NaCac buffer.

For both adults and larvae, 25 µL of haemolymph suspension were used in protein content estimation, 180 µL were used in phenoloxidase response estimation and 180 µL were used in prophenoloxidase response estimation. For prophenoloxidase estimation, buffered haemolymph was mixed with 20 µL 20 mg mL^{-1} chymotrypsin in

NaCac buffer to activate the conversion of prophenoloxidase to phenoloxidase and incubated at 25ºC for 30 min.

Prophenoloxidase samples and phenoloxidase samples were split for duplicate sampling and mixed with 90 µl of 10 mM dopamine and were incubated on a temperature-controlled VERSAmax™ Tuneable Microplate Reader (Molecular Devices, Sunnyvale, CA) at 472 nm for 10 min at 25ºC. Prophenoloxidase and phenoloxidase activity were expressed as the slope of the line over 10 min, which is in the linear phase of the reaction (Povey *et al.*, 2009). Haemolymph protein content was used to standardise the measurements across haemolymph samples. Haemolymph protein content was measured by incubating duplicate samples of 25 µL buffered haemolymph in 175 µL Biuret solution for 30 min and absorbance was read at wavelength 550 nm.

5.3.4 Land use composition

Previous research has established a model for predicting bee bread nutritional composition based on land use composition, estimated by the Countryside Survey 2007 land cover map (LCM07; (Morton *et al.*, 2011); see Chapter 2, section 2.3.3). This model was used to predict the average protein and carbohydrate content of bee bread in 10 km² squares across the UK (Figure 5.4a). Based on the results of the current study, the predicted protein:carbohydrate ratio was then used to estimate the average phenoloxidase immune response of honey bee larvae in these 10 km² squares. For simplicity, only predicted larval phenoloxidase was used because both phenoloxidase and prophenoloxidase showed similar trends, adult immune responses yielded less consistent patterns and are not shown here. Accuracy of predicted

immune function and predicted P:C ratios were verified through comparison with immune function records taken from Hereford and with bee bread nutritional data taken from North-west England and Hereford respectively (see section 5.3.5).

These predictions were then compared with current records of honey bee disease produced by the Food and Environment Research Agency (FERA, https://secure.fera.defra.gov.uk/beebase/index.cfm). The occurrences of European Foulbrood recorded from hive inspections by National Bee Inspectors from 1993 – 2013 were used to compare predicted immune function to actual disease occurrence (Figure 5.4b and 5.4c). Although data exists for both European Foulbrood (*M. pluton*) and American Foulbrood (*Paenibacillus larvae* Ash *et al.* 1994), records of American Foulbrood occurrence in the UK are relatively few, therefore making comparison of geographical patterns between the two not possible.

5.3.5 Statistical analysis

Correlation between bee bread nutritional content and brood food content was analysed using Fisher's correlation coefficients. Analyses of nutritional composition, body chemistry and immune response were performed using a series of generalised linear mixed-effects models (GLMMs) using "*lme4*" package (Bates *et al.*, 2012) in the *R* statistical software (R Core Team, 2013). The sites from which samples were taken were included as random effects as (*1/Hive*). Immune response data (phenoloxidase - *PO* and prophenoloxidase - *proPO*) were analysed as square root transformed dependent variables as an interaction with haemolymph protein content (*HaeProtein*) fitted to a Gaussian error structure.

As the number of bee bread samples was not equal to the number of immune response samples, the mean nutritional contents in terms of protein (*BBProtein*) and carbohydrate (*BBCarb*) were included as independent variables; these variables were altered using the "*jitter*" function in R to add variation to the means based on the standard deviations of the means (*BBProteinsd*) and (*BBCarbsd*). P-values for the F statistics were calculated using the "*lmerTest*" package (Kuznetsova *et al.*, 2014). The results presented represent the output of the most parsimonious models, determined using stepwise deletion based on residual deviance contrasts (Crawley, 2007; Zuur, 2009).

Extrapolation of honey bee nutrition and immune function was based on data provided by the Countryside Survey (Carey *et al.*, 2008a), cartography of these results was produced using ESRI ArcGIS™ version 10.2 (ESRI, 2011). Comparisons between disease incidence and predicted immune function were made using negative binomial zero inflated Poisson (ZIP) regression with logit-link function using the "*pscl*" package (Jackman *et al.*, 2012). Predicted nutritional composition was verified through a Pearson's correlation test to compare nutritional data from North-west England and Hereford datasets with matched areas in the predicted data set.

5.4 Results

The nutritional composition of 168 bee bread samples was analysed. The mean protein content was 30.89 mg g^{-1} ± 12.43 (mean ± S.D.) and mean carbohydrate content was 12.59 mg g^{-1} (± 5.15). The mean protein:carbohydrate ratio of bee bread was 2.99 ± 2.08.

The immune responses from the haemolymph of individual larval and adult honey bees were also analysed. Mean phenoloxidase levels were greater in adults (mean activity ± S.D. 29.56 ± 18.40) than in larvae (20.09 ± 16.36). Similarly, mean prophenoloxidase levels were greater in adults (37.43 ± 22.82) than in larvae (26.41 ± 18.98). When standardising for haemolymph protein content, mean phenoloxidase levels were also greater in adults (mean activity ± S.D. 6.37 mg^{-1} protein ± 5.18) than in larvae (3.65 mg^{-1} protein ± 2.98), a trend that was also noted for prophenoloxidase levels (adults: 7.86 mg^{-1} protein ± 5.66, larvae: 4.93 mg^{-1} protein ± 4.27).

The protein content of brood food, the substance produced by nurse bees within which larval bees incubate, was significantly positively correlated with the protein content of bee bread (Pearson's correlation: $r = 0.226$, t = 2.183, df = 88, P = 0.032, Figure 5.1a), but demonstrated no significant correlation with carbohydrate content (r = -0.011, t = -0.097, df = 88, P = 0.923, Figure 5.1b), nor with protein:carbohydrate ratio (r = -0.125, t = -1.1869, df = 88, P = 0.238). Further, the haemolymph protein levels in larvae were significantly positively correlated with the protein content of the bee breed in their hive (r = 0.185, t = 3.684, df = 385, P < 0.001, Figure 5.1c) and of adults (r = 0.146, t = 3.018, df = 417, P = 0.002, Figure 5.1d).
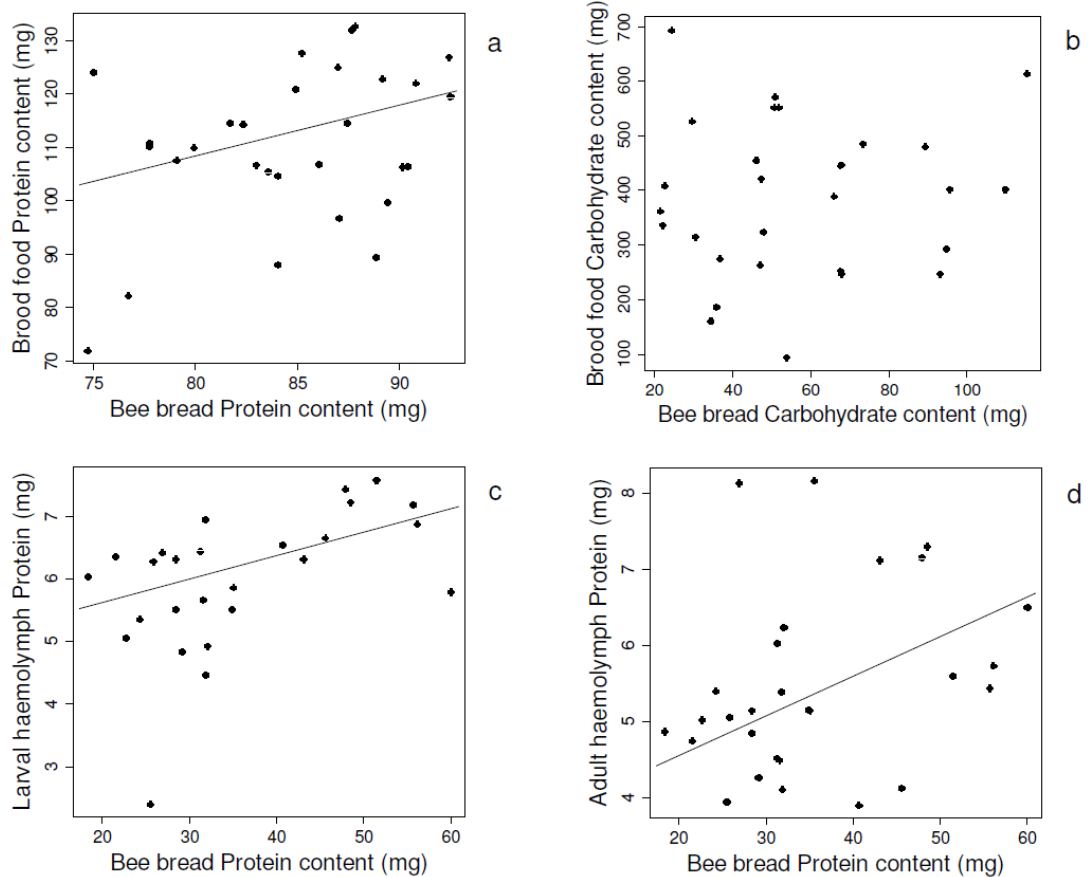
Figure 5.1. Correlations of bee bread nutritional content with brood food and bee haemolymph nutritional contents. Points represent mean values for each hive sampled and demonstrate: (a) significant correlation between brood food and bee bread protein contents; (b) non-significant correlation between brood food and bee bread carbohydrate contents; (c) significant correlation between bee bread and larval haemolymph protein contents; (d) significant correlation between bee bread and adult haemolymph protein contents. Brood food-bee bread correlations (a-b) were performed on data sampled during Chapter 2; significant correlations are indicated by fitted linear regression lines.

