Aspects of the interactions between honey bees (*Apis mellifera*) and propagules of plant pathogens

Jorgiane Benevenute Parish

M. Sc. in Plant Science, Federal University of Viçosa, Brazil

Thesis submitted to The University of Adelaide for the degree of Doctor of Philosophy

School of Agriculture, Food & Wine

Faculty of Sciences, The University of Adelaide

November 2019

I dedicate this thesis to all the people of Australia

Table of Contents

Abstract

The European honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), may actively or passively collect propagules of plant pathogens and transport them to the hive or to flowers, which can contribute to the spread of plant diseases either locally, within and among crops and native vegetation, or between regions due to migratory beekeeping practices.

As honey bee workers defecate outside the hive, transport of hives could give rise to biosecurity concerns if fungal spores remain viable following passage through the digestive tract. To investigate the probability of this mode of transmission of fungal spores, the viability of spores of the plant pathogenic fungi *Botrytis cinerea* and *Colletotrichum acutatum* after they had passed through the digestive tract of newly emerged honey bee workers was studied. With a mean viability of spores of either fungus in faecal suspensions of less than one percent, survival of fungal spores in honey bee faeces was low. Nevertheless, it is a near certainty that foragers and nurse bees that feed on an infected food source will disperse viable spores through faeces in common pollination scenarios, where hundreds of hives are moved between locations. Thus, these findings have implications for biosecurity restrictions associated with the transport of hives to limit the introduction of plant pathogens to new areas.

To explore possible effects of the intake of fungal spores on honey bee health, the effects of the consumption of spores of *Botrytis cinerea*, *Cladosporium* sp. and *Colletotrichum acutatum* on the survival and development of ovaries and hypopharyngeal glands in honey bee workers were investigated. Honey bees failed to consume diets comprising solely fungal spores nor did they consume diets consisting of pollen and fungal spores at the concentrations of 5:1 and 10:1 in vitro. However, mixtures of pollen with spores of any of the three fungi at the concentration of 20:1 increased the lifespan of workers compared to the sole pollen diets, but had no effect on the development of ovaries or hypopharyngeal glands. These findings suggest that the consumption of fungal spores may provide nutritional benefits for honey bees and may compensate for

nutritional imbalances of poor-quality pollen diets.

The potential role of honey bees in distributing plant pathogenic fungal spores and bacteria in the field was investigated by identifying the fungal and bacterial communities associated with honey bees' corbicular pollen collected from almonds, weeds and native vegetation, in five commercial almond orchards. Next generation sequencing of the ITS regions and the 16S rRNA gene showed pollen species and locations differed with respect to the composition of fungal, but not for bacterial, communities. Pathogens of almonds and other plant species were detected in most pollen species, with a higher relative incidence at the end of almond bloom. These findings contribute to the understanding of the microbial ecology involving honey bees and plant species in almond orchards. Further characterisation of these associations may in future help to improve management of these plant pathogens in almond orchard environments.

This research provides new information about the potential nutritional importance of spore collection and consumption by honey bees, the role of honey bees as vectors of plant pathogenic fungi, and fundamental insights into microbial ecological interactions between honey bee workers and plant species in almond orchards. These results have implications for the health of honey bees and the management of hives in agricultural and natural environments in terms of reducing the risk of transmission of plant pathogens among different crops and areas.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Jorgiane Benevenute Parish Date 15/11/2019

Acknowledgements

I sincerely thank my supervisors Dr. Katja Hogendoorn and Prof. Eileen Scott for their guidance, support, advice and friendship. I attribute my growth as a scientist to their encouragement and supervision.

My gratitude is extended to my external advisor Dr. Kelly Hill whose advice guided me through the molecular lab work and the bioinformatics analysis. Thank you to my co-authors Dr. Raymond Correll and Dr. Nathan S. Watson-Haigh for their expert advice and collaboration.

I would also like to thank:

Dr Tijana Petrovic for guidance with the practical work during the early days of my PhD.

Danny Le Feuvre for providing the hives and for his promptness in providing beekeeping advice.

The University of Adelaide, School of Agriculture, Food and Wine and Adelaide Scholarships International for funding my studies and living allowance.

Elisabeth Fung, Trace Akankunda, Scott Groom, Elisabeth Williamson, Jay Iwasaki, and all the members of the Plant Protection group for their support, encouragement and friendship.

My parents, Jorge and Vanda, and all of my family and friends for their love, support and encouragement throughout my life.

My husband, Matt, for his love, patience, support and for keeping me grounded during these studies and at all other times.

The Lord God for life and all blessings received.

Acronyms, abbreviations and symbols

ANOVA	Analysis of variance
ASV	Amplicon sequence variant
AUD	Australian dollar
%	Percentage
°C	Degree Celsius
BCA	Biocontrol agent
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CFU	Colony forming units
cm	Centimetre
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EP	*Eucalyptus* sp. pollen
h	Hour
HPG	Hypopharyngeal gland
ITS	Internal transcribed space
kg	Kilogram
kGy	Kilogray
km	Kilometre
L	Litre
mg	Milligram
min	Minutes
mL	Millilitre
MP	Multifloral pollen
m	Metre

m^2	Square metres
N	Nitrogen
ng	Nanograms
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NSW	New South Wales
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
Psa	*Pseudomonas syringae* pv. *actinidiae*
rRNA	Ribosomal ribonucleic acid
RO	Reverse osmosis
s	Seconds
SA	South Australia
UK	United Kingdom
USA	United States of America
US$	American Dollar
UV	Ultra-violet
V	Volt
Vic	Victoria
v/v	Volume/volume
V3-V4	Region of the 16S rRNA
WA	Western Australia
µL	Microlitre
16S RNA	Ribosomal RNA

Chapter 1

Introduction and Review of Literature

Chapter 1

Introduction and Review of Literature

1.1 Synopsis

The European honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), is considered the most valuable pollinator of crops worldwide (Delaplane, Mayer & Mayer 2000; McGregor 1976) and their pollination can contribute more than 90% to the yields of some fruit, seed and nut crops (Southwick & Southwick 1992). While foraging, honey bees may collect propagules of plant pathogens that are associated with pollen, nectar or honey dew and transfer them to the hive or to other flowers, which can contribute to the spread of plant diseases (Alexandrova et al. 2002a; Pattemore et al. 2014). On occasion, honey bees have been observed to spend long periods of time actively collecting fungal spores, which are transported to the hives and stored in the pollen cells (Deodicar et al. 1958; Shaw 1999). The ability of honey bees to collect propagules of plant pathogens can pose a biosecurity risk associated with the transmission of plant pathogens among areas as honey bee hives are transported and deployed in crops in large numbers (Klein et al. 2007).

The role of honey bees in vectoring plant pathogens has been demonstrated in several studies (Alexandrova et al. 2002a; Gasparoto et al. 2017; Johnson et al. 1993; Pattemore et al. 2014; Pattemore et al. 2018). Some plant pathogenic bacteria and fungi are known to be vectored through honey bee hives, on the bee's body and in association with pollen (Alexandrova et al. 2002b; Pattemore et al. 2014; Pattemore et al. 2018). Also, the bacterium *Erwinia amylovora*, the causal agent of fire blight of apple and pear trees, can remain viable in the bee's intestines (Alexandrova et al. 2002b).

However, it is not known whether spores of plant pathogenic fungi can survive through the digestive tract of honey bee workers, and whether there is a risk associated with the transmission of plant pathogens through honey bee faeces. Further, the effects of the consumption of fungal spores on the health and survival

of honey bee workers need to be explored in order to better understand the behaviour of honey bees regarding the active collection of fungal spores, and its possible consequences for bee health and hive management. Additionally, there is no information available in the literature about communities of plant pathogenic fungi and bacteria potentially vectored by honey bees between plant species in agricultural areas. The identification of associations between communities of plant pathogens and plant species may support the development of sustainable agricultural and ecological management practices to manage plant diseases.

This chapter comprises a review of literature on the interactions between honey bees and propagules of plant pathogens. While the emphasis of this chapter is on honey bees and their associations with fungal spores, a brief description of the importance of honey bees as pollinators, their biology and use in biological control programs is also included, as these aspects contribute to the scientific basis of this research.

1.2 Importance of honey bees for pollination

The European honey bee is the most commonly managed bee in the world and contributes both directly and indirectly to the human economy; through the production of honey and wax, and as the primary pollinator of many wild and crop plants (McGregor 1976; Watanabe 1994). In Australia, honey bee products have an estimated gross value of AUD$ 90 million and pollination services, delivered by managed hives, feral honey bees and other insects contribute AUD$ 4-6 billion annually to the economy (*Bee Aware* 2019).

Honey bees are preferred pollinators for large monocrop plantings for various reasons. They have large colonies, ranging from approximately 10,000 to 50,000 individuals in which approximately 30% are foragers, and that can be housed in standardised hives and transported in large numbers (vanEngelsdorp & Meixner 2010). In addition, they have a long flight season and a broad diet and can hence be used in a wide variety of crops (McGregor 1976; Watanabe 1994). Furthermore, foraging honey bee workers have a high flower constancy, i.e. they will nearly always forage on the same species of flower during a single trip (Free

1963; Grant 1950), which make them very reliable as crop pollinators. Workers can also perform numerous flights per day to average distances of 5 km from the hive (Beekman & Ratnieks 2000). This allows colonies to be placed in central locations within a crop without affecting pollination on the edges of the plantings (vanEngelsdorp & Meixner 2010). Lastly, workers are able to communicate the location of floral resources to other nest members, which makes honey bees efficient pollinators (Seeley 2014).

1.3 Biology of honey bees

The European honey bee, *Apis mellifera*, is a highly eusocial insect composed of more than 26 subspecies which have been grouped into six evolutionary lineages based on behaviour, morphology and molecular characteristics as well as geographic distribution (Franck et al. 2001; Ruttner 1988; Sheppard et al. 1997; Sheppard & Meixner 2003). Honey bee colonies have a complex division of labour between reproductive and worker castes (Page & Robinson 1991). The reproductive castes, queens and drones, do not participate in labour tasks nor do they forage for resources (Lindauer 1952; Rösch 1925). The workers perform all of the tasks except egg laying and they have temporal castes according to their age (Lindauer 1952; Rösch 1925; Sakagami 1953). For three to four days after emergence workers will clean hive cells and feed the brood with secretion from their hypopharyngeal glands (HPGs); for about four to 12 days after that, they will engage in processing the incoming food; then for about nine to ten days, in the last stage of their life, they will become foragers (Lindauer 1952; Page & Robinson 1991; Rösch 1925). However, as noted by Johnson (2008), these age ranges are influenced by genetic and environmental conditions and represent the averages of several studies conducted with different subspecies of *A. mellifera* around the world.

1.3.1 The diet of honey bees

Honey bee workers collect the food for the hive mainly from flowers of a wide variety of plant species. Sources of carbohydrate for honey bees are floral or extrafloral nectar or honeydew produced, for example, by aphids, scales and planthoppers (Santas 1983). These carbohydrates are transported to the hive in the

honey stomach, regurgitated, stored in the comb and form the basis for the production of honey (Brodschneider & Crailsheim 2010). Pollen is the main source of proteins, amino acids, lipids, starch, sterols, vitamins and minerals, and its protein content and nutritive value differ widely according to the plant species and geographic region (Roulston, Cane & Buchmann 2000). According to Kleinschmidt et al. (1974), pollens with >25%, 20-25% and <20% of crude protein are considered respectively of excellent, average and poor quality for bees. Honey bees need ten amino acids in their diets which are described as "most essential" (leucine, isoleucine and valine), "intermediate essential" (arginine, lysine, phenylalanine, threonine) and "least essential" (histidine, methionine, tryptophan) and limitations in any one of these can suppress colony development (De Groot 1953).

In the colony, honey bee workers mix pollen with regurgitated nectar, honey and glandular secretions to produce bee bread, which differs from freshly collected pollen in having a lower pH, less starch and a higher nutritional value (Ellis & Hayes Jr 2009). This alteration in the quality of pollen stored in honey bee colonies has been attributed to fermentation and production of vitamins by microorganisms such as *Lactobacillus* and *Bifidobacterium* species (Vasquez & Olofsson 2009).

Nurse bees feed on bee bread, which allow their HPGs to produce protein-rich royal jelly, which is used to feed to the larvae and the queen. The quality and quantity of protein consumed by honey bees influence their longevity and physiological aspects such as the development of their HPGs and ovaries (Brodschneider & Crailsheim 2010; Di Pasquale et al. 2013; Hoover, Higo & Winston 2006). The HPGs constitute a paired organ, composed of numerous vesicles or acini connected to a duct, in the head of the workers (Crailsheim et al. 1992; Hoover, Higo & Winston 2006). The volume of the acini can serve as an indicator of the nutritional status of a colony (Renzi et al. 2016).

The survival and development of honey bee colonies as a whole are linked to the availability of nutrients in the environment (Brodschneider & Crailsheim 2010). Honey bees require a variety of pollen sources to meet their nutritional requirements by providing a balance of proteins, essential amino acids and fatty acids

(Brodschneider & Crailsheim 2010), as well as reduce any harmful effects of pollen secondary metabolites (Filipiak et al. 2017). The reduction of floral-rich habitats suitable for bee foraging due to the increasing use of monoculture in commercial agriculture can lead to pollen shortage, deficient nutrition and consequently affect the health of individual bees, colony longevity, physiology and resistance or tolerance to pathogens and pesticides (Brodschneider & Crailsheim 2010; vanEngelsdorp et al. 2009).

1.4 Collection of non-floral particles by honey bees

Honey bee foragers have been observed to collect various unusual materials in the size range of pollen grains, including road and coal dust, sawdust, dead wood, fungal spores and cheese mites (Spencer-Booth 1960). Workers have been seen to collect wax covers from the soft scale *Ceroplastes* sp. and transfer them into the hive, but it is unclear whether they use this wax in the hive (Dimou & Thrasyvoulou 2007). In addition, foraging honey bee workers have been seen to burrow in soil (E. Tihelka pers. obs. cited in Tihelka 2018) and forage on cattle dung (K. Crailsheim pers. obs. cited in Tihelka 2018), but what they collected there was not identified. Prasil et al. (2016) observed honey bee workers collecting and storing in the hives dried *Chlorella* algae, which increased honey yield compared to other years in which the collection of the algae did not occur (Prasil et al. 2016). This suggested that the algae may have served as an important source of nutrients for the bees as they contain 40-60% protein, plus sugars, starches, fats, vitamins, antioxidants and substances that promote cell regeneration and growth. Preliminary experiments in vitro also suggested that *Chlorella* algae have a negative impact on the bacterium *Paenibacillus larvae,* the causal agent of American Foulbrood in honey bee larvae (Prasil et al. 2016). Furthermore, honey bees have been observed to collect a range of fungal spores, which is reviewed in detail below.

Apart from the *Chlorella* algae (Prasil et al. 2016), the effects of the materials described above on the health and survival of honey bees have not been experimentally explored. Some of these particles, such as dust from roads and coal, are unlikely to have any nutritional value. Conversely, dead and decaying wood are a rich source of various microorganisms such as fungi (Stokland, Siitonen & Jonsson 2012), and these have

been suggested to be beneficial for honey bees (Tihelka 2018). However, very little information is available in the literature regarding the effects of the consumption of fungi on the health of honey bees.

1.5 Collection of fungal spores by honey bees

Shaw (1990) classified the collection of fungal spores by honey bees into two categories: 'incidental' or 'the collection of spores in lieu of pollen'. In this case, the term 'incidental' refers to passive acquisition on the body or in association with pollen, nectar and honey dew. This is potentially confusing as incidental can also be taken to mean 'on occasion' and is not in direct contrast with 'active collection, i.e. 'the collection of spores in lieu of pollen'. However, the use of these words is in line with the classification of pollen-collecting behaviour of bees into 'active' and 'incidental' (Doull 1971; Inouye et al. 1994; Parker 1926; Portman & Tepedino 2017; Thorp 2000; Westerkamp 1996), where active pollen collection refers to the collection of pollen from anthers or other floral surfaces, while incidental pollen collection refers to pollen that accumulates on bees as they forage for floral resources. To provide a better contrast between the two types of collection behaviour of pollen and fungal spores by bees, we use the terms 'active' and 'passive' collection.

