# Strategies of disease resistance in the superorganism: investigations into the effects of diet on the immunocompetence and behaviour of honey bees.

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

**Signature: ……..**

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## Abstract

As superorganisms, eusocial insect colonies possess both individual and social strategies for epidemic control. Both the physiological immune system within individuals and an array of social behaviours, such as self–quarantine, collectively comprise the colony's immunocompetence. Diet is a modulator of immunocompetence in insects and furthermore insects can self-medicate by ingesting nutrients that promote immunocompetence. However, how diet impacts multiple strategies of epidemic control both physiologically and through social behavioural processes, within a superorganism is not known. Therefore, the central aim of this thesis is to elucidate the role of diet for immunocompetence in the eusocial European honey bee (*Apis mellifera*). In the first data chapter (Chapter 2), I set the framework for measuring honey bee immunocompetence by describing a time course for the expression of two key components of the physiological immune system after challenge; the phenoloxidase pathway and antimicrobial peptides. I establish that only antimicrobial activity is elicited by an experimental pseudo-bacterial challenge and I identify appropriate time points to assess the impact of diet on immunocompetence. I demonstrate that short-term pollen starvation has no impact on physiological immunocompetence. In chapter 3 I show that a pseudo-bacterial challenge causes honey bees to adopt a diet that reduces their intake of pollen, whilst maintaining their intake of carbohydrates and I demonstrate that immunologically challenged honey bees forage more intensively. Based on these two findings, I therefore propose that a dietary mechanism underlies increased foraging intensity, which is adaptive as a form of nutritional targeting for self-removal to reduce colony infection. In chapter 4, I demonstrate that a sustained lack of essential amino acids both promotes antimicrobial peptide

activity and reduces longevity. Furthermore, I show that, like the trend observed with pollen consumption, a pseudo-bacterial challenge causes honey bees to reduce their intake of essential amino acids, Taken together, these results provide new support for the proposition that through dietary modulation, honey bees nutritionally self-medicate at the level of the superorganism in order to reduce in-hive rates of pathogen transmission by increased physiological immunocompetence, self-removal, and mortality in infected individuals.

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## Author's declaration

Unless otherwise stated, the author was responsible for all data collection and analysis. The use of the first person plural (i.e. 'we' as opposed to 'I') reflects the contribution of the supervisors in providing advice on experimental design, analysis and interpretation of data, and of the various internship students and technicians at Fera Science Ltd in providing laboratory support. Method development was done in collaboration with Dr Elizabeth Collison. In chapters three and four, advice on statistical analyses and experimental design was provided by Dr Mark Shirley and Professor Geraldine Wright respectively.

## Abbreviations

| | |
|---|---|
| AMP | Antimicrobial peptide |
| ANOVA | Analysis of variance |
| $\boldsymbol{\beta}$ | The coefficient of disease transmission |
| COXME | Mixed cox proportional hazard model |
| $\boldsymbol{d}$ | The *per capita* rate at which infected individuals are lost from the transmission process |
| *df* | Degrees of freedom |
| EAA | Essential amino acid |
| *F* | F-ratio |
| free PO | Free phenoloxidase |
| g | Grams |
| GLMM | General linear mixed model |
| GOX | Glucose oxidase |
| $\boldsymbol{I}$ | The number or density of infected hosts |
| LME | Linear mixed model |
| LPS | Lipopolysaccharide |
| M | Molar |
| Mg | Milligram |
| ml | Milliliter |
| mM | Millimolar |
| mm | Millimetre |
| *n* | Number |
| *ρ* | Pearson's correlation |

| | |
|---|---|
| PO | Phenoloxidase |
| proPO | Pro phenoloxidase |
| RFID | Radio frequency identification |
| $\boldsymbol{S}$ | The number or density of susceptible hosts |
| SI | Sham-injected |
| $\boldsymbol{t}$ | Time |
| UC | Uninjected control |
| µl | Microliter |
| $^{V}$max | Maximum reaction rate |
| $\chi^2$ | Chi-square |
| $\frac{\Delta \boldsymbol{I}}{\Delta \boldsymbol{t}}$ | The rate of change in the number of infected individuals over time within a population |

# Chapter 1. General Introduction

*"Selfishness beats altruism within groups. Altruistic groups beat selfish groups. Everything else is commentary"*

Wilson & Wilson (2007)

This thesis investigates nutritional modulation of the immune response in honey bees (*Apis mellifera*). Honey bees exhibit both individual and social immunity, including behavioural processes to reduce pathogen transmission within the colony. Therefore, in order to contextualise how their social and behavioural processes affect colony health, in this chapter I firstly describe honey bees as a superorganism. I then introduce the diet of honey bees, the nutritional value of pollen and the various immune functions possessed by honey bees. I review previous work investigating the dependence of immunocompetence on dietary status in honey bees and more widely studied in other insects, as well as the potential for insects to self-medicate through altering their feeding behaviour. I then identify the knowledge gaps in how nutrition affects the various immune responses of honey bees and the consequences of potential self-medication by dietary modulation. Finally, I describe the aims of this thesis and outline how each is addressed in the subsequent chapters.

*1.1. Honey bees as a superorganism*

Eusocial systems comprise related individuals that live in colonies with overlapping generations, exhibit cooperative brood care and are divided into reproductive and non-reproductive castes (Wison & Holldobler 2005). Some sociobiologists subscribe to the notion that natural selection can act at the group level when altruistic groups outperform non altruistic groups (Wilson & Wilson 2007). Analogous to individual cells within multicellular organisms (Seeley 1989), the unit of selection can shift from the individual to the group when the combination of traits allow some groups to survive relative to others, despite disadvantages to individuals within groups. In this context, the phenomenon of eusociality can be usefully understood by formulating the concept of the 'superorganism' (Wheeler 1911).

Wilson & Wilson (2007) & Nowak *et al.* (2010) offer the following scenario for the evolution of eusocial insects: at some point in evolutionary time, freely mixing individuals formed groups, some of which formed cohesive and persistent units as some individuals were more pre-disposed to sociality. Some groups contained offspring that remained with their parents at their nest to care for brood. This gave rise to benefits derived from living in groups by division of labour between reproductive and worker castes, thus pushing species adaptively toward eusociality. Natural selection among colonies could then occur, favouring the *groups* (hereafter colonies) of individuals that contained beneficial traits, such as disease resistance and efficient nutrient acquisition. Natural selection on *colonies* would then shape the life cycle and caste system of the colony. For example, the development of anatomically distinct worker castes that forego reproduction altogether appears to represent a 'point of no return' whereby the colony becomes a stable unit of selection (Wilson & Wilson

2007). In other words, selection shapes the colony by acting upon the queen and her extended genotype within her offspring (Nowark *et al.* 2010). The colonial, or 'eusocial' insects, such as certain bees, ants, wasps and termites, exhibit what can be termed the 'superorganism' state, in which the so-called superorganism takes the place normally occupied by an individual in adaptive evolution by natural selection.

In insect evolution, the earliest bees are thought to have diverged from pollen- and nectar-collecting wasps during the middle Cretaceous, alongside the establishment of the flowering plants (Graham 1992, Winston 1987). Honey bees are classified into the family Apidae, which are characterised by their ability to carry pollen and nest material in a specialised pollen basket on their hind legs. Modern day honey bees (subfamily Apini, genus *Apis*) likely originated in tropical regions in Africa before migrating to Asia and Europe (Graham 1992, Winston 1987). Fossil bees from 30 million years ago show little morphological difference from modern day *Apis* and indicate that eusocial insect societies had already developed 27 million years ago (Winston 1987).

The European honey bee (*Apis mellifera*) lives in large, complex social colonies, containing between 5000 and 50,000 individuals. The colony is comprised of three distinct castes; thousands of sterile female workers, hundreds of male drones and usually one reproductive queen. A wealth of literature describes the social structure and nature of the honey bee colony, reviewed extensively in Graham (1992) and Winston (1987). Below I give a brief description of each caste and their functions within the superorganism from these references.

### *1.1.1. The Queen*

The queen's primary function is egg-laying, although this is somewhat dependent on the colony size, feeding and the time of year. The queen's ovaries are much larger than those of the female workers and she will lay approximately 1500 eggs daily and between 175,000 and 200,000 eggs annually. If the egg is unfertilised (haploid), the egg will develop into a male drone. Fertilized diploid eggs are female and can develop into either workers or queens, depending on the diet fed to the developing larva by the workers. Other queen functions include the production of pheromones such as the so called 'queen pheromone', whose many actions include social control. Queen pheromone is thought to supress rearing of new queens and swarming as well as having roles for colony recognition, attracting drones for mating and orientating workers. Queens are well tended by the workers of the colony; attendant workers surround the queen in groups of six to ten and lick and contact her with their antennae and forelegs. In this way, queens are groomed and her pheromones are picked up and subsequently distributed throughout the colony. In addition, queens have a shortened proboscis (feeding apparatus) and receive their food directly from the workers as a mixture of brood food secretions and honey. The queen's lifespan is determined by her capacity for laying fertilized eggs. Queens usually lay eggs for two to three years until stored sperm gathered from mating flights early in her life runs out, at which point the queen is then killed by the workers and replaced.

### *1.1.2. Workers*

The female adult workers perform the majority of the tasks within the colony. Each adult begins as a single egg (that contains a fertilized zygote) laid by the queen along with some yolk in one cell of wax comb. After approximately three

days, the egg hatches into a larva. Larvae consist essentially of a digestive system and undergo a rapid growth and moulting phase, consuming brood food secretions, pollen and honey from the adult workers for approximately five days. Their cell is then sealed with wax by the adult workers and the larva pupates. Pupae undergo massive developmental changes (holometamorphosis) into the adult phenotype, and adult individuals eclose (emerge) from the cell approximately 12 days later by chewing away the wax capping. The adult workers emerge sterile, with significantly reduced ovaries, a non-functional sperm storage organ (spermatheca) and no genital mating structures. Thus, workers can only lay unfertilized haploid eggs. However, the workers rarely lay eggs in the presence of a queen.

As adults, workers display age-dependent polyethism, which means that they alter their physiology and tasks as they age. The first tasks performed are domestic, such as cleaning wax cells for egg laying, followed by nursing duties, tending to brood and the queen, then wax comb building and receiving and storing food from foragers. The final set of tasks are performed outside the colony and consist of foraging, ventilating the colony and guarding it from robbing by wasps and non-nestmates. Although considerable overlap and flexibility in tasks exists according to colony demands, task specialisation is generally strongest between very young domestic bees and very old forgers. The workers' physiological development and feeding behaviour parallels the tasks they perform. For example, the 'nurse' phenotype is both behaviourally specialised and distinctly different from the similarly specialised and distinct 'forager' phenotype in that nurses eat more pollen in order to fuel development of hypopharyngeal glands, which produce the brood food and its expression is separated in time. Each adult worker adopts the behavioural phenotypes

sequentially, so that a nurse phase normally precedes the forager phases. Sequential behavioural specialisation or ‘temporal polyethism’ will play an important part in this thesis. A worker’s lifespan is highly variable and ranges from a few days to almost a year dependent on the intensity of foraging, the season, and the colony’s nutritional status and race. Average summer lifespans range between 15-38 days, whereas winter bees survive an average of 140 days.

### 1.1.3. Drones

The male drones are adapted entirely for mating with queens and perform no other colony tasks. As brood, drones receive a larger quantity and different composition of brood food than worker larvae. Drones result from unfertilised eggs (they are haploid) and their development time from egg to adult eclosion is 24 days, compared to 21 days for workers. Adult drones are initially fed by the young workers, and they receive brood food, pollen and honey before later feeding themselves directly on the honey stores of the colony. Drones lack many of the physical characteristics for colony tasks possessed by workers, but instead have an endophallus penis and an associated apparatus for clasping the queen during mating. Other adaptations are to locate queens, which include large compound eyes and optic lobes, ten times as many antennal olfactory organs as workers, larger flight muscles and broader wings than workers. On maturity, drones leave the colony and a single mating is performed in flight because the drones die during the mating process in which fatal physical damage is caused by much of the endophallus breaking off and remaining inside the queen. Drones have an adult lifespan of approximately 21-32 days in spring.

### *1.1.4. Swarming*

Swarming is required for the formation of new colonies. Once the population of a colony reaches a certain size, the workers build specialised large wax cells termed ‘swarm cells’. Once the swarm cell contains a fertilised egg, either laid by the queen or moved there by a worker, the workers heavily feed the developing larva with secretions of particular compositions, termed royal jelly, in order to trigger queen development. After capping the cell, swarming commences. Swarms consist approximately 60% of the worker population and the old mated queen. Swarming bees will carry large loads of honey from the original colony in order to fuel the swarming activity and provide the initial food reserves for the new colony. Swarms then settle as a cluster from which scouts will search out potential nest sites. Returning scouts will then communicate distance and directional information with dances. As more bees are recruited to attractive nest sites, more dances are performed, resulting in more recruitment. In this way the cluster relocates to a suitable nest site in order to rebuild a new colony. Following the departure of the swarm, a virgin daughter queen emerges into the vacated colony from her cell. Development of queens takes just 16 days and once emerged, daughter queens kill other un-emerged queens by making small holes in the swarm cells and stinging the developing queen inside. Should two virgin queens emerge into the colony, they will flight by stinging and chewing to the death or the remaining workers may kill the losing queen by surrounding her to sufficiently to raise the temperature to lethal levels (heat balling). Five or six days following emergence, the virgin queen becomes sexually mature and mating flights are then performed, usually enforced by the workers with aggressive behaviour toward the queen. Queens are polyandrous and multiple mating flights may occur, during which time she will expect to mate

with approximately seven to 17 drones. The polyandrous nature of honey bee queens means that each worker is on average more related to a brother than a son of a worker sister and according to the theory of kin-selection, this is the evolutionary driver behind worker policing and destruction of worker-laid eggs. Once mated, the queen will not perform any more mating flights throughout her egg-laying life.

### *1.2. Honey bees as a model system*

The honey bee colony can therefore be considered functioning as an integrated stable unit, or superorganism; the ultimate goal of the workers efforts being the propagation of their genes through the reproductive success of the queen's sexual progeny (Seeley 1989). The key feature relevant to this thesis is that individual workers are expendable and can die for the good of the colony in much the same way that cells might undergo apoptosis (programmed cell death) for the good of the individual organism, because the fate of the queen's genes is tied to the *colony* traits that determine colony survival and reproduction. One such colony-level trait is the ability to resist disease, which is in turn influenced by nutrition and immunocompetence. Thus, honey bees represent an ideal model system to study nutritional strategies for disease resistance in the superorganism.

### *1.3. Colony nutrition*

The honey bee foragers meet the nutritional needs of the colony by collecting nectar, pollen and water (Seeley 1995), which are essential to the survival and growth of the colony (Brodschneider & Crailsheim 2010, Haydak 1970). Once collected and received into the colony, pollen is stored in the wax comb as 'bee bread'. Bee bread is a mixture of pollen, honey, enzymes, glandular antiseptic

factors and some microorganisms. The stored pollen then undergoes a number of biochemical alterations that increase its stability and nutritional value. (Winston 1987).

### *1.3.1. Pollen*

Adult honey bees ingest pollen either directly or from stored bee bread (Crailsheim 1990). Once ingested, pollen is transported to the internal honey crop, before being separated from liquid nectar and passed via the proventriculus into the midgut (Crailsheim *et al.* 1992). Once the pollen is consumed it must be digested and absorbed into the haemolymph to provide the nutritional elements required by the honey bee (Schmidt *et al.* 1987). Pollen has a series of structures surrounding the nutritional cytoplasm composed of the intine, exine and pollenkitt at the outermost layer. These layers present a digestive barrier to be overcome as pollen passes through the digestive tract and the efficiency of digestion differs between different pollens (Keller *et al.* 2005).

In addition to satisfying the colony's requirements for minerals, lipids and vitamins, pollen is the primary source of dietary protein for honey bees (Campana & Moeller 1977) and protein content is most often considered the most important nutritional value of pollen. Indeed, the importance of proteinaceous food for adult bees is demonstrated through its absence, when the adults may resort to cannibalism of brood to meet their dietary requirements (Brodschneider & Crailsheim 2010).

Previous studies have provided valuable data showing the various chemical compositions of pollens from different plant species, demonstrating differing levels of protein, amino acids, moisture, sugars, starch, ash, lipids, pH, fibre, vitamins and minerals from pollens across many plant species (Auclair &

Jamieson 1948, Day *et al.* 1990, Forcone *et al.* 2011, Hanley *et al.* 2008, Herbert & Shimanuki 1978, Keller *et al.* 2005, Martins *et al.* 2011, Oliveira *et al.* 2009, Schmidt *et al.* 1987, Somerville 2005, Somerville & Nicol 2006, Stanciu *et al.* 2011, Standifer *et al.* 1980). The protein content of pollens is commonly found to be largely variable across and within plant families (Hanley *et al.* 2008, Keller *et al.* 2005). For example, bee collected pollens have a protein range of between 15-60% (Roulston *et al.* 2000). However, the amino acid composition appears to be less diverse between species and few plant species seem to lack some of the essential amino acids (EAAs) for honey bees (Roulston & Cane 2002).

### *1.3.2. Protein nutrition of adults*

Dietary pollen and protein promotes the development of the honey bee's hypopharyngeal glands (Crailsheim *et al.* 1992, DeGrandi-Hoffman *et al.* 2010, Di Pasquale 2013, Pernal & Currie 2000), internal organs (Pernal & Currie 2000, Hagedorn & Moller 1968, Haydak 1970, Hoover *et al.* 2006), fat bodies (Alaux *et al.* 2010, Haydak 1970), dry body weight, nitrogen content (De Groot 1953), overall body mass (Crailsheim 1990, Hoover *et al.* 2006) and haemolymph protein levels (Cremonz *et al.* 1998). The addition of dietary pollen (Di Pasquale 2013, Rinderer *et al.* 1977, Schmidt *et al.* 1987, 1995, but see Frias *et al.* 2016), and protein (Archer *et al.* 2014, Altaye 2010, Schmidt *et al.* 1987) is known to increase longevity of healthy and diseased honey bees. However, the addition of protein or EAAs at high concentrations can be detrimental for survival at high concentrations (Paoli *et al.* 2014a,b, Pirk *et al.* 2010).

Mixed pollen in the diet is an important factor determining honey bee longevity. Schmidt *et al.* (1987) found that mixed-pollen diets generally increased individual survival over monospecific pollen diets. Likewise, Di Pasquale (2013) demonstrated that bees infected with the pathogen *Nosema* survived longer when fed a diet of diverse pollen rather than monospecific pollen, except when the monospecific pollen had high protein content. Thus, the nutritional value of pollen may better understood by considering its amino acid content (Alaux *et al.* 2010, Crailsheim 1990, Cook *et al.* 2003).

De Groot (1953) discussed the protein requirements for honey bees for 'normal' growth and longevity and laid the foundations for the understanding of the effects of the dietary amino acids on growth. Ten EAAs were identified for adult honey bees: arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine are essential for growth. Eight apparently non-essential amino acids were identified as tyrosine, cystine, serine, glutamic acid, glycine, alanine, proline and hydroxyproline. If a protein lacking in any one EEA is used as the sole protein diet, then honey bees displayed reduced growth.

### *1.3.3. Nutrient intake targets*

Honey bees are able to regulate their intake of protein, carbohydrates and EAAs to reach a specific ratio or 'nutrient intake target', dependent on age (Archer *et al.* 2014, Paoli *et al.* 2014a). Thus, adult workers are able to regulate the nutrients they eat by varying their diet. The consumption of pollen is dependent on their age and function, with nurse bees consuming the most pollen (Hrassnigg & Crailsheim 1998, Camazine *et al.* 1998, Crailsheim *et al.* 1992) as after emergence, pollen is required for glandular tissue differentiation

(Crailsheim 1990, Pernal & Currie 2000). This time represents the onset of nursing duties when high protein larval food is produced by the hypopharyngeal, mandibular and postcephalic glands. Insofar as pollen intake shapes an individual's phenotype, realised diet composition offers the potential for individuals to express plasticity by behavioural modulation of their feeding preferences, which will be explored later in this thesis.

### *1.3.4. Protein nutrition of larvae*

Larvae receive protein during regular inspections and feeding by the nurse bees. Although some protein is acquired from pollen fed directly to larvae, the majority of dietary protein needed for larval development is acquired from the brood food (Brodschneider & Crailsheim 2010). Upon feeding, a nurse bee will produce a drop of brood food and deposit the food around the larva (Haydak 1970). In this way, the regulation of larval feeding is achieved by the adults (Roulston & Cane 2002). The composition and volume of brood food supplied is dependent on the age and caste of the developing larva (Haydak 1970, Schmikl & Crailsheim 2002) with increasing frequency and feeding duration as the larvae age (Schmikl & Crailsheim 2002). Adults will cease rearing brood (Brodschneider & Crailsheim 2010) or will utilise their own body materials in order to rear brood, resulting in a decreased nitrogen and protein content in the adult's body tissues (Haydak 1970). Furthermore, if the amount of brood is successively reduced, pollen consumption by the nurses is reduced (Hrassnigg & Crailsheim 1998) and the frequency of larval feeding by nurses is correlated with the ratio of available pollen to larvae (Scmickl & Crailsheim 2002).

*1.4. Colonies as targets for disease*

The characteristics of social insect colonies, such as high population densities of genetically similar individuals, coupled with the possibilities of transmission between colonies, present favourable conditions for the proliferation of pathogens. Indeed, honey bees suffer attacks from a well-documented range of bacteria, pests, fungi and virus that spread though contact-based transmission (Budge *et al.* 2015, Cornman *et al.* 2012, Evans *et al.* 2006, 2009, Schmid-Hempel 1995, Wilkins *et al.* 2007). In response, honey bees have evolved an array of immune responses to combat infection (reviewed in Cremer & Sixt 2009 and Wilson-Rich *et al.* 2009). Below I introduce the various components of the insect immune system in the context of honey bees and present individual-based and social defence against pathogens (Alaux *et al.* 2010, 2014, Arathi *et al.* 2006, Dussaubat *et al.* 2013, Goblirsch 2013, Kralj & Fuchs 2006, Laughton *et al.* 2011, Richard *et al.* 2012, Schmid-Hempel 2005, Silici & Kutluca 2005, Starks *et al.* 2000, for reviews see Cremer *et al.* 2007, Wilson-Rich *et al.* 2009 and Evans & Spivak 2010).

At the individual level, should a pathogen overcome the bee's morphological barriers, the pathogen will be challenged by a suite of humoral processes. The humoral immune response within honey bees is governed by four signalling pathways: Toll, Imd, Janus kinase (JAK)/STAT and JNK. Briefly, these pathways are activated when recognition proteins contact non-self, microbial cell wall complexes. The resulting cascade controls upregulation of immune genes that encode effector molecules such as antimicrobial peptides (AMP's) and Phenoloxidase (PO) (Evans *et al.* 2006, Randolt *et al.* 2008).

### *1.4.1. Phenoloxidase pathway*

The phenoloxidase (PO) pathway attacks potential pathogens that have entered the adult honey bee's haemocoel, perhaps after wounding. PO activity results in the deposition of melanin on foreign material from the circulating haemocytes. Upon recognition of invading microbes, haemocytes differentiate and produce melanin by the action of PO. The intermediates produced by PO are themselves toxic to both the host and pathogen and thus PO is present constituitively in the haemolymph as inactive prophenoloxidase (proPO), which is then proteolytically activated into free PO following immune-stimulation. PO oxidises derivatives of tyrosine to form toxic intermediate quinones, which then polymerise to form melanin. On infection, cells known as plasmatocytes aggregate around the foreign body and release the melanin resulting in encapsulation of the invading foreign body. In addition, PO has roles in cuticle melanisation and wound repair. Strong evidence for PO's association with disease resistance is lacking, but some evidence supports PO's role in resistance to viral, bacterial and plant spore pathogens in insects (for reviews see Cerenius & Soderhall 2004, Gillespie *et al.* 1997, González-Santoyo & Córdoba-Aguilar 2011, Soderhall & Cereius 1998 and Wilson-Rich *et al.* 2009).