The relationship between honey bee nutrition and one component of the constitutive immune system of both adult and larval bees, i.e. phenoloxidase activity (*PO* and *proPO*) was examined with haemolymph protein content included as a covariate.

Adult prophenoloxidase activity was not significantly correlated with bee bread protein content (*BBProtein* + *BBProtein^2*: b ± S.E. = 0.067 ± 0.124, $F_{1,94}$ = 0.291, P = 0.594; *b2* ± S.E. = -0.001 ± 0.002, $F_{1,94}$ = 0.801, P = 0.379). Adult phenoloxidase activity was not significantly correlated with nutrition (*BBProtein*: b ± S.E. = -0.204 ± 0.238, $F_{1,94}$ = 0.736, P = 0.399), regardless of the inclusion of haemolymph protein content as a covariate.

Larval prophenoloxidase activity was significantly negatively correlated with bee bread carbohydrate content (*BBCarb*: b ± S.E. = -0.474 ± 0.203, $F_{1,57}$ = 5.469, P = 0.028, Figure 5.2a), but not with bee bread protein content (*BBProtein*: b ± S.E. = 0.005 ± 0.012, $F_{1,57}$ = 0.422, P = 0.675, Figure 5.2b). Although bee bread protein content was not correlated with larval prophenoloxidase activity, haemolymph protein was positively correlated with prophenoloxidase activity (*HaeProtein*: b ± S.E. = 0.104 ± 0.012, $F_{1,372}$ = 79.571, P < 0.001, Figure 5.2f).