Shaw (1999) reviewed the literature regarding the active and passive collection of rust fungi, powdery mildew fungi, *Neurospora* and smut fungi by honey bees (Table 1). Since Shaw's (1999) review, the association between honey bees and fungal spores has received little attention in the literature, and only a few additional observations of fungal spore collection have been published. Spores of *Cladosporium* spp., a fungus commonly found in organic matter, were observed to be actively collected by *Apis mellifera* (Modro et al. 2009). In addition, honey bees have been observed collecting urediniospores of *Puccinia psidii* (since re-named *Austropuccinia psidii*) from infected plants in Australia (Carnegie et al. 2010). *A. psidii* is the causal agent of myrtle rust, a disease that has become established on the Eastern seaboard of Australia but remains a biosecurity threat to natural ecosystems elsewhere in the continent (Berthon et al. 2018; Carnegie et al. 2016; Westaway 2016).

Several hypotheses have been put forward to explain the active collection of fungal spores by honey bees. These include the lack of floral resources (Modro et at. 2009; Shaw 1990; Shaw 1999), the potential nutritional value of spores (i.e. nutrients, amino acids and steroids), attractivity of certain spores due to colour or odour, the possibility that some spores may resemble pollen grains and the fact that spores are available during the whole period of a bee's foraging activity, while pollen may be scarce or only available at certain times of the day (Shaw 1990, 1999). For instance, honey bees may collect and store in the hives yellow/orange spores of rust fungi (i.e. *Austropuccinia psidii, Cronartium conigenum*, *Gymnoconia nitens*, *Melampsora euphorbiae*, *M. larici-populina*, *M. medusa, M. ricini, Phragmidium violaceum, Puccinia graminis, P. oxalidis, Uromyces euphorbiae* and *Zaghouania oleae*, Table 1), because they resemble pollen grains in colour and size range (Shaw 1999). However, the factors that contribute to the motivation of honey bees to collect fungal spores remain to be elucidated. Assessment of the effects of spore consumption on bee and hive health has as yet not been done and may help to understand the function of spore collection.

1.6 Honey bees as vectors of plant pathogens

While foraging on flowers, honey bees may actively or passively collect propagules of plant pathogens and transfer them to the hive (Gilliam, Prest & Lorenz 1989; Pattemore et al 2014; Invernizzi et al. 2018; Table 1) or to other flowers, consequently becoming vectors of phytopathogens (Card, Pearson & Clover 2007; Dedej, Delaphane & Scherm 2004; Johnson et al. 1993). The vectoring of plant pathogenic viruses associated with pollen by honey bees was reviewed by Card, Pearson and Clover (2007) but a recent corresponding review of the role of honey bees in collecting and vectoring plant pathogenic fungi and bacteria is not available. Thus, in this section special attention is given to examples of plant pathogenic fungi and bacteria that are vectored by honey bees.

Honey bees have been documented as a vector of the plant pathogenic fungus *Monilinia vaccinii-corymbosi,* which infects open blueberry flowers and causes mummy berry disease (Dedej, Delaphane & Scherm 2004). The involvement of *A. mellifera* in vectoring *M. vaccinii-corymbosi* poses a dilemma for

blueberry growers as pollinator activity is necessary to ensure adequate fruit set but, at the same time, increasing bee density on farms is likely to increase vectoring of the mummy berry pathogen (Dedej & Delaplane 2003; Sampson & Cane 2000). The absence of more recent research publications suggests that the development of management strategies to limit the spread of plant diseases within the forage range of a hive remains a challenge for agricultural producers.

In addition to vectoring *Monilinia*, honey bees can contribute to the dispersal of conidia of the fungi *Colletotrichum acutatum* and *C. gloeosporioides*, causal agents of postbloom fruit drop in citrus plants (Gasparoto et al. 2017). Workers transferred inoculum of *Colletotrichum* spp. from infected to healthy plants maintained in an insect-proof screenhouse and conidium-like structures of *Colletotrichum* spp. were microscopically identified on the bees' bodies. Whether or not honey bees also disperse these pathogens in open field situations has not been investigated. However, if they do, this could lead to disease outbreaks if environmental conditions are conducive (Agostini, Gottwald & Timmer 1993; Gasparoto et al. 2017; Silva-Junior et al. 2014).

In open orchards, passive collection and dispersal of spores of the plant pathogenic fungus *Botrytis cinerea* has been demonstrated. Following contact with experimentally inoculated kiwifruit flowers, honey bees contributed to the dispersal of spores of *B. cinerea* to other flowers within the same orchard and to other plant species in close proximity to the experimental area (Rose 1996). Kiwifruit flowers visited by honey bees within 2 m of flowers experimentally inoculated with spores of *B. cinerea*, carried more propagules than flowers at the same distance that were not visited by bees. This showed that, over short distances, more propagules of *B. cinerea* were dispersed by honey bees than by wind (Rose 1996).

In addition to fungal spores, honey bees can also vector several plant pathogenic bacteria. The bacterium *E. amylovora*, the causal agent of fire blight in apple and pear plants, is vectored by honey bees (Agrios 2005; Alexandrova et al. 2002a; Johnson et al. 1993). Workers are able to transmit inoculum from infected to healthy flowers, which can lead to systemic infections (Johnson et al. 1993; Nuclo et al. 1998).

It is possible that propagules of plant pathogens occur, and are vectored by pollinators, in consortia, i.e. multi-taxa communities of fungi and bacteria (Aleklett, Hart & Shade 2014), in agricultural systems. Nevertheless, there is a lack of published studies that investigate both fungal and bacterial communities associated with plant species in agricultural landscapes and their possible transfer by pollinators, such as honey bees. Information about communities of plant pathogens vectored by honey bees among pollen species could support development of sustainable disease control methods in agricultural and natural ecosystems.

1.6.1 Survival of plant pathogens in honey bee hives

Theoretically, bees can vector plant pathogens over long distances and periods of time, provided their propagules remain viable on or in the body of the bee, or are deposited in the hive. Understanding the viability of the pathogens in the hive is important as transport in hives can cause threats to biosecurity (Alexandrova et al. 2002b; Pattermore et al. 2014; Pattemore et al. 2018.). To date, propagules of three plant pathogens have been tested for their ability to survive in or on honey bee bodies or in the hive: spores of the myrtle rust fungus, *A. psidii,* and the bacteria *Erwinia amylovora* and *Pseudomonas syringae* pv. *actinidiae* (*Psa*). These studies are reviewed below.

Pattemore et al. (2018) provided evidence that spores of *A. psidii* are collected and transported to the hives by honey bee workers. After experimental inoculation, these spores remained viable in the hives and on the bodies of workers for at least nine days. Therefore, as suggested by Pattemore et al. (2018), spores of *A. psidii* may survive for even longer periods in honey bee colonies and therefore the long-distance movement of hives needs to be considered as a potential risk for the transmission of myrtle rust to other locations.

Similarly, *Psa*, the causal agent of bacterial canker in kiwifruit plants, can survive on the body of honey bees caged in hives for up to six days after the bees were experimentally inoculated (Pattemore et al. 2014). Although it is as yet unknown whether *Psa* carried to flowers by honey bees could lead to an infection, this is very likely as *Psa* has been detected in kiwifruit pollen in Italy and in New Zealand, and contaminated

pollen has been implicated in the spread of the disease in both countries (Gallelli et al. 2012; Vanneste et al. 2011).

The survival of *E. amylovora* in hives, on the bee's body and in pollen experimentally inoculated with suspensions of *E. amylovora* has been assessed. Inoculum survived in beehives and on the bee's body for over 24 hours, and on pollen for 72 hours (Alexandrova et al. 2002b). Furthermore, viable bacteria from the intestines of workers experimentally fed with propagules of *E. amylovora* in sucrose solution were isolated into culture 30 hours after initial contamination. This implies that the transport of hives over long distances can pose a risk to the introduction of this pathogen into other areas. Consequently, movement of beehives in Europe is restricted to limit the long-distance spread of fire blight (e.g. EC Plant Health Directive, 2000/29/EC).

It is also possible that propagules of plant pathogens lose viability after long periods stored in hives due to contact with antimicrobial substances, such as propolis, that coat the interior of the nest (Simone-Finstrom & Spivak 2010). Nevertheless, foragers and nurse bees consume freshly-stored pollen preferentially, which may contain viable plant pathogens (Carroll et al. 2017), and they defecate outside the hive (Winston 1991). This implies that, if propagules of plant pathogens remain viable in the hive, as has been shown for *A. psidii*, *E. amylovora* and *Psa*. workers could consume and transport plant pathogens back to the environment in defecation flights and contaminate both agricultural and natural systems.

While Alexandrova et al. (2002b) have demonstrated the survival of plant pathogenic bacteria in the intestine of workers, the viability of spores of plant pathogenic fungi after defecation by honey bees has not been investigated. Viable propagules of the filamentous fungi *Aspergillus flavus* and *Aureobasidium pullulans*, and the yeasts *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*, *S. rouxii*, *Schizosaccharomyces* sp. and *Zygosaccharomyces* spp. have been isolated from both flowers and the honey stomach of honey bee workers foraging for pollen and nectar on alfalfa plants (Batra, Batra & Bohart 1973). Pathogens of honey bees, such as the microsporidian parasite *Nosema* sp. and several viruses (Chen et al.

2006; Chen et al. 2008; Ribière et al. 2007), are known to be transmitted through honey bee faeces contaminating flowers, nectar, pollen and water (Evans and Schwarz 2011). If spores of plant pathogenic fungi survive through the digestive tract of workers, honey bee faeces could serve as an alternative pathway for transmission of plant diseases among neighbouring crops and other locations, as the transport of hives by beekeepers can reach distances up to 1,400 km in Australia (Benecke 2003). Also, as honey bees can forage at distances of over 5 km from a hive (Beekman & Ratnieks 2000), a single colony could contribute to the spread of disease in an area of 80 km^2 (Pattemore et al. 2014). This poses a further risk to plant biosecurity following an incursion of an exotic pathogen.

Table 1.1: Examples of genera and species of plant pathogenic fungi and bacteria found in (a) honey bee hives, (b) bee bread, (c) nectar or honey dew derived from planthoppers, (d) fungal pellets in pollen traps (e) corbicular loads of fungal spores. Sections of this table are cited in Shaw 1999.

Type	Plant pathogen	Country	Sample type	Plants affected or collection host	References
Fungi	*Austropuccinia psidii* (*Puccinia psidii*)	Australia	a	Myrtaceae	Pattemore et al. 2018
		Jamaica	a	Myrtaceae	Chapman 1964
	Alternaria sp.	USA	b, c	Cereals, oil seeds and fruits	Gilliam, Prest & Lorenz 1989; Invernizzi et al. 2018; Yoder et al. 2013
	Aspergillus flavus	USA	b	Cereal grains, legumes and tree nuts	Egorova 1971; Gilliam, Prest & Lorenz 1989; Yoder et al. 2013
	A. niger	USA	b	Grapes, apricots, onions, peanuts and post-harvest nuts	Gilliam, Prest & Lorenz 1989; Yoder et al. 2013
	Bipolaris sp.	USA	b, c	Poaceae	Invernizzi et al. 2018; Yoder et al. 2013
	Caeoma luminatum	USA	e	Wild blackberry	Cook 1885
	Cladosporium sp.	Uruguay	c, e	Almond and peach	Invernizzi et al. 2018
	Cronartium conigenum	Mexico	a	*Pinus*	Trujillo Flores & Peña Garcia 1989
	Drechslera sp.	Uruguay	c	Turfgrasses	Invernizzi et al. 2018
	Fusarium sp.	USA	b	Cereals, melon, pepper, potato and tomato	Yoder et al. 2013
	Gymnoconia nitens (*Caeoma nitens*)	USA	b	Wild blackberry	Lang 1901

T**able 1:** Continuation

Type	Plant pathogen	Country	Sample type	Plants affected or collection host	References
Fungi	*Melampsora euphorbiae*	Pakistan	a	*Ornamental plants*	Ahmad & Ahmad 1986
	M. larici-populina	Australia	e	*Populus* spp.	Walker et al. 1974
	M. larici-populina	Australia	a, e	*Populus nigra* var. *italica*	Dell 1977
	M. medusae	Australia	e	*Populus* spp.	Walker et al. 1974
	M. ricini	South Africa	e	*Ricinus communis*	Wingfield et al. 1989
	Melampsora sp.	South Africa	e	*Euphorbia geniculata*	Johannsmeier 1981
	Melampsora sp.	USA	e	*Salix* sp.	Williams & Tomlinson 1985
	Oidium farinosum	Germany	e	Apple	Kraus 1920
	Puccinia graminis	New Zealand	e	Agricultural machinery	Bennie 1942
	P. oxalidis	USA	e	*Oxalis* sp.	Wolfenbarger 1977
	Rhizopus sp.	USA	b	Fruits and vegetables	Yoder et al. 2013
	Spegazzinia sp.	Uruguay	c	Citrus	Invernizzi et al. 2018

T**able 1:** Continuation

Type	Plant pathogen	Country	Sample type	Plants affected or collection host	References
	Uredineae sp.	Switzerland	e	ni	Maurizio & Hodges 1950
	Uredo luminata	USA	e	Raspberry and Wild blackcap	Zabriskie 1875
	Uromyces euphorbiae	USA	d	Poinsettias	Schmidt, Thoenes & Levin 1987
	Ustilago sp.	UK	e	ni	Betts 1912
	Zaghouania oleae	India	a, e	*Olea dioica and* brood comb	Deodikar et al. 1958
Bacteria	*Erwinia amylovora*	Italy	a, b	Apple and pear	Alexandrova et al. 2002b
	Pseudomonas sp.	France	b	Fruits and tree nuts	Pain & Maugenet 1966
	Pseudomonas syringae	New Zealand	a	Fruits, vegetables, ornamental plants and tree nuts	Pattemore et al. 2014

ni – No Information

1.7 Entomovectoring

The ability of honey bees to serve as vectors of microorganisms has been used for biological control purposes in a technology called entomovectoring (Mommaerts & Smagghe 2011), which is successfully used in Europe to control plant diseases or insect pests (Kapongo et al. 2008; Kevan et al. 2003; Kevan et al. 2008). This technology consists of setting up bee hives on a farm during the flowering period and sprinkling spores of beneficial microorganisms used as biocontrol agents (BCAs) mixed with a carrier (e.g. wheat flour, cellulose, corn starch) on a daily basis into a dispenser fitted on the front of the hive. Upon leaving the hive, the bees acquire spores of the beneficial microorganisms between their body hairs and distribute them to the flowers (Hokkanen & Menzler-Hokkanen 2007). It is also possible that bees could acquire BCAs from contact with flowers that had been previously visited by other bees (Maccagnani et al. 2006). Entomovectoring is considered sustainable because it allows targeted delivery of the BCA to the place of infection, reducing the need for spraying of pesticides and, consequently, the use of heavy machinery, fuel, water and because it involves the use of BCAs, rather than chemicals that can be harmful to humans and contaminate the environment (Mommaerts & Smagghe 2011). In addition, the use of BCA reduces the probability of the emergence of resistance of plant pathogens and insect pests to pesticides.

1.7.1 Biocontrol agents

Examples of effective BCAs distributed by bees that are commercialised as biofungicides and bioinsecticides are: *Bacillus subtilis*, *Bacillus thuringiensis, Beauveria bassiana, Gliocladium roseum*, *Metarhizium anisopliae* and *Trichoderma* spp. (Mommaerts et al. 2008; Mommaerts et al. 2009; Mommaerts, Jans & Smagghe 2010). In Australia, entomovectoring has been used with strains of *Trichoderma* to prevent brown rot in cherry orchards and core rot in apple as part of the routine orchard practices (Hong, Michailides & Holtz 1998; Roco & Perez 2011). *Trichoderma* is

a fungal genus commonly found in soils and its antagonistic activities have been reported to reduce disease caused by phytopathogenic fungi in a number of crops (Kovach, Petzoldt & Harman 2000; Mommaerts et al. 2008; Shafir et al. 2006; Verma et al. 2007). Nevertheless, to be suitable for entomovectoring, BCAs must have no detrimental effect on the bees (Kevan et al. 2008).

1.8 Effects of biocontrol agents and other fungi on bee health and longevity

The effects of some biofungicides and bioinsecticides, used in entomovectoring, on bee health have been investigated. Certain strains of *Bacillus thuringiensis* can cause mortality to *Bombus terrestris* workers (Mommaerts & Smagghe 2011) but have no effect on adult honey bees (Vandenberg & Shimanuki 1986). Also, the use of *Beauveria bassiana* can cause mycosis in bumble bees but has no negative effect on honey bees (Mommaerts et al. 2009). Further, Binab^{TM} T (WP) Biorational Fungicide, which contain *T. harzianum* ATCC20476 and *T. polysporum* ATCC20475, when used at their recommended concentrations for the control of *B. cinerea*, are not harmful to bumble bee workers via contact or oral ingestion (Mommaerts et al. 2008). However, there is no information available in the literature regarding their effects on honey bees. It has not been demonstrated whether BCAs used in entomovectoring are stored with pollen in the hives and consumed by honey bees. This is considered likely as the food stored in hives have been found to contain plant pathogens which are present on flowers (Gilliam, Prest & Lorenz 1989; Pattemore et al. 2018). It is not known whether propagules of BCAs, for example species of *Trichoderma* or other fungal spores, can influence the health and longevity of honey bees.