In honey bees, PO appears to be activated upon infection with the gut parasite *Nosema* (Antúnez *et al.* 2009, Roberts & Hughes 2014). Some evidence exists for bacterial activation of PO as injection of a pseudo-bacterial challenge, such as the lipopolysaccharides from the bacterial cell walls, results in a reduction in PO levels, possibly due to failure to replenish PO stocks (Laughton *et al.* 2011). The activity of PO responses has been shown to be dependent on the honey bee's developmental stage, caste, body weight (Laughton *et al.* 2011, Wilson-Rich *et al.* 2010), age (Roberts & Hughes 2014), and diet (Alaux *et al.* 2010).

PO activity can be measured by spectrophotometry through its conversion of colourless L-dopa to red/brown dopachrome (Korner & Schmid-Hempel 2004, Laughton & Siva-Jothy. 2010, Moret & Schmid-Hempel 2001). Measurements of PO have inherent difficulties as proPO can be activated by wounding (thus during sample collection) leading to inaccuracies. Thus, detecting activation of PO when the measurements involve collected haemolymph is prone to artefact and interpretation should be made only by reference to differences between 'infection treatments' and appropriate 'Sham-infection' controls, rather than by reference to baseline levels of PO in untreated controls (Korner & Schmid-Hempel 2004).

### *1.4.2. Haemocytes*

In addition to being the main site of synthesis for PO, the haemocytes constitute the cellular arm of the honey bee immune response and are responsible for recognition of invading foreign bodies, neutralisation of pathogens and phagocytosis. If the foreign body is too large for phagocytosis, such as a large accumulations of bacteria, haemocytes aggregate and form 'nodules' around the invading body for subsequent encapsulation or excretion (for reviews see Gillespie *et al.* 1997, Wilson-Rich *et al.* 2009).

### *1.4.3. Antimicrobial peptides*

Antimicrobial peptides (AMP's) and lysozymes form part of the inducible immune system in honey bees. Upon recognition of microbial surface molecules, AMP's are synthesised and released into the haemolymph from the fat body cells. In addition, some AMP's are synthesised in the haemocytes, pericardial cells, Malpighian tubules, and midgut. Honey bees possess six AMP's: Hymenoptaecin, Apidaecin, Abaecin, Defensin-1 and -2 and Apisimin in

addition to three lysozymes (Evans *et al.* 2006) and these are effective against a range of gram positive and gram negative bacteria through various modes of action, including hydrolysis of cell wall structures, prevention of cell division, cell membrane disintegration, formation of membrane channels and increasing bacterial cell permeability (Bulet *et al.* 1999, reviewed in Gillespie *et al.* 1997 and Mylonakis *et al.* 2016). AMP's are thought to represent a secondary response that clears persistent microbes that survive the initial cellular and PO response (Dunn & Drake 1983, Gätschenberger *et al.* 2013, Haine *et al.* 2008), thereby protecting the host from persistent infections.

Phenotypic AMP activity can be artificially elicited by injecting honey bees with bacteria or by a pseudo-bacterial challenge (Azzami *et al.* 2012, Chan *et al.* 2009, Gätschenberger *et al.* 2013, Laughton *et al.* 2011, Mallon *et al.* 2003, Randolt *et al.* 2008). To assay AMP activity, haemolymph is collected and placed into an agar plate that is covered with a bacterial lawn. After incubation, the zone of inhibition of bacterial growth can be measured as a proxy for AMP activity (with a larger zone of inhibition corresponding to greater AMP activity).

### *1.4.4. Glucose oxidase*

An important social immune response in honey bees is the production of Glucose oxidase (GOX) in the hypopharyngeal glands and subsequent secretion into the brood food and honey. GOX catalyses the oxidation of glucose into gluconic acid and hydrogen peroxide ($H_2O_2$) (Alaux *et al.* 2010, Bucekova 2014, White 1963), the former having antimicrobial properties, thereby protecting kin. Therefore GOX activity can be measured as a parameter for social immunity (Alaux *et al.* 2010).

The activity of GOX can be measured using a spectrophotometer, using a commercially available assay kit (The Amplex© Red Glucose/Glucose Oxidase Assay Kit A22189). Briefly, the assay kit contains a reagent that catalyses the reaction of $H_2O_2$ and horseradish peroxidase to produce Resazurin, resulting in a colour change.

### *1.4.5. Behavioural strategies*

In addition to the individual defenses described above, honey bees engage in social behaviours to combat pathogen spread, such as grooming of nest mates infected with parasites (allo-grooming) (Richard *et al.* 2012); the physical removal of infected nest mates from the colony (Arathi *et al.* 2006); nest construction using antimicrobial propolis (Silici & Kutluca 2005) and behavioural fever, where adults sufficiently raise the temperature of the colony to combat brood infections (Starks *et al.* 2000). Furthermore, health comprised individuals can exhibit quarantine-like behaviours such as self-removal and reduced contact with the queen and brood (Alaux *et al.* 2012, Rueppell, *et al.* 2010). These self-removal behaviours appear to be associated with accelerated temporal polyethism (Lecocq *et al.* 2016, Natsopoulou *et al.* 2016, Wang & Moeller 1970), For example, infected and immune stimulated workers rapidly progress to tasks normally done outside the colony by older bees and exhibit the forager phenotype, including an increased expression of foraging genes and reduced hypopharyngeal glands (Alaux *et al.* 2012). In addition, infected workers perform more flights (Dussaubat *et al.* 2013, Goblirsch *et al.* 2013 but see Lach, *et al.* 2015) and exit the colony for longer foraging periods (Alaux *et al.* 2014, Kralj & Fuchs 2006).

### *1.4.6. Disease transmission theory*

According to classic disease transmission theory, two central factors that reduce the spread of disease through a population are the removal of infected individuals and decreased transmission efficiency (Lloyd-Smith *et al.* 2005, McCallum *et al.* 2001). This can be demonstrated in simple model describing the changes in the numbers of infected individuals over time in a population that has been invaded by an epidemic (Equation 1.1).

$$\frac{\Delta I}{\Delta t} = \beta SI - dI \qquad \textbf{(Equation 1.1)}$$

The rate of change in the number of infected individuals over time within a population, $\Delta I/\Delta t$, where $I$ = the number or density of infected hosts, $t$ = time, $S$= the number or density of susceptible hosts, $\beta$= the coefficient of disease transmission (or transmission efficiency) when an infected individual contacts a susceptible individual and $d$ = the *per capita* rate at which infected individuals are lost from the transmission process (through recovery, quarantine or death).

As $dI$ increases, through recovery, quarantine or death of infected individuals, and $\beta SI$ decreases, through less contact between infected and susceptible individuals, then the rate of infection decreases.

Although information on disease transmission in insect colonies is lacking, in the context of social insect colonies, strategies to combat epidemics may be better understood by comparing the role of individuals with the role of individual cells or organs of multicellular organisms (Cremer & Sixt 2009). Therefore, although individual honey bee workers may recover through immunocompetence, mortality in infected workers or behaviours analogous to quarantine, such as self-removal, may have beneficial fitness consequences for

the queen by reducing disease transmission within the colony. An important part of this thesis will therefore be to explore how honey bees modify the possible spread of in-colony infection by modifying $d$ and $I$.

*1.5. Nutritional effects on immunocompetence in insects*

Whilst providing protection, mounting the immune response is costly (reviewed in Schmid-Hempel 2005). Direct energetic expense of immune activation is demonstrable in insects through their $CO_2$ production (Ardia *et al.* 2012). In addition to the direct expense of immune activation, immune cells demand amino acids for the synthesis of proteins involved in immune pathways and thus nutrition is a limiting factor (reviewed in Li *et al.* 2007). The links between immunity and nutrition have long been recognised (Chandra 1996) and the impact of diet on insect immunity has been the focus of numerous studies (Alaux *et al.* 2010, 2011, Cotter *et al.* 2011, De Block & Stoks 2008, Feder *et al.* 1997, Fellous & Lazzaro 2010, Klemola *et al.* 2007, Koella & Sorensen 2002, Lee *et al.* 2006, 2008, Mckean & Nunney 2005, Ojala *et al.* 2005, Povey *et al.* 2009, 2014, Rantala *et al.* 2003, Rolff *et al.* 2004, Schmid-Hempel & Schmid-Hempel 1998, Siva-Jothy & Thompson 2002, Srygley *et al.* 2009, Szymaś & Jedruszuk 2003, reviewed in Ponton *et al.* 2013). Whilst these studies have demonstrated that diet can impact immune function, many have focused on generalist diet treatments or limited food access (Alaux *et al.* 2011, Szymaś & Jędruszuk 2003, De Block & Stoks 2008, Feder *et al.* 1997, Klemola *et al.* 2007, Koella & Sorensen 2002, Ojala *et al.* 2005, Rantala *et al.* 2003, Schmid-Hempel & Schmid-Hempel 1998, Siva-Jothy & Thompson 2002).

Results vary for different arms of the immune system, species and dietary regime. For example, Ojala *et al.* (2005) attributed differences in the

encapsulation ability of artificial implants (nylon filaments) in Arctiid moth larvae to differing antioxidant secondary metabolites across different plant diets. In the blood sucking insect *Rhodnius*, AMP activity and haemocyte activity, but not PO, were found to increase when fed on human blood meals compared to blood plasma (Feder *et al.* 1997). Schmid-Hempel & Schmid-Hempel (1998) found no difference in the encapsulation ability of nylon filaments in *Bombus* between environments where access to pollen and nectar were altered temporally to produce poor or variable environments. The authors offer an explanation that immunocompetence is more dependent on the needs of physiological tasks, e.g. foraging, in the short term rather than the outward availability of resources. Rantala *et al.* (2003) and Siva-Jothy & Thompson (2002) demonstrated that PO activity was increased, but not the encapsulation ability, in *Tenebrio* beetles given access to apple feed and rat chow respectively, when compared to starved controls. Furthermore, PO activity returned to normal levels when starved individuals were allowed to subsequently feed (Siva-Jothy & Thompson 2002). Likewise, Rolff *et al.* (2004) and De Block & Stoks (2008) found increases in PO activity in *Lestes viridis* when fed compared to semi-starved controls. Koella *et al.* (2002) demonstrated an increased melanisation response to latex beads in the mosquito *Anopheles* fed diets of increased sugar concentration. However, this increase was observed only in individuals that had previously fed on a blood meal. In contrast to the previous studies, Klemola *et al.* (2007) found that the encapsulation ability on nylon filaments was reduced in the autumnal moth *Epirrita* when fed high quality plant diets.

The impact of diet on immunocompetence may be better understood by the consideration of specific dietary components. For example, dietary protein has been demonstrated to promote antibacterial activity, PO, encapsulation and

haemocytes in the Egyptian cotton leafworm *Spodoptera littoralis* and Mormon crickets, *Anabrus simplex* (Cotter *et al.* 2011, Lee *et al.* 2006, 2008 Povey *et al.* 2009, 2014, Srygley *et al.* 2009).

Immunocompetence and the possession of other fitness-related traits have been linked in invertebrates (Armitage & Siva-Jothy 2005, Rantala *et al.* 2000). For example, larger ornamental wing spots are associated with the encapsulation ability of the damselfly (Ranala *et al.* 2000). However, immunocompetence itself is an important fitness trait that conflicts with other life history traits and thus may not be 'bound' indicatively to other fitness related traits (Siva-Jothy & Thompson 2002). Furthermore, trade-offs have been demonstrated between different immune responses and between immunity (Cotter *et al.* 2004, 2008, 2011, 2013, Moret & Schmid-Hempel 2001, Povey *et al.* 2009) and fitness related traits in bees such as longevity, foraging ability and learning (Konig & Schmid-Hempel 1995, Mallon *et al.* 2003, Moret & Schmid-Hempel 2000, Riddell & Mallon 2006). Thus, insects may be able to detach pathogen resistance from fitness related traits if they are able to ingest the appropriate nutrients (Ojala *et al.* 2005). This plasticity has been demonstrated in *Drosophila* (Mckean & Nunney 2005). When allowed to mate, excess food increased the ability of female flies to mount an antibacterial immune response when challenged with *E. coli* relative to males, suggesting an immunological cost of mating behaviour in males. However, when not mated, both male and female flies demonstrated similar levels of immunity. The authors argue that, if diet did not affect immunocompetence, then females would show a 'fixed' increased immunity relative to males across various conditions of food availability as a female biased immunological difference would arise due to promoting longevity in females and thus egg production, or an immunological

trade off in males of producing sexual traits. In addition, immunological dietary effects can be independent from growth and development (Fellous & Lazzaro 2010, Klemola *et al.* 2007, Mckean & Nunney 2005, Ojala *et al.* 2005, Schmid-Hempel & Schmid-Hempel 1998). Therefore, hosts may compensate for the costs of trading off other fitness components by increasing resource uptake (Povey *et al.* 2009).

Cotter *et al.* (2011) elegantly demonstrated the effects of both the quality and quantity of ingested nutrients on the trade-offs between different immune responses and other life history traits in *Spodoptera*, using a geometric approach for dietary regimes. Individuals were given diets that varied in both their ratio of protein to carbohydrate and the proportion of digestible nutrients. In this way, a large range of the 'nutritional landscape' was covered, allowing comparisons over many combinations of ingested protein and carbohydrates. The authors demonstrated that different arms of the immune response (Lysozyme and PO) peak at different combinations of protein and carbohydrates, so that no single diet can promote all immune responses.

### *1.5.1. Nutritional effects on immunocompetence in honey bees*

The addition of pollen to the diet of honey bees is known to increase resistance to pathogens (Di Pasquale 2013, Rinderer *et al.* 1974, 1977). However, only three studies to date have investigated the direct impacts of diet on immunocompetence (i.e. the ability to mount an immune response) in honey bees (Alaux *et al.* 2010, 2011, Szymaś & Jędruszuk 2003).

Alaux *et al.* (2010) investigated the role of pollen diversity and protein content on activity of PO, GOX and haemocytes in honey bees fed polyfloral and monofloral diets of differing protein content. In immunologically unchallenged

honey bees, the activity of PO increased when bees were fed pollen. However, this increase was only observed in bees fed a high protein polyfloral diet. PO activity was not significantly different between bees fed monofloral protein diets and pure sugar solution or between polyfloral protein diets and the monofloral protein diets, even those containing equal levels of protein.

Activity of GOX was increased in bees fed pollen, with higher GOX activity produced by bees fed the polyfloral diets, when compared with bees fed monofloral pollen diets of the same protein content and control bees fed a pure sugar solution. The effect of pollen feeding on GOX is perhaps not surprising, given that pollen is required for development of hypopharyngeal glands; the site of GOX synthesis. However, the effect of pollen diversity, rather than dietary pollen, indicates that additional factors within pollen, such as amino acid content, may limit immunocompetence. The clear increase in GOX activity resulting from pollen feeding may reflect an investment in social immune processes rather than individual responses. Indeed, honey bees possess a reduced complement of immune genes and AMP's when compared to the non-social species, *Drosophila* and *Anopheles* (Evans *et al.* 2006).

In addition, Alaux *et al.* (2010) found a counter-intuitive reduction in haemocytes in bees fed pollen when compared to pollen starved controls, indicating a possible trade-off between a more costly type of haemocyte and total supply of haemocytes. Alternatively, honey bees may compensate for a reduction in available nutrients by increasing haemocyte supply (Alaux *et al.* 2010). In an earlier study, an increase in the total number of circulating haemocytes, and changes in the proportions of different types of haemocytes

were observed in bees in response to pollen deprivation (Szymaś & Jedruszuk 2003).

The effects of diet on AMP activity in honey bees have been investigated only in terms of pollen availability and the level of gene expression of individual AMP's. Alaux *et al.* (2011) demonstrated that genes encoding Lyzozyme-2 *and* -3 and Defensin-1 and Toll and Imd pathway activators were upregulated when bees had access to dietary pollen. However, upregulation was negated when the bees suffered a challenge with the parasitic mite *Varroa* and a series of vectored viruses. Parasitism had a general down-regulatory effect on genes involved in protein metabolism. The fat bodies represent the main site of synthesis of AMP's and fat bodies have been shown to decrease with age in bees (Alaux *et al.* 2010, Doums *et al.* 2002, Wilson-Rich *et al.* 2010) and increase with availability of pollen in the diet (Alaux *et al.* 2010). However, no data exist showing how fat body size correlates with immunocompetence in bees.

*1.6. Self-medication in insects*

The concept of dietary self-medication in animals was first postulated by Janzen (1978). However, more recently, evidence for dietary self-medication in insects has come to light (for reviews see, de Roode *et al.* 2013 and Abbott 2014). For a behaviour to be classified as self-medication, four criteria must be met; the substance must be deliberately contacted; the substance must have detrimental effects to the parasite; the detrimental effects on the parasite must increase the hosts fitness and the substance must normally have detrimental effects on the host (in the absence of the parasite) (Abbott 2014).

Self-medication can take the form of altering the consumption of a particular nutrient normally consumed (Abbot 2014). Therefore, insects may govern the trade-offs between immune responses at the point of ingestion, given that different immune responses optimise with different nutrient combinations. Indeed, when pathogen challenged individuals were offered an unrestricted dietary choice in the above mentioned trials, they adjusted their intake of specific nutrients so that the resulting diet would promote immunocompetence and longevity (Lee *et al.* 2006, Povey *et al.* 2009, 2014 but see Cotter *et al.* 2011). Similarly, another study demonstrated that individuals of the caterpillar *Grammia incorrupta* altered their diet when parasitised or injected with Sephadex beads. The encapsulation response was promoted by increased carbohydrate consumption and individuals reduced their protein intake when challenged (Mason *et al.* 2014).

Dietary self-medication in insects can also take the form of ingestion of non-nutritive or toxic plant compounds (Baracchi *et al.* 2015, Bos *et al.* 2015, Millan *et al.* 2012, Singer *et al.* 2004, 2009, Smilanich *et al.* 2011). Millan *et al.* (2012) found that *Drosophila* larvae prefer ethanol containing food after exposure to endoparasitic wasps. The resulting larval diet leads to toxic levels of ethanol in the haemolymph for the endoparasitoid wasp larvae developing within the fly larvae. Singer *et al.* (2004, 2009) and Smilanich *et al.* (2011) found that caterpillars (*Grammia incorrupta* & *Grammia geneura*) that survived attack by endoparasitoids increased consumption of food containing plant compounds toxic to the endoparasite. Furthermore, consumption increased in later stage infections, suggesting that caterpillars may rely on immune defences such as encapsulation in early infections and resort to self-medication once the immune system is overwhelmed (Smilanich *et al.* 2011). Two recent studies

have found similar behaviours in social hymenoptera. Bos *et al.* (2015) demonstrated that ants (*Formica fusca*) preferentially fed on diets containing $H_2O_2$ when infected with a fungus. $H_2O_2$ is toxic and detrimental to healthy individuals, however when infected, the resulting diet increased resistance and survival. Likewise, bumblebees (*Bombus terrestris*) demonstrate a slight preference for non-nutritive nicotine when infected with *Crithidia*, however nicotine diets failed to fully clear infections and did not incur a survival benefit (Baracchi *et al.* 2015).

Curiously, dietary self-medication in insects appears to extend to kin. Kacsoh *et al.* (2013) found that *Drosophila* exposed to an endoparasitoid wasp results in adults preferentially laying eggs on dietary mediums that contain ethanol, thereby providing offspring with a diet toxic to the endoparasites. Another study demonstrated that Monarch butterflies (*Danaus plexippus*) display similar behaviour. Adults infected with a protozoan parasite pass on infective spores to developing larvae during oviposition on plants. Adults cannot clear infections from themselves but prefer to lay eggs on plant species that provide larvae with dietary compounds toxic to the protozoan parasite developing within the larvae (Lefèvre *et al.* 2010, 2012).

### *1.7. Conclusions and knowledge gaps*

Honey bees are reliant on protein nutrition for physiological development and evidence now exists demonstrating that pollen nutrition promotes certain components of honey bee immunocompetence. However, of the few studies to investigate the role of diet in honey bee immunocompetence, none have assessed the impact of individual nutrients such as amino acids. The ability of an insect to mount an immune response may depend more upon the balance of

specific dietary components, rather than an increased general nutrient intake. In addition, only one study has addressed this question using an immune challenge (exposure to *Varroa* parasitism), potentially masking specifically elicited immune responses such as AMP's or giving rise to uncontrolled variation in vectored virus loads and doses between individuals.

Self-medication through selection of diets based on medicinal properties is known to occur in insects and selection of nutrients that promote immunocompetence has been demonstrated in insects. Whilst these studies demonstrate that insects can self-medicate, none have investigated how eusocial insects alter their feeding behaviour for different nutritive components when immunologically challenged.

Honey bees must be considered a superorganism, whereby the efforts of each sterile worker benefit the colony and thus propagate her genes through the success of the queen. However, only one study has considered the impact of diet on a single social physiological immune response (GOX), and no studies have considered how honey bees self-medicate via feeding behaviour and the consequences for the social behavioural processes possessed by honey bees to combat epidemics, such as self-removal.

Honey bees feed on an almost exclusively floral diet with pollen and nectar providing all of their naturally available nutrients. In particular, pollen is the main source of dietary protein and essential amino acids available to the honey bee colony. Thus, environmentally realistic dietary treatments can be prepared. Furthermore, honey bees possess individual and social immune responses displayed both physiologically and through behavioural processes. Therefore

honey bees are an ideal model system to study the impact of diet on multiple strategies of immunocompetence within a superorganism.

My preceding review indicates that further investigations are needed to elucidate; (i) whether pollen feeding and specific nutrients impact honey bee immunocompetence in the context of a controlled immunological challenge, (ii) whether honey bees self-medicate at the individual and social level via dietary selection and finally (iii) whether it is appropriate and fruitful to apply the adaptive paradigm of the superorganism to epidemic control in a eusocial insect society, namely that of the honey bee.

*1.8. Thesis aims*

This thesis has three main aims:

1) To establish a framework within which to investigate dietary modulation of immunocompetence in honey bees.

2) To investigate dietary modulation of physiological immunocompetence in honey bees.

3) To explore the ability of honey bees to self-medicate for reduced spread of infection at the both the individual and social level.

The first aim is addressed in chapter two, where I describe a physiological time-course for the induction and expression of the several immune pathways. I utilize the various immune assays described in the literature to investigate impacts of colony variation and immunological challenge on both the amplitude and speed of the immune response in laboratory trials. In order to gain a comprehensive understanding of immunocompetence and account for trade-offs between multiple immune pathways, physiological investigations are conducted on two immune responses (PO and AMP's). Throughout this thesis,

I use a non-pathogenic pseudo-bacterial challenge (injection of LPS) to elicit the immune system, thus allowing me to detangle the effects of immunological challenge from those of an active pathogen, which is an important factor previously absent from investigations into links between diet and immunocompetence in honey bees.

The second aim is addressed in chapters two and four. In chapter two, I investigate the impacts of access to dietary pollen on various immune pathways (GOX, PO and AMP's) over 24 hours and show that pollen starvation does not impact the physiological immunocompetence. In chapter four, I investigate the longer term impact of dietary EAAs on immunocompetence (activity of AMP's) and longevity by tracking the immune response of honeybees either provided with or starved of dietary EAAs and a diet devoid of EAAs promotes the AMP response but decreases longevity in immunologically active honey bees.