Larval phenoloxidase activity was marginally significantly correlated with bee bread carbohydrate content (*BBCarb*: b ± S.E. = -1.192 ± 0.579, $F_{1,23}$ = 4.235, P = 0.050, Figure 5.2c), and not with bee bread protein content (*BBProtein*: b ± S.E. = 0.039 ± 0.025, $F_{1,23}$ = 2.365 P = 0.137, Figure 5.2d). Larval haemolymph protein was also significantly correlated with phenoloxidase activity (*HaeProtein*: b ± S.E. = 0.211 ± 0.024, $F_{1,366}$ = 75.939, P < 0.001, Figure 5.2e).
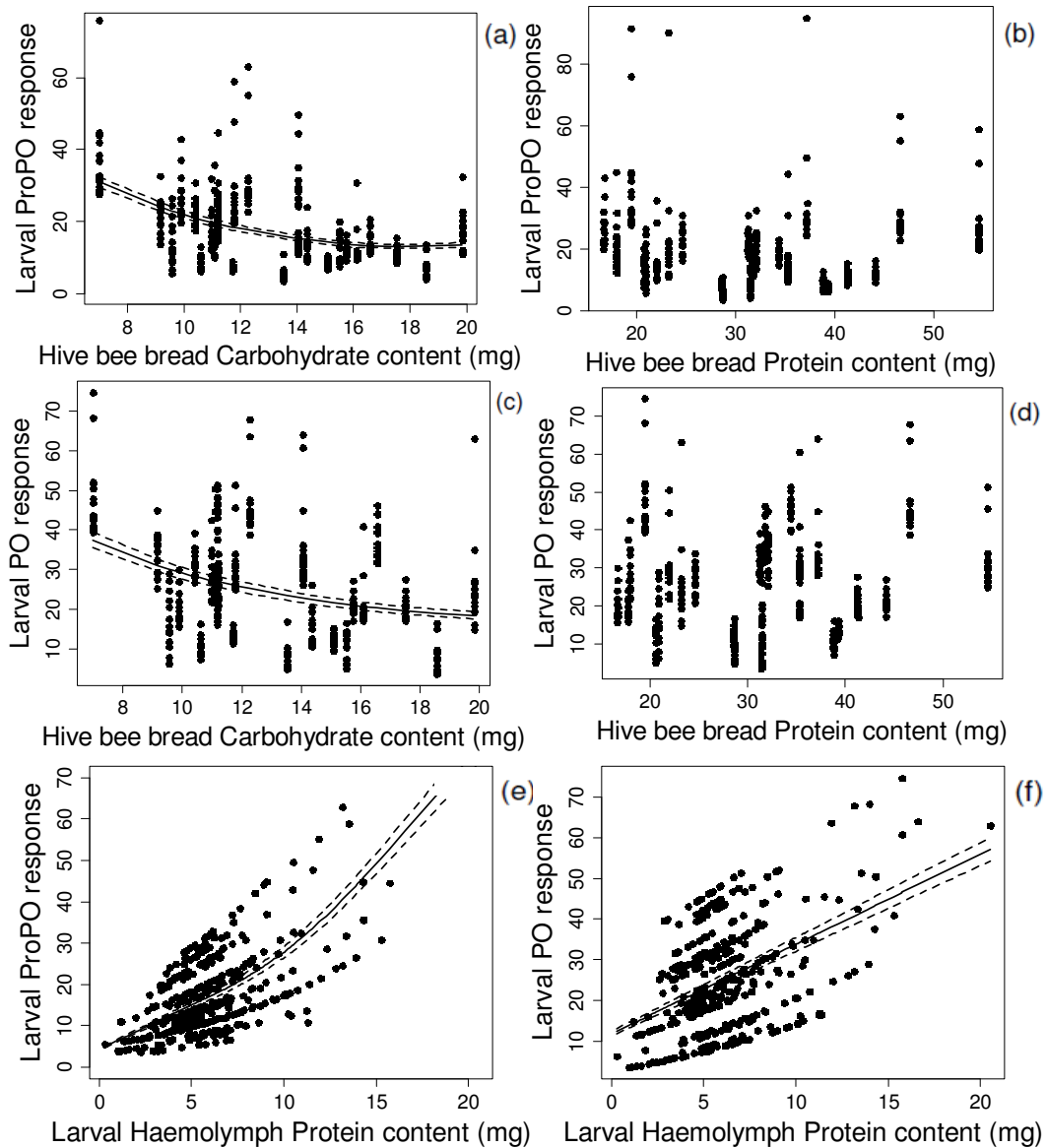
Figure 5.2. Variation in bee bread nutritional content and honey bee immune responses. Fitted data from minimal models showing (a) negative correlation between hive mean bee carbohydrate content and larvae prophenoloxidase activity; (b) non-significant relationship between hive bee bread protein and larval prophenoloxidase activity; (c) negative correlation between hive bee bread carbohydrate content and larval phenoloxidase activity; (d) non-significant relationship between hive bee bread protein and larval phenoloxidase activity; (e) significant correlation between larval haemolymph content and larval phenoloxidase activity; (f) significant correlation between larval haemolymph content and larval prophenoloxidase activity.
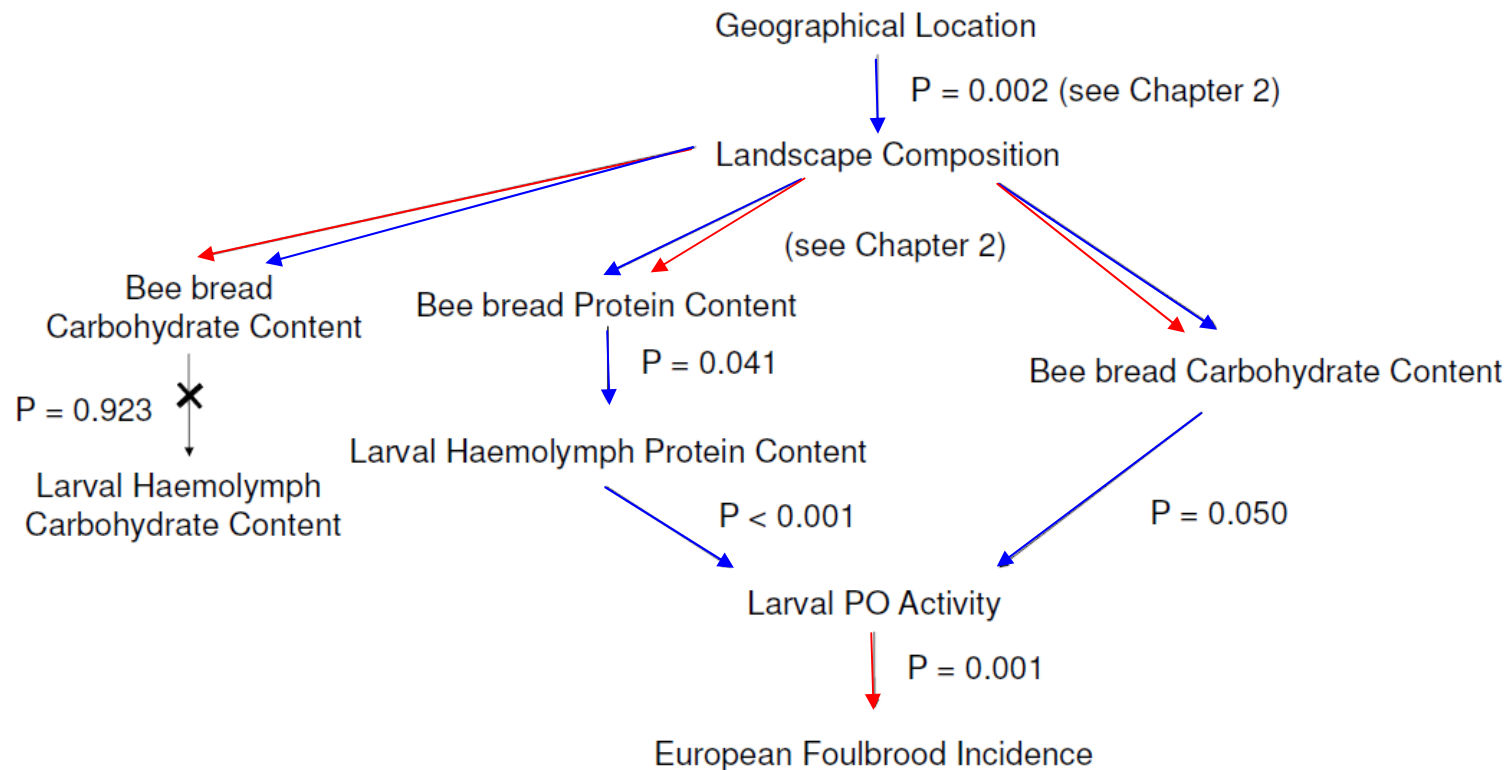
Figure 5.3. Schematic diagram of results of honey bee nutrition and immune function statistical analysis. (Blue for positive, red for negative). Results from Chapter 2 (section 2.3.4) show that bee bread nutritional content is linked to landscape composition; in the present study a link between nutritional content and immune function (in this case larval phenoloxidase expression) has been demonstrated.

The observed links between environment and nutrition (see Chapter 2), and between nutrition and larval immune function (Figure 5.3) suggest that a link between environment and immune function could be made. Nutritional values for bee bread were predicted from data provided by the Countryside Survey 2007 Land Cover Map (Morton *et al.*, 2011), and highlighted possible areas of the United Kingdom where bee bread nutrition is likely to be significantly skewed towards high or low protein content, based on locally-available pollen sources (Figure 5.4a). Most notably, the areas with the highest predicted protein content included northern England, Wales and western Scotland; whilst central areas such as London and southeast England showed the lowest predicted bee bread protein content.

These predicted nutritional values were then used to predict larval bee phenoloxidase immune response, based on the significant interaction between bee bread carbohydrate content and larval phenoloxidase activity (Figure 5.2d and Figure 5.3) and between bee bread protein content, larval haemolymph protein content and phenoloxidase activity (Figure 5.3 A phenoloxidase value was generated for each of the 10 km² squares across the UK for which landscape composition data are available (Figure 5.4b).