The quantity of fungal spores actively collected by honey bee workers during occasional foraging trips and the fact that spore collection may take considerable periods of time led Shaw (1990) to infer that some nutritional benefit might be obtained from the collection of spores of certain fungal species. Contrary to this suggestion, negative effects of consumption of fungal spores by honey bee workers have been demonstrated by Schmidt, Thoenes & Levin (1987). These authors reported that the consumption of negligible amounts of spores of the rust fungus, *Uromyces euphorbiae*,

collected from pollen traps, can cause rapid mortality of honey bee workers in vitro (Schmidt, Thoenes & Levin 1987).

Negative effects on honey bee larvae have also been shown after feeding larvae with honey dew secretions from the planthopper *Epormenis cestri* in *Sebastiania schottiana* trees. The honey dew contained spores of the fungi *Alternaria*, *Bipolaris, Cladosporium*, *Drechslera*, *Gonatodobotrium*, *Gonoderma*, *Spegazzinia* and *Tetracladium* spp., *Metacapnodium spongiosum* and *Trichomerium foliicola,* and these spores were found stored in the hives (Invernizzi et al. 2018). Consumption of diets containing nectar contaminated with these honey dew secretions led to mass death of honey bee larvae in vitro and in hives and was linked with a disease called "River disease" which has been reported only in Uruguay (Invernizzi et al. 2018). These authors attributed the mortality of larvae to the possible presence of toxic substances such as xanthoxyline and its derivatives commonly produced by the genus *Sebastiana* (Calixto et al. 1990; Filho et al. 1995) but they did not investigate the role of fungal spores, collectively or individual, in larval mortality.

On the other hand, the benefits of the consumption of fungal extracts by honey bees have been demonstrated (Stamets et al. 2018; Stevanovic et al. 2018). Stamets et al. (2018) administrated 50% sucrose solution mixed with ethanolic extracts of the Basidiomycete species *Fomes fomentarius*, *Ganoderma applanatum, G. resinaceum*, separately and at different concentrations to honey bee workers naturally infected with Deformed Wing Virus and Lake Sinai virus in vitro and to hives. These extracts were produced from mycelia which were grown first on sterile brown rice, then in birch (*Betula papyrifera*) sawdust and then homogenised. The consumption of these extracts by the workers was shown to reduce the titre of both viruses in vitro and extracts of species of *Ganoderma* also reduced the titre of both viruses in workers from hives (Stamets et al. 2018). Furthermore, aqueous extracts from fresh fruiting bodies of the mushroom *Agaricus brasiliensis* slightly increased the strength (i.e. brood and adult population) of experimentally fed honey bee colonies (Stevanovic et al. 2018).

In summary, honey bees are likely to have associations with multiple plant pathogens in many crops, only some of which have been documented. In addition, the risk of transmission of plant pathogenic fungi through the digestive tract of workers, has not been explored. Furthermore, there is limited and contradictory evidence about why workers actively collect fungal spores and the effects of the consumption of spores by bees. It is also unclear whether workers would consume diets comprised solely of fungal spores. The consumption of fungal spores could have positive or negative effects on bee health and survival, and on the physiological development of honey bee ovaries and HPGs, for example, as these are affected by the type of diets consumed by bees (DeGrandi-Hoffman et al. 2010). This thesis explores the association of bees with plant pathogenic fungi, fungal spores as a food source and impacts of their consumption of bee survival and health.

1.9 Crop and fungi chosen for this study

1.9.1 Almond

Almond (*Prunus dulcis* (Mill.) D. A. Webb) production in Australia is located in the Northern Adelaide Plains and the Riverland of SA, Sunraysia in Vic, the Riverina in NSW and the Swan Valley in WA (Almond Board of Australia 2019). All varieties currently grown commercially in Australia are self-incompatible and rely completely on cross-pollination by honey bees to produce the crop (Cunningham, FitzGibbon & Heard 2002; Hill, Stephenson & Taylor 1985). In orchards, the varieties are arranged in alternating rows to maximise exposure of each variety to the pollinizer, ensuring nut set (Connell 2000) and yield (Hendricks 1996). Nonpareil is the most popular variety in Australia (45.9% of all almond trees) due to its consistent yield and good market acceptability, followed by Carmel (26.6%), Price (9.7%), Monterey (7.1%) and others (9.9%) (Almond Board of Australia 2019).

The almond industry contributes approximately AU$429 million to the Australian economy and is currently undergoing a large investment in expansion which will require an increased number

of healthy honey bee hives for pollination (*Bee Aware* 2019). Besides the financial benefits of placing large numbers of hives in almond orchards during bloom, which lasts for a month, this crop is attractive to beekeepers because almond pollen comprises over 25% crude protein (Somerville 2005) and therefore is considered a resource of excellent quality for bees.

Some Australian almond orchards are managed as bare-ground monocultures, because living ground cover has been considered to have a negative influence on moisture availability, orchard microclimate and harvest efficiency (Wilkinson 2012). However, an increasing number of orchards are being planted with cover crops as these benefit pollinators by providing an alternative food source (Saunders, Luck & Mayfield 2013), and improve soil fertility, pest control and productivity (Altieri 1995, 2004; Reintjes, Haverkort & Waters-Bayer 1992).

A number of diseases affect almond production causing yield loss (Adaskaveg & Förster 2000; Horsfield, Wicks & Wilson 2010; McKay et al. 2014; Ram and Bhardwaj 2004). Important fungal diseases in Australian orchards include; anthracnose (caused by *Colletotrichum acutatum*), brown rot (*Monilinia laxa)*, rust (*Tranzschelia discolor*), scab (anamorph *Fusicladium carpophilum*, synonym *Cladosporium carpophilum*) and shot hole (*Wilsonomyces carpophilum*). Research on lower limb dieback has been initiated recently and possible causal agents isolated to date are; Botryosphaeriaceae spp., *Cytospora* spp., *Eutypa lata* and *Pleurostoma richardsiae* (Oswald et al. 2018). Important bacterial diseases of Australian almond orchards include: bacterial canker (*Pseudomonas syringae* pv. *syringae*), bacterial spot (*Xanthomonas campestris* pv. *pruni*) and crown gall (*Agrobacterium tumefaciens* synonym *Rhizobium radiobacter*), *Xylella fastidiosa* presents a quarantine threat (Stefani 2010; Ram and Bhardwaj 2004; Rathé et al. 2012). In Australia, fungicide and occasional bactericides are applied to almond trees over night to minimise the occurrence of disease and exposure of bees and other beneficial insects to the pesticides. However, there is a restricted number of registered products available for use in almond orchards (APVMA 2019; Horsfield, Wicks & Wilson 2010).

Thus, due to the increasing importance of this industry to the Australia economy, almond orchards were chosen to study the diversity profile of fungal and bacterial communities associated with pollen of almonds, weeds and native vegetation that are potentially vectored by honey bees.

1.9.2 Important fungi potentially vectored by bees in Australia

Among the plant pathogenic fungi noted in section 1.6, three are of particular relevance to this research; *Botrytis cinerea* and *Colletotrichum acutatum,* which can infect the flowers and have been demonstrated to be vectored by honey bees (Gasparoto et al. 2017; Rose 1996), and *Cladosporium* spp. which has been observed to be actively collected by honey bees (Modro et al. 2009).

1.9.2.1. *Botrytis cinerea*

The necrotrophic fungus *Botrytis cinerea* Pers. Fr. is the causal agent of diseases such as blossom blight, soft rot and blossom-end rot as well as numerous post-harvest diseases in over 200 crop species (Williamson et al. 2007). Infection caused by *B. cinerea* commonly begins on attached senescent flowers and then, as a soft rot, it spreads to colonise the adjacent developing fruit, for example, in almonds, apples, pears, cucumbers, French beans and strawberries (Asadollahi et al. 2013; Williamson et al. 2007). *B. cinerea* is able to infect green tissues and remain latent until depletion of natural antifungal compounds and accumulation of sugars and organic acids during ripening provide conditions suitable for its germination or growth (Droby & Lichter 2004; Williamson et al. 2007). Flowers are particularly susceptible to infection because they contain pollen which is a nutrient-rich resource that provides ideal conditions for germination of conidia and growth of *B. cinerea*. A combination of methods is typically used in the control of infections caused by *B. cinerea* because of its genetic plasticity, variety of modes of attack, diverse hosts as inoculum sources and ability to survive for long periods as sclerotia in crop debris (Hua et al. 2018;

Leroux 2004; Pertot et al. 2017; Sun et al. 2018). Thus, *B. cinerea* was chosen for this study because of its ubiquity and its importance as a pathogen of numerous crops.

1.9.2.2 *Colletotrichum acutatum*

Species of *Colletotrichum* spp. cause anthracnose in a wide range of crops including almond, apple, citrus, strawberry and stone fruit (Adaskaveg & Förster 2000). Disease symptoms vary from fruit rots to shoot, leaf, and flower blights (Adaskaveg & Hartin 1997; Bernstein et al. 1995; Maas & Howard 1985; Peres et al. 2002). *C. acutatum* JH Simmonds has been identified as a species complex which includes around 30 species, however this name is still used in a broad sense for anthracnose pathogens of fruits in many countries (Braganca et al. 2016). Symptoms caused by *C. acutatum* have been described on blossoms, fruit, leaves and woody tissue at all stages of development in almond orchards (Adaskaveg & Förster 2000). Similar symptoms have been observed in Australia, except for blossom blight, although the fungus has been recovered from asymptomatic blossoms (McKay et al. 2014). *C. acutatum* can cause lesions to the stigmas of experimentally inoculated citrus plants, infecting the citrus pollen grains and producing conidia (Marques et al. 2013). Also, as described above, it has been shown to be vectored by honey bees as workers carried conidia from infected to healthy citrus plants maintained in a greenhouse and conidium-like structures of *Colletotrichum* spp. were observed on the bees' bodies. (Gasparoto et al. 2017). Thus *C. acutatum* was chosen for this study because it can occur in flowers, be vectored by honey bees and cause great economic loss to almond orchards (Gasparoto et al. 2017; Marques et al. 2013; McKay et al. 2014).

1.9.2.3 *Cladosporium* sp.

The genus *Cladosporium* contains over 772 species (Dugan et al 2004), which are cosmopolitan and commonly occur on exposed surfaces (Bensch et al. 2012). *C. cladosporioides* (Fresen) G.A. de Vries and *C. herbarum* Pers. Link, for example, occur ubiquitously as saprophytes or as secondary invaders plant parts (Holliday 2001). The former has also been reported to be a

biological control agent (Paul & Park 2013). Other species of this genus are primary phytopathogens, causing leaf spots and other lesions on plants (Schubert 2005).

Venturia carpophila E.E. Fisher (anamorph *Fusicladium carpophilum* Thum (Ouden); synonym *Fusicladosporium carpophilum* Partridge and Morgan Jones; *Cladosporium carpophilum* Thüm.; *Megacladosporium carpophilum* Thüm. (Vienn-Bourg); *Fusicladium carpophilum* Thüm (Oudem.); *Cladosporium americanum* H.C. Greene) is the causal agent of scab disease in almond, apricot, peach and plum orchards (Fisher 1961) and has relatively restricted distribution worldwide (González-Domínguez, Armengol & Rossi 2017). Scab occurs on leaves and fruit as black freckles of variable size (Bock et al., 2011; Chen, Bock & Wood 2017) and severe infections can result in early defoliation of trees (Ogawa & English 1991). *V. carpophila* overwinters as mycelia in lesions on one-year-old twigs and produces primary inoculum from these lesions (Scherm et al. 2008). However, pseudothecia (sexual fruiting bodies) of *V. carpophila* have also been reported to overwinter in apricot leaves in Australia (Fisher 1961). *V. carpophila* is commonly transported by wind and rain, which may result in infection of fruit during spring (Lan & Scherm 2003). As noted above, Modro et al. (2009) documented that honey bees actively collected spores of *Cladosporium* sp. but did not identify the fungus to species. A culture of an unidentified *Cladosporium* sp. was isolated from bee bread and, in view of the above information, was used in some of the experiments conducted during this study.

1.10 Communities of microorganisms in pollen

Most natural habitats on earth are colonised by a large diversity of microorganisms, the vast majority of which are deemed "unculturable" (Willey, Sherwood & Woolverton 2017). Metabarcoding is an approach that allows the identification of a more complete profile of microorganisms in complex samples compared to conventional culture-based approaches (Anderson et al. 2013; Korpelainen, Pietiläinen & Huotari 2016). Metabarcoding has been extensively used to study the bacterial communities associated with floral nectar, the alimentary

tract of honey bee workers, bee bread from hives placed in different landscapes (i.e. grasslands, urban, broadleaf woodlands, and arable land cover) and pollen from the corbiculae of foragers returning to hives (Anderson et al. 2013; Corby-Harris, Maes & Andeson 2014; Donkersley et al. 2018).

In addition, metabarcoding approaches have been used in studies of bacterial communities of flowers and pollen (Ambika Manirajan et al. 2016; Shade, McManus & Handelsman 2013; Purahong et al. 2018). Comparisons of bacterial communities of apple flowers over time and apple trees experimentally treated or not with the antibiotic streptomycin (Shade, McManus & Handelsman 2013) revealed a succession of microorganisms during flower development and only a slight reduction in the microbial population caused by the antibiotic. Further, bacterial communities of leaves and flowers of kiwifruit plants identified consortia of *Psa* with two other pathogens: *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava* (Purahong et al. 2018). The interactions among these pathogens have not been studied but led these authors to suggest that it may increase the capability of disease development. Research on bacterial communities of pollen of plants that are wind or insect pollinated, showed that the bacterial diversity is strongly influenced by plant species and pollination type (Ambika Manirajan et al. 2016). Pollen of plants that are pollinated by insects had microbiota similar to wind pollinated plants, suggesting the vectoring of microorganisms by pollinators (Ambika Manirajan et al. 2016).

Metabarcoding has also been applied to study the impact of the fungicides metconazole and penthiopyrad on the fungal and bacterial community of almond nectar (Schaeffer et al. 2017). These fungicides reduced fungal richness and diversity but had no influence on the bacterial community composition. Nevertheless, fungal communities associated with honey bees and or pollen species have received little attention. There is no information available in the literature about both fungal and bacterial communities, especially plant pathogens, of pollen species vectored by honey bees in almond orchard landscapes. The identification of such communities in almond orchards may reveal new insights and dynamics between microbes, honey bees and pollen

species and may support the development of management strategies to reduce the transmission of plant pathogens by honey bees.

1.11 Aims and significance of the project

The interactions between honey bees and spores of plant pathogenic fungi are largely unexplored. There is limited and contradictory evidence of the effects of the consumption of fungal spores on the health and longevity of honey bees and the potential for transmission of phytopathogenic fungi through honey bee faeces has not been demonstrated. In addition, there is a lack of information about fungal and bacterial communities, especially plant pathogens, potentially vectored by honey bees among plant species in almond orchards.

Understanding of the effects of the consumption of fungal spores by honey bee workers has implications for the management of honey bee hives, especially in light of the increased number of hives required for pollination of Australian almonds orchards. Additionally, the identification of pathways of transmission of plant pathogenic fungi by honey bees can lead to improvements in decisions about managing agricultural and natural ecosystems and reduction in the risk of transmission of plant pathogen between crops and regions.

Therefore, the aims of this research were to investigate: i) the survival of spores of the plant pathogenic fungi *B. cinerea* and *C. acutatum* through the digestive tract of honey bee workers; ii) the effects of the consumption of spores of *B. cinerea*, *Cladosporium* sp. and *C. acutatum* on the health and longevity of honey bee workers and iii) the identification of fungal and bacterial communities associated with pollen species collected by honey bees in almond orchards.

1.12 Linking statement

The research in this thesis is presented in six chapters, including four research chapters, one of which was accepted for publication in the peer reviewed journal Apidologie (Chapter 2) on the 5th

of September 2019, and one of which was submitted to Scientific Reports (Chapter 4) on the 1st of October 2019.

Chapter 1 consists of an introduction to the thesis and review of literature which focuses mainly on the unexplored aspects of the collection of fungal spores by honey bee workers, honey bees as vectors of plant pathogens and the possible effects of the consumption of fungal spores on bee health. The aims and significance of the research are presented at the end of this chapter.