The third aim is explored in chapters three and four. To evaluate the potential alteration of feeding behaviour by honey bees following immunological challenge, I investigated how immune activation (AMP activity) alters honey bees feeding behaviour. By altering their diet, honey bees may alter their foraging behaviour, or specifically increase foraging intensity. Increased foraging outside the colony could be construed as a form of altruistic self-removal from the colony, thereby reducing the spread of a contagious disease. Thus, I test whether dietary alteration by immune activated honey bees could potentially provide a unifying explanatory mechanism for the self-removal phenomenon. In chapter three I use a laboratory 'feeding choice' trial to establish whether honey bees alter their consumption of pollen and carbohydrates when challenged with LPS and subsequently use Radio

Frequency Identity Tag (RFID) technology to track the life time foraging activity of healthy and immune activated honey bees following re-introduction into field colonies. Pollen is the primary source of EAAs for honey bees. Thus, in chapter four, I investigate how immunologically active honey bees regulate their intake of EAAs when immunologically challenged. I then use an enforced diet trial to track the AMP and longevity responses of immunologically active honey bees supplied with nutritionally complete or incomplete diets to elucidate the endpoints for immunocompetence and longevity after dietary alteration, in the context of combating epidemics according to classic disease transmission theory.

In the final chapter, I collate and evaluate the evidence that the expendability of individual bees proposed by the superorganism paradigm provides a valuable perspective for explaining the collection of behaviours and physiological responses that I have observed my experiments.

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# Chapter 2. Factors to consider when investigating dietary effects on immunocompetence in honey bees (*Apis mellifera*).


*2.1. Abstract*

1. Social insects are able to mount an array of individual and social immune responses to combat pathogens. Furthermore, immune responses are metabolically costly. Thus, dietary intake can impact immunocompetence. However, few studies have investigated simultaneous investment in multiple physiological immune strategies in social insects. Here, I investigate both the dynamics of individual immunity and the short-term role of dietary pollen in honey bee immunocompetence. When studying this relationship, it is necessary to take account of numerous confounding factors governing the various components of the immune responses of insects because responses are temporal, can be traded off against each other and vary between colonies.

2. My objectives were to measure the temporal activity of phenoloxidase and antimicrobial peptides over 48 hours. In addition, we measured the impact of dietary pollen on both social immunity (Glucose oxidase) and individual immunity (the Phenoloxidase cascade system and antimicrobial peptides) in cohorts of caged honey bees when healthy or subject to immune activation by injection of lipopolysaccharide (LPS) over 24 hours. I carefully studied temporal and provenance-based factors.

3. I found no evidence that short-term pollen feeding affects immunocompetence, even when bees are immune activated, but clearly show that the measured immune responses are temporally dynamic and vary across colonies as we found evidence for a physiological trade-off between the

phenoloxidase and the antimicrobial peptide immune responses within one colony.

4. My results highlight the importance of consideration of the colony variation and the temporal nature of immune responses for studies investigating immunity in honey bees.

*2.2. Introduction*

Social insect societies consist of high population densities of genetically similar individuals presenting favourable conditions for pathogen spread (Schmid-Hempel 1995). In response, social insects have evolved social immune defenses in addition to individual immunity (reviewed in Cremer *et al.* 2007, Wilson-Rich *et al.* 2009).

Honey bees engage in hygienic social behaviours such as allo-grooming (Richard *et al.* 2012); removal of infected nest mates from the colony (Arathi *et al.* 2006); nest construction using antimicrobial propolis (Silici & Kutluca 2005) and behavioral fever, whereby honey bees elevate within colony temperatures to facilitate disease resistance (Starks *et al.* 2000). In addition, antiseptic glandular secretions are distributed throughout the colony. Glucose oxidase (GOX) is produced in the hypopharyngeal glands and secreted into honey during process of converting nectar to honey, thereby providing protection to others in the colony. GOX catalyzes the oxidation of glucose into gluconic acid and antimicrobial hydrogen peroxide ($H_2O_2$) (Alaux *et al.* 2010, Bucekova *et al.* 2014, White 1963). At the individual level, the immune system of insects is well documented. Major cellular and humoral responses include innate enzyme cascade systems, phagocytosis and synthesis of antimicrobial peptides upon recognition of microbial infection (Cremer *et al.* 2007, Evans *et al.* 2006, Wilson-Rich *et al.* 2009). Melanisation is a process that results in the encapsulation or nodulation of foreign material that is too large for phagocytosis Melanin is produced when the inactive enzyme, pro-phenoloxidase (proPO), is activated into phenoloxidase (PO) in the haemocytes. PO oxidizes derivatives of tyrosine to form toxic intermediate quinones. These then polymerise to form melanin which is then deposited on the invading foreign body (Söderhäll &

Cereius 1998). In addition, an array of antimicrobial peptides (AMP's) can be synthesised upon recognition of microbial infection and subsequently released into the haemolymph (Bulet *et al.* 1999).

Mounting this immune response is metabolically costly (Ardia *et al.* 2012) and under resource allocation (Alaux *et al.* 2010, Cotter *et al.* 2011). Furthermore, the various arms of the immune response can be traded off against each other (Cotter *et al.* 2004, 2008, 2011, 2013, Moret & Schmid-Hempel 2001, Rao *et al.* 2010). The cost of mounting an immune response may be mediated by acquisition of nutrients. Immune cells demand amino acids for the synthesis of proteins involved in immune pathways and cell division and thus immune function has a dietary demand, given that protein or amino acid deficient diets are known to compromise immune function in animals and humans (Li *et al.* 2007). Generalist diet treatments such as access to a protein source or limited food supply are known to affect the immune system of insects (Alaux *et al.* 2010, 2011, De Block & Stoks 2008, Feder *et al.* 1997, Klemola *et al.* 2007, Koella & Sorensen 2002, Mckean & Nunney 2005, Ojala *et al.* 2005, Rantala *et al.* 2003, Rolff *et al.* 2004, Schmid-Hempel & Schmid-Hempel 1998, Siva-Jothy & Thompson 2002, Szymaś & Jedruszuk 2003). More specifically, variations in the diet composition such as protein and carbohydrate ratios have been shown to promote insect immunocompetence (Alaux *et al.* 2010, Cotter *et al.* 2011, Fellous & Lazzaro 2010, Lee *et al.* 2006, 2008, Povey *et al.* 2009). However, no studies have investigated how dietary variations impact immunocompetence in honey bees after a challenge with a non- pathogenic immune elicitor, thereby decoupling the demands of the immune system and the pathogen.

Whilst nectar collection and subsequent conversion into honey provides the main dietary carbohydrate source for honey bees, pollen satisfies the dietary requirements for minerals, lipids and vitamins (Herbert & Shimanuki 1978). In addition, pollen is the main source of dietary protein for honey bees (Hrassnigg & Crailsheim 1998, Pernal & Currie 2000) and pollen consumption has been demonstrated to affect honey bee immunocompetence by increasing both PO and GOX activity but decrease haemocyte numbers in unchallenged honey bees (Alaux *et al.* 2010, Szymaś & Jedruszuk 2003). Therefore, honey bees provide an excellent model to study the effects of diet on multiple strategies of immunity. However, various factors govern the immune responses in insects. For example, the immune responses of bees vary across colonies, age and are temporally dynamic (Azzami *et al.* 2012, Gätschenberger *et al.* 2013, Korner & Schmid-Hempel 2004, Laughton *et al.* 2011, Randolt *et al.* 2008, Roberts & Hughes 2014). I therefore need to account for such variation in order to achieve our broader research objective of diet-IC studies.

In order to determine the optimum time to asses a response, I investigated the temporal dynamics of two arms of the individual immune response in honey bees by measuring AMP activity, proPO and PO in a time trial over 48 hours. To begin to investigate the diet-IC question, I investigate how short-term pollen availability impacts the immune system in individuals challenged with an immune elicitor (Lipopolysaccharide or LPS). In order to gain a comprehensive understanding of the immune response, multiple parameters of immunity at both the individual (proPO, PO & AMP activity) and social level (GOX activity) were measured over two time points (seven and 24 hours) in cohorts of caged honey bees that were provided with both pollen and syrup or were pollen starved (syrup only). If all elements of the immune response covary in coordination,

using a single component as a proxy in detecting overall immunocompetence may be possible.

I hypothesise that nutrients gained from pollen feeding can mediate costs of the immune responses of honey bees and thus predict that pollen feeding will promote immunocompetence either by increasing the amplitude or the speed of the immune response.

## *2.3. Methods*

### *2.3.1. Temporal changes in immune expression*

In order to control for any age related and colony immune effects, newly emerged adult bees from a single colony were used. A single brood comb was collected in October 2013 and incubated in constant darkness at 34°C and 60% humidity to mimic colony conditions. Honey bees were allowed to emerge over 24 hours before being caged in groups of six in plastic containers (11.4 cm diameter x 7.7 cm). Caged bees were incubated as above and had access to syrup (50% w/v sucrose in distilled $H_2O$). The trial was run in two blocks (one for each collection day) consisting of 45 cages each.

In order to activate the AMP response, the bees' immune system was challenged according to Laughton *et al.* (2011) and Mallon *et al.* (2003) with an injection of LPS. LPS is a cell surface complex derived from *E. coli* that provides a standardized passive challenge that will elicit an immune response without interacting pathogenically with the host (Korner & Schmid-Hempel 2004).

In order to account for variation between grouped cohorts of bees, cages (n=90) were randomly assigned to one of three immune treatments and five time points; an LPS injected and Sham-injected group and an unchallenged control group to control for the effects of chilling the bees on ice. In the LPS and Sham-injected groups, 2µl of either insect Ringer's solution containing LPS (0.5mg/ml) (Sigma) or insect Ringer's solution was injected into the haemolymph of ice anesthetised bees 24 hours after emergence. Samples were harvested immediately after the immune treatment or 5, 7, 24 and 48 hours post immune treatment.

### *2.3.2. Dietary pollen trial*

Newly emerged adult bees were obtained in the same way as the time course trial from three colonies in May 2013. Each cage contained six bees from the same source colony. Forty eight hours after emergence, three bees from each cage received one of the three immune treatments from the time course trial. Bees were then colour marked as injected or Uninjected in all cages. Cages (n=72) were then assigned to a 'Pollen fed' group (*ad libitum* access to both pollen and syrup (50% w/v sucrose/distilled $H_2O$)) and a pollen starved group with *ad libitum* access to syrup only. Pollen was prepared as a 'dough' made by homogenizing corbicula loads of mixed pollen species (BodyMe® Organic Spanish Bee Pollen) with distilled water into a paste (2.68 g/ml pollen:$H_2O$). The feeders were weighed and replaced every 24 hours.

### *2.3.3. Sample Harvesting*

For measurements of PO activity, haemolymph collected via perfusion bleeds (Laughton & Siva-Jothy 2010). Individuals were perfusion bled by cutting the 4$^{th}$ abdominal tergite of ice anesthetised bees before being flushed through with 0.5ml of Sodium Cacodylate solution (NaCac) via injection. Samples were immediately frozen in liquid nitrogen to disrupt haemocytes before being frozen at -20C. For measurements of antimicrobial activity, neat haemolymph was collected from an abdominal segment wound. Heads were severed and frozen for measurements of GOX activity in the dietary pollen trial. The time trial data confirmed that the AMP immune response peaked at 24 hours, but was detectable at similar levels 48 hours post injection (Figure 1). Therefore for the trial investigating the effect of dietary pollen on immunocompetence, the immune response was measured at 7 and 24 hours post immune stimulation,

allowing us to detect potential effects on the amplitude or speed of the immune response.

### *2.3.4. Prophenoloxidase and free phenoloxidase*

Activity of proPO and PO was assayed photospectromically following Laughton & Siva-Jothy (2010). Samples were thawed on ice and centrifuged at 4°C for 10 minutes. 20µl of the supernatant was added to a chilled 96 well plate. Replicate aliquots of bee haemolymph were used on PO and proPO. For measurements of total PO, 5µl of α-Chymotrypsin solution (5mg/ml) (Sigma C4129) was added for activation of proPO to PO, whereas for measurements of free PO, 5µl of $dH_2O$ was added. Following incubation at room temperature (25°C ± 2°C) for 5 mins, a master mix was added containing, 20µl of filtered L-Dopa in Phosphate Buffered Saline (PBS) (11 mg/ml), and 135µl $dH_2O$ per well. Readings were then taken at 490 nm every 15 seconds for one hour in a microplate plate reader. Each plate contained two negative control wells where NaCac containing haemolymph was substituted for $dH_2O$. Enzymatic activity was calculated as the slope during the steepest linear phase of the reaction curve (the $^{V}$max value) for 15 minutes, using Softmax ©Pro 4.1 software. The average of the negative control values were subtracted from the average of the two replicate samples from individual bees. In addition, plate to plate variation was controlled for by adjusting values to via positive control (obtained from a 'bulk' sample of *ad libitum* perfusion bleeds) on each plate. Inactive proPO values were calculated by subtracting the free PO values from the total PO values.

### *2.3.5. Glucose oxidase (GOX)*

For GOX, heads were thawed, homogenised and centrifuged in 100 µl of PBS. 20ul of the supernatant was then assayed photospectromically using the

commercially available Amplex® Red Glucose/Glucose Oxidase Assay Kit. Absorbance readings were taken at 560nm every 45 seconds for 30 minutes. Enzymatic activity was then calculated as the maximum slope of the reaction over 15 minutes using Magellan™ software.

To control for a plate effect during GOX and PO readings, treatments were balanced across plates and included a positive control. Plate values were then converted using one positive control value as a numerator and one as a denominator (Armitage & Boomsma 2010).

*2.3.6. Antimicrobial peptide activity*

AMP activity was assessed with a clearance zone assay using neat haemolymph. Bees were bled by cutting the 4th abdominal tergite of ice anesthetised bees and collecting the resulting haemolymph. Agar plates were prepared with a bacterial lawn were prepared using 6 ml of 1% agar in PBS solution containing 0.2 mg/ml lyophilized *Micrococcus luteus* (Sigma M0508), thereby standardizing the bacterial component of the plates. 2ul of haemolymph was randomly assigned to 2mm wells cut into the agar and plates were incubated at 27°C for 24-48 hours until clear zones were visible. The diameters of the resulting zones of bacterial clearance were measured as a proxy for AMP activity.

*2.3.7. Statistical analyses*

The effect of the immune treatment, colony, time and access to pollen on proPO, activated PO and GOX were analysed with ANOVA's. Significance was assessed using backwards stepwise model selection, if the model indicated significant variation, pairwise treatment means were compared using Tukey tests using the package 'lsmeans' in R (Lenth 2014). For both trials, values of

free PO were log transformed and in the time course trial, levels proPO were square root transformed to meet the assumptions of normality. Variation in AMP activity among immune treatments, time point, colonies and whether bees had access to pollen was analyzed with Kruskal-Wallis tests due a highly skewed distribution of data and means separated with Bonferroni corrections. The time course trial was run in two blocks, each consisting of 45 cages. Data were combined for analysis when no interaction was found between the day of collection and either time or immune treatment. All statistical analyses were performed in R 3.0.2 (R Core Team 2014).

## *2.4. Results*

### *2.4.1. Time course trial*

#### *2.4.1.1. Antimicrobial peptide activity*

AMP activity varied across both time and the immune treatment (Kruskal Wallis tests, time: $\chi^2$= 22.4, *p<0.001*, immune treatment: $\chi^2$ = 23.6, *p<0.001*; Fig. 2.1). Overall, AMP activity was significantly higher in LPS injected (LPS) compared to the Sham-injected (SI) and Uninjected control bees (UC) (Immune treatment: LPS vs Sham, *p=0.008*, LPS vs Uninjected control, *p<0.001*, Sham vs Uninjected control, *p=0.03*). At 24 hours following the immune treatment, AMP activity was significantly higher than 0 and 5 hours and was detected at similar levels 48 hours post immune treatment (0 & 5 hours vs 24 hrs, *p<0.05*). AMP activity did not vary across blocks (Kruskal Wallis test: $\chi^2$= 0.004, *p=0.95*).

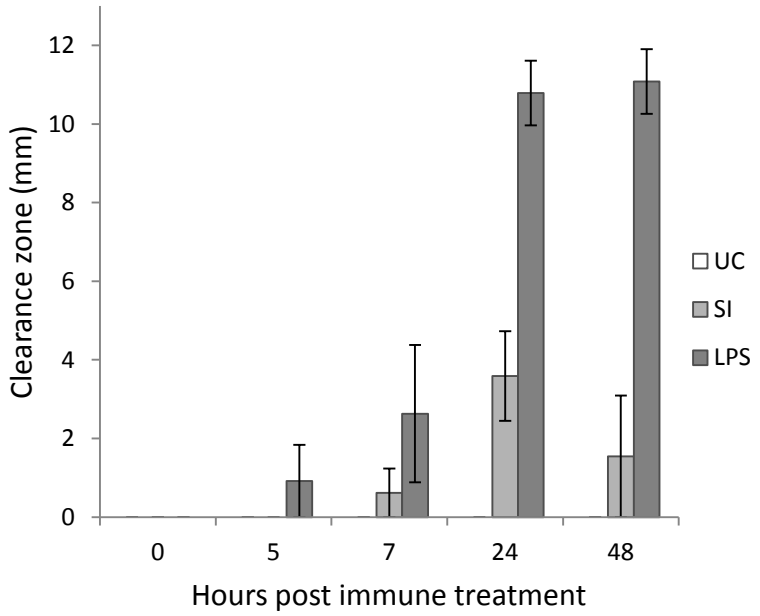


**Figure 2.1.** Changes in antimicrobial peptide (AMP) activity over 48 hours in honey bees exposed to various levels of immune-stimulation. Dark grey bars represent LPS injected bees (LPS), light grey bars represent Sham-injected bees (SI). No activity was seen in the Uninjected control bees. Values are

averages (mean) of two bees from each cage (n=90 cages from a single colony (six cages per time point/treatment)) and each cage contained six bees. Error bars denote SE.

*2.4.1.2. Prophenoloxidase and free phenoloxidase*

Activity of free PO varied across time (ANOVA, $F_{4,85}$= *6.09, p <0.001,* Fig. 2.2). The activity of free PO increased at 24 and 48 hours post immune treatment compared to all previous time points. Activity was higher at 48 hours compared to all previous time points except immediately after and 24 hours post immune treatment (Turkey's pairwise comparison: 48 hours vs 5 and 7 hours, *p<0.05*, all other pairwise comparisons, *p>0.05*). Activity of free PO did not vary across block (ANOVA, $F_{1,82}$= *0.92, p=0.33*), the immune treatments ANOVA, $F_{2,83}$= *1.38, p=0.26*) or any two way interactions (Likelihood ratio tests p>0.05).

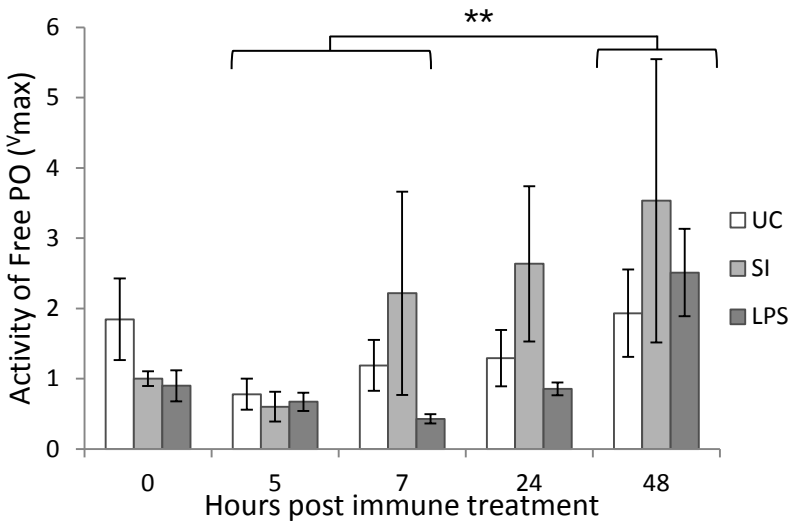


**Figure 2.2** Changes in PO activity activity over 48 hours in honey bees exposed to various levels of immune-stimulation. Dark grey bars represent LPS injected bees (LPS), light grey bars represent Sham-injected bees (SI), white bars represent Uninjected control bees (UC). Values are averages (mean) of two

bees from each cage (n=90 cages from a single colony (six cages per time point/treatment)) and each cage contained six bees. Error bars denote SE and stars show significant differences (*p<0.05*).

Activity of ProPO did not vary across time (ANOVA, $F_{4,84}$= *0.58, p=0.68*), the immune treatments (ANOVA, $F_{2,82}$= *0.17, p=0.85*) or any two way interactions (likelihood ratio tests, *p>0.05,* Fig. 2.3.). ProPO varied across the experimental blocks (ANOVA, $F_{1,88}$= *6.06, p=0.02*). However, the blocks were combined for analysis due to the non-significant interaction between time and the immune treatments with block.

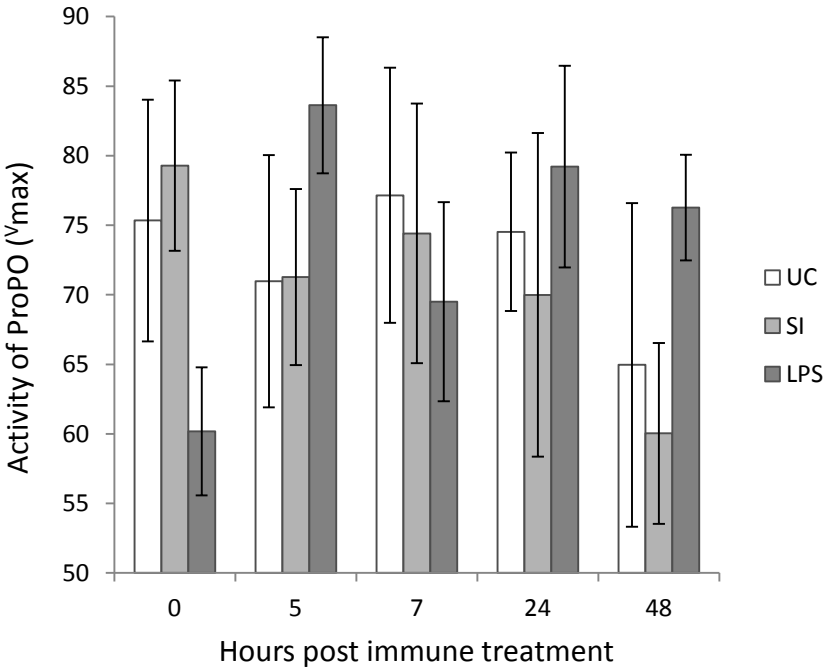


**Figure 2.3.** Changes in proPO activity activity over 48 hours in honey bees exposed to various levels of immune-stimulation. Dark grey bars represent LPS injected bees (LPS), light grey bars represent Sham-injected bees (SI), white bars represent Uninjected control bees (UC). Values are averages (mean) of two bees from each cage (n=90 cages from a single colony (six cages per time

point/treatment)) and each cage contained six bees. Error bars denote SE and stars show significant differences (*p<0.05*).

### *2.4.2. Availability of dietary pollen*

#### *2.4.2.1. Prophenoloxidase and free phenoloxidase*

Levels of proPO in individual honey bees responded to immune-stimulation but the magnitude of this response varied among source colonies (ANOVA: Colony, $F_{2,63}$*=15.32, p<0.001,* Immune treatment, $F_{2,63}$*=5.3, p=0.008,* Colony x Immune treatment, $F_{4,63}$*=3.2, p= 0.02,* Fig. 2.4). Post hoc test testing with Tukey corrections revealed that the injection treatment (LPS) reduced proPO, compared to the Uninjected control group (UC) in colony C and in the Sham-injected group (SI) compared to the Uninjected control group in colony B (Turkey's pairwise comparison: (i) Colony C, LPS vs UC, *p=0.006,* LPS vs SI, *p=0.56*, SI vs UC, *p=0.07*, (ii) Colony B, LPS vs UC, *p=0.34*, LPS vs SI, *p=0.12*, SI vs UC, *p=0.003*). The activity of proPO did not vary among diets (ANOVA*:* $F_{1,61}$*=0.55, p=0.46*), the time points (ANOVA*:* $F_{1,62}$*=2.67, p=0.10*) or any other two-way interactions (Likelihood ratio tests tests p>0.05).