The frequency of European Foulbrood (*M. pluton*) infected hives in each of the 10 km² squares was significantly correlated with lower larval phenoloxidase activity (zero-inflated Poisson GLM: $b \pm$ S.E. = -0.012 $\pm$ 0.004, z = -3.216, df = 4, 2996, P = 0.001) and prophenoloxidase activity (ZIP: $b \pm$ S.E. = -0.009 $\pm$ 0.003, z = -3.100, df = 4, 2996, P = 0.001). Furthermore, areas with high phenoloxidase responses did not show records of infection by *M. pluton*, such as central Wales and west Scotland (Figure 5.4c).
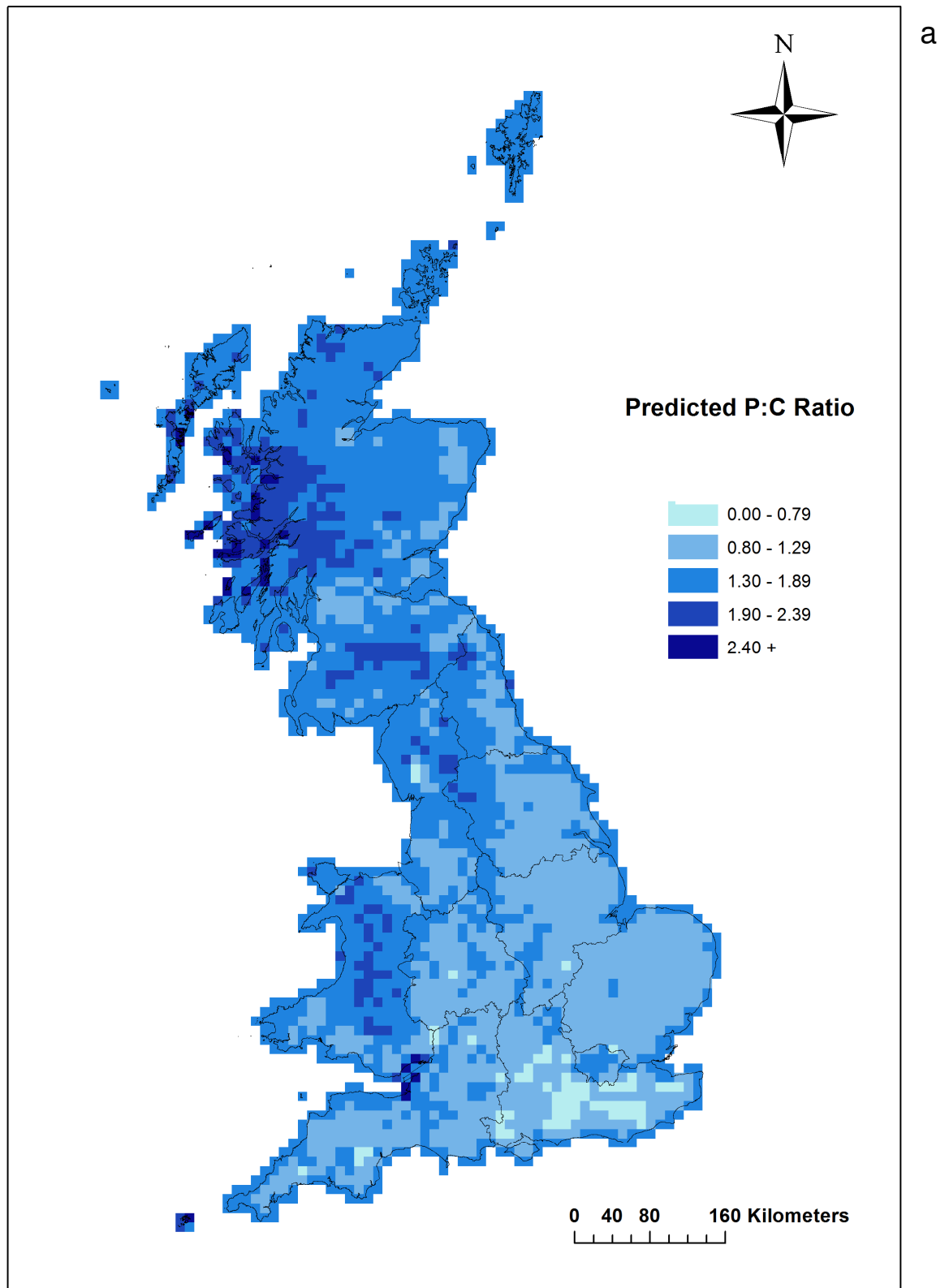
Figure 5.4. Predicted honey bee nutrition and immune response across the United Kingdom. (a) Protein:carbohydrate values of bee bread determined by landscape composition values from the Countryside Survey land cover map;
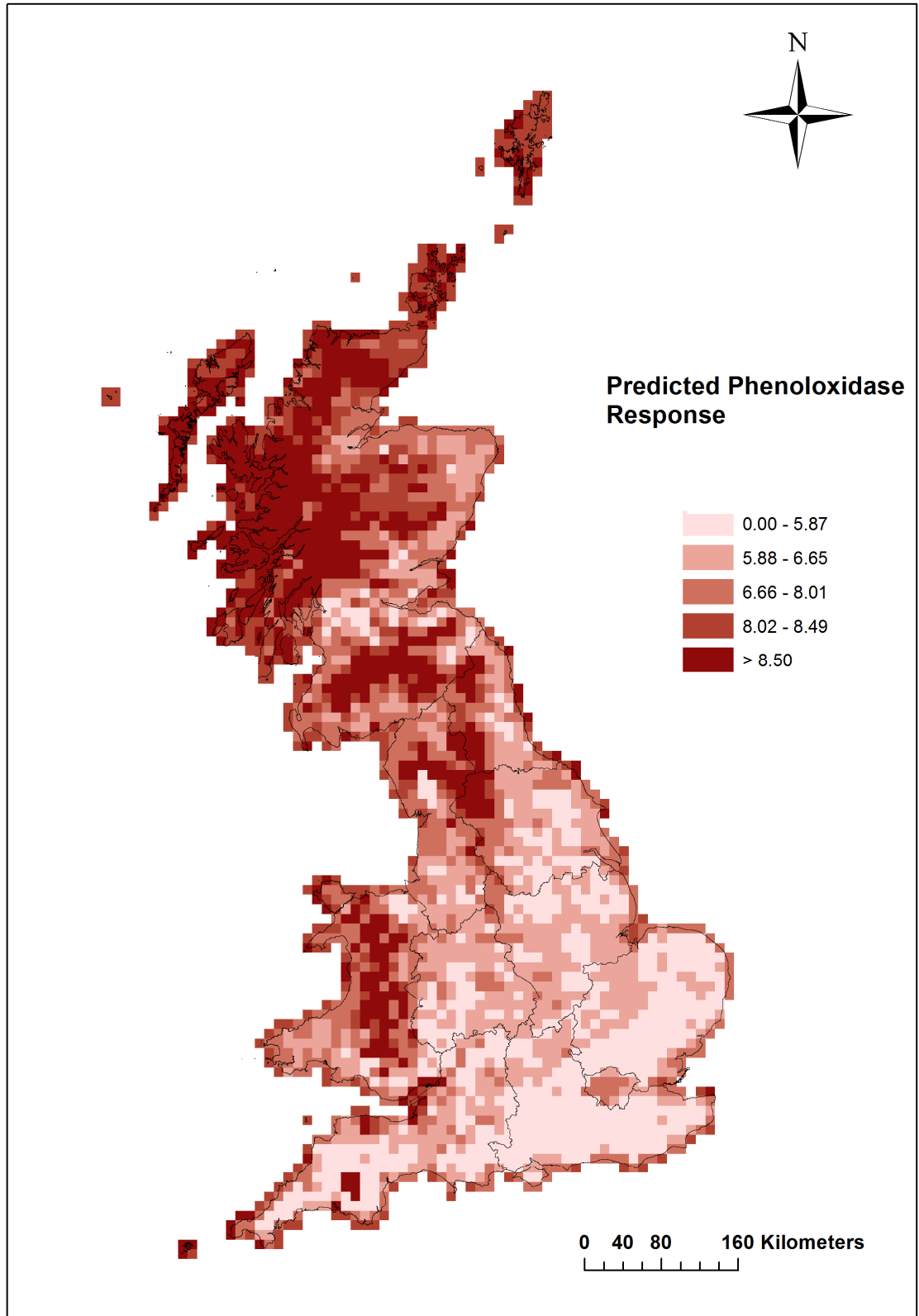
(b) Immune response values for larval honey bees based on results of the current study;
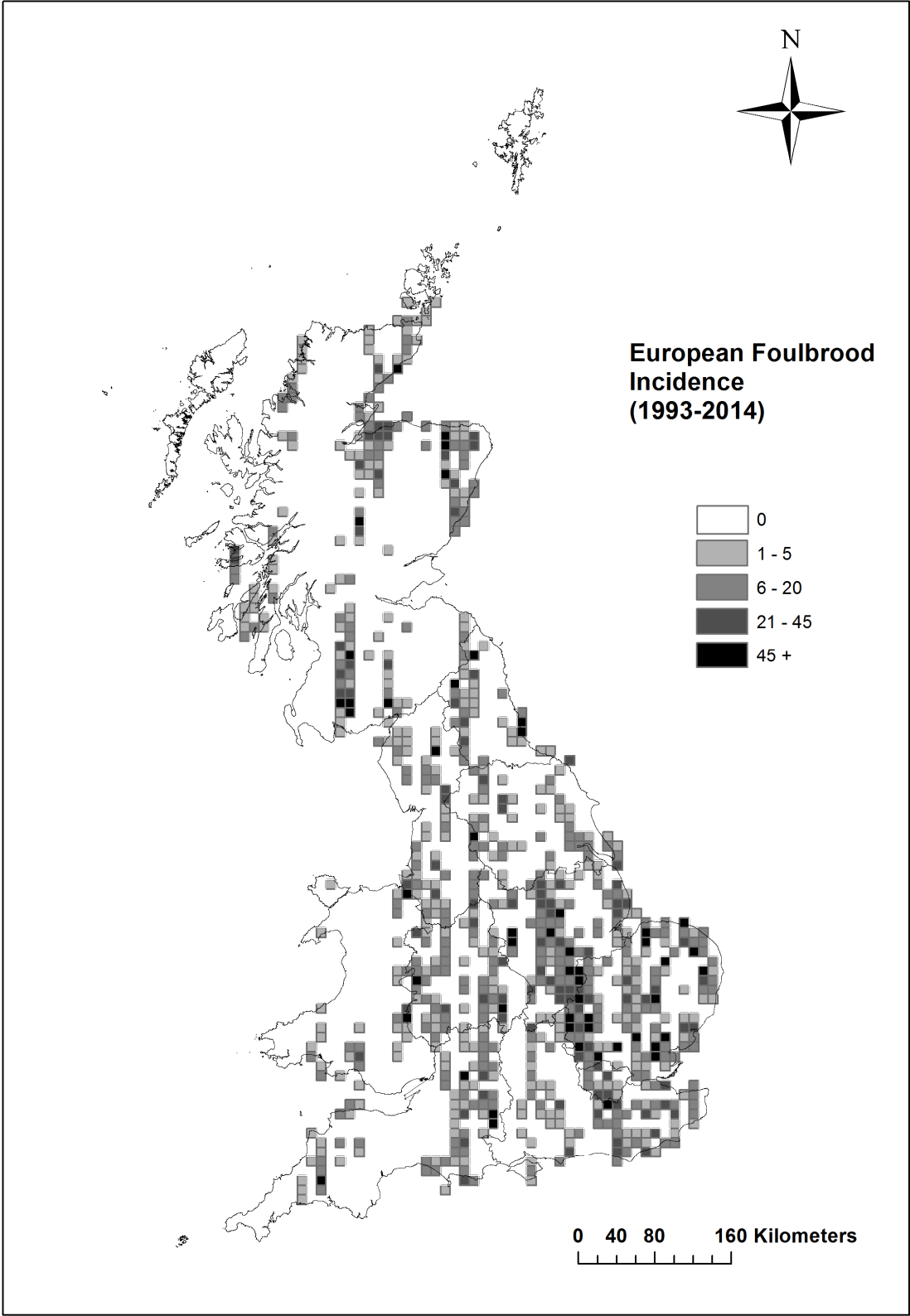
(c) Actual disease records for European Foulbrood (*M. pluton*) within the 10 km²
squares reported by the National Bee Inspectors of FERA between 1999-2014.