In Chapter 2 a new route of transmission of spores of plant pathogenic fungi is identified. The research in this chapter shows that spores of the generalist phytopathogenic fungi *B. cinerea* and *C. acutatum* can survive through the digestive tract of summer and autumn honey bee workers for at least 24 h after initial contact with an infected food source and there is a high risk of the dispersal of propagules of these fungi through honey bee faeces. The findings presented in this chapter have biosecurity implications associated with the transport of hives between crops and regions to limit the dispersion of plant pathogens.

While it is not known what factors motivate honey bees to collect fungal spores as sole loads, Chapter 3 comprises of a short communication which reports an observation of the active collection of spores of *Podosphaera xanthii,* the causal agent of powdery mildew on cucurbits, by honey bee workers on zucchini plants in a domestic garden in Adelaide, South Australia during autumn of 2018.

Research on the effects of the consumption of spores of *B. cinerea*, *Cladosporium* sp. or *C. acutatum* in association with a monofloral or multifloral pollen sources is presented in Chapter 4. The consumption of spores of *B. cinerea*, *Cladosporium* sp. or *C. acutatum* in association with pollen extended the lifespan of workers, which provides evidence that honey bees can benefit from the active collection of certain fungal spores. These results have implications for the health and behaviour of honey bees.

Chapter 5 presents further examination of the role of honey bees in vectoring plant pathogenic

fungi and bacterial with pollen among almonds, weeds and native vegetation in almond orchards. This research is the first to report the diversity of plant pathogens associated with pollen of species of plants in and around almond orchards, and has implications for microbial ecology and management of agricultural systems.

Chapter 6 provides a general discussion, integration of the research findings and consideration of future research into the pathways of transmission of plant pathogens, the understanding of the behaviour of honey bees to actively collect fungal spores and its possible consequences on bee health and hive management.

The chapters in this thesis may contain some repetition as they were intended to be published and read separately. References cited in the introduction and review of literature (Chapter 1) and general discussion (Chapter 6) are listed at the end of the thesis.

Chapter 2

Survival and probability of transmission of plant pathogenic fungi through the digestive tract of honey bee workers

Manuscript accepted on 5th September 2019 by Apidologie

Statement of Authorship

Title of Paper	Survival and probability of transmission of plant pathogenic fungi through the digestive tract of honey bee workers
Publication Status	Published ☐ Submitted for Publication ☐ Accepted for Publication **X** Unpublished and unsubmitted work written in manuscript style ☐
Publication Details	Jorgiane B. Parish, Eileen S. Scott, Raymond Correll, Katja Hogendoorn

Principal Author

Name of Principal Author (Candidate)	Jorgiane Benevenute Parish		
Contribution to the Paper	Designed and performed the experiment, analysed the data, and wrote the manuscript.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/11/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Eileen S. Scott		
Contribution to the Paper	Contributed to research ideas and design, provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	12/11/2019

Name of Co-Author	Raymond Correll		
Contribution to the Paper	Contributed to data analysis, and writing of the manuscript		
Signature		Date	11/11/2019

Name of Co-Author	Katja Hogendoorn		
Contribution to the Paper	Contributed to research ideas and design, provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	12/11/2019

Chapter 2

Survival and probability of transmission of plant pathogenic fungi through the digestive tract of honey bee workers

Jorgiane B. Parish[1], Eileen S. Scott[1], Raymond Correll[2], Katja Hogendoorn[1]

[1]School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, South Australia 5005, Australia

[2]Rho Environmetrics, Highgate, South Australia 5063, Australia

Abstract

Honey bees, *Apis mellifera*, have been implicated as vectors of plant pathogens. However, the survival of spores of plant pathogenic fungi through the digestive tract of workers has not been investigated. As workers defecate outside the hive, transport of hives could give rise to biosecurity concerns if fungal spores remain viable following passage through the digestive tract. To determine the likelihood that honey bees serve as vectors, this study investigated the viability of spores of *Botrytis cinerea* and *Colletotrichum acutatum* after passing through the digestive tract of summer and autumn worker bees. For both fungi, the mean viability of spores in faeces suspensions was less than one percent of the initial dose fed to the bees. Although survival was low, the large number of workers per hive implies a high probability of transmission of viable spores through honey bee faeces. Hence, in the case of economically important fungal diseases, transported hives could be a source of inoculum and quarantine restrictions should be considered.

Keywords: *Apis mellifera*, plant pathogenic fungi, honey bee faeces, quarantine, *Botrytis*

cinerea, *Colletotrichum acutatum*

1. INTRODUCTION

Honey bees (*Apis mellifera* L.) have been implicated as vectors of plant pathogens by several authors (Alexandrova et al. 2002a; Dedej et al. 2004; Gasparoto et al. 2017; Pattemore et al. 2014; Shaw 1999). Honey bees are known to disperse pathogens, such as the bacterium *Erwinia amylovora* (the causal agent of fire blight of apple and pear trees), from inoculum placed in hives to flowers, leading to the establishment of the bacteria on the surface of the stigmas and, consequently, infection (Johnson et al. 1993). In addition, *E. amylovora* can remain viable in beehives and on the bee's body for over 24 h, on pollen for 72 h and for a period of 36 h in the bee's intestine (Alexandrova et al. 2002b). In Europe, these findings have led to the implementation of biosecurity restrictions associated with the transport of beehives to prevent the long-distance transport of the pathogen (e.g. EC Plant Health Directive, 2000/29/EC). Additionally, spores of *Austropuccinia psidii,* the causal agent of myrtle rust, survived in honey bee hives and on the body of workers for at least 9 days after they had been experimentally inoculated (Pattemore et al. 2018). These authors provided evidence that myrtle rust spores are brought to the hives by foragers, even when there were no signs of workers actively collecting spores, and neither the hive environment nor the grooming behaviour of the bees killed or removed the spores. In addition to the suggestion by Pattemore et al. (2018) that spores of *A. psidii* could survive in hives for extended periods of time, and that long distance movement of hives needs to be considered as a potential risk for the transmission of myrtle rust spores to other locations, it is likely that the transmission of plant pathogens could also occur between neighbouring crops within the forage range around a hive.

While attention has been given to the presence of plant pathogenic bacteria and fungi on the body of honey bees, the survival of fungal spores after consumption and passage through the

digestive tract has not been studied. However, species of yeasts commonly found in nectar can survive and coexist in the gastrointestinal tract of bumble bees during several weeks of winter hibernation when the availability of flowers is severely reduced, thereby using the gastrointestinal tract as a reservoir (Pozo et al. 2018). Although Pozo et al. (2018) did not show the routes and rates by which yeasts are transmitted from the gastrointestinal tract of bumble bees to flowers in the following spring, this may involve a faecal route. Faecal transmission of viruses has been demonstrated (Ribière et al. 2007) but the faecal transmission of plant pathogenic fungi is as yet unstudied, and there is limited information on whether the transport of hives could lead to the dispersal of plant pathogenic fungi between regions.

It has been documented that honey bees actively collect spores of phytopathogenic fungi and transport them to the hives (Shaw 1999) or to other flowers (Altizer et al. 1998). As workers defecate outside of the hive (Winston 1991), consumption of such spores could raise a biosecurity concern if they remain viable in the hives and survive through the digestive tract of honey bee workers. This is particularly important for generalist phytopathogenic fungi, such as *Botrytis cinerea* and *Colletotrichum acutatum*, which cause disease on a wide range of horticultural crops (Adaskaveg and Hartin 1997; Hatcher 1995; Williamson et al. 2007).

Therefore, this study aimed to (i) investigate the viability of spores of *B. cinerea* and *C. acutatum* after passage through the digestive tract of honey bee workers; and (ii) estimate the probability of honey bees dispersing spores of plant pathogenic fungi through their faeces. This information is crucial to understand this potential pathway of plant disease transmission by honey bees and whether the movement of hives for crop pollination purposes poses a potential biosecurity threat in terms of spread of plant pathogenic fungi.

2. MATERIALS AND METHODS

2.1. Preparation of bees

Feeding experiments were conducted in mid-February and late March of 2018 using summer and autumn workers, respectively. These two types of workers were chosen because environmental factors related to season can influence the worker's metabolism due to changes in the levels of microbiocidal enzymes in the digestive tract (Orčić et al. 2017), which could damage or digest fungal spores. For each experiment, newly emerged workers were obtained by placing brood frames with late stage pupae, originating from three hives, in an incubator in the dark at 34 ^{o}C and approximately 50-70% relative humidity (Hatjina et al. 2013). Colonies were kept at the Waite Campus, University of Adelaide, Adelaide, Australia and were regularly inspected for symptoms of disease. Newly emerged workers contain few or no bacteria in their gut (Martinson et al. 2012) and acquire gut microbes by feeding on the supplies in the comb and contact with its surfaces or by trophallaxis of nectar with older foragers (Powell et al. 2014). Therefore, workers were collected directly after emergence to minimize potential contamination. Equal numbers of workers were collected from each comb.

Individuals were harnessed in 500 μL plastic centrifuge tubes by placing a paper collar between their heads and thoraces (Hori et al. 2006). Small amounts of cotton wool were placed at the bottom of each tube to support the weight of the abdomen. Preliminary experiments showed that this support prolonged survival of the harnessed workers. The tubes containing the workers were placed in a tube holder and incubated in the conditions described above.

2.2. Feeding experiment

Bees were fed in the morning and afternoon for 3 days. As newly emerged workers have poor learning and memory performance (Masson and Arnold 1984), on the first day bees were fed by using a sterile needle to gently unfold their probosces and offered 50% sucrose solution. As demonstrated by Behrends and Scheiner (2009), after 24 hours of conditioning, a few workers began to show response to gustatory stimuli and were subsequently fed using the proboscis extension reflex method (Bitterman et al. 1983; Takeda 1961), others were fed as described above. Only bees that participated in all feeding schedules were used in the experiment. Each worker received 10 μL of 50% sucrose solution containing 0.01% (v/v) Tween 20 with or without fungal spores at the concentration of 10^6 spores/mL at each feeding time. This amount of liquid is less than their average daily intake of 33 μL (Decourtye et al. 2003) and was chosen to prevent early defecation. In the control treatments, workers received sterile sucrose solution only. Each treatment and control consisted of 21 bees.

The concentration of fungal spores administrated to the bees was based on the amount of colony forming units (CFU) of plant pathogens found on bees foraging on infected flowers, which commonly ranges from 10^3 to 10^6 CFU per bee (Dedej et al. 2004; Pattemore et al. 2014).

2.3. Preparation and assessment of spore suspensions

Two plant pathogenic fungal species were used in the bioassays, *Botrytis cinerea* (CBS 140599) and *Colletotrichum acutatum* (EU670080*)*, which had been isolated from grape and almond, respectively. The isolates were obtained from the Plant Pathology laboratory of the School of Agriculture, Food and Wine, University of Adelaide. Mycelia were cultured on potato dextrose agar (PDA, Difco) in 9-cm diameter Petri plates at 25 °C under cool white fluorescent and black light with a photoperiod of 12 h for 10 days. Cultures were established

on three consecutive days to generate suspensions with spores of the same age in sterile sucrose solution, the latter to provide a source of carbohydrates for the workers. Spores were harvested by flooding the cultures with 15 mL of sterile 50% sucrose solution containing 0.01% (v/v) Tween 20 and dislodging spores from the mycelia using a sterile plastic spreader. The resulting suspensions were filtered through three layers of sterile cheesecloth to remove any hyphal fragments present (Scheuerell and Mahaffee 2006). The concentration of spores in each initial stock suspension was determined using a Neubauer haemocytometer and a compound microscope, and the suspension diluted with 50% sucrose solution to obtain the target concentration. To determine the viability of spores, aliquots of 0.1 mL of the dilution 1:1000 of spore suspension in sucrose solution were spread onto three replicate plates of PDA and incubated as described above. CFU were counted 36 h after plating. Suspensions were prepared using fresh cultures every day.

2.4. Faeces collection and plating

Approximately 24 h after the bees were last fed, the faeces were collected by placing the abdomen of individual bees over a sterile 1.5 mL tube and gently applying pressure to the abdomen (Bailey et al. 1983; Ribière et al. 2007). Prior to faeces collection, individual workers were surface disinfected for one minute using 1% sodium hypochlorite solution to remove any superficial contaminants, and then rinsed three times in 1 mL of sterile water. A volume of 1 mL of sterile water containing 0.01% (v/v) Tween 20 was added to each tube containing faeces. The suspensions of faeces for each bee were vortexed for 1 minute, and three replicate aliquots of 0.1 mL from each tube were spread on PDA medium containing streptomycin sulfate (50 mg/L) and chloramphenicol (250 mg/L) in 9-cm diameter Petri plates and incubated as described above. The antibiotics were used to inhibit the growth of bacteria. To control for

surface contamination, the final rinse from the surface disinfection was also collected and plated in the same manner. After 36 h, CFU were determined for all plates. Additionally, the faecal material of a group of 21 newly emerged workers that had not participated in any of the feeding schedules was collected and plated as described above to assess the initial microbial composition of the intestinal contents of the newly emerged workers. None of the bees used in the experiments defecated naturally during the period of 3 days.

2.5. Confirmation of identity of fungal isolates

In order to ascertain the identity of the colonies obtained from plates spread with the faeces suspensions, representative colonies were transferred to PDA amended with streptomycin sulfate (50 mg/L) and incubated for 10 days at 25 ^{o}C. To obtain pure cultures, plates were flooded with sterile water containing 0.01% (v/v) Tween 20, and spore suspensions were prepared as described above. Aliquots of 0.1 mL of each suspension were spread over reverse osmosis water agar (20 g/L, Difco) in a Petri dish. After 8 h, single germinating spores were located using a dissecting microscope in a laminar flow cabinet and transferred to three replicate plates of PDA and incubated at 25 °C under cool white fluorescent and black light for 10 days as described previously.

Mycelia were collected by scraping the surface of single spore-derived cultures using a sterile scalpel. DNA was extracted and purified using the Wizard Genomic DNA Purification Kit (Promega, USA), following the manufacturer's instructions. The spacer regions ITS1 and ITS2 were amplified and sequenced using the primers ITS1 and ITS4 (White et al. 1990).

Polymerase chain reaction (PCR) was carried out with 2 µL DNA (25 ng/µL), 8.5 µL nuclease-free water, 1 µL of each primer and 12.5 µL GoTaq Colorless Master Mix 2x (Promega, USA) in a total volume of 25 µL. The PCR cycles consisted of 2 min initial denaturation at 94°C,

followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 1 min of elongation at 72°C, and a final 10 min elongation at 72°C. PCR was performed in a TC-512 thermal cycler (Techne, UK). PCR amplification products were analysed by electrophoresis at 110 V for 30 min in a 2% agarose gel containing 0.003% nucleic acid staining solution (GelRed, Intron Biotechnology) in 1 x Tris borate EDTA buffer and viewed under UV light. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) and sent to the Australian Genome Research Facility for bidirectional Sanger sequencing. The forward and reverse sequences were edited using Geneious software version 9.1.4 (Kearse et al. 2012). Consensus sequences were compared with sequences deposited in GenBank using the Basic Local Alignment Sequence Tool (BLAST) (Boratyn et al. 2013). The GenBank sequences of the original isolates were also selected and aligned with the sequences obtained from this study.

2.6. Data analysis

All statistical analyses were executed in R version 3.3.2 (R-Project 2019). Normality and homoscedasticity assumptions were tested for all variables by QQ plot and Shapiro test, and by Bartlett test, respectively. The daily data for the viability of spore suspensions of *B. cinerea* and *C. acutatum* in 50% sucrose solution were subjected to generalized linear models (GLM) with binomial error distribution. Data for survival of spores of *B. cinerea* and *C. acutatum* in honey bee faeces during summer and autumn were log transformed and analysed using linear models. The ggplot2 package was used for plotting the figures (Wickham 2011).

The probability of dispersal of spores of plant pathogenic fungi through honey bee faecal matter can be estimated by: $P = 1 - (1 - p)^{(tmn)}$, where p is the probability of a bee carrying a viable spore, t is the average number of trips per bee, m the number of workers per hive and n the number of hives leading to the probability of at least one viable spore being transferred through

honey bee faecal matter.

3. RESULTS

None of the controls, i.e. i) the intestinal contents of newly emerged workers that did not participate in either feeding experiment, ii) the suspensions from the last rinses of bees and iii) the sterile sucrose solutions used to feed bees in the control treatments, yielded microorganisms that would grow on PDA containing antibacterial antibiotics in the conditions imposed.