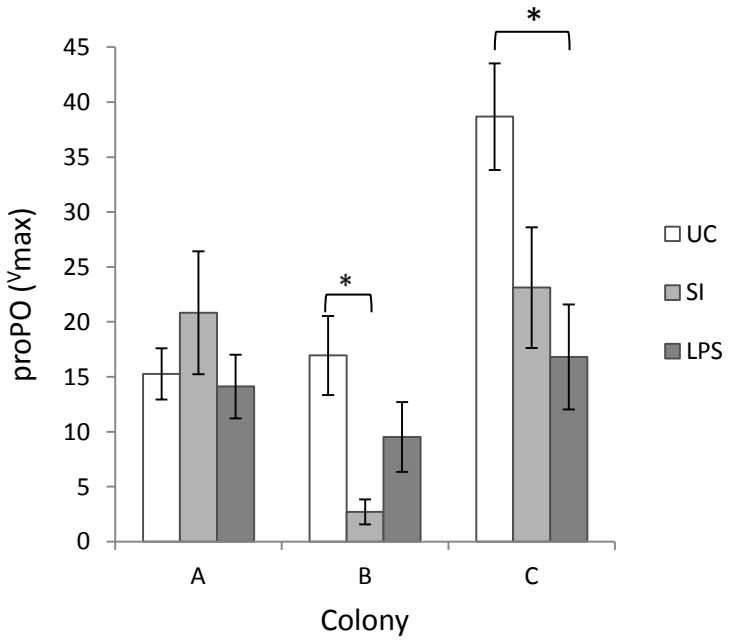


**Figure 2.4.** The activity of proPO in three colonies exposed to various levels of immune-stimulation. Dark grey bars represent LPS injected bees (LPS), light grey bars represent Sham-injected bees (SI), white bars represent Uninjected control bees (UC). Values are averages (mean) from one bee per cage (eight cages per colony/immune treatment). Errors bars denote SE and stars show significant differences.

The activity of freePO did not vary between diets, (ANOVA*: $F_{1,65}$=0.60, p=0.44*), time, (ANOVA*: $F_{1,68}$=1.13, p=0.30*), the immune treatment (ANOVA*: $F_{1,66}$=0.15, p=0.86*), colony (ANOVA*: $F_{1,69}$=1.09, p=0.34*) or any two-way interactions (Likelihood ratio tests p>0.05, Fig. 2.5).

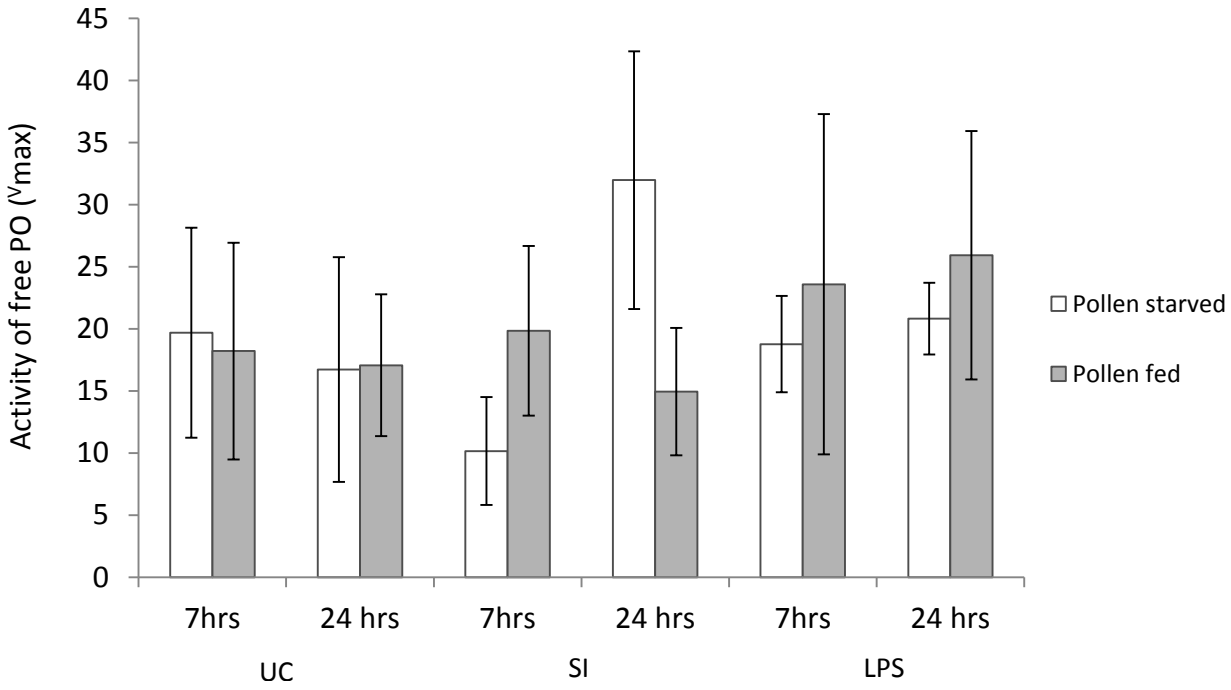


**Figure 2.5**. The activity of free PO in pollen fed (pollen + syrup) and pollen starved (syrup only) honey bees 7 hours (white bars) & 24 hours (grey bars) post exposure to various levels of immune-stimulation: LPS injected (LPS), Sham-injected (SI) and Uninjected control bees (UC). Values are averages from one bee per cage (6 cages per time point/immune treatment/diet). Error bars denote SE.

*2.4.2.2. Glucose Oxidase (GOX) activity*

Activity of GOX did not differ the immune treatments (ANOVA*:* $F_{1,61}=0.09$, $p=0.91$), time (ANOVA*:* $F_{1,63}=0.35$, $p=0.56$), the colonies (ANOVA*:* $F_{1,64}=0.76$, $p=0.47$), the diets (ANOVA*:* $F_{1,66}=3.1$, $p=0.08$), , or any two-way interactions (Likelihood ratio tests p>0.05, Fig. 2.6).

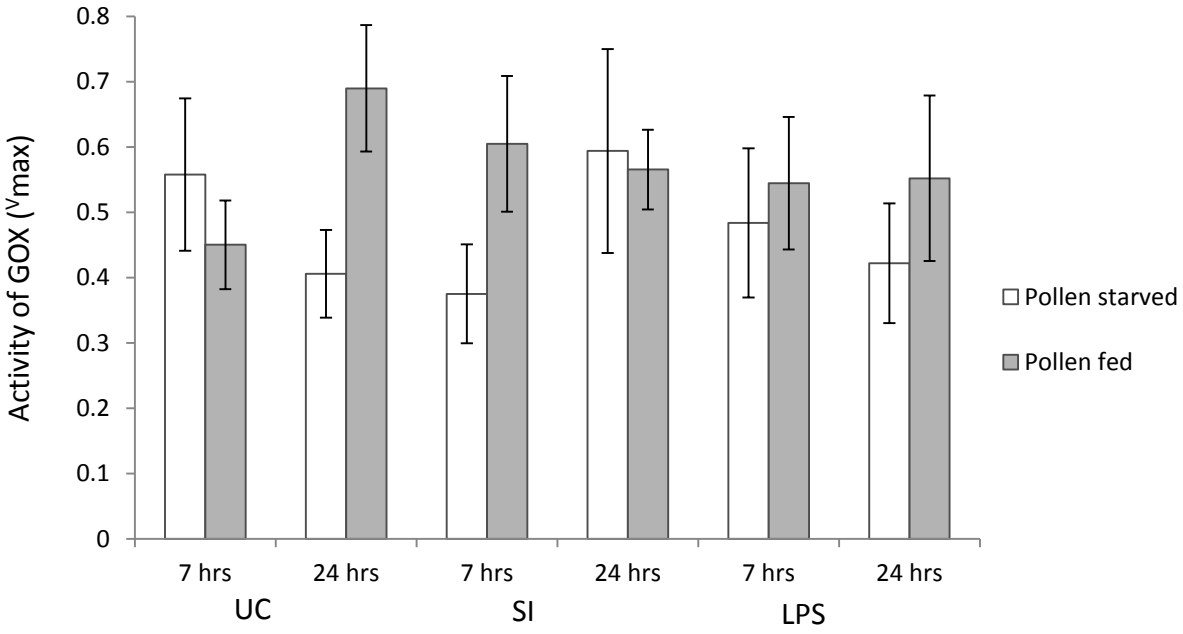


**Figure 2.6**. The activity of GOX in pollen fed (pollen + syrup) and pollen starved (syrup only) honey bees 7 hours (white bars) & 24 hours (grey bars) post exposure to various levels of immune-stimulation: LPS injected (LPS), Sham-injected (SI) and Uninjected control bees (UC). Values are averages from one bee per cage (4-6 cages per time point/immune treatment/diet). Error bars denote SE.

*2.4.2.3. Antimicrobial peptide activity*

AMP activity varied between both time and the immune treatments. There was no effect of diet or colony on the activity of AMP's (Kruskal Wallis tests: Time: $\chi^2 = 16.57$, $df = 1$, $p<0.001$, Immune treatment, $\chi^2 = 19.08$, $df = 2$, $p<0.001$, Pollen, $\chi^2 = 0.13$, $df = 1$, $p=0.71$, Colony, $\chi^2 = 0.59$, $df = 2$, $p=0.74$). AMP activity was greatest 24 hours post injection and in both the Sham and LPS injected groups (Control vs Sham, $p=0.006$, Control vs LPS injection, $p<0.001$, Sham vs LPS injection, $p=0.25$, Fig. 2.7). When the 24 hour group was analysed separately, AMP activity differed amongst all three immune treatments (Kruskal Wallis test:

$\chi^2 = 22.70$, *df = 2, p<0.001*, Control vs Sham, *p=0.002,* Control vs LPS injection, *p<0.001,* Sham vs LPS injection, *p=0.03*).

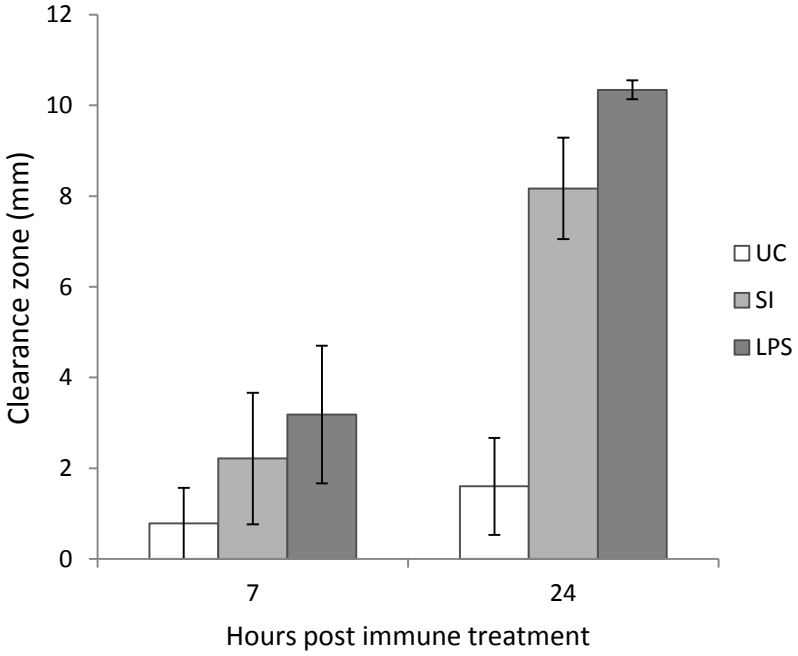


**Figure 2.7**. The clearance activity of honey bee haemolymph (AMP activity) 7 & 24 hours post exposure to various levels of immune-stimulation. Dark grey bars represent LPS injected bees (LPS), light grey bars represent Sham-injected bees (SI), white bars represent Uninjected control bees (UC). Values are averages (mean) of one bee per cage (10-12 cages per time point/immune treatment) are given in bars). Activity varied across the immune treatments and time (*p<0.05*). Error bars denote SE.

*2.4.2.4. Correlation between syrup consumption and activity of PO*

Curiously, the activity of free PO was positively correlated with the amount of sugar solution consumed, indicating a link between carbohydrate consumption and levels of activated PO (*ρ= 0.30, n=62,* p=0.02, Fig. 2.8). However, although

free PO did not vary detectably over the time points, sugar solution consumption was greatest at 24 hours, therefore confounding the effects of the time point and sugar solution consumption. Thus, interpretation must be done with caution and further work is needed to establish if carbohydrate consumption is linked with activation of PO, perhaps because of a metabolic cost of PO activation.

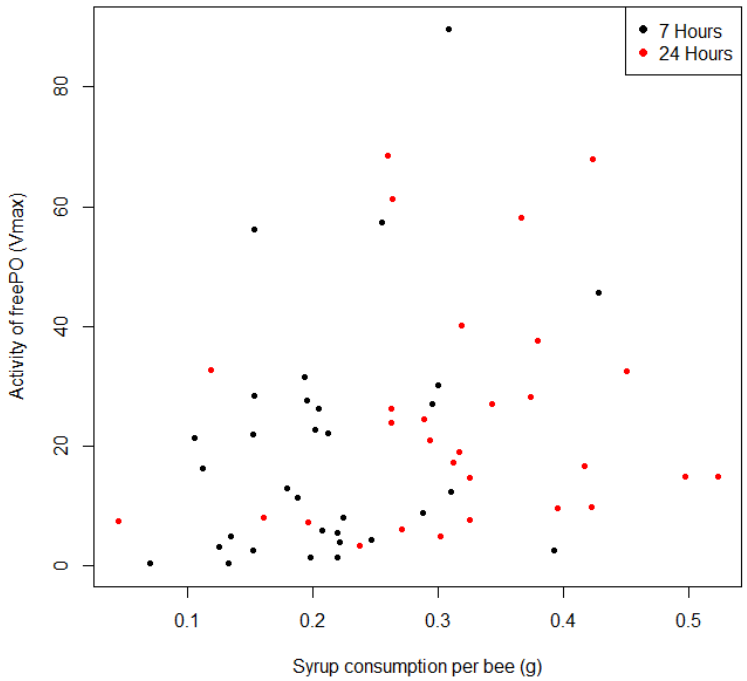


**Figure 2.8.** Activity of free PO and sugar solution consumption per bee. Colours show different time points: 7 hrs (n=32 bees) and 24 hours (n=29 bees) post exposure to various levels of immune-stimulation: (LPS injection, Sham-injection and Uninjected controls). Activity of free PO was positively correlated with the amount of consumed sugar solution (*ρ= 0.30, n=62,* p=0.02).

*2.4.2.5. Consumption*

Consumption of pollen did not vary between the immune treatments, time or their interaction. However, whilst not significant, there appeared to be a trend in feeding behaviour in the pollen feed group, LPS injected bees appeared to eat less pollen than controls (ANOVA: $F_{2,26}$= *2.31, p=0.11*, Fig. 2.9). Consumption of sugar solution varied across time in both the pollen fed and starved groups, with higher consumption at 24 hours (ANOVA: Pollen fed: $F_{1,23}$=*21.6, p<0.001,* Pollen starved: $F_{1,31}$=*6.8, p=0.01*), however sugar solution consumption did not vary between the immune treatments.

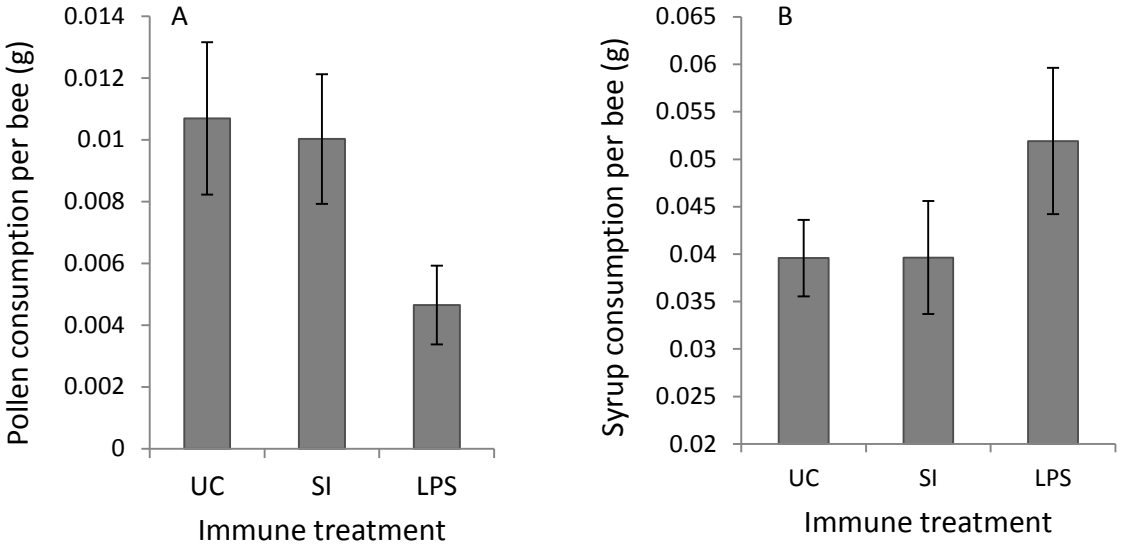


**Figure 2.9**. Consumption of pollen (A) and syrup (50%w/v), (B) (g bee$^{-1}$ 24 hours$^{-1}$) post exposure to various levels of immune-stimulation in the pollen fed group. We observed a non-significant trend suggesting LPS injected bees ate less pollen than the Sham-injected (SI) and Uninjected control group (UC). Error bars denote S.E..

*2.4.2.6. Mortality*

Overall, mortality was extremely low, with only three Sham-injected bees dying after 24 hours in the time course trial and all bees surviving in the pollen feeding trial.

*2.5. Discussion*

I demonstrate that the immune system of honey bees varies both temporally and between colonies. As expected, AMP activity was highest in LPS injected bees, although some AMP activity was detected in Sham-injected bees, possibly due to bacterial infection following wounding or as a pre-emptive defence against future infection of the wound (Korner & Schmid-Hempel 2004). My results are similar to those of Gätschenberger *et al.* (2013), who detected AMP activity in honey bees injected with *E coli* after six hours, and found that AMP activity peaked after 24 hours, and remained high for 72 hours. My data identifies optimal time points for measuring the AMP response in honey bees as seven hours and 24 hours following LPS injection, as AMP activity was first observed at seven hours, but peaked 24 hours and remained at similar levels 48 hours post immune treatment. My results differ from those of Siede *et al.* (2012), who found that injection of Ringers solution alone was sufficient to produce levels of AMP activity similar to that of LPS injected bees. A possible explanation is that samples were pooled in the study by Siede, *et al.* (2012), thereby masking individual variation.

I found no clear temporal pattern of PO activity or in response to the immune treatment. In contrast, Laughton *et al.* (2011) found that total PO activity (proPO + free PO) was reduced in LPS injected honey bees. I observed a reduction in proPO as a potential physiological trade of between the activity of PO and AMP's, however this reduction varied amongst colonies. The injection treatments induced AMP activity whilst levels of proPO were decreased. Trade-offs between the different arms of the insect immune system can occur (Cotter *et al.* 2004, 2008, 2011, 2013, Moret & Schmid-Hempel 2001, Povey *et al.* 2009, Rao *et al.* 2010) and evidence of trade-offs between PO and AMP

activity have been previously demonstrated in insects (Cotter *et al.* 2011, 2013, Moret & Schmid-Hempel 2001, Povey *et al.* 2009). I observed this pattern between AMP's and proPO in one colony only. However, my results may be explained by the importance of the source colony, given that proPO activity differed between colonies.

### *2.5.1. Pollen and Immunocompetence*

The absence of any effect of pollen feeding on our measured immune traits is surprising, given the protein rich nature of the immune system. Diet has previously been demonstrated to impact immunocompetence in non-social insects. An increase in PO activity and AMP activity with increasing dietary protein has been demonstrated in *Spodoptera*, caterpillars (Lee *et al.* 2006, 2008, Povey *et al.* 2009) and *Anabrus* crickets, even after 7 hours (Srygley 2009). Mckean & Nunney (2005) demonstrated increased lysozyme-like activity in *Drosophila* when individuals were given ad libitum access to a yeast meal (protein). Similarly, Feder *et al.* (1997) found increased lysozyme-like activity in *Rhodnius* when fed whole blood meals compared to blood plasma meals. In addition, the availability of dietary pollen and protein has been demonstrated to affect immunocompetence in bees (Alaux *et al.* 2010, 2011, Brunner *et al.* 2014). Our results differ from those of Alaux *et al.* (2010, 2011) and Brunner *et al.* (2014). Alaux *et al.* (2011) demonstrated that expression levels of genes encoding AMP's are increased in pollen fed bees. Alaux *et al.* (2010) found a slight increase in the activity of overall PO activity in honey bees fed a high protein pollen diet compared no pollen controls, but not when fed a low protein pollen diet. Likewise, Brunner *et al.* (2014) found that pollen starved bumblebees failed to upregulate AMP genes when infected with *Crithidia bombi.* However, in my study, it is possible that bees in my study had insufficient time

for physiological development for detection of a more sustained AMP or GOX response due to pollen feeding. Alternatively, AMP and GOX activity may only be promoted by pollen feeding when bees have more time to assimilate the acquired nutrients. In the study by Alaux *et al.* (2010), the authors also report an increase in the activity of GOX in bees fed pollen, regardless of protein content. However, bees were fed pollen for five and ten days and GOX activity increased with age, independent of diet. Likewise, the differences in AMP gene expression reported by Alaux *et al.* (2011) were in eight day old bees.

My findings may indicate a relative importance of the larval life stage on immunity in the resulting adult. For example, Fellous & Lazzaro (2010) demonstrated increased expression of two antimicrobial peptide genes (*Diptericin A* and *Metchnikowin)* in *Drosophila* adults, but not larvae, when the larvae were fed diets of increased protein ratios and found that this expression was independent of individuals general condition. Thus, the composition of the honey bee larval diet may influence immunocompetence in newly emerged adult bees relative to their adult diet. Alternatively, investigations into how diet affects immunity may be better understood by considering combinations between different nutritional components. Cotter el at (2011) used a geometric 'nutritional matrix' design to demonstrate that no single blend of protein:carbohydrate:calorie ratio could satisfy all immune responses simultaneously in *Spodoptera* caterpillars and that different immune responses 'peak' in different regions of a nutritional matrix. Therefore the dietary requirements can be non-complimentary between different arms of immunity. In our study, specific nutritional component ratios and combinations were not controlled. Thus, bees may have been forced to over or under consume

particular nutrients thereby masking any ‘peaks’ in the different immune responses.

Curiously, I observed a non-significant trend in feeding behaviour across the immune treatments (*p=0.11)*, as immune activated bees tended to consume less pollen. Previous work has demonstrated that infected individuals can adjust their nutrient intake ratio to increase survival and immunocompetence (Lee *et al.* 2006, 2009) and that healthy honey bees regulate their dietary intake to achieve a particular ratio of amino acids (Paoli *et al.* 2014). Thus, honey bees may alter their ratio of dietary nutrients when immune activated. In my study, only half of the bees in each cage received the immune treatment, potentially allowing untreated bees to consume more pollen and diluting any measureable differences between the feeders in LPS treated and control cages. Further research is needed to establish whether honey bees alter their diet in response to immune activation and what, if any benefits are incurred from such a dietary change. In addition, our results highlight the importance of consideration of; (i) the temporal nature of the immune response, (ii) measuring multiple arms of the immune response to account for potential trade-offs and (iii) using individuals from multiple colonies in studies with a focus on insect immunity.