Predicted bee bread nutritional composition was verified using Pearson's correlation tests according to section 5.3.5; these showed that predicted protein content was significantly positively correlated with recorded protein content (Pearson's correlation: $r = 0.787$, t = 6.109, df = 23, P < 0.001), as was predicted carbohydrate content with recorded values ($r = 0.398$, t = 2.082, df = 23, P = 0.0487).

5.5 Discussion

In this study the effects of naturally occurring variation in diet composition on a honey bee immune trait were studied. Consistent with previous studies, these analyses here of both larval and adult honey bees within multiple hives have demonstrated that they both are capable of initiating a variable phenoloxidase response (Schmid *et al.*, 2008). This study found that honey bees that collect higher protein pollens and produce bee bread with higher protein contents produce larvae that have a higher haemolymph protein content. Adults express stronger constitutive immune responses (phenoloxidase and prophenoloxidase activity) than larvae, but were also more variable resulting in a lack of significant correlation between nutrition and immune function in adults.

Links between immune responses and diet have been demonstrated frequently in many invertebrates, including honey bees when studied in a laboratory setting (Alaux *et al.*, 2010b; DeGrandi-Hoffman *et al.*, 2010; Cotter *et al.*, 2010; Lee *et al.*, 2006; Ponton *et al.*, 2011). These previous studies delivered diets in discrete compositions, meaning that they did not address the naturally occurring variability in dietary composition (see Chapter 2). Even when mixed diets are offered, as with the geometric framework approach to nutrition analysis (Altaye *et al.*, 2010; Cotter *et al.*,

2010; Simpson and Raubenheimer, 2012), these mixes comprise a limited number of discrete diets. Due to the link between diet and immune responses, it is important to consider the naturally occurring variation in diet quality and the potential effects on immune responses.

This study examined not only the phenoloxidase immune pathway, but also accounted for variability of nutrition in diets, which allowed for more realistic predictions of differences in immune response to changing diet quality. Honey bee immune responses were estimated in terms of phenoloxidase and prophenoloxidase expression in both adult and larval bees. Phenoloxidase is an enzyme responsible for catalysing key steps in the synthesis of melanin, a pigment that is found in the cuticle (Ashida and Brey, 1995) and is involved in the encapsulation of foreign bodies. Phenoloxidase is thought to be a particularly important component of the immune response and is involved in resistance to a range of pathogens (Washburn *et al.*, 1996; Vega and Kaya, 2012). Therefore, variation in the nutrients available for maintaining this response may be important when considering variation in the susceptibility of honey bees to disease.

In Chapter 2, it was demonstrated that bee bread nutritional composition reflects the landscape composition in local areas around hives (see section 2.3.4), and in Chapter 3 it was suggested that this is linked to the varying composition of available forage in different areas (see section 3.4.4). Analysis in the present chapter has shown that this variation in bee bread nutritional composition is reflected in the nutritional composition of brood food; haemolymph composition and phenoloxidase/prophenoloxidase activity in larvae (see Figure 5.3). This correlation occurs despite differences in bee bread nutritional composition within the hive (see Chapter 2, section 2.4.2).

Consistent with the findings of this study, previous research has demonstrated that honey bee hives fed specific pollens, had haemolymph protein titres that reflected the protein contents of the pollens in their diets (Basualdo *et al.*, 2013). The results presented here further support the findings of Chapter 3 and imply that specific floral resources have a quantifiable impact on honey bee nutrition in the form of bee bread and brood food (Di Pasquale *et al.*, 2013), and therefore honey bee immunity (see Figure 5.3).

The results of our study are consistent with previous research that suggest bee immune responses are linked to the protein and carbohydrate levels in their diets; since bee bread (the protein food stores in the hive) are significantly correlated with brood food (the diet produced by nurse bees to feed larvae in the hive). Similar to previous studies, this research has demonstrated that as the protein concentrations of diets increase, the haemolymph protein concentrations and phenoloxidase/prophenoloxidase activity of honey bees also increase (DeGrandi-Hoffman *et al.*, 2010). Conversely that increasing carbohydrate levels were found to be correlated with a decrease in immune function. Previous research has indicated that this may be due to increasing carbohydrate effectively diluting the available dietary protein resulting in a weaker immune system (Povey *et al.*, 2009). However, it may equally be due to higher carbohydrate contents in the haemolymph resulting in increased fitness of pathogens and therefore reduced fitness of the host bee (Mayack and Naug, 2010). Due to a lack of significant direct interactions between P:C ratio and immune function however, either of these possibility remains likely.