The spores of both fungi had a mean viability of greater than 85% in 50% sucrose solution (Fig. 1). There was a significant difference between days in the viability of spores in the suspensions used to feed summer bees (*B. cinerea*, X_2^2=32.42, P<0.01; *C. acutatum*, X_2^2= 38.45, P<0.01), but not in the suspensions used to feed autumn bees (*B. cinerea*, X_2^2=1.06, P>0.05; *C. acutatum*, X_2^2=0.04, P>0.05).

No colonies of *B. cinerea* or *C. acutatum* grew on the plated faeces of bees that had been fed sterile sucrose solution containing Tween 20. There were significant differences in the survival of spores through the digestive tract between the seasons and fungal species ($F_{(3, 80)}$=12.32, P<0.001, Fig. 2). Survival of spores through the digestive tract was significantly higher for autumn workers than for summer workers (*B. cinerea*, $F_{(1,40)}$=5.30, P<0.05; *C. acutatum*, $F_{(1, 40)}$ =0.22, P<0.01) and the mean number of CFUs of *C. acutatum* in faeces suspensions was significantly greater than *B. cinerea* for both summer ($F_{(1, 40)}$=5.88, P<0.05) and autumn bees ($F_{(1, 40)}$=11.81, P<0.05). There was no evidence of proportion changes for the survival of *B. cinerea* and *C. acutatum* among the seasons ($F_{(1, 80)}$= 2.66, P>0.05). Overall, less than one percent of the conidia in the initial dose fed to the bees over a period of 3 days resulted in colonies on PDA supplemented with antibacterial antibiotics.

The predominant fungal colonies isolated from the faeces of the bees resembled *B. cinerea* and

C. acutatum in morphology and were confirmed to be identical to those provided in the feeding suspensions. In addition, in the experiment with summer bees, single colonies of *Aspergillus* sp. were detected from the faeces of two workers and a single colony of *Penicillium* sp. was detected from the faeces of a single worker that received suspensions of *B. cinerea*. Furthermore, a single colony of *Penicillium* sp. was detected from the faeces of a single worker that received suspensions of *C. acutatum*. In the experiment with autumn bees a single isolate of *Cladosporium* sp. was detected from the faeces of a worker from the *C. acutatum* treatment. The sequences generated by PCR ranged from 297 to 513 bp in length and the accession numbers are given in the supplementary files (Table S1).

Given that defecated spores had a viability of approximately 1%, the probability of dispersal of spores of plant pathogenic fungi through honey bee faecal matter, *P*, approaches 1 when 1, 10 or 100 workers from multiple hives foraging in an area consume 6 x 10^4 spores (Fig. 3).

DISCUSSION AND CONCLUSIONS

Spores of two common plant pathogenic fungi, *B. cinerea* and *C. acutatum,* can survive passage through the digestive tract of honey bee workers for at least 24 h, and viable spores have a high probability of being dispersed through faecal matter. These findings concur with the report by Alexandrova et al. (2002b) that the bacterium *E. amylovora* remained viable in the workers' intestines 36 h after initial contamination. The period of time for which propagules of plant pathogens remain viable in the intestines of workers is critical as the duration of transport of hives between crops and regions is typically less than 24 h. In addition, there is potential for long range dispersal due to migratory beekeeping practices, which extend to distances of up to 4,500 km in the USA (Simone-Finstrom et al. 2016) and 1,400 km in Australia (Benecke 2003).

Although the survival of plant pathogen propagules through the digestive tract of workers will

be affected by conditions such as pH, osmotic pressure, enzymes, and oxygen and nutrient availability imposed on the spores in the digestive tract (Dillon and Charnley 1991), it is likely that the majority of the spores were digested. In honey bees, enzymes such as the antioxidant superoxide dismutase and glutathione S-transferase are known to have microbiocidal activity (Nikolenko et al. 2011). The activity of these enzymes is influenced by environmental factors associated with different seasons and in winter workers their levels decrease (Korayem et al. 2012; Orčić et al. 2017). This may explain the larger proportion of spores that survived in autumn than in summer bees, which could, consequently, increase the risk of infection of autumn or winter flowering crops such as almonds and canola. Nevertheless, it is possible that different outcomes would have been obtained if the experiments had been conducted using older workers as their gut microbiota (Martinson et al. 2012) could potentially enhance or interfere with digestion of fungal spores.

In healthy bee colonies, workers will defecate outside their hives. In theory, this could spread spores of plant pathogenic fungi over a typical forage range of 5 km from the hive (Beekman and Ratnieks 2000) if workers feed on spores or infected food sources and the spores survive through their digestive tract. Such vectoring of generalist plant pathogenic fungi through faeces could affect agricultural and natural systems as these species of fungi can cause disease in a large range of host plants, including almond, apple, citrus, stone fruit and strawberry (Adaskaveg and Hartin 1997; Hatcher 1995; Williamson et al. 2007). Furthermore, dispersal of viable spores would occur even if only a small percentage of spore-fed bees defecate, because of the large number of bees per hive, the number of hives per unit area and the number of foraging trips made by each bee. As a single spore can induce infection in favourable conditions (Deverall and Wood 1961), if foragers and nurse bees feed on an infected food source in or outside of the hive, dispersal of viable spores through faeces is a near certainty in common pollination scenarios where 100 or more hives are moved from one location to

another. However, the incidence of contamination through hive transport depends on whether the spores can cause infection on plants after defecation. While such investigation was beyond of the scope of our study, it needs to be considered for future work.

The survival of spores of plant pathogenic fungi other *B. cinerea* and *C. acutatum* has not, to our knowledge, been documented. Of particular concern for Australia is *Austropuccinia psidii*, the causal agent of myrtle rust, which is considered a biosecurity threat (Berthon et al. 2018; Carnegie et al. 2016; Westaway 2016). Honey bees are known to actively collect rust spores (Chapman 1964; Shaw 1990) and have been observed collecting urediniospores of *Puccinia psidii* (since re-named *Austropuccinia psidii*) from infected plants in Australia (Carnegie et al. 2010). Spores of *A. psidii* and many other plant pathogenic fungi typically are spread by wind, often many kilometres from the initial infected area (Makinson 2018). Movement of honey bee hives may pose a potential pathway for the spread of myrtle rust against the prevailing wind direction and beyond its current distribution along the eastern seaboard and the North Territory of Australia. As noted by Pattemore et al. (2014), little can be done to manage the transport of plant pathogens within the foraging range of a hive but quarantine restrictions associated with movement of bee hives from currently infected to uninfected areas should be considered.

Spores of *B. cinerea* and *C. acutatum* in 50% of sucrose solution showed high viability when plated on PDA (i.e. >80%). However, limited variability (approximately 10%) was observed among the suspensions used to feed summer bees, which might reflect genetic variation resulting from the multinucleate nature of spores of *B. cinerea* (Roper et al. 2011), which could translate into differences in viability in the conditions imposed. Similarly, several species in the genus *Colletotrichum* can occasionally produce multinucleate spores (TeBeest et al. 1989). In addition, although the colonies used in this study were of the same age and were maintained in the same conditions, it is possible that some spores had reduced viability such that they failed

to germinate within 36 h.

This research was conducted using microbiological culture methods, which has the advantage of providing quantitative results that take into account the viability of the spores that survive through the digestive tract of honey bee workers and grow in the conditions imposed. By contrast, molecular techniques such as real time PCR would have quantified both viable and non-viable spores. Apart from implications for biosecurity and epidemiology, the findings that the majority of fungal spores consumed lose their viability inside the gut, combined with the information that honey bee workers collect fungal spores, may indicate that these spores are digested and are an alternative nutrient source for bees. Large scale experiments are needed to further understand the complexities of the interactions among different species of microorganisms in the digestive tract of honey bee workers, to determine how different factors influence the survival of spores in the digestive tract of honey bees, for how long workers will continue to produce infective faeces after initial contamination and whether honey bee hives can serve as long term reservoirs of phytopathogenic fungi.

ACKNOWLEDGEMENTS

We acknowledge Dr Tijana Petrovic and Dr Ricardo Feliciano dos Santos for guidance with laboratory work, the Adelaide Scholarships International (ASI) scheme for supporting the first author and the School of Agriculture, Food and Wine for operating support.

AUTHOR CONTRIBUTIONS

JBP: design and execution of the experiment, data analyses, and writing; ESS, KH: experimental design, technical assistance, and manuscript editing; RC: data analyses and manuscript editing; All authors read and approved the final manuscript.

REFERENCES

Adaskaveg JE, Hartin RJ (1997) Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California Phytopathology 87:979-987 doi:10.1094/phyto.1997.87.9.979

Alexandrova M, Cimini B, Bazzi C, Carpana E, Massi S, Sabatini AG (2002a) The role of honeybees in spreading *Erwinia amylovora* Acta Horticulturae 590:55-60

Alexandrova M, Porrini C, Bazzi C, Carpana E, Bigliardi M, Sabatini AG (2002b) *Erwinia amylovora* longevity in beehives, beehive products and honeybees Acta Horticulturae 590:201-205

Altizer SM, Thrall PH, Antonovics J (1998) Vector behavior and the transmission of anther-smut infection in *Silene alba* The American Midland Naturalist 139:147-164

Bailey L, Ball B, Perry J (1983) Association of viruses with two protozoal pathogens of the honey bee Annals of Applied Biology 103:13-20

Beekman M, Ratnieks FLW (2000) Long-range foraging by the honey-bee, *Apis mellifera* L Functional Ecology 14:490-496 doi:10.1046/j.1365-2435.2000.00443.x

Behrends A, Scheiner R (2009) Evidence for associative learning in newly emerged honey bees (*Apis mellifera*) Animal Cognition 12:249-255

Benecke FS (2003) Commercial beekeeping in Australia. A report for the Rural Industries Research and Development Coorporation. RIRDC Publication, ACT, Australia

Berthon K, Esperon-Rodriguez M, Beaumont L, Carnegie A, Leishman M (2018) Assessment and prioritisation of plant species at risk from myrtle rust (*Austropuccinia psidii*) under current and future climates in Australia Biological Conservation 218:154-162

Bitterman M, Menzel R, Fietz A, Schäfer S (1983) Classical conditioning of proboscis extension in honeybees (*Apis mellifera*) Journal of Comparative Psychology 97:107-119

Boratyn GM et al. (2013) BLAST: a more efficient report with usability improvements Nucleic Acids Research 41:W29-W33 doi:10.1093/nar/gkt282

Carnegie AJ, Kathuria A, Pegg GS, Entwistle P, Nagel M, Giblin FR (2016) Impact of the invasive rust *Puccinia psidii* (myrtle rust) on native Myrtaceae in natural ecosystems in Australia Biological Invasions 18:127-144

Carnegie AJ, Lidbetter JR, Walker J, Horwood MA, Tesoriero L, Glen M, Priest MJ (2010) *Uredo rangelii*, a taxon in the guava rust complex, newly recorded on Myrtaceae in Australia Australasian Plant Pathology 39:463-466

Chapman G (1964) Urediospore collections by honey bees from *Puccinia psidii* Annals of the Entomological Society of America 57:264

Decourtye A, Lacassie E, Paham-Delegue M (2003) Learning performances of honey bees (*Apis mellifera* L.) are differentially affected by imidacloprid according to the season Pest Management Science 59:269-278 doi:10.1002/ps.631

Dedej S, Delaphane KS, Scherm H (2004) Effectiveness of honey bees in delivering the biocontrol agent *Bacillus subtilis* to blueberry flowers to suppress mummy berry disease Biological Control 31:422-427 doi:10.1016/j.biocontrol.2004.07.010

Deverall BJ, Wood RKS (1961) Infection of bean plants (*Vicia faba* L.) with *Botrytis cinerea* and *B. fabae* Annals of Applied Biology 49:461-472

Dillon RJ, Charnley AK (1991) The fate of fungal spores in the insect gut. In: Cole TG, Hoch CH (eds) The fungal spore and disease initiation in plants and animals. Plenum Press, New York and London, pp 129-156 doi:https://doi.org/10.1007/978-1-4899-2635-7

Gasparoto MCG, Lourenco SA, Tanaka FAO, Sposito MB, Marchini LC, Silva GJ, Amorim L (2017) Honeybees can spread *Colletotrichum acutatum* and *C. gloeosporioides* among citrus plants Plant Pathology 66:777-782 doi:10.1111/ppa.12625

Hatcher PE (1995) 3-Way interactions between plant pathogenic fungi, herbivorous insects and their host plants Biological Reviews 70:639-694 doi:10.1111/j.1469-185X.1995.tb01655.x

Hatjina F, Papaefthimiou C, Charistos L, Dogaroglu T, Bouga M, Emmanouil C, Arnold G (2013) Sublethal doses of imidacloprid decreased size of hypopharyngeal glands and respiratory rhythm of honeybees in vivo Apidologie 44:467-480

Hori S, Takeuchi H, Arikawa K, Kinoshita M, Ichikawa N, Sasaki M, Kubo T (2006) Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L Journal of Comparative Physiology A 192:691-700 doi:10.1007/s00359-005-0091-4

Johnson KB, Stockwell VO, Burgett DM, Sugar D, Loper JE (1993) Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by honey bees from hives to apple and pear blossoms Phytopathology 83:478-484 doi:10.1094/Phyto-83-478

Kearse M et al. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data Bioinformatics 28:1647-1649

Korayem AM, Khodairy MM, Abdel-Aal A-AA, El-Sonbaty AA (2012) The protective strategy of antioxidant enzymes against hydrogen peroxide in honey bee, *Apis mellifera,* during two different seasons Journal of Biology and Earth Sciences 2:93-109

Makinson RO (2018) Myrtle rust in Australia - a draft action plan, presented at the Plant Biosecurity Cooperative Research Centre's National Science Exchange. Melbourne, Australia

Martinson VG, Moy J, Moran NA (2012) Establishment of characteristic gut bacteria during development of the honeybee worker Applied and Environmental Microbiology 78:2830-2840

Masson C, Arnold G (1984) Ontogeny, maturation and plasticity of the olfactory system in the workerbee Journal of Insect Physiology 30:7-14

Nikolenko AG, Saltykova ES, Gaifullina LR (2011) Molecular mechanisms of antioxidant protective processes in honeybee *Apis mellifera*. In: Farooqui T & Farooqui AA (eds) Oxidative stress in vertebrates and invertebrates: molecular aspects of cell signaling. John Wiley & Sons, Hoboken, NJ, USA, pp 279-293

Orčić S et al. (2017) Seasonal variation in the activity of selected antioxidant enzymes and malondialdehyde level in worker honey bees Entomologia Experimentalis et Applicata 165:120-128

Pattemore DE, Bateson M, Buxton M, Pegg G, Hauxwell C (2018) Assessment of the risks of transmission of myrtle rust (*Austropuccinia psidii*) spores by honey bees (*Apis mellifera*). A Plant & Food Research report prepared for: Ministry for Primary Industries.

Pattemore DE, Goodwin RM, McBrydie HM, Hoyte SM, Vanneste JL (2014) Evidence of the role of honey bees (*Apis mellifera*) as vectors of the bacterial plant pathogen *Pseudomonas syringae* Australasian Plant Pathology 43:571-575 doi:10.1007/s13313-014-0306-7

Powell JE, Martinson VG, Urban-Mead K, Moran NA (2014) Routes of acquisition of the gut microbiota of *Apis mellifera* Applied and Environmental Microbiology 80:7378-7387

Pozo MI, Bartlewicz J, van Oystaeyen A, Benavente A, van Kemenade G, Wäckers F, Jacquemyn H (2018) Surviving in the absence of flowers: do nectar yeasts rely on overwintering bumblebee queens to complete their annual life cycle? FEMS microbiology ecology 94:fiy196

R-Project (2019) R Core Team. R: A Language and Environment for Statistical Computing., 1.2.1335 edn., Vienna, Austria.