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# Chapter 3. Immune-stimulation alters feeding behaviour and increases foraging intensity in honey bees

*3.1. Abstract*

In honey bees (*Apis mellifera*), adult workers normally forage outside the hive only after a domestic in-hive period as a nurse bee. Immune-stimulated individuals exhibit forager-like behaviour that reduces contact with brood and also may precipitate altruistic 'self-removal' from the colony, thereby reducing the spread of pathogens. In healthy individuals, a nutritional mechanism underlies the transition from nurse to forager phenotype, whereby bees reduce pollen intake and adopt a carbohydrate-biased diet. We therefore hypothesized that immune-stimulation would cause a carbohydrate biased dietary shift and would increase foraging intensity in honey bees. By using a common immune-elicitor in complementary laboratory and field experiments, we demonstrate that immune stimulation by injection of lipopolysaccharides (LPS) causes bees to adopt a carbohydrate-biased diet and that the same immune treatment increases foraging among individuals kept in colonies. We therefore propose that increased foraging intensity is mediated by the shift to a carbohydrate-biased diet. Our proposed mechanism provides a coherent and parsimonious explanation for a wide variety of previous observations and lends further support to the growing evidence that honey bees adopt self-removal as a strategy for social immunity.

*3.2. Introduction*

The evolution of sociality in insects has produced favorable conditions for the proliferation of pathogens. Social insect colonies typically contain populations of genetically similar individuals at high density that provide ideal conditions for epidemics and offer dispersal processes for colony-to-colony transmission (Schmid-Hempel 1995). In response, social insects have evolved individual and social immune responses, which collectively comprise cellular, humoral and behavioural defenses (Alaux *et al.* 2010, Evans *et al.* 2006, Richard *et al.* 2008, 2012, Schmid-Hempel 2005, Schmidt & Buchmann 1992, Soderhall & Cerinius 1998, reviewed in Cremer *et al.* 2007 and Wilson-Rich *et al.* 2009). Infected individuals can exhibit behavioural modifications that reduce contact with nest mates, including extreme expressions of social immunity such as altruistic self-removal from the colony (Alaux *et al.* 2012, Bos *et al.* 2012, Heinze & Walter 2010, Rueppell *et al.* 2010, Ugelvig & Cremer 2007).

In honey bees (*Apis mellifera*), altruistic self-removal by adults is associated with the premature development of a forager-like phenotype because experimentally infected bees exhibit accelerated temporal polyethism (Lecocq *et al.* 2016, Natsopoulou *et al.* 2016, Wang & Moeller 1970), perform more flights (Dussaubat *et al.* 2013, Goblirsch *et al.* 2013 but see Lach *et al.* 2015) and exit the colony for longer foraging periods (Alaux *et al.* 2014, Kralj & Fuchs 2006). Experimentally, immune stimulation with an artificial immune challenge causes increased expression of foraging-related genes, a decrease in size of the brood food producing hypopharyngeal glands and the development of certain forager-like behaviours, such as a decreased tendency to make contact with the queen (Alaux *et al.* 2012).

Pollen is the dietary source of protein, lipids, minerals and vitamins for honey bees (Campana & Moeller 1977). Several studies of honey bees have also demonstrated nutritional modulation during the transition to the forager phenotype (Crailsheim & Stolberg 1989, DeGrandi-Hoffman *et al.* 2010, Pernal & Currie 2000, Sagili *et al.* 2005, Toth *et al.* 2005) as, compared to nurse bees, foragers reduce pollen intake whilst increasing carbohydrate intake and precocious foraging appears in healthy, pollen-starved colonies (Ament *et al.* 2010, Crailsheim *et al.* 1992, Haydak 1970, Paoli *et al.* 2014).

However, the mechanism by which immune stimulation can induce the forager phenotype has previously remained unresolved. We therefore hypothesized that immune stimulation causes both adoption of the carbohydrate-biased forager like diet and increased foraging. We used complementary laboratory and field experiments to determine whether a common immune-elicitor (injection with bacterial lipopolysaccharides (LPS)) would induce both a change in diet and foraging intensity. While not constituting a critical test of whether a carbohydrate-biased diet increases foraging activity, we hope to establish whether the previously seen immunologically induced forager phenotype translates into increased foraging behaviour in field colonies and propose an underlying dietary mechanism.

*3.3. Methods*

*3.3.1. Dietary choice after immune-stimulation*

In order to obtain young adult honey bees, brood combs were collected from three colonies kept at the National Bee Unit (York, UK) in September 2013 and subsequently incubated in constant darkness at 34°C and 60% humidity to mimic colony conditions. Adult honey bees that emerged over 24 hours were then placed in plastic cages (11.4 cm diameter x 7.7 cm) so that each cage contained six bees from the same colony. The caged bees had access to a feeder containing sugar solution (50% w/v) prior to immuno-treatment. In total, 50 cages were randomly assigned to three treatment groups, with a balanced stratification across the original colonies. Cages were incubated as described above and randomly placed within the incubator to control for any effect of position. Forty eight hours after emergence, bees were exposed to immune-stimulation by an injection of lipopolysaccharide (LPS) solution (Sigma L2630) (Alaux *et al.* 2012, Laughton *et al.* 2011, Mallon *et al.* 2003, Siede *et al.* 2012*)*. LPS is a cell surface complex derived from *E. coli* that provides a standardized challenge that will elicit an immune response without interacting pathogenically with the test subject (Korner & Schmid-Hempel 2004). LPS (2 µl of 0.5 mg/ml LPS in Ringers solution) was injected between the $4^{th}$ and $5^{th}$ abdominal tergites of ice-anesthetized bees using a fine needle (n = 16 cages). Control groups were also established after chilling by injecting ringers solution without LPS (Sham injection; n = 17 cages) or by leaving the bees untreated (Uninjected control; n = 17 cages).

After treatment, all cages were simultaneously supplied with pre-weighed pollen and a feeder of syrup (sugar 50% w:v). Pollen was available to each cage of

bees as a paste made by homogenizing corbicula loads of mixed organic pollen (BodyMe® Bristol, UK) with distilled water (2.68 g pollen per ml). Dead bees were counted and removed at 24 and 48 hours post-injection. Pilot data had previously revealed that LPS injection induced peak AMP activity at 24 hours and that activity remained high for at least 48 hours post injection (See Fig. 2.1 in Chapter 2); therefore dietary consumption of pollen and sugar solution was calculated after 48 hours by subtracting the final weight of the feeders from the pre-weight.

### *3.3.2. Foraging behaviour after immune-stimulation*

In July 2015 combs were collected from five colonies in order to obtain young adult honey bees in the same way as in the previously described experiment. On the day of emergence, groups of 9-11 bees were caged and allocated into the same three treatments described above: LPS-injection; Sham-injection; and non-injected control. Immediately after treatment, all bees were tagged with a unique Radio Frequency Identifier (RFID) transponder (Mic3-Tags 1.0 x 1.6 x 0.5 mm (Microsensys Ltd, Erfurt, Germany)). Tags were fixed to each individual's dorsal thorax with shellac adhesive before the bees were re-caged, provided with pollen and sugar solution feed and incubated as described above. Twenty four hours after treatment, dead individuals were removed and the live bees were reintroduced into colonies (Fig. 3.1.). Each receiving colony housed bees from all three treatment groups (50 bees per treatment per colony), which enabled us to compare treatment effects between colonies.

The foraging activity of individual bees was monitored using paired RFID-reader units Microsensys Ltd, Erfurt, Germany) positioned at each hive entrance to provide a directional reading of each tagged bee passing through the readers.

The date and time of each exit and entry at each colony were continually logged and stored over a period of 44 consecutive days, thus allowing us to obtain the flight time, duration and the number of flights for each tagged bee (Appendix S3.6). The foraging intensity of bees in each treatment was described by the cumulative total of their exits. Individual bees were considered as potential foragers until their last detected exit, after which they were presumed dead (Decourtye *et al.* 2011).

**Figure 3.1.** A honey bee (*Apis mellifera*) tagged with a Radio Frequency Identity Tag (RFID).

### *3.3.3. Assaying the immune response*

To confirm that the LPS injection used on the bees in our study induced AMP's at the expected levels (Alaux *et al.* 2012, Laughton *et al.* 2011, Mallon *et al.* 2003, Siede *et al.* 2012), we used a 'zone of clearance' assay that involved placing extracted haemolymph into an agar plate covered with a bacterial lawn. After incubation, the zone of clearance due to lysed bacteria was measured as a proxy for AMP activity.

In the feeding experiment, haemolymph was bled from a random sample of nine surviving bees (three from each colony) from each treatment group 48 hours after injection and assayed for AMP activity. For the foraging experiment, we assayed AMP activity in bees from 15 replicate sacrificial cages that were set up on the same day as the experimental cages. Haemolymph was bled from a random sample of 15 bees (three from each colony) from each treatment group 24 hours after injection.

Ice-anesthetized bees were bled by cutting the 4$^{th}$ abdominal tergite and collecting the emerging haemolymph. Bacterial lawns were produced in petri dishes (90 mm) by pouring 6 ml of 1% agar in phosphate-buffered saline solution containing 0.2 mg/ml lyophilised *Micrococcus luteus* (Sigma M0508). We applied 1 µl of neat haemolymph from each bee to a randomly assigned well (2 mm depth & width) in the agar. After inoculation, the plates were incubated at 27°C and the diameters of the resulting clearance zones were subsequently measured once visible.

In the foraging experiment, similarly high levels of AMP activity were recorded in both the Sham-injected and LPS-injected bees. In addition, bees in the Sham-injected group suffered greater mortality than both LPS injected and Uninjected bees (Figs S3.7 & S3.8). Therefore, statistical comparisons of foraging behaviour were made only between the LPS-injected and Uninjected control groups.

*3.3.4. Statistical analyses*

All statistical analyses were performed in R 3.0.2 (R Core Team 2014) as follows. The effect of the immune stimulation treatment on consumption of pollen and syrup was analyzed using linear mixed effects (LME) models with

‘colony’ treated as a random effect within the ‘nlme’ package (Bates *et al.* 2014). If the model indicated significant variation, pairwise treatment means were compared using Tukey tests using the package ‘lsmeans’ in R (Lenth 2014). The effect of the immune treatment on the cumulative foraging rate was investigated by a ‘multiple time-to-event’ analysis. Each exit from the hive was classed as an ‘event’ and the individual bee’s identity was used as a ‘frailty’ term within the ‘coxme’ package (Therneau 2012), thereby allowing us to include all exit events throughout each bee’s lifetime in the measure of foraging intensity. Variation in the duration of foraging trips and the number of flights per bee among treatments was analysed with Kruskal-Wallis test due a highly skewed distribution of data. In both experiments, variation in AMP activity among treatments was analyzed with a Kruskal-Wallis test due a highly skewed distribution of data and means separated with Bonferroni corrections. Laboratory survival was analysed as a mixed COX hazard model using the ‘coxme’ package with cage treated as a random effect (Therneau 2012).

## *3.4. Results*

### *3.4.1. Dietary choice after immune-stimulation*

Immune-stimulation affected pollen consumption (LME: $F_{2,45}$=*17.86, p<0.001*, Fig. 3.2A), which was lowest in immune-stimulated bees (LPS vs. Sham, *p<0.001*; LPS vs. Uninjected, *p<0.001,* Sham vs. Uninjected, *p=0.05*). In contrast, immune-stimulation did not affect consumption of sugar solution regardless of whether analyzed by cage (LME: $F_{2,45}$ *= 0.45, p=0.63*) or *per capita* (LME: $F_{2,45}$ *= 0.957, p=0.39,* Fig. 3.2B). In effect, immune-stimulation caused a shift towards a carbohydrate-biased diet.

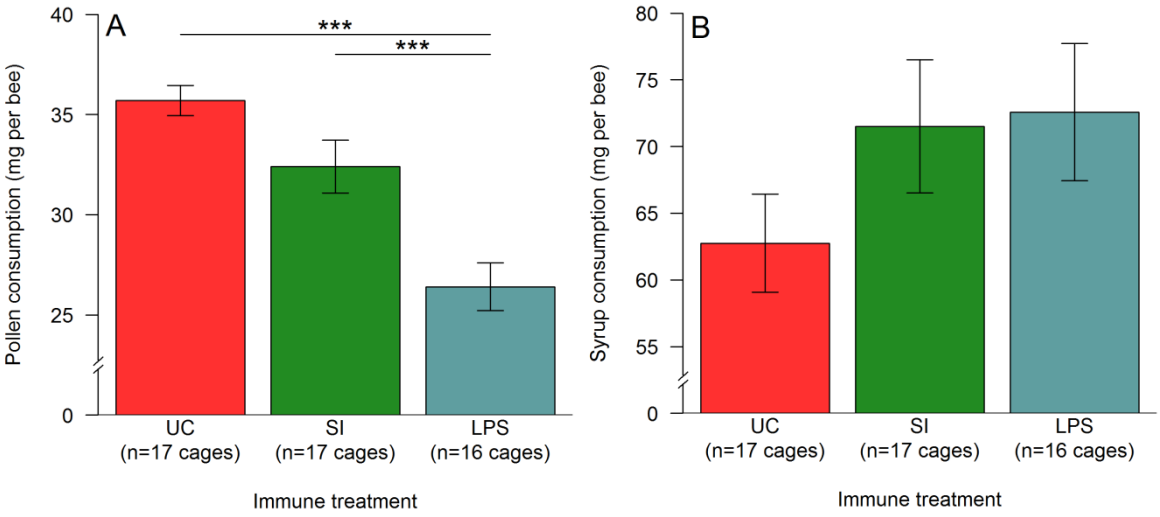


**Figure 3.2.** Consumption of pollen (A) and syrup (50% w/v sugar solution) (B) per surviving honey bee (g bee$^{-1}$ 48 hours$^{-1}$) (Uninjected control (UC); Sham-injection (SI); and lipopolysaccharide injection (LPS)). Error bars denote SE and stars show significant differences (*p<0.05).*

### *3.4.2. Foraging behaviour after immune-stimulation*

Immune-stimulated bees accumulated foraging bouts more quickly than non-injected controls (COXME: Immune treatment: $\chi^2$ *= 13.07, df = 1, p<0.001*), which indicates that immune-stimulation caused a small but detectable increase

in foraging intensity (Fig. 3.3), and the magnitude of this effect varied amongst colonies (Colony: $\chi^2 = 16.71$, $df = 4$, $p=0.001$; Colony x Immune treatment: $\chi^2 = 56.58$, $df = 4$, $p<0.001$).

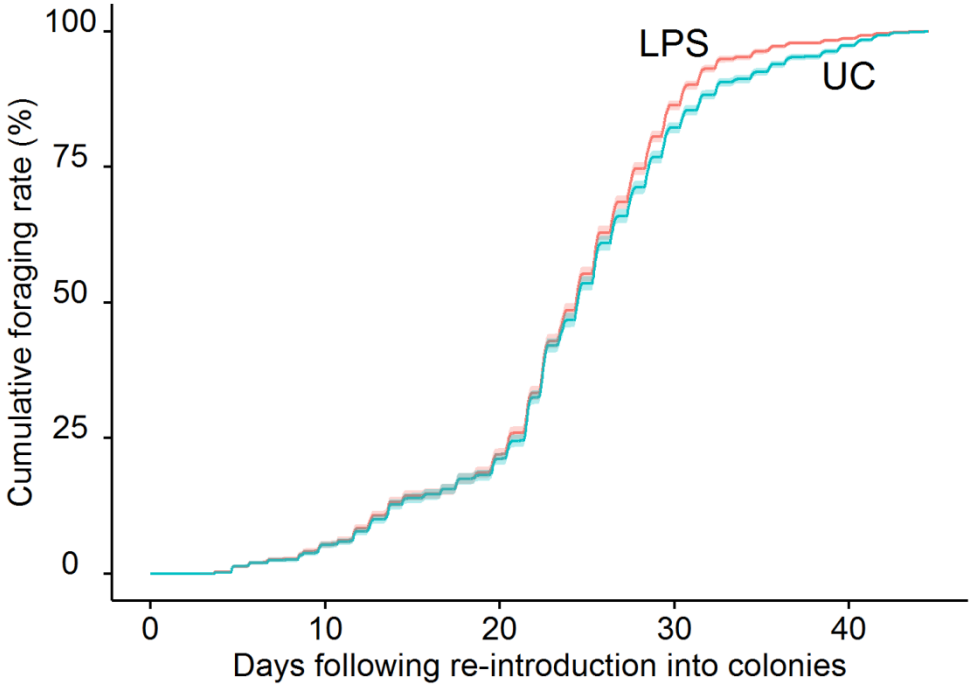


**Figure 3.3.** Cumulative foraging rate of LPS injected and Uninjected honey bees. 95% C.I. bands are shown for the number of exits only and do not account for the individual bees (LPS injected (LPS), n=5293 exits by 199 bees, Uninjected control (UC), n=5290 exits by 197 bees).

Immune treatment did not affect either the duration of foraging bouts (Kruskal Wallis test, $\chi^2 = 0.12$, $df = 1$, $p=0.72$, Fig. 3.4A) or the number of trips per bee (Kruskal Wallis test, $\chi^2 = 0.02$, $df = 1$, $p=0.88$, Fig. 3.4B). Both the duration and number of foraging bouts varied between colonies (Kruskal Wallis tests, duration, $\chi^2 = 15.91$, $df = 4$, $p=0.003$, number of trips, $\chi^2 = 109.39$, $df = 4$, $p<0.0001$ , Fig. 3.4C &D).

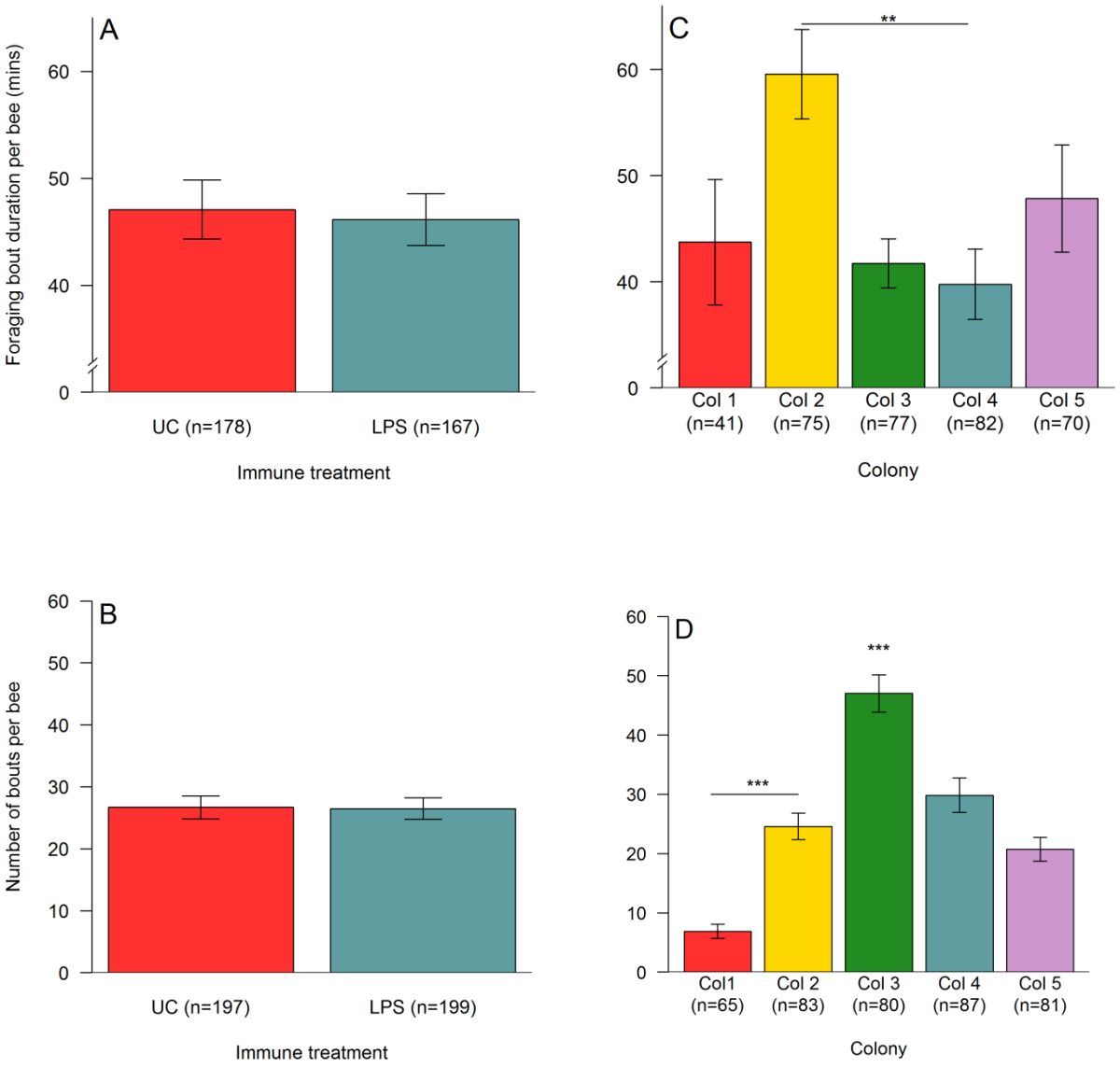


**Figure 3.4.** Average foraging duration (A) and number of foraging trips per bee (B) for immune stimulated lipopolysaccharide injected (LPS) and Uninjected control (UC) groups. Respective data are also presented by colony (C and D). The number of foraging trips and duration of foraging trips varied between colonies (Kruskal Wallis tests, $p<0.05$). The number of foraging bees are shown below each group. Error bars denote SE and stars show significant differences ($p<0.05$).

*3.5. Discussion*

Our study demonstrates for the first time that immune-stimulation causes honey bees to reduce their pollen consumption while maintaining carbohydrate consumption, meaning they in effect adopted a forager like, carbohydrate-biased diet. Specifically, LPS injected and Sham-injected bees reduced their pollen consumption by 31% and 22% compared to the Uninjected control bees, respectively. In addition, LPS injected bees accumulated foraging bouts more quickly than Uninjected bees in field colonies.

It is not clear why our Sham-injected honey bees activated AMP's to the same level as the LPS-injected group and experienced the highest mortality of all three groups; observations not seen in any other experiments of this thesis (Section: 2.4.1.1. & Fig. 2.1, Section: 2.4.2.3, & Fig 2.7, Section 2.4.2.6, Section 3.7.2 & Fig S3.7A, Section 3.7.3 & Fig S3.8A, Section 4.8.3 & Fig. S4.6). Indeed, previous work has demonstrated that sham-injection controls alone can illicit an AMP response in honey bees (Alaux *et al.* 2012, Richard *et al.* 2008). However, LPS consistently induces increased levels of AMP activity relative to sham-injection controls (Alaux *et al.* 2012, Richard *et al.* 2008). Therefore, we cannot rule out contamination in the Sham-injection immune treatment, possibly resulting in uncontrolled microbial infection and leading to AMP activation and increased mortality.

Previous studies have established a link between infection and foraging activity. For example, young honey bee workers experimentally infected with the microsporidian *Nosema apis* increased foraging activity compared to uninfected controls when reintroduced into field colonies (Goblirsch *et al.* 2013, Dussaubat *et al.* 2013, Alaux *et al.* 2014). In addition, honey bees challenged by

pathogens leave the colony for extended periods and exhibit accelerated age dependent polyethism (Lecocq *et al.* 2016, Kralj & Fuchs 2006, Natsopoulou *et al.* 2016, Wang & Moeller 1970, but see Lach *et al.* 2015). Individual honey bees possess a reduced complement of immune genes and thus may rely on social defense strategies to combat pathogen spread (Evans *et al.* 2006). Potentially therefore, increased foraging represents a mechanism for altruistic social isolation to reduce the risk of pathogen transmission at the colony level (Bos *et al.* 2012, Heinze & Walter 2010, Rueppell e*t al.* 2010, Ugelvig & Cremer 2007).