The relationship between diet and immune function is complex: there is evidence that increasing dietary carbohydrate results in an increase in pathogen fitness. Rather than this being due to immunosuppression as a result of carbohydrate

in the diet, it may be due to pathogens exploiting the additional metabolic material made available in high carbohydrate diets (Lee *et al.*, 2006). Here, it has been shown that increasing dietary carbohydrate results in a significant reduction in the phenoloxidase/prophenoloxidase activity in both larval and adult honey bees. The microsporidian *Nosema apis*, parasitizes honey bees by consuming trehalose sugar in the host haemolymph (Mayack and Naug, 2010). The resulting reduction in haemolymph carbohydrate levels could result in an increase in phenoloxidase/prophenoloxidase activity in the host honey bee; however, it was not possible to obtain haemolymph carbohydrate data within this study and so this remains speculative. Parasitism by *N. apis* has further implications beyond the fitness of individual bees though, as it can lead to a reduction in foraging capacity in parasitized individuals, while also increasing feeding behaviour (Mayack and Naug, 2013; Martín-Hernández *et al.*, 2011), which has significant implications for colony level stability (Botías *et al.*, 2013).

Honey bee larvae demonstrated increasing phenoloxidase activity when they had higher haemolymph protein content (see Figure 5.2f) in hives that store higher protein content bee bread (see Figure 5.3). Some invertebrates demonstrate a reduction in fitness when fed diets with a high protein:carbohydrate (P:C) ratio. High protein diets with P:C < 5:1 have been shown to reduce lifespan in *Drosophila melanogaster* (Lee *et al.*, 2008) and can lead to colony collapse within the black ant (*Lasius niger* L.; (Dussutour and Simpson, 2012). A decline in larval immune function was not observed in hives with higher protein bee bread, indicating that the issues associated with high P:C ratios in larvae may be relevant for life-history traits such as lifespan (Lee *et al.*, 2008) and development rates (Cotter *et al.*, 2008a).

In addition to larval immune function, the relationship between bee bread nutritional composition and adult immune function was investigated. Adult immune responses were less significantly correlated with diet nutritional composition than when correlating larval immune function. Young adult bees are nurse bees that consume bee bread as a source of protein. Although high protein diets have been linked to higher haemolymph protein levels in adult bees (Basualdo *et al.*, 2013), adult nurse bees use a high proportion of their dietary protein in the production of brood food in their hypopharyngeal glands (Sagili and Pankiw, 2007; Hrassnigg and Crailsheim, 1998). Larval honey bees do not produce brood food, they use their dietary protein for development and in the maintenance of their immune system (Page and Peng, 2001). The protein intake of a hive is partitioned in favour of the larvae (Cremer *et al.*, 2007; Page and Peng, 2001) and adults may have less protein with which to maintain immune systems (Alaux *et al.*, 2010b).

There are a number of potential factors that must be considered to understand the lack of significant correlation between adult immune function and nutrition. The most important caveat being that the sampling of bee bread was performed on the same date as sampling of bees for immune testing. Previously (see Chapter 4, section 4.52), it was noted that pollen takes approximately 3-4 weeks to mature into bee bread; therefore, the nutritional data collected in this paper is from 3-4 weeks previous to the corresponding immune function data. If the non-linear temporal variation in bee bread nutritional composition (see Chapter 2, section 2.4.3) is considered, then it is evident that the composition will be significantly different from that which the adult bees had been feeding on before sampling.

Furthermore, the immune systems of adult bees are distinct from those of larvae: numerous studies have indicated that adult honey bees immunosenesce,

meaning that they deactivate several of their immune pathways upon reaching adulthood (Wilson-Rich *et al.*, 2008; Amdam, 2011; Amdam *et al.*, 2005; Scharlaken *et al.*, 2008). After immunosenescence, adult bees still maintain the phenoloxidase immune pathway, but do not produce haemocytes or antimicrobial peptides (Schmid *et al.*, 2008). Here, it was demonstrated that adult honey bees expressed a higher mean level of phenoloxidase and prophenoloxidase; other studies have suggested that adult honey bees have a weaker immune system due to the smaller selection of immune pathways available (Behrens *et al.*, 2010; Bull *et al.*, 2012). From the results of this study, it could be suggested that adult honey bees increase their expression of phenoloxidase/prophenoloxidase in order to compensate for immunosenescence. Adults may have evolved to immunosenesce because they are 'disposable', as the major focus of the bee colony is rearing and maintenance of the larvae (Evans and Pettis, 2005; Seeley, 2008). Honey bee colonies focus on supplying a high quality of food to their larvae, in order to maintain a high level of fitness in the next generation of workers (Fellous and Lazzaro, 2010).

Consistent with the findings of Chapter 2, it was shown here that protein and carbohydrate composition in bee bread vary significantly between hives. Further, it was shown that as protein increases, protein titres in the haemolymph increase, which are in turn linked to an increase in larval phenoloxidase activity. If phenoloxidase activity reflects general immune function in honey bee larvae, then it might suggest that in areas with low protein content bee bread, susceptibility to some immune stressors such as pathogens might be elevated, and possibly could contribute to increased colony decline in these areas. This hypothesis was tested by comparing a predicted immune response (phenoloxidase) with UK Government records of a honey bee pathogen (European Foulbrood, see Figure 5.4).

Predicted areas of high protein content bee bread were significantly correlated with areas of higher predicted phenoloxidase immune response (see Figure 5.4a and 5.4b). When compared with the records of European Foulbrood (*M. pluton*) incidence, areas with a higher predicted phenoloxidase immune response were located in areas with low incidence of this disease, and areas with a lower predicted immune response were located in areas with higher incidence (see Figure 5.4b and 5.4c). Previous research indicates that honey bees, bumble bees and solitary bees respond to landscape level changes in composition and configuration in terms of their foraging and nesting site choice (Visscher *et al.*, 1985; Visscher and Seeley, 1982; Steffan-Dewenter *et al.*, 2002; Steffan-Dewenter and Tscharntke, 2000). However, it must be noted that there are many other factors not addressed in this study that impact the plant communities from which bees can forage on the national scale, such as temperature gradients, rainfall, presence of competitor species. Given these constraints, the results presented here may suggest that honey bee immune function is significantly linked to landscape composition through its impact on the nutritional composition of bee bread (see Chapter 2). A broader landscape-scale analysis of honey bee phenoloxidase activity would provide further evidence confirming this relationship. Further research into the predictive ability of the Countryside Survey dataset to accurately determine plant community composition, as well as the relative effects of improved immune function and fitness on bees' abilities to forage would greatly strengthen the conclusions that can be drawn here.

The infection records of European Foulbrood (*M. pluton*) in honey bee colonies are based on inspections by FERA employed National Bee Inspectors. Using this data, this study determined that areas of low predicted immune response were correlated with areas demonstrating a high frequency of European Foulbrood

occurrence/outbreaks, and high predicted immune response with a low frequency of the disease (see Figure 5.4b and 5.4c). However, there are issues/caveats/limitations with the data used to establish this relationship, which must be noted and may limit the accuracy of the conclusions drawn. Although National Bee Inspectors are legally permitted to inspect any bee hive in UK, these records are limited in their scope by the number of inspectors and the remoteness of hives. FERA currently employs only 60 inspectors for England and Wales and the Scottish Beekeepers Association employs 10 inspectors for Scotland, which manage 22250 ± 12287 inspections per year and have undertaken a total of over 400,000 hive inspections since 1993 (FERA, 2014). Unfortunately, there are no accurate records for the total number of bee hives in UK either currently or historically, predominantly due to the voluntary nature of the scheme, the occurrence of unregistered beekeepers, and annual fluxes in colony numbers due to winter losses, restocking apiaries and swarming (House of Commons Public Accounts Committee, 2009). Approximately 80,000 hives are currently registered with FERA, but these numbers are likely underestimates of the actual number of hives (House of Commons Public Accounts Committee, 2009). There are also no data available on hive density across UK; it may be that infection free zones are where there are no beehives.