Ribière M, Lallemand P, Iscache AL, Schurr F, Celle O, Blanchard P, Oliver V, Faucon JP (2007) Spread of infectious chronic bee paralysis virus by honeybee (*Apis mellifera* L.) feces Applied and Environmental Microbiology 73:7711-7716 doi:10.1128/aem.01053-07

Roper M, Ellison C, Taylor JW, Glass NL (2011) Nuclear and genome dynamics in multinucleate ascomycete fungi Current Biology 21:R786-R793

Scheuerell SJ, Mahaffee WF (2006) Variability associated with suppression of gray mold (*Botrytis cinerea*) on geranium by foliar applications of nonaerated and aerated compost teas Plant Disease 90:1201-1208

Shaw DE (1990) The incidental collection of fungal spores by bees and the collection of spores in lieu of pollen. Bee World 71:158-176

Shaw DE (1999) Bees and fungi, with special reference to certain plant pathogens Australasian Plant Pathology 28:269-282 doi:10.1071/ap99044

Simone-Finstrom M, Li-Byarlay H, Huang MH, Strand MK, Rueppell O, Tarpy DR (2016) Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees Scientific Reports 6:32023

Takeda K (1961) Classical conditioned response in the honey bee Journal of Insect Physiology 6:168-179

TeBeest D, Shilling C, Riley LH, Weidemann G (1989) The number of nuclei in spores of three species of *Colletotrichum* Mycologia 81:147-149

Westaway JO (2016) The pathogen Myrtle Rust ('*Puccinia psidii*') in the Northern Territory: First detection, new host and potential impacts NT Nat. 27:13

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics PCR Protocols: a guide to methods and applications 18:315-322

Wickham H (2011) ggplot2 Wiley Interdisciplinary Reviews: Computational Statistics 3:180-185

Williamson B, Tudzynsk B, Tudzynski P, van Kan JAL (2007) *Botrytis cinerea*: the cause of grey mould disease Molecular Plant Pathology 8:561-580 doi:10.1111/j.1364-3703.2007.00417.x

Winston ML (1991) The biology of the honey bee. Harvard University Press, Cambridge MA

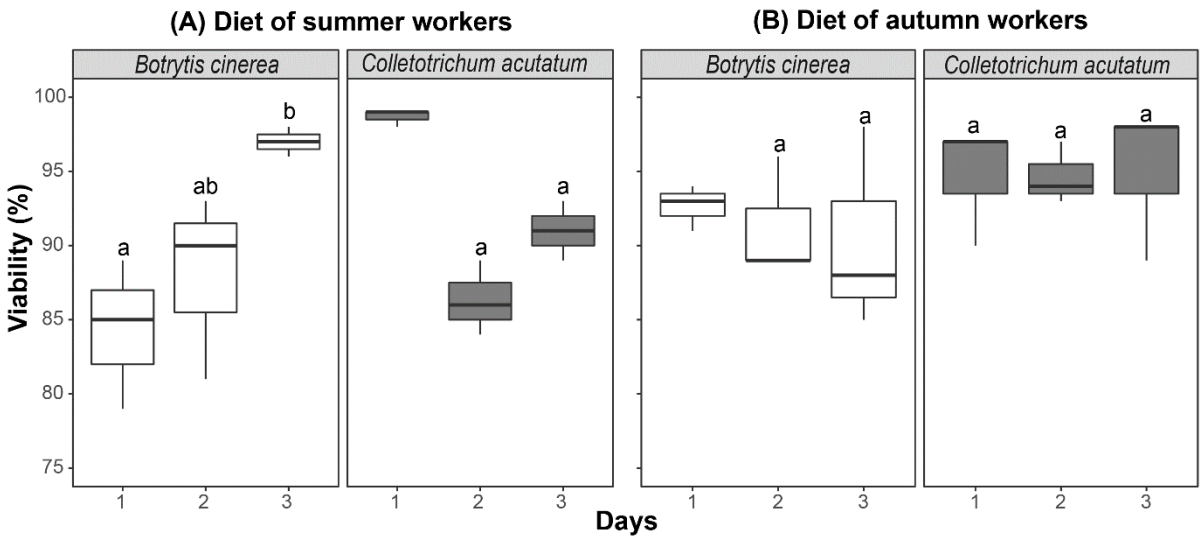

Figure 1. Viability of spores of *Botrytis cinerea* and *Colletotrichum acutatum* in 50% sucrose solution used to feed summer (A) and autumn (B) workers over 3 days. Colony forming units were determined on potato dextrose agar amended with streptomycin sulfate (50 mg/L) and chloramphenicol (250 mg/L). The lower-case letters indicate significance (Generalized Linear binomial model, $P<0.05$) among the suspensions for each fungus.

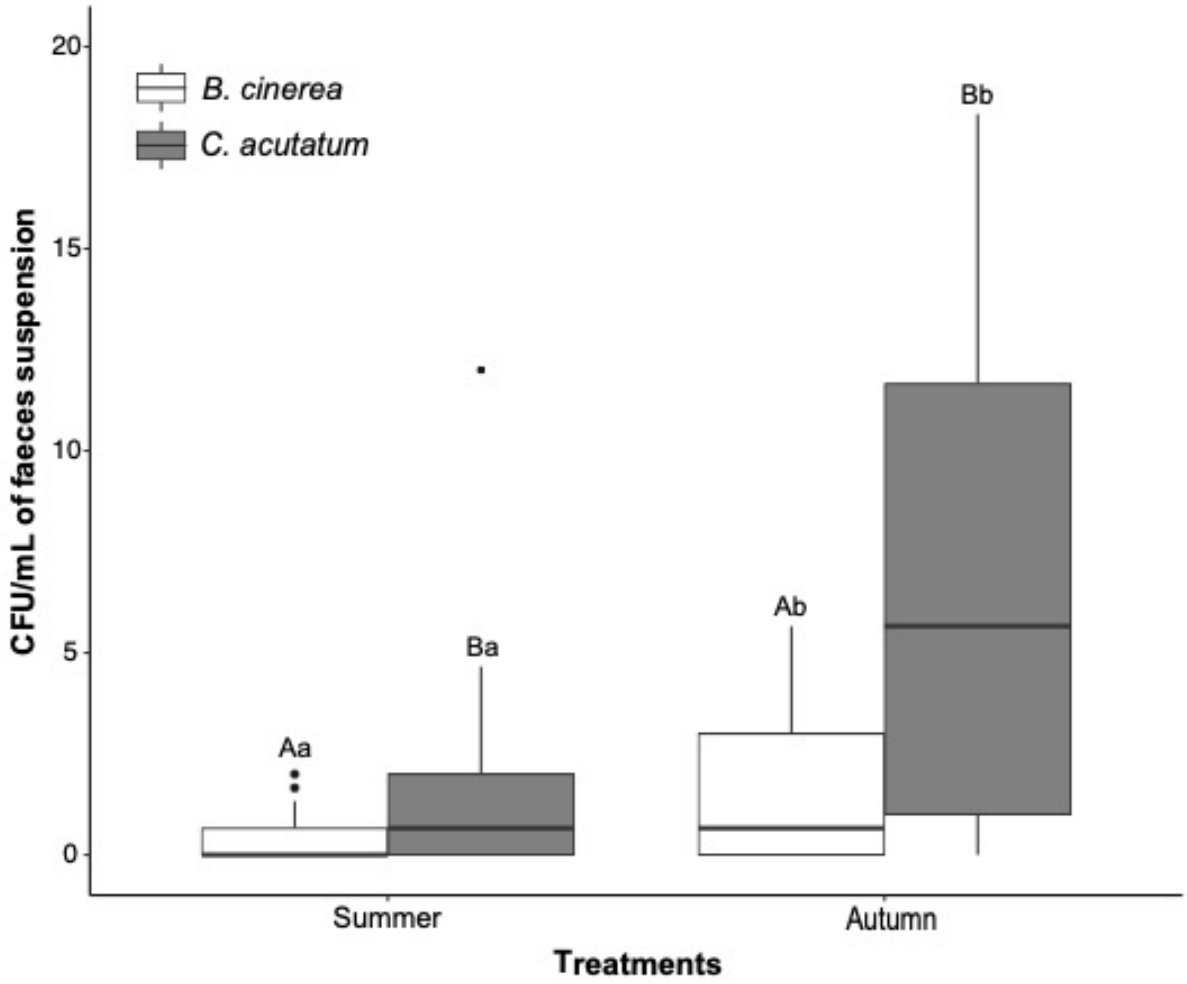

Figure 2. Abundance (CFU, colony forming units) of *Botrytis cinerea* and *Colletotrichum acutatum* in diluted faeces of summer and autumn workers fed with 10^6 spores/mL of either fungus. CFU were determined on the potato dextrose agar plates amended with streptomycin sulfate (50 mg/L) and chloramphenicol (250 mg/L). Control treatments are not shown as no colonies arose from the faeces of workers fed with sterile sucrose solution. Upper-case letters indicate significant differences between the fungal species for each season and lower-case letters indicate significant difference between the seasons for each fungal species (Linear Model, $P<0.05$).

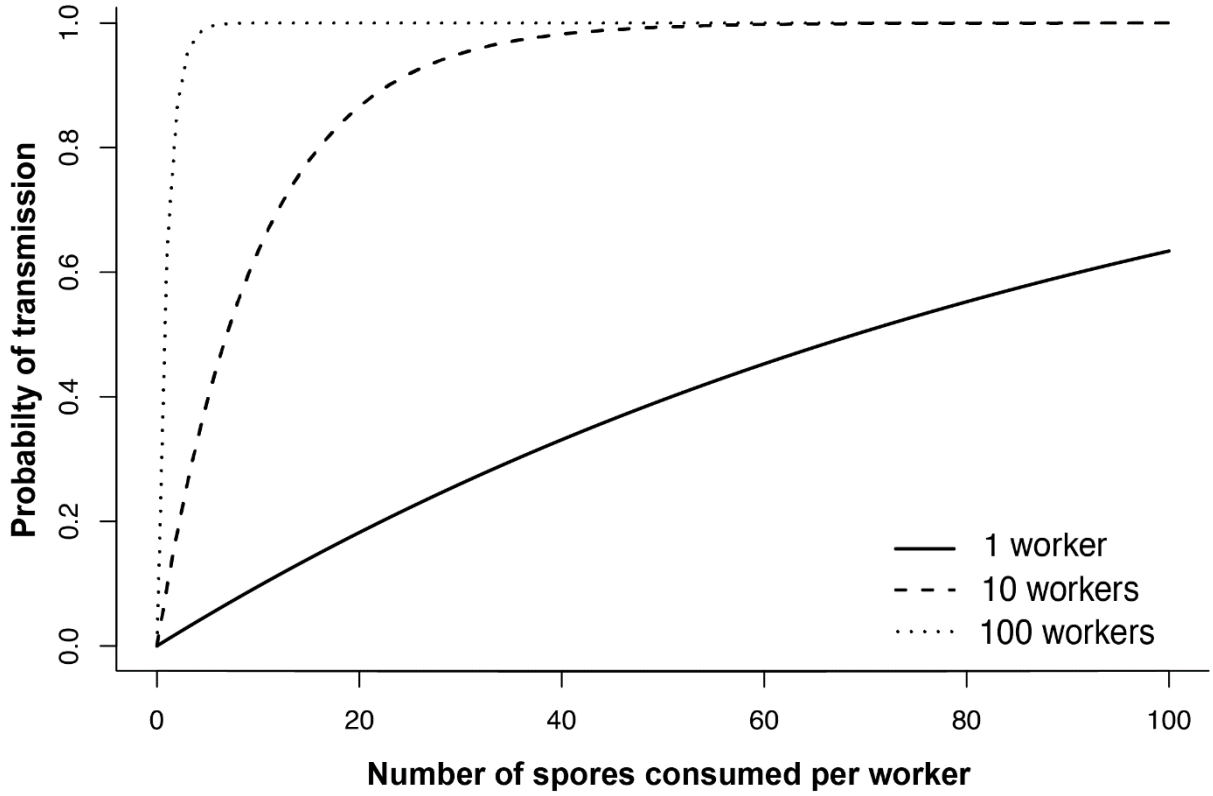

Figure 3. The probability of dispersion of spores of plant pathogenic fungi through honey bee faecal matter in relation to the number of workers consuming 6 x 10^4 spores.

Supplementary Information

Table 1: GenBank accession numbers of original fungal cultures and fungi isolated from worker bee faeces**.**

Species	Season	Isolate code	Source	GenBank accession number (ITS)
Botrytis cinerea	Summer	S1	Original culture	MK402089
		S2	Original culture	MK402090
		S3	Faeces	MK402091
		S4	Faeces	MK402092
		S5	Faeces	MK402093
Colletotrichum acutatum		S6	Original culture	MK402094
		S7	Original culture	MK402095
		S8	Faeces	MK402096
		S9	Faeces	MK402097
		S10	Faeces	MK402098
Aspergillus sp.		S11	Faeces	MK402099
Penicillium sp.		S12	Faeces	MK402100
B. cinerea	Autumn	S13	Original culture	MK402101
		S14	Original culture	MK402102
		S15	Faeces	MK402103
		S16	Faeces	MK402104
		S17	Faeces	MK402105
C. acutatum		S18	Original culture	MK402106
		S19	Original culture	MK402107
		S20	Faeces	MK402108
		S21	Faeces	MK402109
		S22	Faeces	MK402110
Cladosporium sp.		S23	Faeces	MK402111

Chapter 3

Collection of conidia of *Podosphaera xanthii* by honey bee workers

Brief communication prepared to submit to Australasian Plant Pathology

Statement of Authorship

Title of Paper	Collection of conidia of *Podosphaera xanthii* by honey bee workers
Publication Status	Published ☐ Submitted for Publication ☐ Accepted for Publication ☐ Unpublished and unsubmitted work written in manuscript style **X**
Publication Details	Jorgiane B. Parish, Eileen S. Scott, Katja Hogendoorn

Principal Author

Name of Principal Author (Candidate)	Jorgiane Benevenute Parish		
Contribution to the Paper	Designed and performed the experiment, analysed the data, and wrote the manuscript.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/11/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Eileen S. Scott		
Contribution to the Paper	Contributed to research ideas and design, provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	12/11/2019

Name of Co-Author	Katja Hogendoorn		
Contribution to the Paper	Contributed to research ideas and design, provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	12/11/2019

Chapter 3

Collection of conidia of *Podosphaera xanthii* by honey bee workers

Jorgiane B. Parish, Eileen S. Scott, Katja Hogendoorn*

School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, South Australia 5005, Australia

Abstract

Honey bees, *Apis mellifera*, actively collect a range of materials including pollen and fungal spores. However, the interactions between honey bees and fungal spores is largely unexplored. Therefore, this study aimed to assess the corbicular contents of workers foraging on zucchini leaves severely affected by powdery mildew. The dimensions (length and width) of conidia removed from the corbiculae of honey bee workers and from the zucchini leaves were similar and conidia matched the description of *Podosphaera xanthii*, the causal agent of powdery mildew of cucurbits. Our findings that corbicular loads comprised only conidia of *P. xanthii* confirm that honey bees exhibited constancy, just as they almost always forage on a single species of flower per foraging trip, and highlight the importance of understanding the behaviour of honey bee workers in regards to the collection of fungal spores.

Keywords *Apis mellifera*, powdery mildew, *Cucurbita pepo*

Honey bees, *Apis mellifera,* are known to collect fungal spores actively or passively (Shaw 1990; 1999). Passive collection of fungal spores occurs in association with pollen, nectar and honeydew and has, for example, been documented for *Botrytis cinerea*, *Colletotrichum acutatum* and other species (Gasparoto et al., 2017; Shaw 1999). Active collection of sole loads

of spores has been documented for a range of fungal genera and species, such as *Cladosporium*, *Melampsora*, *Neurospora* spp., *Uromyces euphorbiae* and *Zaghouania oleae* (Deodikar et al., 1958; Modro et al., 2009; Schmidt, Thoenes & Levin 1987; Shaw 1999). Various other materials, including wax covers of the soft scale *Ceroplastes* sp. (Dimou and Thrasyvoulou 2007), sawdust (Spencer-Booth 1960) and dried *Chlorella* algae (Prasil et al., 2016), are also occasionally actively collected by honey bees.

Early in March of 2018, honey bee workers were seen collecting material from the surface of leaves of zucchini plants (*Cucurbita pepo*) that were severely affected by powdery mildew (*Podosphaera xanthii*, synonym *P. fusca)* (McGrath & Staniszewska 1996; Sapak et al., 2017), in a domestic garden in a suburban area in Adelaide, South Australia (Figure 1). The origin of the bees, i.e. managed or feral hive, was not identified but could have been either, as both home gardens with honey bee hives and several feral hives in hollows of *Eucalyptus* trees could be found in the immediate surroundings. This study aimed to establish whether the loads collected by honey bee workers foraging on the zucchini leaves contained spores of the powdery mildew fungus.

Figure 1. Collection of spores of *Podosphaera xanthii* by honey bee workers on zucchini (*Cucurbita pepo*) plants.

To collect the pellets from the corbiculae of honey bees, a total of 10 workers with two replete (or full) corbicular loads were collected in sterile Falcon tubes and killed by freezing at -20^{o}C for approximately 2 hours. The pellets were then removed from the corbiculae in a laminar flow cabinet using a sterile needle and placed in 1.5 mL plastic centrifuge tubes. The contents of each tube were diluted in 200 µL of sterile water containing 0.01% (v/v) Tween 20 and vortexed for 1 minute. A drop of each suspension was placed on a microscope slide and a cover slip was placed over it.