It is already known that the ontogeny of foraging is influenced by an individual's nutritional status. For example, honey bees display precocious foraging when they cannot synthesise dietary lipids (Toth *et al.* 2005) and hypopharyngeal gland development is reduced when bees are deprived of dietary protein (Crailsheim & Stolberg 1989, DeGrandi-Hoffman *et al.* 2010, Pernal & Currie 2000, Sagili *et al.* 2005) and this nutritional causality may be adaptive in that it strengthens recruitment of nurse bees to the foraging force in times of pollen shortage, which has a homeostatic effect.

In addition to these previous findings, our present study has now established that an identical immune-stimulation induces both the adoption of a forager-like diet and increased foraging intensity of honey bees. A potential parsimonious and coherent interpretation is that a dietary shift links foraging activity to initial infection (Fig. 3.5).

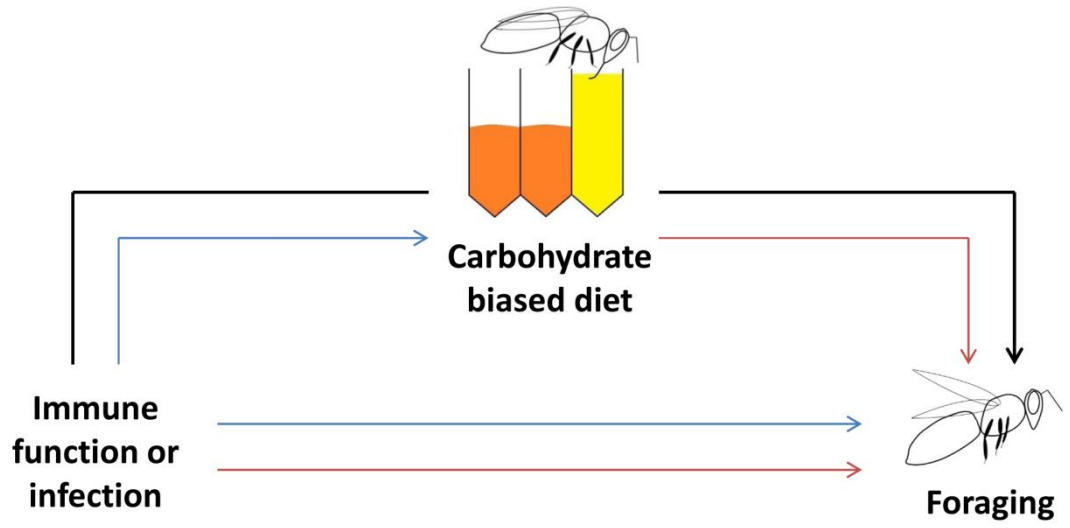


**Figure 3.5**. Incorporation of various new and previous observations into a conceptual model that postulates a role for dietary shift/nutritional targets as an adaptive response to infection by social immunity in honey bees. Blue arrows – our results; red arrows – previous studies; black arrow – proposed model of biological causality.

Our demonstration of an LPS induced dietary shift is consistent with the observation that insects in general are known to regulate their food intake in response to a pathogen challenge (Adamo 2005, Adamo *et al.* 2007, 2010, Baracchi *et al.* 2015, Bos *et al.* 2015, Lee *et al.* 2006, Goldsworthy 2010, Mason *et al.* 2014, Milan *et al.* 2012, Singer *et al.* 2004, 2009, Smilanich *et al.* 2011, Povey *et al.* 2009, 2014). Furthermore, the resulting diet can promote

immunity and survival (Adamo *et al.* 2007*,* 2010, Bos *et al.* 2015, Lee *et al.* 2006, Mason *et al.* 2014, Milan *et al.* 2012, Singer *et al.* 2004, 2009, Povey *et al*. 2009, 2014). Therefore, immunologically active honey bees may also reduce pollen intake in order to combat the pathogen or to compensate for its effects. A reduction in protein intake when immunologically challenged has been previously demonstrated in an insect. Mason *et al.* (2014) found that individuals of the caterpillar *Gramma incorrupta* reduced their intake of protein, whilst maintaining carbohydrate intake when challenged with both a parasitoid and an artificial parasitoid challenge (latex bead injection). Furthermore, carbohydrate intake was positively correlated with their melanisation response, a key component of insect immunity. Alternatively, increased foraging may be a response to an increased demand for nutrients at the colony level when under pathogen attack. However, altruistic self-removal, bolstered immunity and increased foraging intensity are by no means mutually exclusive, as all three may result from the same dietary change.

It may be posited that increased foraging intensity may be caused by an adaptive intervention by the pathogen rather than as a direct result of immune stimulation. Parasites are under selection for successful disease transmission and so may influence foraging ontogeny to improve dispersal. However, our use of an inactive pathogen to stimulate immune function demonstrates that increased foraging behaviour can be instigated in the absence of an active pathogenic influence, which implies that it originates in the bees themselves. Indeed, Alaux *et al.* (2012) also demonstrated that honey bees exhibited other behavioural responses that are arguably counter to the interests of the pathogen, namely decreased queen attendance when immune-stimulated by LPS injection, reduced hypopharyngeal gland size and an increase in

expression of foraging genes, which further supports the proposition that these phenomena are adaptations of the honey bees themselves.

Our results are consistent with the growing consensus that honey bees perform altruistic self-removal as a general strategy of defense against pathogen spread. Further research should investigate whether LPS induced dietary change and increased foraging behavior are directly linked and are consistent following experimental manipulation of other aspects of honey bee immunity such as the phenoloxidase pathway, and whether altruistic self-removal by infected individuals actually reduces pathogen transfer to nest mates.

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*3.7. Supporting Information*

### **3.7.1. Appendix S3.6.** RFID data preparation

For the analysis of the cumulative foraging rate, data was filtered to include all exit events only (10523 exit events by 396 bees). A small proportion of unusually long flights (1%), (6-96hrs) were observed where bees presumably entered un-monitored colonies and subsequently returned to the study hives. Therefore, for the average foraging duration per bee, data was filtered to include foraging times of up to six hours only (345 bees).

**3.7.2. Figure S3.7.** Antimicrobial peptide activity

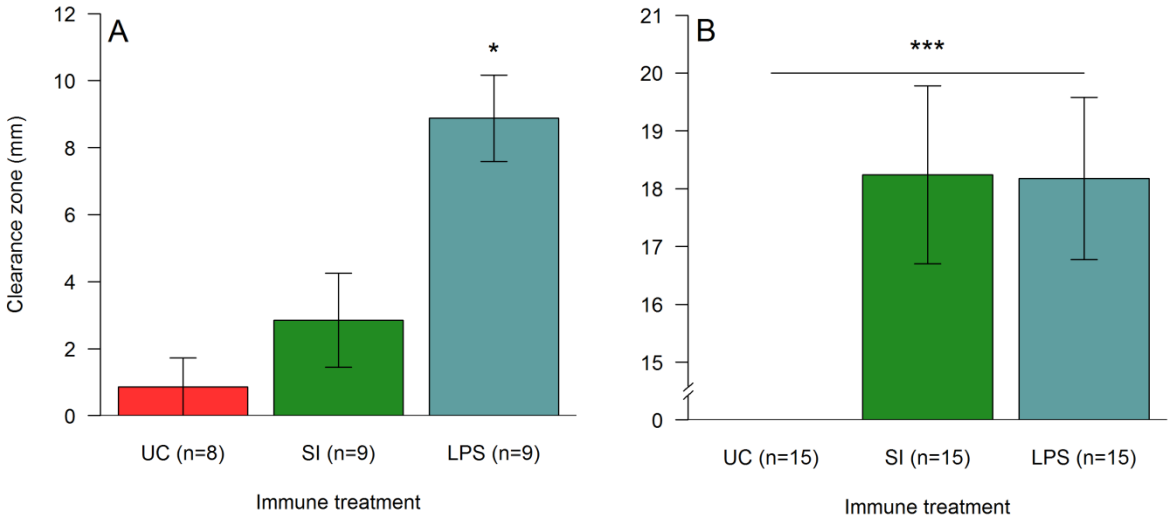


**Figure S3.7.** Antimicrobial peptide activity (AMP) activity 48 hours post immune treatment in the feeding experiment (A) and 24 hours post immune treatment in the foraging experiment (B). AMP activity was affected by the immune treatment (Kruskal Wallis tests: Feeding experiment: $\chi^2 = 13.19$, *df = 2, p=0.001,* Foraging behaviour experiment: $\chi^2 = 27.03$, *p<0.001*). In the feeding experiment, AMP activity was significantly higher in LPS-injected (LPS) bees compared to Sham-injected (SI) and Uninjected controls (UC) (*LPS vs SI, p=0.03, LPS vs UC, p=0.005, SI vs UC, p=0.81*). However, in the foraging behaviour experiment, AMP activity was similar in both LPS and Sham-injected groups, with no clearance activity observed in the Uninjected controls (*LPS vs SI, p=1, LPS vs UC, p<0.001, SI vs UC, p<0.001*), Error bars denote S.E. and stars show significant differences (p<0.05). The numbers of bees are given below each treatment group.

### 3.7.3. **Figure S3.8** Laboratory mortality

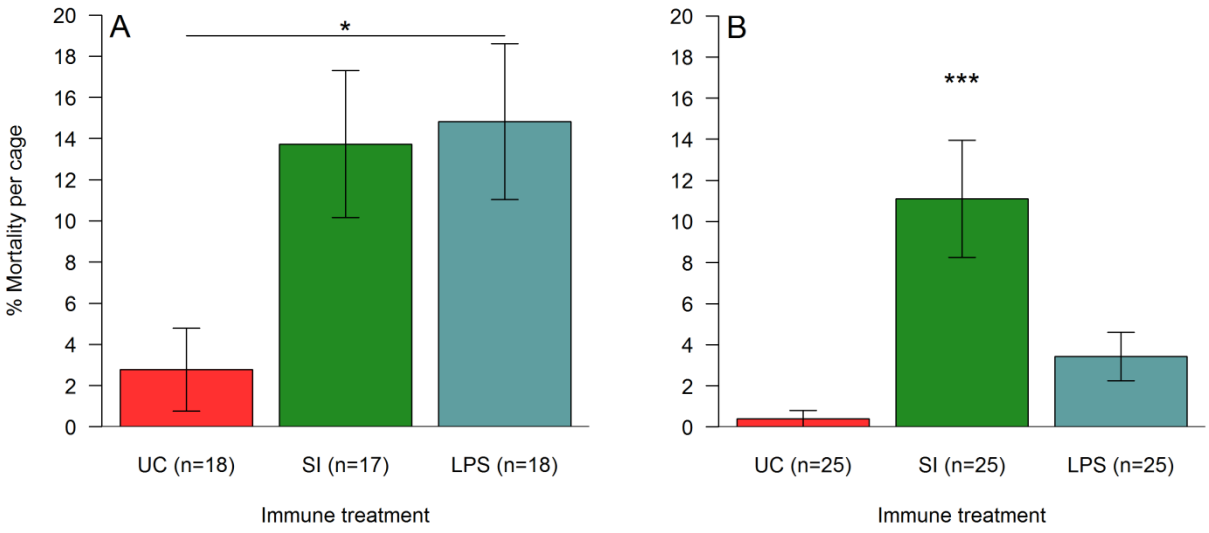


**Figure S3.8.** Laboratory mortality varied between the immune treatments in both the feeding and foraging experiments (Feeding experiment: $\chi^2 = 12.48$, $df = 2$, $p=0.002$, Foraging experiment: $\chi^2 = 22.77$, $df = 2$, $p<0.001$). In the feeding experiment, mortality rates were significantly elevated in both LPS-injection (LPS) and Sham-injection (SI) treatments compared to Uninjected controls (UC) over 48 hours, indicating that the injection had a small but consistent detrimental impact (*LPS vs UC, p=0.01, SI vs UC, p=0.02; LPS vs SI, p=0.96,* (A). However, in the foraging experiment, mortality rates were significantly elevated only in the Sham-injected group over 24 hours (*LPS vs UC, p=0.18, SI vs UC, p=0.001; LPS vs SI, p=0.02,* (B). Error bars denote S.E. and stars show significant differences ($p<0.05$). The number of cages is given below each treatment group.

# Chapter 4. Modulation of individual diet as a strategy for epidemic control in honey bees.

*4.1. Abstract*

Large colonies of social insects are potentially highly vulnerable to epidemic diseases that are transmitted by contagion. However, the rate of disease transmission is reduced when infected individuals either recover or die. Here we show that immuno-stimulated honey bee workers prefer a carbohydrate-biased diet that both boosts their immunocompetence and reduces their longevity. We therefore propose that honey bees possess an individually- based, diet-modulated strategy for epidemic control. Our results pave the way for the development of in-hive feeds that promote disease resistance.

*4.2. Introduction*

The theory of epidemic outbreaks (Anderson & May 1985, John & Samuel 2000, Lloyd-Smith *et al.* 2005, McCallum *et al.* 2001) links the rate of spread of a contagious disease through a population with the relative abundance of infected individuals because this governs the frequency with which uninfected individuals are exposed to transmission. In human populations, two key strategies for controlling epidemics include vaccination (increased immunocompetence) and quarantine (reducing the frequency of transmission events). Like human societies, colonies of social insects are vulnerable to epidemic diseases and are therefore likely to benefit from similar adaptive strategies. Indeed, behaviours analogous to self-imposed quarantine are evident in social hymenoptera, including honey bees (Bos *et al.* 2012, Heinze & Walter 2010, Rueppell *et al.* 2010, Ugelvig & Cremer 2007).

The immune response of social insects comprises an array of individual-based and social immune responses, including cellular, humoral and behavioural modifications that defend against pathogens (Cremer *et al.* 2007, Wilson-Rich *et al.* 2009). Dietary nutrition plays an important role in supporting an effective immune system (Li *et al.* 2007) and is also a modulator of longevity in insects (Alaux *et al.* 2010, 2011, Di Pasquale 2013, Cotter *et al.* 2011, Lee *et al.* 2006, 2008, Mason *et al.* 2014, Povey *et al.* 2009, 2014, Schmidt *et al.* 1987, 2005, Srygley *et al.* 2009). Therefore, it is possible that dietary modulation by infected individuals could form the basis for a dual strategy of epidemic control because it can enhance immunocompetence, thereby resembling vaccination. In eusocial insects, unlike other organisms, non-reproductive individuals can be sacrificed. In functional terms, the individual's fatality thereby resembles quarantine in reducing transmission through social contact. Hypothetically,

therefore, contagious transmission in the colony can be combatted by strategies analogous to both immunisation and quarantine that are produced through dietary modulation. In honey bees (*Apis mellifera*), these strategies could be initiated through dietary modulation because insects in general are known to be capable of modifying their feeding behaviour towards a nutritional optimum, dependent on age, development, nutritional protein source and health status (Altaye *et al.* 2010, Lee *et al.* 2006, Paoli *et al.* 2014a, Povey *et al.* 2009, 2014, Mason *et al.* 2014, Simpson & Raubenheimer 1993, Stabler *et al.* 2015). Further, it is already established that, individual insects under immunological challenge will adopt a diet that affects their level of immunocompetence or longevity by both changing the amount of food consumed and preferentially consuming protein or carbohydrate rich diets or substance normally harmful to healthy individuals (Adamo *et al.* 2010*,* Bos *et al.* 2015, Lee *et al.* 2006, Mason *et al.* 2014, Millan *et al.* 2012, Singer *et al.* 2004, 2009, Smilanich *et al.* 2011, Povey et al. 2009, 2014, for review see Abbot 2014 and Kyriazakis *et al.* 1998).

In summary, it is clear that social insects are under natural selection for improved epidemic control and it is theoretically possible that infected individuals could modulate their feeding to achieve phenotypic endpoints that minimise the potential for disease transmission. We therefore hypothesized that the individual adult workers of the eusocial honey bee will, on immunological challenge, adopt a diet that both increases immunocompetence and reduces longevity.

The diet of honey bees derives almost exclusively from floral forage, which comprises nectar and pollen. Honey, or processed nectar, is the bees' source

of carbohydrate and pollen provides essential amino acids (EAAs), which are necessary for growth and cannot be synthesised by honey bees (De Groot 1952). Potentially, individual honey bees therefore can modulate the composition of their diet by adjusting the ratio of carbohydrate and EAAs in their diet (Paoli *et al.* 2014a). To test the hypothesis that dietary modulation can affect the critical determinants of disease transmission, we first used an 'enforced diet' trial to establish the phenotypic outcome of dietary modulation. Specifically, by forcing individuals to consume syrup diets that differed in the relative richness of essential amino acids (EAAs), we determined the consequences for an individual's immunocompetence and longevity. We then used 'dietary choice' trials to test whether infected individuals modulated their diet towards the postulated adaptive optima for disease control. Specifically, we investigated whether a simulated pathogenic challenge elicited feeding from EAA vs. carbohydrate diets appropriate for achieving increased immunocompetence and reduced survival. In order to monitor the immunocompetence of individual bees, we measured the levels of anti-microbial proteins (AMPs) in the haemocoelic fluid because AMPs are known to upregulated during bacterial and viral infection in honey bees (Chan *et al.* 2009, Evans 2004, Gätschenberger *et al.* 2013, Steinmann *et al.* 2015) and their heightened levels are strongly associated with disease resistance in insects (Bahrndorff *et al.* 2014). Furthermore, AMPs are postulated to defend insects against persistent bacteria that survive the initial immune response (Dunn & Drake 1983, Haine *et al.* 2008), thereby effectively immunizing against persistent infection. For example, bacterial clearance has been demonstrated after just 30 minutes in honey bees, yet a heightened AMP activity continues for several days thereafter (Gätschenberger *et al.* 2013).

## *4.3. Methods*

### *4.3.1 Honey bee provenance and husbandry*

Frames of sealed brood were collected from colonies kept at the National Bee Unit (York, UK) and incubated over 24 hours at 34C and 60% humidity to mimic colony conditions. The experimental replicates were batches of 20 freshly emerged honey bees from the same colony, which were collected and caged together in plastic containers (11.4 cm diameter x 7.7 cm). The experiments described below used adult workers from either two colonies (enforced diet) collected in October 2014 or three colonies (dietary choice) collected in July 2014.

In order to simulate an encounter with an infectious microbial disease, individuals were immune-stimulated by injection of bacterial lipopolysaccharide, LPS (Sigma L2630) (Alaux *et al.* 2012, Laughton *et al.* 2011, Mallon *et al.* 2003, Siede *et al.* 2012*).* LPS is a cell surface complex derived from *E. coli* that provides a standardized challenge that will elicit an immune response without interacting pathogenically with the test subject. Using a fine needle, 2μl of 0.5mg/ml LPS in insect Ringer's solution was injected between the $4^{th}$ and $5^{th}$ abdominal tergite of ice-anesthetized bees. In order to establish that LPS-injected bees increased their immunocompetence, we measured the antimicrobial activity of the haemolymph as follows. Honey bees were ice-anesthetized until immobile and then the forth abdominal tergite was cut and the emerging haemolymph collected. In order to quantify antimicrobial activity, bacterial lawns were established on petri dishes (90 mm) that were prepared by pouring 6 ml of 1% agar in phosphate-buffered saline solution containing 0.2 mg/ml lyophilised *Micrococcus luteus* (Sigma M0508). Aliquots (2 μl) of neat

haemolymph from each bee were randomly assigned to one of a series 2 mm wells created in the agar. Plates were incubated at 27°C for 96 hours and we recorded the resulting zones of bacterial clearance as indicative of AMP activity.

*4.3.2. Enforced diet trial*

Cages of bees were restricted to either a nutritionally complete diet that contained both carbohydrate as a pure sugar solution (1M, 342mg/ml $dH_2O$) and solutes comprising all ten of the honey bees EAAs [molar ratio EAA:Carbohydrate of either 1:57 (n=12 cages) or 1:50 M (n=12 cages)] or a nutritionally incomplete diet containing only carbohydrate solute (1M sugar syrup, n=12 cages) (see supporting information, Table S4.4). We chose an EAA:carbohydrate ratio of 1:50 M because it is adopted by young, queenless bees (Paoli *et al.* 2014a).

One honey bee was removed from each cage per day for assessment of AMP activity for eight days, as, under natural conditions, honey bees of this age reduce their digestive proteolytic activity. (Moritz & Crailsheim 1987). The feeders were replenished and corpses were removed daily.

*4.3.3. Unrestricted dietary choice after immune-stimulation*

Cages were incubated as above and the bees were allowed to feed *ad libitum* on sugar solution (1M) for 24 hours before being randomly assigned to one of three immune treatments: injection of LPS as above (n=15 cages); control Sham-injection with insect Ringers solution (n=20 cages); and Uninjected control (n=16 cages). The injection treatments were implemented on ice-immobilised individuals. Thereafter, all cages were provided with two feeders, an EAA feed and a pure sugar solution feed, where consumption was separately monitored in order to determine the effect of immune challenge on

diet. In order to enable the bees to regulate the amount of EAAs ingested, the EAA feed was prepared in a 1M sucrose solution and each of the ten EAAs was present at 4.5 mM, giving a final EAA:carbohydrate ratio of 1:23 (see supporting information, Table S4.5). The pure sugar solution feed was solely 1M sucrose solution, so that the concentration of sugar was the same in both feeds. Our set-up was designed so that any dietary modulation of EAA uptake would force honey bees to alter their carbohydrate intake unless they were able to compensate by uptake from the pure sugar solution. Therefore, we characterized feeding behaviour was by the relative consumption from the two feeders rather than by the actual amount of nutrients ingested (see Mason *et al.* 2014). Cages were incubated as described above with three control cages to measure evaporation.

Feeders were replaced daily and consumption was calculated as the difference in the mass of the feeders minus the average daily evaporation. The actual amount of nutrients consumed was calculated from the mass of feed consumed and the known concentrations of the carbohydrates and EAAs. Both the cumulative consumption from each feeder and the actual amount of nutrients consumed per surviving bee was calculated per cage after eight days. Confirmation of AMP activity was achieved from one randomly selected bee from each cage 24 hours following the immune treatment.

#### *4.3.4. Statistical analyses*

In the unrestricted diet trial, the effect of the immune treatment on consumption was analyzed with linear mixed effects models within the *nlme* package (Bates *et al.* 2014a) with colony treated as a random effect. The effect of

immunostimulation on AMP activity was analyzed with a Kruskal-Wallis test because the data were not normally distributed.

In the enforced diet trial, we analyzed AMP activity after scoring the level of bacterial clearance at each well as a binary variable, either active or inactive, as the data were not normally distributed. We therefore tested the effect of diet, day and colony on AMP activity by using a Binomial GLMM that was implemented in the *lme4* package (Bates *et al.* 2014b). The effect of diet and colony on survival was analyzed with a COX proportional hazard model. Pairwise comparisons for the surveil analysis were completed with Tukey corrections within the *Multcomp* package (Hothorn *et al.* 2008). In both the AMP and survival analysis, ‘cage’ was entered into the model as a random effect.

All models were initially fitted with all two-way interactions and final significance of effects was assessed after backwards model selection. All statistical analyses were performed in R 3.0.2 (R Core Team 2014).