The present study provides the first evidence that honey bee diets, produced by the bees themselves, have a significant effect on the immune function of larval and adult bees. These results are distinct from those of previous studies, as they account for the significant variation that occurs in diet composition in honey bee hives. Honey bee diets are determined by the quality of forage available in the environment around their hives. The effects of landscape composition on honey bee immune systems that were demonstrated here may be used to propose potential optimal locations for raising

bees with a higher immune function; it may also go someway towards explaining the variability in susceptibility to disease in bees and help to inform epidemiological models of honey bee disease.

Chapter 6. General discussion

Working with beekeepers was a key part of this research, their knowledge and experience was invaluable in designing and implementing my studies.

6.1 Introduction

The key findings of the research presented in this thesis have described the dynamics of honey bee nutrition in terms of consumption of pollen. Herein, I have studied floral and microbial diversity, landscape ecology, immunology and behavioural ecology associated with honey bee nutrition. To address these, I focused on bee bread, a material that honey bees derive from pollen. Bee bread is an essential component of nutrition in the honey bee hive, providing dietary components necessary to develop the brood as well as stimulating egg laying by the queen (Oliver, 2007b; Herbert and Shimanuki, 1978).

Previous research has indicated that nutrition may have significant impacts on the biology (i.e. immune function, fecundity or longevity) of invertebrates, in particular honey bees, fruit flies and armyworm (*Spodoptera exempta*); however, these studies have been primarily laboratory based (Alaux *et al.*, 2010b; Povey *et al.*, 2014a; Lee *et al.*, 2008). Extrapolating the results of these studies is problematic as they can control for the significant variation that occurs in the insect's natural environment. An example of this would be a study of honey bee dietary protein quantity and diet diversity (diversity of pollens in diet) affecting immune responses (Alaux *et al.*, 2010b). This previous study used combinations of pollen from seven species (*Acer*, *Castanea*, *Cistus*, *Erica*, *Quercus*, *Salix* and *Taraxacum*), whereas here in Chapter 3 the results suggest that there could be more than 30 different pollen species found in bee hives in the North-west of England. The results of this thesis may hold a greater relevance than the previous research because the patterns that are emerging are in spite of the extent of environmental variation, which may therefore presents an alternative view that may be more applicable to the real world.

6.2 Pollination services

Invertebrate mediated pollination occurs during 35% of global food production, pollination results in both higher production levels and greater food quality and is important in maintaining global food supply. Pollinating insects include honey bees, bumble bees, solitary bees and several genera of flies. Honey bees alone have an estimated annual value of at least £190 million per annum in the United Kingdom (Knight *et al.*, 2009). The ability of honey bees to pollinate is modulated by the fitness of a colony, which is itself a product of numerous factors, amongst which is the nutrition of the bees (Brodschneider and Crailsheim, 2010).

The European Commission recently published results of a European Union-wide monitoring scheme of honey bee colony mortality for over-winter losses and losses during the beekeeping season (Marie-Pierre *et al.*, 2014). The report included data on farming practices and records of infectious and parasitic diseases to explain patterns of losses. Similarly, OPERA (Observatory for Productivity and Efficient use of Resources in Agriculture) have released a report of threats to honey bee health (Capri and Marchis, 2013). Both of these reports present agricultural practices and land use change as integral threats to honey bee health across Europe (Figure 6.1). However, these reports have focused on the causes of honey bee mortality, but not the sources of pressures on bees that lead to mortality, such as poor nutrition; neither have suggested a causal relationship between honey bee health, nutrition and land use. Therefore, I suggest that a significant knowledge gap exists; this thesis has attempted to address it through examination of the link between honey bee nutrition, environmental composition of land use and honey bee biology in terms of immune responses.
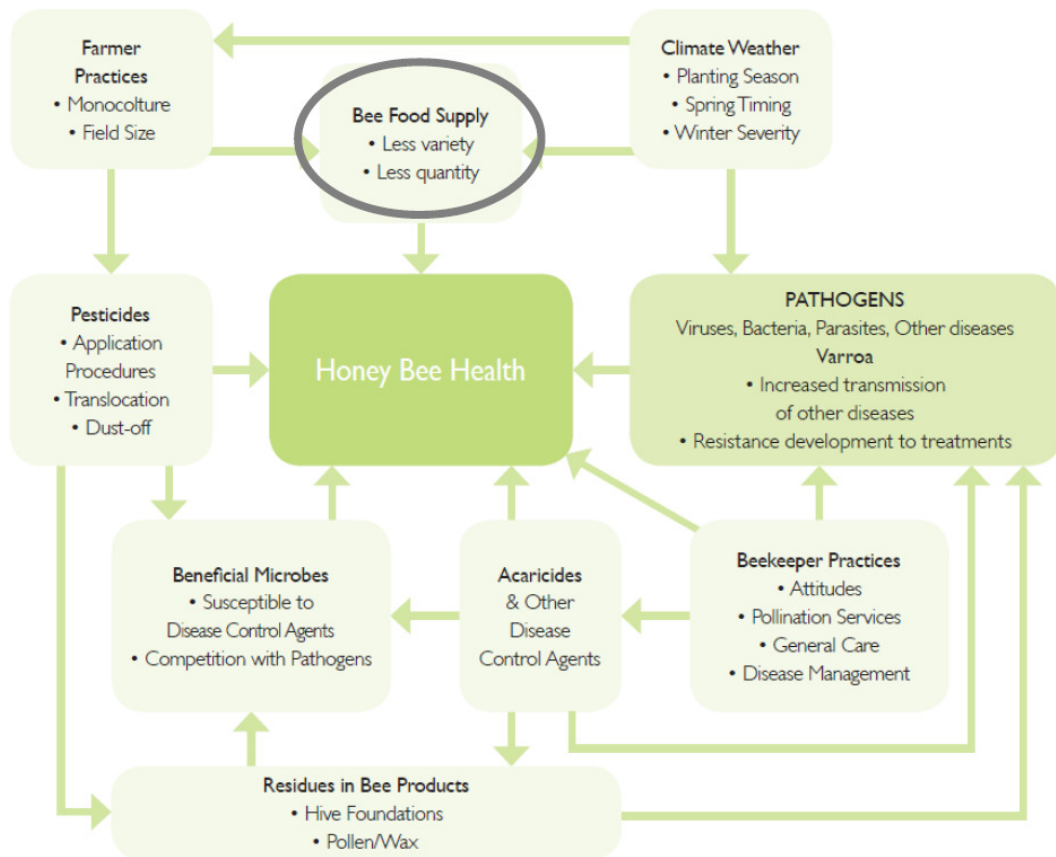
Figure 6.1. Interrelationship of bee health Stressors; from OPERA bee health report (Capri and Marchis, 2013), honey bee nutrition has been highlighted. The figure and the accompanying report describe the important factors that are theorised to be determining patterns in honey bee mortality across Europe.

6.3 Foraging, pollen and bee bread

Bee bread is made from pollen stored on frames, honey bees are extensive foragers, capable of collecting pollen and nectar from anywhere within 0.5 km and 10 km of their hive (Beekman and Ratnieks, 2000). Within the foraging range, plants that honey bees target for foraging will vary according to the composition of the landscape, as certain landscapes select for different floral assemblages (Keller *et al.*, 2005a). Indeed, this thesis has demonstrated that a sample of bee bread taken from one area can vary significantly in the number and composition of species of pollens that comprise it when compared to bee bread from a hive in another area (see Chapter 3).

Pollen nutritional contents vary depending on the species of plant from which it derives: previous studies suggest that pollen protein content could vary from around 12% (*Tanacetum vulgare*: Asteraceae) to 62% (*Dodecatheon clevelandii*: Primulaceae; (Roulston and Cane, 2000; Somerville, 2005). This thesis has determined that bee bread nutritional content varies significantly in nutritional composition between bee breads within the same hive (see Chapter 2), and this was suggested to be derived from variability in which pollen species the foragers of a given hive are collecting. Furthermore, the number and composition of pollens in bee bread were found to have a significant impact on the nutritional composition of bee bread (see Chapter 3).