To allow visual comparison of the corbicular loads with the material on the leaf surface, five leaves with sporulating powdery mildew were collected in plastic bags. Using a method modified from Langvad (1980), material on the surface of the zucchini leaves was collected by gently applying strips of adhesive tape (approximately 2 x 1 cm). Each piece of tape was placed on a microscope slide (adhesive side up) and a drop of lactoglycerol and a cover slip were placed on top. Ten replicate slides were prepared from material collected from the surface of the zucchini leaves.

All slides were observed at 400x magnification using a Leica DM750 optical microscope (Leica, Germany). In each replicate, the length and width of 20 spores were measured using an ocular micrometer. Measurements of spores from the corbiculae of ten workers were compared with ten replications of spores removed from the zucchini leaves using analysis of variance after testing normality and homoscedasticity assumptions by QQ plot and Shapiro test, and by Bartlett test, respectively. Statistical analyses were performed in R statistical software version 3.3.2 (R-Project 2019).

The length and width of spores from the corbiculae of honey bee workers and from the zucchini leaves with powdery mildew did not differ (length: $F_{(1, 18)}=2.87$, P=0.09; width: $F_{(1, 18)}=3.02$, P=0.08; Figure 2). Spores were hyaline, ellipsoid-ovoid and had a mean length and width (±

se) of 26.9 ± 2.4 x 16.7 ± 1.9 µm for the samples from the corbiculae of workers and 27.4 ± 2.9 x 16.1 ± 2.4 µm for samples from the zucchini leaves. No other materials, spores of other shapes and sizes, pollen grains nor hyphal fragments were observed in the samples collected from the corbiculae of the workers.

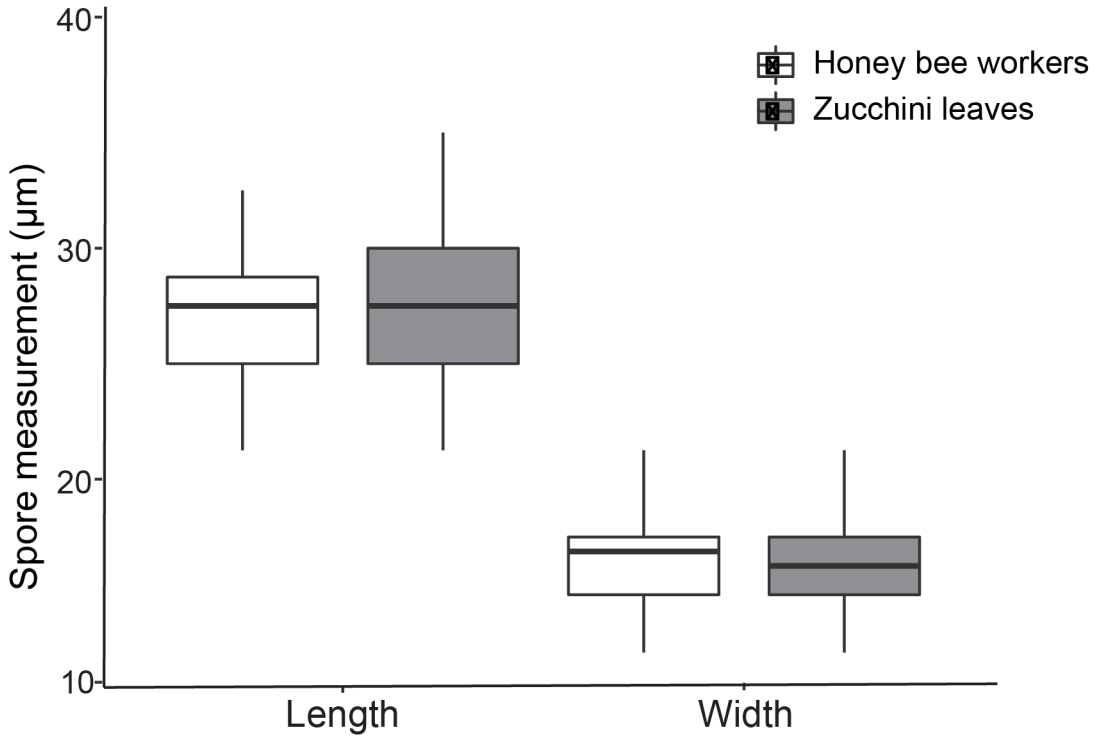

Figure 2. Length and width of spores of obtained from the corbiculae of *Apis mellifera* workers or from zucchini (*Cucurbita pepo*) leaves with powdery mildew.

The spores removed from the corbiculae of honey bees and directly from the surface of the zucchini leaves matched those described for conidia of *P. xanthii* (Nayak & Babu 2017). Therefore, our observations confirmed that honey bees collected pure loads of conidia of *P. xanthii* from the surface of the zucchini leaves covered by sporulating powdery mildew.

Previous reports of honey bees foraging on leaves covered with sporulating powdery mildew include several behavioural observations conveyed to Shaw (1999) as personal communications. However, these observations did not confirm whether the material collected

by the bees consisted of pure spores. In addition, Shaw (1999) reported that Kraus (1920) found pellets containing mixtures of apple pollen and spores of *Oidium farinosum* (anamorph of *P. leucotricha* Ell. & Ev.), the causal agent of powdery mildew on apple trees (Kraus 1920, cited in Shaw 1999). Similarly, Shaw & Robertson's (1990) observations that pellets of *Neurospora* sp. also contained small amounts of mycelia, pollen grains and spores of other fungi. These observations contrast with the pure conidial pellets we found. Thus, our observations show that honey bees may exhibit spore constancy just as they almost typically collect single species of pollen per trip (Free 1963).

The collection of fungal spores by honey bee workers has been attributed to the poor quality and availability of pollen sources within the forage range of a colony (Modro et al., 2009; Shaw 1990; Shaw 1999), however this has not been experimentally demonstrated. The availability of pollen can vary substantially in time and space (Di Pasquale et al., 2013) and, given the dry hot summers in Adelaide, it is possible that honey bees are locally confronted by pollen shortages at the start of autumn, even in areas that contain suburban gardens and native vegetation as for instance species of Eucalyptus and ornamental plants in flowering.

As noted by Shaw (1990), fungal spores could be a source of proteins, amino acids and steroids that may supplement nutritionally poor diets. In addition, they may have various colours and volatile compounds that may be attractive to bees. It is unclear what motivates honey bees to collect fungal spores. Demonstration of nutritional value of the spores collected would indicate functionality of this behaviour. However, it is also possible that, during times of pollen scarcity, bees simply collect other particles available in the landscape, and that have no nutritional benefits, but are of size and shape similar to fungal spores. Therefore, further research is warranted to explore the behaviour of honey bees in regard to the collection of fungal spores.

References

Deodikar GP, Thakar CV, Shah PN, Salvia SR, Chitale PS (1958) Foraging of honeybees on fungal rust spores (*Cytopsora oleae*) on *Olea dioica*. Bee World 39:120-121

Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, Suchail S, Brunet JL & Alaux C (2013) Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? Plos One 8:e72016

Dimou M, Thrasyvoulou A (2007) Collection of wax scale (*Ceroplastes* sp.) by the honey bee *Apis mellifera*. J Apic Res 46:129

Free J (1963) The flower constancy of honeybees. J Anim Ecol 32:119-131

Gasparoto MCG, Lourenco SA, Tanaka FAO, Sposito MB, Marchini L, Silva GJ & Amorim L (2017) Honeybees can spread *Colletotrichum acutatum* and *C. gloeosporioides* among citrus plants. Plant Pathol 66:777-782

Kraus PX (1920) (White apple mildew as bee bread). Dtsch Imker Bohemen 33:120-122

Langvad F (1980) A simple and rapid method for qualitative and quantitative study of the fungal flora of leaves. Can J Microbiol 26: 666-670

McGrath MT, Staniszewska H (1996) Management of powdery mildew in summer squash with host resistance, disease threshold-based fungicide program, or an integrated program. Plant Dis 80:1044-1051

Modro AFH, Silva IC, Message D, Luz CFP (2009) Saprophytic fungus collection by africanized bees in Brazil. Neotropical Entomol 38:434-436

Nayak A, Babu B (2017) First report of powdery mildew on *Lagenaria siceraria* caused by *Podosphaera xanthii* in India. J Plant Pathol 99:289

Prasil O, Urajova P, Kristufek V, Frydrychova R (2016) Vcely a rasy. Modernı vcelar 13:23-24

R-Project (2019), R Core Team, R: A Language and Environment for Statistical Computing., version 1.2.1335 edn., Vienna, Austria

Sapak Z, Salam M, Minchinton E, MacManus G, Joyce D, Galea VJ (2017) POMICS: A simulation disease model for timing fungicide applications in management of powdery mildew of cucurbits. Phytopathol 107:1022-1031

Schmidt JO, Thoenes SC, Levin MD (1987) Survival of honey bees, *Apis mellifera* (Hymenoptera: Apidae), fed various pollen sources. Ann Entomol Soc Am 80:176-183

Shaw DE (1990) The incidental collection of fungal spores by bees and the collection of spores in lieu of pollen. Bee World 71: 158-176

Shaw, D. E. (1999), Bees and fungi, with special reference to certain plant pathogens. Australas Plant Pathol 28:269-282

Shaw DE, Robertson DF (1980) Collection of *Neurospora* by honeybees. Trans Br Mycol Soc 74:459-464

Spencer-Booth Y (1960) Feeding pollen, pollen substitutes and pollen supplements to honeybees. Bee World 41: 253-263

Chapter 4

Nutritional benefit of fungal spores for the development of honey bee workers

Manuscript submitted to Scientific Reports on 1st October 2019

Statement of Authorship

Title of Paper	Nutritional benefit of fungal spores for the development of honey bee workers
Publication Status	Published ☐ Submitted for Publication X Accepted for Publication ☐ Unpublished and unsubmitted work written in manuscript
Publication Details	Jorgiane B. Parish, Eileen S. Scott and Katja Hogendoorn

Principal Author

Name of Principal Author (Candidate)	Jorgiane Benevenute Parish		
Contribution to the Paper	Designed and performed the experiment, analysed the data, and wrote the manuscript.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/11/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Eileen S. Scott		
Contribution to the Paper	Contributed to research ideas and design, provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	12/11/2019

Name of Co-Author	Katja Hogendoorn		
Contribution to the Paper	Contributed to research ideas and design, provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	12/11/2019

Chapter 4

Nutritional benefit of fungal spores for the development of honey bee workers

Jorgiane B. Parish, Eileen S. Scott, Katja Hogendoorn*

School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, South Australia 5005, Australia

*Correspondence: Katja Hogendoorn

School of Agriculture, Food & Wine, Waite Campus, The University of Adelaide

PMB 1, Glen Osmond, SA, 5064

Phone: +61 8 8313 6555

Email: katja.hogendoorn@adelaide.edu.au

ABSTRACT

The collection of fungal spores by honey bees, *Apis mellifera*, can be classified as active or passive, the latter when spores are associated with pollen, nectar or honey dew. While low quality and shortage of pollen have been raised as hypotheses for fungal spore collection, the nutritional value of fungal spores for honey bees is poorly understood. Here we investigated the effect of consumption of fungal spores on survival, ovarian activation and the development of the hypopharyngeal glands (HPGs) in honey bee workers. Two pollen diets (*Eucalyptus* sp. pollen and a multifloral pollen) supplemented or not with spores of *Botrytis cinerea*, *Cladosporium* sp. or *Colletotrichum acutatum* were used. Consumption of diets that contained fungal spores increased the longevity of honey bee workers but had no significant effect on the development of their HPGs and ovaries. This demonstrates that fungal spores may have nutritional value for honey bees and that the consumption of fungal spores may compensate for nutritional imbalances of poor-quality pollen diets.

Keywords: *Apis mellifera*, fungal spores, *Eucalyptus* sp. pollen, multifloral pollen, longevity

INTRODUCTION

Honey bees, *Apis mellifera*, are primary pollinators of crops worldwide [1,2] and without these pollinators, the yields of many fruit, seed and nut crops would decrease by more than 90% [3]. Hence, factors that affect bee health, such as the Varroa mite, associated viruses, pesticide use, and nutritional stress, are causes of concern [4-6]. Nutritional stress in honey bee hives can arise either through a lack of floral resources, for example due to drought, urbanisation or planting of crops that do not provide adequate food for bees. While nutrient deficiency can be obvious during pollination events in monocultures [7-9], even polyfloral scenarios may be composed of combinations of floral resources that are incapable of mitigating nutritional imbalances for

bees.

Spore collection has been described as 'incidental' or 'the collection in lieu of pollen' [17], where the term 'incidental' refers to passive acquisition on the body, while 'the collection in lieu of pollen' refers to the occasional active collection by the bees. The use of these terms differs slightly from the historic classification of pollen-collecting behaviour of bees into 'active' and' incidental'[11-16]. In the former case, active pollen collection refers to the purposeful uptake of pollen directly from anthers or other floral surfaces, while incidental pollen collection refers to pollen that accumulates on bees as they forage for nectar. To achieve clarity with regard to the use of these terms, we suggest that both pollen and spore collection be classified as 'active' and 'passive'.

Shaw [10] suggested that bees might be motivated to collect fungal spores by a lack of floral resources, the chemical composition of spores (i.e. nutrients, amino acids and steroids), attractants such as colour or odour, and because certain spores may resemble pollen grains. However, none of these hypotheses has been experimentally tested. Based on the fact that honey bees actively collect fungal spores as sole load, it has been suggested that the bees might obtain some nutritional benefit from their consumption [10]. Seemingly contrary to this suggestion, detrimental effects of consumption of pure spores of a rust fungus have been experimentally demonstrated [18]. However, bee bread, which is consumed by workers, is unlikely to consist solely of spores, as field-collected spore pellets can become mixed with pollen during storage in the hive. Therefore, the health effects of consumption of fungal spores should be investigated.

To investigate the functional significance of spore collection, we experimentally tested the effects of consumption of fungal spores in combination with pollen on bee health. We investigated whether diets of pollen plus fungal spores negatively or positively affected

survival of workers, ovary activation and development of hypopharyngeal glands (HPGs). Both worker survival and HPG development are related to hive health. HPGs constitute a paired organ, composed of numerous vesicles or acini connected to a duct, in the head of workers [19]. Nurse bees feed the larvae with secretions from these glands [20]. The volume of the acini can serve as an indicator of the nutritional status of a colony [20], and diets [6] and fungicides [21] have been observed to affect HPG development. Similarly, the nutritional value of forage has been linked to ovarian activation [22]. However, the impact of fungal spores on ovary activation in workers has not been investigated. Here we investigate the effect of consumption of spores of three common fungi, *Botrytis cinerea*, *Cladosporium* sp. and *Colletotrichum acutatum,* in association with *Eucalyptus* sp. pollen (EP) and multifloral pollen (MP) on longevity, HPG acinus size and ovarian activation in honey bee workers.

RESULTS

Establishment of the spore concentration in the diet

Honey bee workers did not consume diets solely comprising spores of any of the three species of fungi tested. In addition, workers did not consume diets comprising EP supplemented with spores of *B. cinerea*, *Cladosporium* sp. or *C. acutatum* at weight ratios of 5:1 and 10:1. There was no significant difference in the diet consumption rate among the treatments involving the monofloral EP with or without spores of *B. cinerea, Cladosporium* sp. or *C. acutatum* at the ratio of 20:1 ($F=2.34$, $df=3$, $P=0.07$, Table S1). Diet consumption rates during the 7-day experiments in which the effects on the hypopharyngeal glands and ovaries were assessed, were similar among the treatments ($F=0.005$, $df=3$, $P=0.99$). There was no significant interaction between treatments and cages. Hence, as the ratio of 20:1 of pollen to spores allowed assessment of the effects of spore consumption in association with EP, it was used for further assessments.

Effects of spore consumption on the survival of workers

In the initial experiment in which several ratios of pollen to spores were examined, the survival curves for the workers fed with the monofloral EP with and without fungal spores differed significantly among the treatments (log-rank test: X^2 =32, df =3, P<0.001; Fig.1, Table 1). Workers fed on EP as the sole diet died sooner than bees that consumed the mixture of pollen and fungal spores. Overall, the maximum survival of the bees that consumed EP only, or EP supplemented with spores of *B. cinerea, Cladosporium* sp. or *C. acutatum* was 39, 40, 39 and 46 days, respectively.