## *4.4. Results*

### *4.4.1. Enforced diet trial*

The diet affected immunoactivation (Binomial GLMM: Diet, $\chi^2_1$= 8.87, *p=0.01*, Day, $\chi^2_1$= 13.66, *p<0.001*, Fig. 4.1A, Colony, $\chi^2_1$= 10.53, *p<0.001*). Relative to individuals fed on the 1:57 nutritionally complete diet, honey bees fed on the pure sugar solution diet more frequently exhibited immunoactivation after immuno-challenge (pure sugar solution vs 1:57 diet, *p=0.01*; pure sugar solution vs 1:50 diet; *p=0.22*, 1:57 diet vs 1:50 diet, *p=0.26*). However, when the two nutritionally complete diets were combined due to the similarity in EAA concentration, honey bees fed on the pure sugar solution diet more frequently exhibited immunoactivation compared to bees fed EAAs (Binomial GLMM: Diet, $\chi^2_1$= 6.33, *p=0.01*, Day, $\chi^2_1$= 99.0, *p<0.001*, Fig. 4.1A, Colony, $\chi^2_1$= 12.79, *p<0.001*). Honey bees fed pure sugar solution also exhibited reduced longevity compared to bees fed EAAs regardless of whether the EAA diets were analysed separately (COXPH: Diet, $\chi^2$ = *28.5, df = 2, p<0.001*, Colony, $\chi^2_1$= 18, *p<0.001*, pure sugar solution vs 1:57 diet, *p<0.001*; pure sugar solution vs 1:50 diet; *p<0.001*, 1:57 diet vs 1:50 diet, *p=0.16*) or combined (COXPH: Diet, $\chi^2$ = *25.2, df = 1, p<0.001*, Fig. 4.1B, Colony, $\chi^2_1$= 17.77, *p<0.001*).

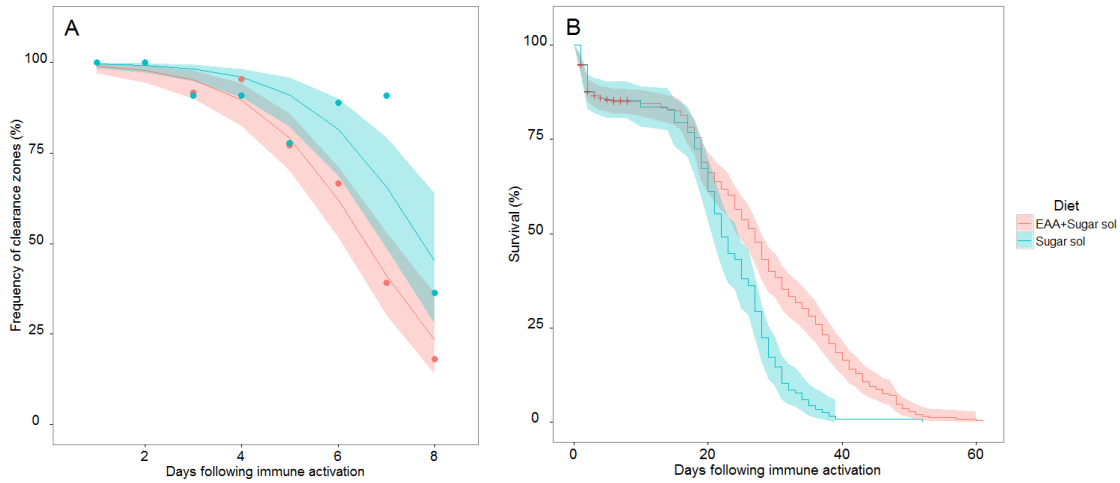


**Figure 4.1.** Levels of immunocompetence and longevity in immunostimulated honey bees. Panel A: the frequency of clearance zones (AMP activity) produced over eight successive days by honey bees restricted to diets of either pure sugar syrup (blue symbols, n=9-12 bees per day) or nutritionally complete syrup (pink symbols, n=21-24 bees per day). Panel B: survival of honey bees (% survival) when restricted to diets of either pure sugar syrup (blue, n=216 bees) or nutritionally complete syrup (pink, n=473 bees). Shaded areas indicate 95% C.I. based on individual bees as replicates and crosses indicate censoring caused by the removal of individuals for assays of AMP activity.

### *4.4.2. Diet modulation under simulated pathogenic challenge*

Our immunostimulus (LPS injection) was successful because AMP activity varied across the immune treatments (Kruskal Wallis tests: $\chi^2 = 29.57$, $df = 2$, $p<0.001$, Fig. S4.6). AMP activity was greatest in LPS-injected bees compared to the Sham-injected bees and Uninjected control bees (LPS vs SI $p=0.001$; LPS vs UC $p<0.001$; SI vs UC $p=0.006$).

Overall, immune-stimulated bees consumed less syrup than the Uninjected controls (LME: $F_{2,46}$ = 5.99, *p=0.005,* LPS vs SI *p=0.3*, LPS vs UC *p=0.001*, SI vs UC *p=0.05*), resulting in a corresponding decrease in uptake of both carbohydrates and EAAs (LME: EAAs: $F_{2,46}$ =3.99, *p=0.03*; carbohydrates: $F_{2,46}$ = 5.99, *p=0.005*; Fig. 4.2).

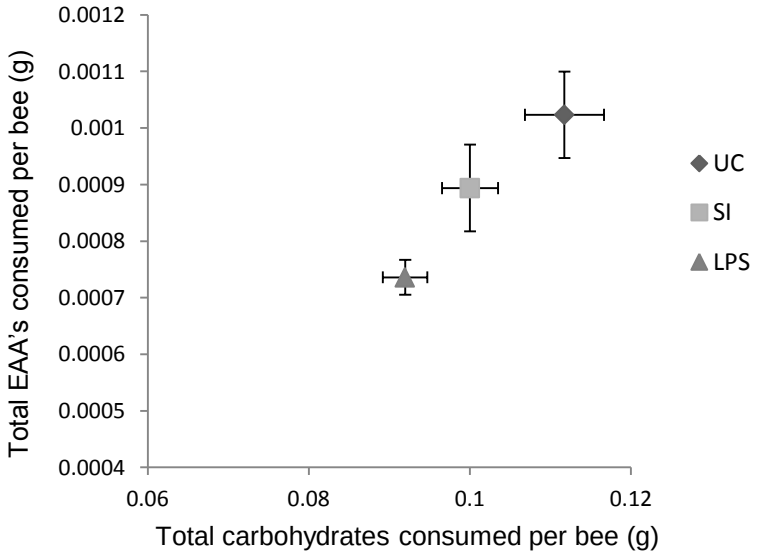


**Figure 4.2.** Overall levels of uptake of carbohydrates and essential amino acids (EAAs) by honey bees in immunostimulated and control treatments. The dietary quantities were calculated for surviving honey bee eight days after the immune treatment was administered (Uninjected control, UC: n=16 cages; Sham-injection, SI: n=20; and lipopolysaccharide injection, LPS: n=15 cages). Error bars denote S.E.

The overall reduction was due to immune-stimulated bees consuming less from the EAA feeder whilst maintaining uptake from the pure carbohydrate feeder (LME: EAA feeder: $F_{2,46}$ =3.99, *p=0.03*, Sugar solution feeder: $F_{2,46}$ = 0.90,

*p=0.41*, Fig. 4.3A & B), thereby exhibiting a modulation of feeding behaviour. (LPS vs. UC, *p=0.01*; LPS vs. SI, *p=0.21*; SI vs. UC, *p=0.34*).

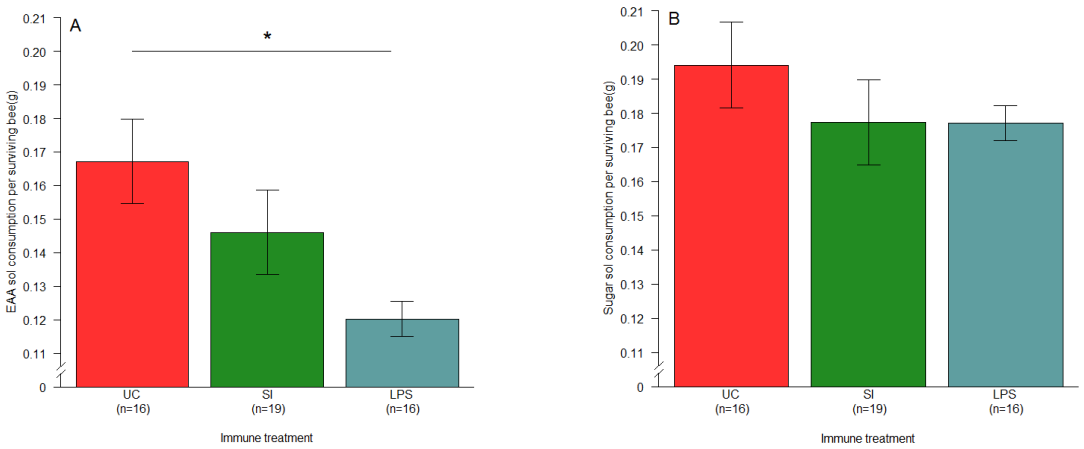


**Figure 4.3.** *Ad libitum* consumption of nutrionally complete and incomplete sugar syrups (g bee$^{-1}$ 8 d$^{-1}$) by immunostimulated and control honey bees. Panel A: consumption of sugar syrup containing essential amino acids (EAAs); Panel B: consumption of pure sugar syrup. Histogram bars represent three treatment groups: Uninjected control, UC (n = 16 cages); Sham-injected control, SI (n = 20 cages); and lipopolysaccharide injection, LPS (n = 15 cages). Error bars denote S.E. The starred bar spans treatments that differ statistically ($p$<0.05).

## *4.5. Discussion*

### *4.5.1. Evidence for epidemiological adaptation in honey bees*

*C*lassic disease transmission theory tells us that removal of infected individuals through recovery or mortality reduces the potential of a disease to spread through a population (Lloyd-Smith *et al.* 2005, McCallum *et al.* 2001). Our study demonstrates that forcing honey bees to feed on a pure sugar solution diet led to individuals exhibiting increased AMP activity in response to immunostimulus and reduced longevity relative to those fed a nutritionally complete diet with EAAs, which we propose realises effects analogous to vaccination and quarantine.

We detected relatively long-lasting AMP activity that persisted eight days immunostimulus. Eight days constitutes a substantial portion of an adult honey bee's life span (normally c. 15-38 days) (Winston 1987), which suggests that our analogy with vaccination is reasonable. Individuals in the immune-stimulated treatment also adopted a diet that reduced their chance of survival relative to controls, which under realistic in-hive conditions would reduce an individual's lifetime number of contacts with uninfected individuals. Our findings suggest that immunostimulated honey bees freely adopted a diet that would modulate their phenotype towards characteristics that could reduce the spread of epidemic disease. We therefore propose that, in the context of eusocial insect societies, alteration of feeding behaviour by infected non-reproductive workers may represent an adaptive strategy to self-medicate the superorganism. We recognise, however, that further research is needed to establish that the effects that we have detected have efficacy in supressing disease transmission under realistic in-hive conditions and where infection is by

a live pathogen. Nevertheless, our findings begin to indicate that some feeding behaviours by individual social insects can be construed as self-medication. If the alteration of feeding behaviour in LPS injected bees is indicative of a response to a real pathogen, our findings would potentially meet the four criteria for attribution of self-medication; (i) the substance must be deliberately contacted, (ii) the substance must have detrimental effects to the pathogen, (iii) the detrimental effects on the pathogen must increase the hosts fitness and (iv) the substance must normally have detrimental effects on the host (in the absence of the pathogen) (Abbott 2014). The preference for a pure syrup diet was observed only after immunological challenge (satisfying the first criteria criteria) and a pure sugar solution diet promoted AMP activity (potentially satisfying the second and third criteria). In addition, young healthy honey bees normally consume EAAs through pollen feeding, without which, they cannot develop their hypopharyngeal glands, needed for feeding brood (Crailsheim *et al.* 1992), therefore satisfying the fourth criteria.

### *4.5.2. How do our results integrate with previous work?*

#### *4.5.2.1. Dietary modulation*

Our results are consistent with previous studies demonstrating that insects can alter feeding behaviour when immunologically challenged (Adamo 2005, Adamo *et al.* 2007, 2010, Baracchi *et al.* 2015, Bos *et al.* 2015, Lee *et al.* 2006, Goldsworthy 2010, Mason *et al.* 2014, Millan *et al.* 2012, Singer *et al.* 2004, 2009, Smilanich *et al.* 2011, Tyler *et al.* 2006, Povey et al. 2009, 2014, for review see Kyriazakis *et al.* 1998). Furthermore, like other studies of insects at both the larval and adult stage, the honey bees that we studied reduced the overall consumption in response to an immune challenge (Adamo 2005, Adamo

*et al.* 2007, 2010, Goldsworthy 2010, Mason *et al.* 2014) and altered their uptake of nutrients such as protein in order to promote immunocompetence (Lee *et al.* 2006, Povey et al. 2009, Mason *et al.* 2014). For example, *Spodoptera* larvae have been shown to prefer protein rich diets in order to bolster their AMP response when immunologically challenged (Lee *et al.* 2006, Povey et al. 2009). Our results are similar to those of Mason *et al.* (2014) who found that larvae of *Grammia incorrupta* reduced their intake of high protein/low carbohydrate food whilst maintaining their intake of low protein/high carbohydrate food when parasitized or subject to a pseudo parasitic challenge (injection of latex beads) and that their activity of phenoloxidase, a key immune enzyme involved in cellular immunity was positively correlated with carbohydrate intake.

#### *4.5.2.2. Increased AMP activity on the pure sugar solution diet*

In contrast to our findings in honey bees, previous studies have found that dietary protein increases AMP activity in other non-social insect species (Cotter *et al.* 2011, Lee *et al.* 2006, 2008, Povey et al. 2009, 2014). However, it is likely that honey bees do not conform to the general trend among insects. Specifically, a previous study of honey bees has also found that levels of AMP-related gene expression were unresponsive to the presence of dietary protein when honey bees suffered a challenge with the parasitic mite *Varroa* and an associated array of vectored viruses (Alaux *et al.* 2011).

There is a possible artefactual explanation for the patterns exhibited by honey bees as follows. Honey bee adult workers are females and can develop their ovaries in the absence of a queen (Altaye *et al.* 2010, Pernal & Currie 2000, Frias *et al.* 2016). The caged individuals that we studied were orphaned

workers, so it is therefore possible that they might have prioritised physiological development over immune function in our study if they were provided with access to dietary EAAs. However, this potential artefact seems unlikely to have affected our study because the individuals in the study by Alaux *et al.* (2011) were exposed to queen pheromone and pollen feeding nevertheless had no effect on the levels of AMP gene expression in the immunochallenged honey bees. These individuals would have been able to elevate their AMPs without competition from the resource-demanding task of ovary development, but they did not. Therefore, we interpret our findings to mean that effect of diet on the levels of immunocompetence would not have been influenced by using queenless honey bees.

In the above mentioned study, pollen feeding resulted in increased gene expression for the AMP's Lyzozyme-2 *and* -3 and Defensin-1 in unchallenged honey bees, which were not investigated here, due to a lack of AMP activity in unchallenged bees (Figure S3). It therefore remains unclear how dietary EAAs would affect phenotypic AMP activity unchallenged honey bees. However, the AMP response is generally activated upon challenge, (Chan *et al.* 2009, Evans 2004, Gätschenberger *et al.* 2013, Steinmann *et al.* 2015) rather than being constitutive. Therefore, our results remain valid in the context of AMP activiation upon epidemic invasion.

The mechanism by which a pure syrup diet increased AMP activity may be explained by energy allocation trade-offs between the immune system and digestion, as honey bees that were forced to consume EAAs would incur a metabolic cost of digesting EAAs (for review see Kyriazakis *et al.* 1998). In addition, direct trade-offs between the immune system and digestion are known

to occur in insects. For example, although our nutrients of interest were EAAs, trade-offs between the transport of ingested lipids and bacterial resistance have been demonstrated in the adult cricket *Gryllus texensis*, owing to a common lipoprotein used for both lipid transport and binding to pathogens for subsequent immune activation (Adamo *et al.* 2010). It is also possible that the honey bees restricted to the pure sugar solution diet may have simply consumed more than the honey bees restricted to the EAA diet, given that honey bees consumed less of the diet containing EAAs when immunologically active. Indeed, adult bumblebees (*Bombus*) increase consumption of carbohydrates when injected with LPS (Tyler *et al.* 2006). Thus, enhanced AMP activity from the pure sugar solution diet may have resulted from a relative increase in dietary calories.

*4.5.2.3. Diet and longevity*

Our findings that EAAs promote longevity in honey bees likewise suggests that they do not conform to the widespread pattern among insects that dietary protein reduces longevity (for review see, Simpson & Raubenheimer 2009). Likewise, previous studies with bumblebees and honey bees have shown that dietary protein and EAAs have no effect on, or can be detrimental for survival at high concentrations (Pirk *et al.* 2010, Paoli *et al.* 2014a,b, Stabler *et al.* 2014). However, like our study, previous work on honey bees have found that dietary pollen (the primary source of dietary EAAs) and protein increases honey bee's longevity (Archer *et al.* 2014, Altaye 2010, Di Pasquale 2013, Rinderer *et al.* 1974, 1977, Schmidt *et al.* 1987, 1995, but see Frias *et al.* 2016). The apparent contrast between the responsiveness of longevity to dietary protein that we observed in honey bees and the collection of other results may be explained by the dietary ratio of EAA:carbohydrate that we used in our experiments and differences in the way that survivorship was monitored. A

previous study (Paoli *et al.* 2014a) determined the EAAs to carbohydrate intake target of young bees as 1:50 and found that this ratio had no effect on survival compared to honey bees fed on pure sugar solution, whereas high EAA:carbohydrate ratios reduced survival. However, the investigators censored the longevity data after 14 days. In our trial, by contrast, we monitored longevity for the honey bee's entire life time. Therefore a possible explanation is that we unmasked longer-term beneficial effects of dietary EAAs when supplied at optimal ratios (1:50-1:57). Alternatively, decreased survival in honey bees fed on the pure sugar solution diet may have resulted from the cost of increased AMP activity, as previously demonstrated in bumblebees (*Bombus)* (Moret & Schmid-Hempel 2000).

*4.5.2.4. Future work*

Taken together, our results offer evidence to support the paradigm that malaise behaviours such as suicidal quarantine through dietary change have evolved in eusocial insects to reduce pathogen transmission within groups (Shakhar & Shakhar 2015). Therefore, future work should; (i) investigate whether honey bees avoid dietary EAA' s following challenge with a real pathogen and (ii) directly investigate the dynamics of pathogen transmission to nest mates following the consequences of decreased survival and increased immunocompetence through dietary change.

*4.6. References*

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*4.8. Supporting information*

**4.8.1. Table S4.4.** Essential amino acid (EAA) and carbohydrate feed concentrations in the enforced diet trial. Each EAA was present at 2mM and 1.7 mM in a 1M carbohydrate solution, giving a final EAA:Sugar ratios of 1:50 M and 1:57 M. The pure carbohydrate feed consisted of the 1M sucrose solution devoid of any EAAs.

| | **1:50M Diet** | **1:57M Diet** |
|---|---|---|
| **Essential Amino Acid (EAA)** | **mg/ml (2mM)** | **mg/ml (1.7mM)** |
| Methionine | 0.1460 | 0.1287 |
| Tryptophan | 0.1998 | 0.1762 |
| Arginine | 0.1655 | 0.1460 |
| Histidine | 0.1518 | 0.1339 |
| Phenylalanine | 0.1616 | 0.1425 |
| Isoleucine | 0.1284 | 0.1132 |
| Threonine | 0.1165 | 0.1028 |
| Leucine | 0.1283 | 0.1132 |
| Valine | 0.1146 | 0.1011 |
| Lysine | 0.1787 | 0.1576 |

| **Carbohydrate** | **g/ml (1M)** |
|---|---|
| Sucrose | 0.3423 |

**4.8.2. Table S4.5.** Essential amino acid (EAA) and carbohydrate feed concentrations in the dietary choice trial. Each EAA was present at 4.5mM in a 1M carbohydrate solution, giving a final EAA:Sugar ratio of 1:23. The pure carbohydrate feed consisted of the 1M sucrose solution devoid of any EAAs.

| **Essential Amino Acid (EAA)** | **mg/ml (4.5mM)** |
|---|---|
| Methionine | 0.6632 |
| Tryptophan | 0.9077 |
| Arginine | 0.7520 |
| Histidine | 0.6896 |
| Phenylalanine | 0.7342 |
| Isoleucine | 0.5831 |
| Threonine | 0.5294 |
| Leucine | 0.5830 |
| Valine | 0.5207 |
| Lysine | 0.8118 |

| **Carbohydrate** | **g/ml (1M)** |
|---|---|
| Sucrose | 0.3423 |

**4.8.3.** Antimicrobial peptide activity (AMP) in the unrestricted diet trial.

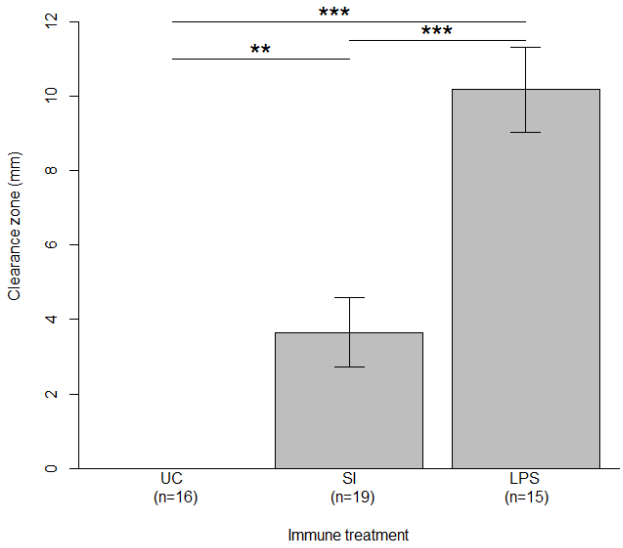


**Figure S4.6.** Antimicrobial peptide activity (AMP) activity 24 hours post immune treatment in the Dietary choice trial. AMP activity varied across the immune treatments (Kruskal Wallis tests: $\chi^2 = 29.57$, $df = 2$, $p<0.001$). AMP activity was greatest in LPS injected (LPS, n=16 bees) bees compared to the Sham-injected (SI, n=19 bees) and Uninjected control bees (UC, n=15 bees) (LPS vs SI $p=0.001$, LPS vs UC $p<0.001$, SI vs UC $p=0.006$). Error bars denote SEM and stars show significant differences.

# Chapter 5. General Discussion

Honey bee health has recently been the focus of much scientific research, prompted in part by reports of honey bee declines in the USA and Europe, coupled with an agricultural reliance on honey bees as pollinators (reviewed in Goulson *et al.* 2015 and Potts *et al.* 2010). Honey bees are the dominant managed pollinator for agricultural crops and are important pollinators of wild flowers. Therefore, research has spanned multiple drivers of honey bee health such as environmental, genetic and disease related factors (reviewed in Goulson *et al.* 2015 and Potts *et al.* 2010). In recent years, attention has turned to the importance of nutrition for honey bees in order to fight infections (DeGrandi-Hoffman & Chen 2015).

The notion that eusocial insects employ multiple strategies of individual and social immunocompetence is well established (reviewed in Cremer *et al.* 2007, Wilson-Rich *et al.* 2009 and Evans & Spivak 2010). Likewise, the impact of diet on immunocompetence has been widely studied in insects (Cotter *et al.* 2011, Lee *et al.* 2006, 2008, Povey *et al.* 2009, 2014). However, few studies have investigated the role of dietary nutrients for immunocompetence in the context of the superorganism (Alaux *et al.* 2010, 2011, Szymaś, & Jędruszuk 2003, Kay *et al.* 2014), taking into account the numerous individual and social, behavioural and physiological strategies of immunocompetence exhibited by eusocial insects.

The central aim of this thesis was therefore to investigate the impact of diet on the both the behavioural and physiological immune strategies of honey bees. Specifically, this thesis investigated the interplay between diet, immune-stimulation, feeding behaviour and the phenotypic endpoints achieved by honey

bees in order to fight infection. Below, I evaluate my findings and discuss their implications.

*5.1. Evaluation of the main findings*

*5.1.1. Characterisation of the time course of immune response in honey bees*

My first objective was to establish a framework within which to investigate dietary modulation of immunocompetence in honey bees. I therefore described a time course for the dynamics of the physiological immune responses PO and AMP's and found that, in line with previous findings, AMP's responded strongly to injection with LPS (Alaux *et al.* 2012, Laughton *et al.* 2011, Mallon *et al.* 2003). Optimal time points to measure maximum AMP activity were identified as seven hours and 24-48 hours post injection. AMP activity was first detectable seven hours post-injection, and it increased slightly at seven hours, peaked at 24 hours and remained high for 48 hours post injection with LPS.