Bee bread nutritional composition was also found to vary significantly between hives located in different areas (see Chapter 2); and this was found to be linked to changes in the landscape composition in these different areas. As stated above, different landscapes select for differential floral assemblages, with pollens of certain nutritional values. The composition of diets can have impacts on the fitness of

honey bees, and this thesis demonstrated that honey bee colonies vary significantly in the ability of their workers and larvae to mount an immune response (see chapter 5). Immune responses are an effective proxy for the overall fitness of an invertebrate (Cotter *et al.*, 2010) and here it was demonstrated that variation in immune responses was linked to differences in the nutritional composition of bee breads within the hive (see Chapter 5). The results presented here support the idea that landscape composition around a hive has significant impacts on the immune response that honey bee larvae can mount, and hence fitness, of honey bee colonies. This evidence was further supported by testing the predicted immunocompetence, based on landscape composition; against actual disease records for honey bees (see Chapter 5). Here, significant similarities in areas of low predicted immune response and areas of high honey bee disease incidence were found. These results have begun to address the significant knowledge gap linking honey bee mortality due to disease with environmental composition and land use identified previously (Figure 6.1). However, it must be noted that the results presented here are subject to numerous constraints, most importantly being consideration of other factors beyond landscape composition that are certainly having a significant impact on plant community composition and bee nutrition.

This thesis has studied the relationship between honey bees and aspects of their environment; it has also examined the microbial communities that are associated with them. A diverse assemblage of bacteria in bee bread was both isolated by culture and detected using molecular methods (see Chapter 4). Although several previous studies have suggested that these organisms may serve several important functions for honey bees, the diversity of organisms found here highlights the lack of knowledge of the part microbes play in this system. Future studies should explore the origin of this

community, their potential functions in bee bread and the consequences for honey bee fitness.

6.4 Implications for findings

The findings presented in this thesis have significant implications for insect pollinator management. A recent POST note, published by the UK government suggests that insect pollinator decline may be due to, land use change, agricultural intensification and increased pests and diseases (Wentworth, 2013). The results of this thesis suggest that these three factors may be linked, as landscape composition may determine both honey bee nutrition and susceptibility to disease and specifically it was shown that arable agricultural land has a significant negative impact on nutrition (see Chapter 2). Furthermore, data from the Food and Environment Research Agency (FERA, https://secure.fera.defra.gov.uk/beebase/index.cfm) indicates that disease incidence in UK has increased recently and the EPILOBEE report indicated that honey bee mortality in the UK is amongst the highest in Europe (Marie-Pierre *et al.*, 2014).

In the future, local Government or land managers could be approached with recommendations on landscape management, planning policy and municipal planting regimes based on the results of this thesis and future studies. And hence, significant changes could be made to increase the proportion of bee-beneficial landscape types that generate higher protein diets and bees with greater immune response, in order to counter the increase in disease-related mortality in England (Marie-Pierre *et al.*, 2014).

6.5 Directions for future research

In Chapter 2, the composition of the environment around a hive was shown to influence honey bee nutrition. Colonies are limited to forage <10 km from the hive (Beekman and Ratnieks, 2000), as colonies cannot change their hive location, they are limited in the potential forage they can access. Honey bees disperse by swarming (Zeng *et al.*, 2005). Site selection by a swarm is based on combined decisions of scout bees finding "optimal" locations for the swarm (Rittschof and Seeley, 2008). Scouting bees are as likely to select a single site for relocation as they are multiple sites (Seeley and Buhrman, 1999). When multiple potential sites are selected, the process by which scout bees select a landing site for the swarm is believed to be based on a "weighted additive strategy"; whereby the swarm evaluates the alternatives in terms of all the relevant attributes, weight each attribute according to its importance, sum the weighted attributes for each alternative, and finally choose the alternative whose total valuation is the highest (Schmidt, 1995).

A major issue with this theory is that the "attributes" by which scout bees evaluate potential sites are poorly understood. Studies have shown that swarm scouts response to certain chemicals (geranic acid, citral, geraniol and rose oil), which are all plant volatiles (Papachristoforou and Ilanidis, 2013). Based on bee swarm responses to plant volatiles, it is conceivable that scout bees are responding to certain cues in the environment from plants and therefore that swarms may respond to landscape composition cues when searching for a landing site. By identifying swarm origins and

comparing the landing site landscape composition with origin site, we may begin to understand dispersal mechanisms for foraging in honey bees.

In Chapter 3, we attempted to identify the plant species whose pollen result in higher protein and carbohydrate contents in bee bread. The techniques used here to determine the plant diversity in the bee bread food stores were relatively coarse and may have missed many species of plant that rarely occur in the pollen stores. Additionally, we were not able to generate sequence identifications for plant species using the techniques employed.

Recently developed advanced DNA sequencing techniques (next generation sequencing) can be used to examine the diversity of plants in bee stored pollen with greater accuracy, detail and fidelity (Lee *et al.*, 2012). Unfortunately, the cost of these assays (up to £120 per sample) prohibited extensive use in this thesis. Using next generation sequencing that targets highly variable regions of the plant 18S ribosomal DNA (Hollingsworth *et al.*, 2009), a future study will be able to both identify individual species of plant that are associated with higher protein content bee bread and the complexity of the plant communities that comprise bee bread. The results of this study should reveal which plants are most beneficial for bees to forage on in terms of benefiting their diets. By identifying which plant species increase honey bee protein nutrition, these findings could be used to recommend new planting regimes for municipal, agricultural, horticultural and individual based wildflower schemes.

Finally, in Chapter 4, this thesis examined the diversity and stability of the microbial community associated with bee bread. The genera identified in this study included *Lactobacilli*, *Enterobacter* and *Acinetobacter*, which have been suggested by previous studies to be integral to the production of bee bread (Vásquez and Olofsson, 2009; Martinson *et al.*, 2012; Mattila *et al.*, 2012). No study has yet conclusively

stated the contribution these particular organisms make to the production of bee bread, although it has been eluded that it may be through lactic acid fermentation. A proteomics based approach to culturable isolates of these microbes may provide an empirical basis to these suggestions, but identifying which of them are capable of digesting the chemical components of pollen, but which are absent in bee bread. Sporopollen, a polymer of phenylpropanoid and lipid monomers covalently coupled by ether and ester linkages (Bubert *et al.*, 2002), is believed to be the primary target of degradation by these microorganisms. Sporopollenin makes pollen grains highly resistant to physical and chemical degradation (Grienenberger *et al.*, 2010). The resistance of sporopollenin to chemical degradation makes it difficult to analyze by chemical methods and hence the precise composition of it has yet to be established (Bubert *et al.*, 2002; Grienenberger *et al.*, 2010; Mao *et al.*, 2013). Before studying the activity of microorganisms in the digestion of pollen grains, the chemical components of sporopollenin should be determined.

6.5 Conclusion

This thesis sought to supplement the primarily laboratory based studies on the effects of nutrition on invertebrate fitness with data collected from the field. By using data sourced in such a way, the conclusions that have been drawn here are subject to many more caveats, but the stories it tells may be truer to reality that previous studies. It has considered some of the factors that determine and modify the nutritional composition of diets that honey bees produce and consume within their hives, revealing the importance of the environment, floral resources and bacterial communities in determining nutritional composition of bee bread. Furthermore it has

examined the relationship between this nutritional composition and bee fitness during both the larval and adult life stages, identifying significant relationships between larval immune responses and dietary composition, but not for adult honey bees. In doing so this thesis contributes further to our understanding of the interaction between the environment and the nutrition of honey bees and how this impacts the fitness of the bees. By examining this interaction in a wild study system, rather than based on laboratory studies, the results of this thesis have addressed a significant knowledge gap in honey bee ecology, but have also highlighted the issues that can occur when working in these systems.

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