The immune response of insects varies both temporally and according to different pathogenic challenges (Lemaitre *et al.* 1997, Haine *et al.* 2008 a,b). In comparison, my AMP-time course results are in general agreement with studies of AMP activity in other insects, demonstrating peaks in physiological activity after 24 hours (Bulet *et al.* 1991, Haine *et al.* 2008 a,b, Korner & Schmid-Hempel 2004). Although I measured AMP's only for 48 hours in my initial investigations, the AMP response of honey bees may endure beyond this interval because among insects generally it is known to be long lasting and begin after the initial bacterial clearance from haemolymph. AMP's have therefore been postulated to act as a defence against persistent infection (Haine *et al.* 2008 a,b) and thus may be thought of as analogous to

vaccination. Indeed, I found that AMP activity continued for up to eight days in honey bees in later trials (discussed below).

Contrary to previous findings, I observed that PO activity in honey bees was not consistently decreased by LPS injection. In other studies, the activity of PO has been found to reduce in honey bees following LPS injection, possibly due to a trade off with AMP activity or an inability to replenish stocks following activation (Laughton *et al.* 2011). In my investigation, the decrease in proPO activity after immunostimulation varied amongst colonies. Consequently, the conventional response may have been observed had I sampled more widely across colonies. Likewise, Siede *et al.* (2012) found no effect of LPS injection on the expression of a gene encoding proPO in honey bees from a single colony.

### *5.1.2. Dietary modulation of immunocompetence in honey bees*

My second aim was to investigate dietary modulation of physiological immunocompetence in honey bees. Contrary to expectation, I found no evidence that access to dietary pollen affected any components of honey bee immunocompetence measured here, at least in the short term (24 hours). However, when investigating the longer term role of dietary EAAs, I found that honey bees that I had restricted to a pure carbohydrate diet exhibited increased immunocompetence (AMP activity) and reduced longevity compared to those restricted to a diet containing EAAs.

Crucially, I found evidence that dietary EAAs did not promote both AMP activity and survival in honey bees. An avoidance of dietary EAAs when under immunological challenge may be a trade between the two traits of made at the point of ingestion. It would be interesting to investigate the optimal nutritional

ratios of EAA:carbohydrates for performance of the other components of the immune response in honey bees.

One possible approach that I did not pursue has been suggested by Raubenheimer & Simpson (1993) and Simpson & Raubenheimer (1993), who outlined a 'geometric framework' approach to nutrition to investigate how animals regulate their intake of different nutrients to achieve a specific ratio (the intake target) in order to maximize fitness. Confining test animals to a diet that offers a particular ratio of two nutrients allows one to plot the amount of food consumed on a graph where each axis represents the two different nutrients, which define a so-called 'nutrient space'. The levels of nutrients consumed will fall upon the slope (or 'rail') through nutrient space that represents the ratio of nutrients offered in the food. When restricted to nutritionally imbalanced diets, animals will consequently under and over ingest particular nutrients in order to achieve a target amount of another nutrient. By confining test animals to multiple rails through the nutrients space, one can determine the intake target and where an individual's performance peaks within the nutrient space. In this way a large area of nutrient space can be explored and the fitness peaks and troughs can be mapped as a response surface over the nutrient space. By offering test animals a choice of nutritionally imbalanced diets whereby the animals can feed differently from each and therefore regulate intake of the nutrients of interest, one can determine the intake target within the nutrient space (Raubenheimer & Simpson 1993, Simpson & Raubenheimer 1993). Cotter *et al.* (2011) demonstrated that the peak responses for body mass, lysozyme activity, melanisation and PO for *Spodoptera littoralis* larvae differed over nutrient space. Therefore demonstrating that no single diet can optimise all of the measured traits.

In future, I propose that a similar experiment could be performed to explore the nutritional space of honey bees, allowing fitness-related performance of immune traits such as PO and GOX to be mapped onto a EAA:Carbohydrate nutrient space much wider than explored in this thesis. In addition, by exploring both other immune responses and longevity, the extent to which honey bees reach a compromise between the nutritional needs of competing immune pathways and longevity could be investigated more fully.

Immunocompetence may vary between the different arms of the immune system on different dietary ratios depending on whether the individual is infected, due to trade-offs within the immune system. For example, trade-offs between PO and AMP activity have been previously reported in insects (Cotter *et al.* 2011, 2013, Moret & Schmid-Hempel 2001, Povey *et al.* 2009). I found some evidence for a trade-off between PO and AMP's in honey bees. However, this trade-off varied amongst colonies (see chapter two) and so the generality and governing factors of the PO-AMP inter-relationships are not yet fully determined. In order to resolve this uncertainty, it would be necessary to conduct a larger study including more colonies and using the geometric framework approach described above. Eventually, this kind of investigation may reveal that this is a common trade-off for honey bees and also evaluate in full whether the trade-off has an underlying dietary basis, which would be manifested should PO an AMP activity map onto different regions of the nutrient space.

### *5.1.3. Self-medication at the both the individual and social level.*

My third aim was to explore the ability of honey bees to self-medicate at the both the individual and colony level. Based on the trend in feeding behaviour

observed in chapter two, I established that honey bees consume a carbohydrate-biased diet when immune-stimulated and demonstrated that dietary alteration resulted in increased foraging behaviour. Based on these findings, I argued that diet is a driver of self-removal in healthy honey bees. Previous studies have interpreted an increase in foraging activity by infected individuals as a form of self-removal in order to reduce potential disease transmission within the colony. Immune-stimulated honey bees maintained intake of carbohydrates but reduced pollen feeding in laboratory trials, and accumulated foraging flights more rapidly than controls in field colonies (Chapter three). Based on my findings, I argue that the altered feeding behaviour of infected bees is explicable by reference to epidemic control theory at the colony level. Specifically, it appears that a pure carbohydrate diet is adaptive because it both increased the AMP response in honey bees over eight days (i.e. decreases the number of susceptible individuals, $S$) and decreases longevity (i.e. decreases the number of infective individuals, $I$). Taken together, these effects reduce the rate of spread of a contagious disease according to epidemic control theory by reducing the magnitude of the product $SI$ in Eq 1.

The feeding behaviour of immune-stimulated bees towards EAAs suggested that their nutritional target was to emulate pollen feeding. Immune activated bees reduced their intake of EAAs whilst maintaining intake of a pure carbohydrate diet, therefore demonstrating a preference for a diet that resulted in higher immunocompetence and lower longevity. Therefore, honey bees fed as predicted by epidemic control theory (Chapter Four).

For a behaviour involving consumption of a given substance to be considered as dietary self-medication, four criteria must be met (Abbott 2014); (i) the

substance must be deliberately contacted, (ii) the substance must have detrimental effects to the parasite, (iii) the detrimental effects on the parasite must increase the host's fitness and (iv) the substance must normally have detrimental effects on the host in the absence of the parasite. In the case that I have studied, the first criterion is met by the quantitative adjustment of pollen and EAA intake when under immunological challenge. Should the responses of bolstered immunocompetence, self-removal and reduced survival through dietary change in LPS injected bees represent responses with a real pathogen, and these responses translate to reduced disease transmission potential within the colony, the second and third criteria would be satisfied. The fourth is satisfied by the fact that pollen-starved honey bees have a reduced capacity to rear brood as they cannot develop their hypopharyngeal glands (Crailsheim *et al.* 1992). Consequently, it is appropriate to interpret the dietary modulation that I have observed as a potential form of self-medication.

Therefore, this thesis reveals that diet can affect both individual immunity and feeding behaviour when honey bees are immunologically active and that reduced feeding on pollen and EAAs when challenged has multiple health consequences, which may be considered as self-medication at both the individual and social levels.

It remains unclear whether the observed dietary change in immunologically challenged honey bees is most efficacious though increasing individual immunocompetence, by causing self-quarantine by increased foraging or death, or by increasing the foraging force to boost resources for offsetting losses due to infection. Nevertheless, any of these consequences may be construed as beneficial strategies for a superorganism. Specifically, it is coherent to

postulate that natural selection may favour queens that produce workers that, when infected, select a diet that promotes individual immunocompetence, increased foraging behaviour and in early death in the infected individual. The overall potential effect is to reduce disease transmission within the colony, whilst also increasing the foraging force and therefore increase the queen's fitness. The reality is likely some combination of all. In this way, so-called 'malaise behaviour' (in this case, reduced feeding on EAAs or pollen) may have evolved to reduce pathogen transmission in eusocial insects (Shakhar & Shakhar 2015). For example, one can imagine that in the evolutionary past, the survival cost of increasing individual immunocompetence through dietary change resulted in fitter colonies better able to deal with epidemics, whilst also benefiting from increased food stores when under pathogen attack.

An alternative explanation for reduced pollen intake when honey bees are immunologically activated is that avoidance of pollen and increased foraging activity may be an attempt to reduce further infection from nest mates or pollen (Shakhar & Shakhar 2015). Indeed, pollen represents a disease transmission route for honey bees (Higes *et al.* 2008, Mazzei *et al.* 2014). However, LPS-injected honey bees also forage more intensively, making them more likely to contact pollen. Furthermore, reduced pollen feeding reduces the development of their hypopharyngeal glands, which are needed for brood feeding (Alaux *et al.* 2012) and there is no evidence that bacterial brood diseases affect the health of adult honey bees. For example, although the adults can carry the two primary bacterial diseases of honey bee brood, American and European foulbrood, no pathological effects are observed and the bacteria do not multiply within adults (Forsgren 2010, Wilson 1971). Therefore it is unlikely that avoiding

brood due to increased AMP activity would have any beneficial effects in terms of avoiding contracting bacterial disease.

### *5.2. Theories that can explain dietary alteration*

Illness-induced anorexia is a malaise behaviour commonly observed in animals (reviewed in Kyriazakis *et al.* 1998) and insects (Adamo 2005, Adamo *et al.* 2007, 2010, Goldsworthy 2010, Mason *et al.* 2014). As reduced feeding occurs in response to both pathogens and artificial immune challenges, it assumed to be adaptive for the host, rather than manipulation by the pathogen. Likewise, my results demonstrate that honey bees exhibited reduced feeding behaviour in response to a pseudo-bacterial challenge. Many hypotheses have been proposed to explain the adaptive significance of illness induced anorexia for the host (Kyriazakis *et al.* 1998), including; (i) a reduction dietary nutrients for the host results in the starvation of the invading pathogen, (ii) reduced food intake reduces the energy needed for processing nutrients in order to counter the energy needed for mounting an immune response, (iii) a reduction in feeding bolsters the immune response and (iv) a reduced overall intake allows animals to be more selective in their diet composition. My results provide support for hypotheses three and four, given that immunologically active honey bees both reduced their overall intake, but did so selectively, by reducing only their intake of pollen and EAAs whilst maintaining uptake from a pure carbohydrate diet. It would be interesting to directly test this hypothesis experimentally. Future work should aim to ascertain whether experimental restriction of overall intake allows honey bees to be more selective between two or more nutrients when immunologically active.

*5.3. Other nutritional components of pollen that bees may have been modulating*

Potentially, there are other nutritional components of pollen that may potentially drive nutritional modulation (i.e. differential consumption) by infected honey bees. The role of lipids in insect immunity has recently gained attention as an effector of immune pathways. Bareletta *et al.* (2016) demonstrated that the formation of lipid droplets was induced by bacterial and viral challenge in *Aedes aegypti* in both adults and cell lines, indicating a role of lipid droplets in antimicrobial defence. Furthermore, trade-offs between the processing of digested lipids and disease resistance has been demonstrated in an insect (Adamo *et al.* 2010). Lipids may be especially important for honey bees if increased foraging represents a means of social immunocompetence, in that a reduction in dietary lipids accelerates development to the forager phenotype (Toth *et al.* 2005). However, pollen universally provides the majority of dietary nutrients that are absent from honey and reduced pollen intake by infected honey bees would by definition also reduce lipid intake. Therefore, reduced pollen intake when immunologically activated could occur whether driven by a single or multiple nutrients.

*5.3.4. Caveat regarding the use of a pseudo-pathogenic challenge*

Throughout this thesis, I used an LPS injection as a pseudo-bacterial challenge with the intention of thereby untangling the effects of immune activation from the effects of a live pathogen, which could impose dietary modulation for its own adaptive ends and thereby confound the interpretation of dietary preferences as adaptive in honey bees themselves.

### *5.3.5. Conclusion*

Here, I demonstrate evidence of self-medication through dietary change in honey bees. When allowed to self-compose their diet, honey bees altered their diet when immunostimulated by reducing their consumption of pollen and EAAs. Immunostimulated honey bees selected a forager-like diet and engaged in intensive foraging; suggesting that the altruistic self-removal strategy of infected honey bees observed in previous work has an underling dietary mechanism. Furthermore, diets devoid of EAAs promoted immunocompetence by increasing AMP activity, suggesting that honey bees self-medicate at both the individual and social level to prevent disease spread within the colony.

In future research, the obvious progression is to investigate whether infection with real pathogens results in the same reduction in feeding pollen and EAA feeding, and the consequences for immunocompetence, increased foraging intensity and early mortality observed in my trials. Furthermore, the immune responses of honey bees are pathogen specific, as demonstrated in other insects (reviewed in Siva-Jothy *et al.* 2005). For example, AMP's are upregulated in honey bee larvae upon infection with *Paenibacillus larvae* (Chan *et al.* 2009). However, no humoral immune responses are activated in adults upon viral infection (Azzami *et al.* 2012). Adults infected with the gut parasite, *Nosema* infection seem to activate their PO response but their AMP response is suppressed, although results vary between trials using *N. Apis* and *N. ceranae* (Antúnez *et al.* 2009, Chaimanee *et al.* 2012, Roberts & Hughes 2014). Therefore, different pathogens and the associated specific immune responses may exert different pressures on the honey bees feeding behaviour. In addition, the effects of increased foraging activity and early mortality in infected individuals, on the transmission of real pathogens between colony members

warrants further investigation. In line with my findings that LPS injection results in the rapid accumulation of foraging bouts, previous studies have demonstrated that honey bees infected with real pathogens alter foraging acuity, generally resulting in more flights or increased time spent outside the colony (Alaux *et al.* 2014, Dussaubat *et al.* 2013, Goblirsch *et al.* 2013, Kralj & Fuchs 2006). Transmission rates between 'in house' honey bees and experimentally infected honey bees that are allowed to forage or killed could be investigated directly.

Both the peaks in immune activity and the transmission potential of real pathogens resulting from increased foraging or early mortality could be mapped onto nutritional space using the geometric framework approach. Combining such an approach with investigations of feeding behaviour would allow one to elucidate both the dietary effects on immunocompetence in response to multiple pathogens/mixed infections and the impacts of those diets on disease transmission between individuals brought about through dietary change.

*5.4. References*

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# 6. Appendix. Notes on method development

## *6.1. Antimicrobial peptides (AMPs)*

### *6.1.2. Zone of inhibition:*

A series of small pilot trails revealed that AMP activity could be detected via a zone of clearance assay, using lyophilised *Micrococcus luteus* (Sigma M0508) seeded agar plates. However, initial trails using live bacteria and attempting zone of inhibition assays, rather than zone of clearance assays that used lyophilised bacteria, yielded negative and inconsistent results:

No inhibition zones were observed from haemolymph samples collected via perfusion bleeds (see section 2.3.3.) or homogenised thoraxes from LPS injected honey bees (0.5mg/ml LPS in Phosphate-buffered Saline (PBS)), using agar plates spread with *Escherichia coli* or *M luteus* lawns, regardless of whether haemolymph was pipetted onto absorbent discs placed on the agar or into wells cut into the agar. Likewise, no inhibition zones were observed from haemolymph collected from neat bleeds or homogenised thorax samples from LPS injected honey bees (0.5mg/ml LPS in PBS), using agar plates that were spread with *Arthobacter globuformis* lawns. All plates were incubated for 24-48 hours at 37°C.

Zones of inhibition were first observed in a small trail using neat haemolymph from LPS injected honey bees (0.5mg/ml LPS in Insect Ringers Solution), comparing the sensitivity of three different bacterial lawns:

Honey bees were exposed to an immune treatment of either LPS injection (0.5mg/ml LPS in Insect Ringers Solution) (n=10 bees) or left as uninjected controls (n=3 bees). Twenty four hours post injection, bees were ice

anesthetised before a small cut was made on the dorsal side of the second abdominal segment allowing 2-6ul of haemolymph to be collected with a pipette before being transferred to a tube washed out Phenylthiourea (PTU) to inhibit melanisation (Ardia *et al.* 2012). Agar plates were spread with one of three bacterial lawns; *E. coli*, *Staphylococcus aureus,* or *M. luteus a*nd was tested for sensitivity to the zone of inhibition from haemolymph. Haemolymph from each honey bee was tested against all three bacteria, expect in one case where a single honey bee could not be tested be tested against *E. coli* and a replacement honey bee was used. Plates were incubated for 24 hours at 37°C.

Two inhibition zones were observed from LPS injected honey bees in the agar plate seeded with *M. lutues.* No other zones were observed.

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*6.1.3. Zone of clearance:*

It was hypothesised that using lyophilized *M. luteus* in a zone of clearance assay rather than live *M. luteus* in a zone of inhibition would standardize plate variation due to differential plate growth. Four plate concentrations of *M. lutues* in 1% agar in PBS (0.5mg/ml, 0.1mg/ml, 0.2mg/ml and 5mg/ml) were tested against haemolymph from honey bees exposed to three levels of immune activation (Uninjected control; n=15, 0.5mg/ml LPS in insect ringers; n=18 and 5mg/ml LPS in insect ringers; n=14 injections). Honey bees were individually bled 24 hours following the immune treatment and 2-4ul of haemolymph was added to 2ul of PTU to inhibit melanisation.

Samples were bulked per immune treatment/*M luteus* plate concentration and replicated three times on each plate. 2ul of haemolymph sample was added to 2mm wells and plates were incubated at 27°C.

Zones of clearances were visible after incubation for 24 hours, but clearest after 72 hours, in all samples from LPS injected honey bees and in one sample from the uninjected control bees, except in the plate concentration of 0.05ml *M. luteus,* where no zones were present. Clearance activity was most apparent in the plate concentration 0.2mg/ml *M. luteus* and clearance activity was similar in samples from honey bees injected with 0.5mg/ml and 5mg/ml LPS (Fig 6.1A & B).

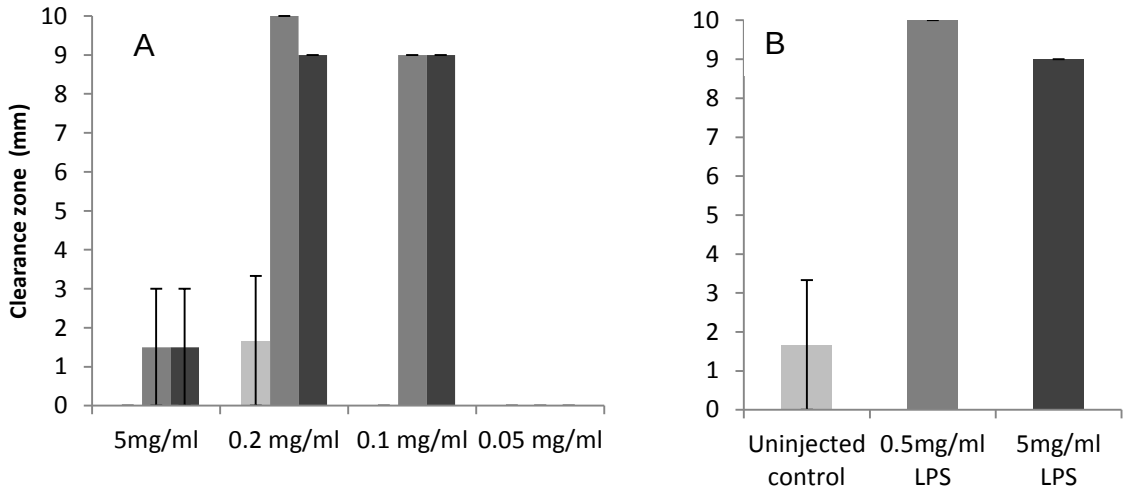


**Figure 6.1.** Clearance activity (zones of clearance) of honey bee haemolymph 24 hours post immune treatment (light; Uninjected control, shaded;0.5mg/ml LPS, dark; 5 mg/ml LPS) on agar plates seeded with different concentrations of lyophilised *M. luteus* (A) and from honey bees injected with three concentrations of LPS (B). Error bars denote S.E.

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*6.2. Glucose Oxidase (GOX).*

Comparisons were made between the results obtained using a commercially available kit (Amplex® Red Glucose/Glucose Oxidase Assay Kit) and the method of Alaux *et al*. 2010, except one modification where we increased the

sample load to 20ul from 10ul per well (see section 2.3.5. for sample preparation). Curves from a standard dilution series and sample dilution series were produced and compared using both methods.

Nine dilutions in a 50% dilution series were prepared from:

1) A stock 100ul/ml GOX solution obtained from the Amplex Red Assay Kit,

2) A bulked sample of ten homogenised bee heads in 1 ml of PBS.

Although the standard dilutions gave negative readings in both cases, possibly due to inhibitory effects of high GOX concentrations (advisory material: Amplex® Red Glucose/Glucose Oxidase Assay Kit), the Amplex Red Kit gave the expected curve for the sample dilution series. We were unable to obtain the expected curve using the method modified from Alaux *et al.* 2010 (Fig 6.2A & B).

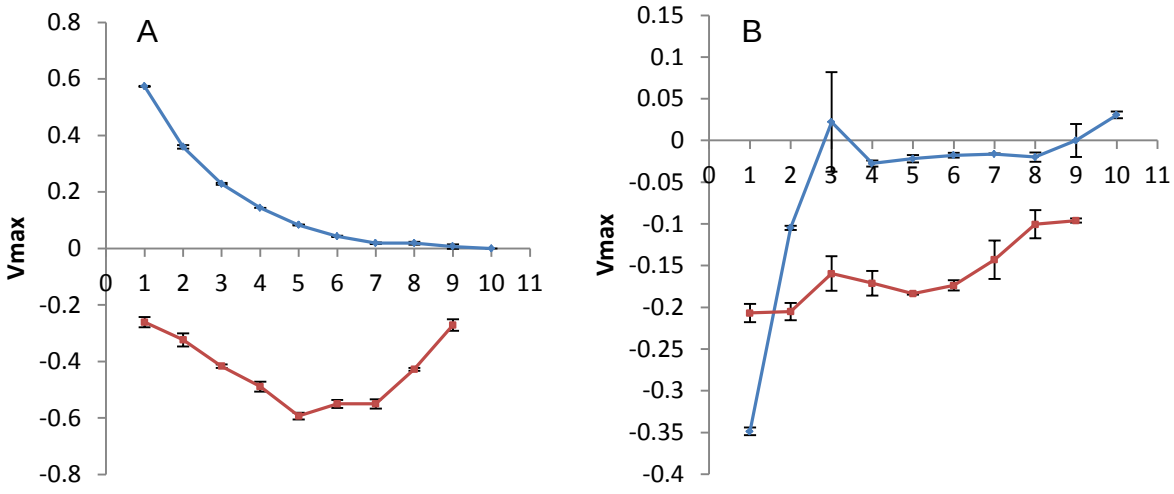


**Figure 6.2.** A 50% dilution series from a bulked sample of ten honey bee heads/ml PBS (blue) and 100ul/ml GOX standard (red) using the Amplex® Red Glucose/Glucose Oxidase Assay Kit (A) and an adaptation of the methods used

by Alaux *et al.* (2010) (B). Error bars denote S.E. between duplicate sample replicates.

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*6.3. References*

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