Similar results were obtained in the subsequent comparative experiment using the two pollen diets (i.e. EP and MP) with or without spores of *B. cinerea, Cladosporium* sp. or *C. acutatum*. The survival curves differed significantly between the treatments (log-rank test: X^2 =53.8, df =7, P<0.001; Fig. 2; Table 1). Survival of workers that were fed with the pollen-only diets was significantly shorter than of those that consumed the mixtures of pollen and fungal spores, except for the combination of the MP with spores of *B. cinerea*. Overall, the maximum survival of workers that were fed EP only and EP supplemented with spores of *B. cinerea, Cladosporium* sp. or *C. acutatum* was 25, 28, 43 and 40 days, respectively. The maximum survival of workers that were fed MP only and MP supplemented with spores of *B. cinerea, Cladosporium* sp. or *C. acutatum* was 29, 27, 30 and 36 days, respectively. In all cases, the survival of bees that were fed MP was shorter than the bees fed on EP. This corresponds with the generally poorer nutritious value of the multifloral pollen (Table 2). There were no significant differences among the treatments: EP, MP and MP supplemented with spores of *B. cinerea*; EP with spores of *B. cinerea* and all of the diets with exception of EP alone; EP supplemented with spores of *Cladosporium* sp., MP with spores of *Cladosporium* sp. and MP with spores of *C. acutatum*; EP with spores of *C. acutatum*, MP supplemented with spores of

either *B. cinerea*, *Cladosporium* sp. and *C. acutatum;* or MP supplemented with spores of either *B. cinerea*, *Cladosporium* sp. and *C. acutatum*. There was no significant difference among the diets involving the EP and the MP with or without fungal spores in terms of amount consumed per bee (F=0.91, df=7, P=0.49, Table S1).

Effects of diets on the development of HPGs and ovaries

Neither the volume of the acini in the HPGs nor the ovary activation of workers that consumed EP only or EP supplemented with spores of *B. cinerea*, *Cladosporium* sp. or *C. acutatum* differed significantly among the treatments (HPGs - F = 0.24, df= 3, P =0.26, Fig. S1; ovaries – F =1.66, df=3, P=0.17). The acini appeared normal in shape, which was similar for all treatments. The effects on HPG and ovary activation were investigated for the combination of spores with EP only.

Gamma irradiation and nutritional factors of pollen samples

The plated suspensions of gamma-irradiated pollen did not yield any microorganisms, whereas non-irradiated monofloral and multifloral pollen yielded approximately 10^5 CFU of viable fungi/mL of suspension (i.e. gram of pollen) and sporadic bacterial colonies. The nutritional content of the pollen samples (i.e. amino acids, ash, carbohydrates, lipids and protein) and the microorganisms naturally associated with them varied between the pollen samples, except for sugar and sodium levels (Table 2). Pesticide residues were not detected in the samples (Table S2).

DISCUSSION

Our results demonstrate that consumption of spores of *B. cinerea*, *Cladosporium* sp. and *C. acutatum* in association with pollen can extend the lifespan of honey bee workers but had no

effect on the volume of the acini in the HPGs or activation of the ovaries. HPG development and ovary activation are influenced by the quality and quantity of protein ingested by honey bee workers [6,20,23,24]. Although fungal spores generally have a low protein content [25], spores could conceivably serve a source of additional nutrients for bees when combined with pollen. This is supported by our data, as bees that fed on pollen supplemented with spores survived longer than those that did not. In addition, the diets with fungal spores contained 5% less pollen than the EP-only diet. As the development of HPGs and ovary activation were similar in diets that did and did not contain spores, the nutritional value of spores may be equal to the value of the EP used in our experiments. It would have been informative to investigate whether spore consumption had a positive effect on these traits in combination with MP. Unfortunately, we did not assess this, as, we did not, *a priori*, expect the MP to be less nutritious than the EP. The differences between the first and subsequent experiments with respect to the survival of honey bee workers that were fed EP may be due to genetic variation [26].

Pollen of different plant species varies greatly in protein content, amino acid composition, lipid, starch, vitamin and mineral content. This can affect individual bee health and colony longevity, physiology, and resistance to or tolerance of disease [6,27,28]. The quality of pollen for bees can be classified [29] according to their levels of crude protein (c.p.) as excellent (>25% c.p.), average (20-25% c.p.), and poor (and <20%;). Our finding that EP was richer in c.p. than the MP was unexpected, but it may explain the finding that workers fed with EP diets survived longer than those on the MP diets.

Furthermore, the MP contained the species *Arctotheca calendula* and *Echium plantagineum,* which belong to the families Asteraceae and Boraginaceae, respectively. The pollen of some species of the Asteraceae and Boraginaceae contains pyrrolizidine alkaloids [30,31], plant secondary compounds, which confer protection from herbivory [32]. These alkaloids possess

chemical properties that interfere with their digestion by non-specialized bees, including *A. mellifera* [33]. Thus, if pyrrolizidine alkaloids were present in the pollen of *A. calendula* and *E. plantagineum* this might, in part, explain the difference in survival rates between EP and MP. Nevertheless, in both experiments, the survivorship of workers was, overall, extended by the consumption of spores of *B. cinerea*, *Cladosporium* sp. or *C. acutatum* combined with pollen. The consumption of spores of *Cladosporium* sp. and *C. acutatum* in association with either pollen sample used in our experiments provided the longest lifespan, substantiating the hypothesis that nutritional benefits can be obtained by the collection of fungal spores by honey bees.

In addition to the nutritional contribution of pollen and fungal spores tested to the health and survival of honey bee workers, the microorganisms naturally present in the pollen samples, i.e. fungal spores and bacterial cells, could be an additional source of nutrients that enriches the quality of the diets consumed by the bees. Our results highlight the importance of knowing the composition of the microbiota associated with food sources fed to bees to understand their role and impact on the health and lifespan of bees. Thus, in our gamma-irradiated diets these dead microorganisms may have played a role in the nutritive value of the pollen, a factor that has so far been overlooked in other honey bee feeding experiments [6,19,24]. However, only the pollen samples were analysed for nutrient composition due to limited resources and the challenges of producing sufficient quantities of fungal spores for experimentation. Accordingly, fungal spores were allocated to the feeding experiments.

During our experiments, honey bees failed to consume diets consisting solely of spores of any of the three species of fungi used, nor did they consume spores of the cereal rust fungus, *Puccinia graminis* (data not shown). Comparable results have been reported by Schmidt et al. [18], in that honey bee workers in cages consumed negligible amounts of spores of *Uromyces* sp.

however, even those small amounts were toxic, causing rapid mortality, in contrast to our experiments. When spores are collected by bees in the field, it is likely that they are mixed with pollen from different sources and various microorganisms during the formation of bee bread. This would dilute the risk of any possible toxic effects and could enrich the nutritional quality of the food stored in the hives.

Honey bees have been observed to actively collect spores of *Cladosporium* sp. [34], and our results show that these spores can significantly increase the longevity of the workers. Cell walls of *Cladosporium* sp. contain β- 1,3-1,6 – glucan, a member of β-D-glucose polysaccharides [35,36], which are known to be involved in the immune stimulatory activity in many vertebrate and invertebrate species [37]. Although the effects of β-glucan depend on the target organism and the dose administered [37], it has been shown to extend the lifespan of honey bee workers [38]. In addition, extracts of the mushroom *Agaricus brasiliensis*, which is rich in glucans, have been shown to increase slightly the strength of experimentally fed honey bee colonies [39]. Hence, the β-glucans contained in the cell walls of fungi might have contributed to the outcome of our experiments.

In comparison with our findings that spores of *Cladosporium* sp. in association with pollen extended the survival of honey bee workers in vitro, experimental feeding of honey bee larvae with nectar contaminated with honey dew secretions from the planthopper *Epormenis cestri* in *Sebastiania schottiana* trees, which contained spores of several fungi including *Cladosporium*, caused mass death of the larvae [40]. Although any negative effect of the fungi cannot be excluded, it is likely that the larval mortality in the latter experiment was associated the presence of toxic substances, such as xanthoxyline and its derivatives, commonly produced by the genus *Sebastiana* [41,42].

In summary, this study provides the first experimental evidence that consumption of spores of

some fungal species can extend the lifespan of honey bee workers, presumably by providing nutritional benefits. This supports the notion that foraging honey bee workers are likely to actively collect fungal spores. Further research is needed to identify specific interactions between honey bees and different species and strains of fungi; and to investigate the impact of the consumption of fungal spores on the learning, memory, resistance to and tolerance of diseases of bees. Furthermore, it needs to be established whether bee colonies preferentially collect fungal spores when other nutrient sources are in low supply, and whether β-glucans are responsible for the extended longevity of the workers.

MATERIAL AND METHODS

Pollen samples

Two pollen mixtures were used in the experiments. The first pollen sample was composed mainly of *Eucalyptus* sp. and was collected in Nannup, Western Australia. During the collection of this pollen the main species in flower was *E. baxteri*. The second pollen sample was collected in Adelaide, South Australia, and consisted of a mixture of *Arctotheca calendula* (capeweed), *Eucalyptus* sp., *Echium plantagineum* (salvation Jane) and *Oxalis pes-caprae* (soursob). Pollen grains were morphologically identified by the acetolysis method described by Erdtman [43] and glycerine slides were prepared as described in Kearns and Inouye [44]. Photographs showing polar and equatorial views of the pollen grains at 400x magnification were obtained using a Leica DM750 optical microscope coupled to a ICC50W Camera (Leica, Germany) and compared with the APSA [45] reference key. Both EP and MP samples were obtained from the same beekeeper by placing a pollen trap in front of the hive entrances, subsequently dried and stored at -20 ^{o}C after arrival at the laboratory.

Gamma irradiation and nutritional quality of pollen samples used in the experiments

Pollen samples were sterilised by subjecting to gamma irradiation of 25 kGy [46,47] at Steritech (Dandenong, Victoria). To ascertain effectiveness, the pollen samples were plated before and after irradiation. For each pollen sample, 1 g was diluted in 0.9 ml of sterile water containing 0.01% (v/v) Tween 20, the suspension was mixed by vortex and a 10-fold serial dilution was performed. After mixing each dilution by vortex for 1 minute, aliquots of 0.1 ml of the dilutions 1:100 and 1:1000 were spread onto three replicate plates of potato dextrose agar (PDA, Difco) in 9-cm diameter Petri dishes and incubated at 25°C under white fluorescent light with 12-h photoperiod. Observations were made 36 hours post-plating to determine the number of colony forming units (CFU).

Nutritional quality and pesticide residues of the pollen samples were tested at Agrifood Technology (Werribee, Victoria). Duplicates of each pollen sample was assessed for ash, carbohydrate, lipid, protein, sodium and sugar contents. The protein (N x 6.25) content was determined by the Kjeldahl method [48] and total lipids were analysed after the disruption of pollen walls by hydrolysis with hydrochloric acid. The presence of pesticide residues in the two pollen diets was assessed via gas and liquid chromatography with a limit of quantification of 0.01 mg/kg and a limit of detection of 0.005 mg/kg. Amino acid profiles were determined by analysis of duplicate samples at the Australian Proteome Analysis Facility (Sydney, New South Wales).

Fungal material and spore production

Three fungal species were used in the bioassays: *Botrytis cinerea* (CBS 140599, GenBank accession number: KX710078), *Colletotrichum acutatum* (European Molecular Biology Laboratory accession number: EU670080*)* and *Cladosporium* sp. (GenBank accession number:

MK402112), which had been isolated from grapes, almond, and bee bread, respectively. The *Cladosporium* isolate, which was not identified to species level, has 100% of sequence similarity with *C. halotolerans*, *C. sphaerospermum*, *C. parahaloterans* and *C. cladosporioides* [49,50]. The first two isolates were obtained from the Plant Pathology laboratory and the third from a honey bee hive located at the School of Agriculture, Food and Wine, University of Adelaide, South Australia, Australia. Mycelia were cultured on PDA in 9-cm diameter Petri plates for 10 to 14 days and incubated as described above. Cultures were established in consecutive weeks to generate spores of similar age.

Spores were harvested by flooding 10-14 day-old cultures with 15 mL of sterile reverse osmosis (RO) water containing 0.01% (v/v) Tween 20 and dislodging spores from the mycelia using a sterile plastic spreader. The resulting suspensions were filtered through two layers of sterile cheesecloth to remove hyphal fragments [51]. Spore suspensions from three plates of the same fungus were combined in a sterile 50-mL tube and then centrifuged at 4500 rpm, 25 ^{o}C for 10 minutes. The supernatant was removed and the remaining suspensions (approximately 0.3 - 0.4 mL) were placed in sterile lids of 2 mL cryogenic tubes (Simport, Canada) in a biohazard laminar flow cabinet and left to dry overnight. Spores in lids were kept in sterile sealed Petri dishes at 4 ^{o}C until diet preparation.

Bee rearing and feeding

Experiments were conducted with newly emerged workers collected from three different hives during March of 2018 and 2019. Colonies were maintained in a bee enclosure located at the Waite Campus, University of Adelaide and were regularly inspected for symptoms of diseases. Workers were obtained by placing single frames with late stage pupae in an incubator at 34 ^{o}C and 50 - 70% relative humidity [6]. Groups of 10 individuals were placed in metal cages (6.5 x 8.5 x 4.5 cm; Small-Life Supplies, England) and fed with gamma-irradiated pollen and fungal

spores offered in a sterile cryogenic lid; and sterile 50% (*w/v*) sucrose solution provided in a 5-mL syringe.

Pollen was mixed with sterile RO water at a mass ratio of 7:3 and subsequently with fungal spores. To establish the optimal concentration of spores, workers were offered spores of each fungus as a sole diet, or in a mix with EP at a weight ratio of 5:1, 10:1 and 20:1. In a subsequent experiment the effects of fungal spores were tested in mixes with EP and MP, mixed separately with spores of each fungus at a mass ratio of 20:1. Diets were freshly prepared every day, measured and provided *ad libitum*. The rates of diet consumption per bee per day were also assessed. Caged bees were incubated as described above. Each treatment consisted of six replications. Dead bees were removed from the cages every day until 100% mortality was reached for every cage.

Hypopharyngeal gland development and ovary activation

To assess whether fungal spores can affect the development of the HPGs, workers were fed with EP with or without spores of *B. cinerea*, *Cladosporium* sp. or *C. acutatum* at a mass ratio of 20:1. In the same manner as for the longevity experiment, groups of ten newly emerged workers were placed in cages and reared for 7 days. On day 8, bees were placed in 1.5-mL Eppendorf tubes and stored at -80^{o}C until dissection. The paired HPG were dissected in 100 µL of physiological saline (0.9% NaCl) and stained with a drop of methyl blue dissolved in Ringer's solution [19]. Glands were mounted on slides and examined as described above. The development of HPGs was assessed by measuring the length and width of 30 randomly chosen acini per bee using the Leica Acquire 3.4.1 software. The total number of bees assessed for diets of EP, or EP supplemented with spores of *B. cinerea*, *Cladosporium* sp. or *C. acutatum* was 59, 58, 57 and 58, respectively. The volume of each acinus was calculated by applying the following equation: 1/6 x 3.14 x length x width [52].

The ovaries of five bees per cage were dissected under a binocular dissecting microscope. Their development was classified into five stages according to Pernal and Currie [24] as; 0: undeveloped ovaries, 1: slight enlargement, early stages of differentiation; 2: slight development (presence of distinct cells leading to swellings and constrictions), 3: moderate development (egg volume exceeding that of the nutritive follicle), 4: highly developed (presence of fully formed eggs and ovaries with mature oocytes).

Statistical analysis

All statistical analyses were executed in R version 3.2.1 [53]. The survival data were analysed using the Kaplan-Meier method and log-Rank test. Bonferroni correction was applied to the comparison sets to limit the risk that significant differences would be obtained by chance [54]. The HPG volume data were subjected to analysis of variance followed by Tukey's honestly significant difference (HSD) test, when appropriate. Diet consumption and ovary activation data were compared using a generalized linear model (GLM) with a Gaussian family distribution and Tukey's HSD test, when appropriate. Normality and homoscedasticity assumptions were tested by QQ plot and Shapiro test, and by Bartlett test, respectively. Only the acini volume data required transformation [log(x)] prior to analyses of variance.

ACKNOWLEDGEMENTS

The first author was supported by the Adelaide Scholarships International (ASI).

AUTHOR CONTRIBUTIONS

JBP: designed and performed experiment and data analyses, and wrote the paper. EES, KH: experimental design, technical assistance, and editing of the paper. All authors read and approved the final manuscript.

REFERENCES

1 Gallai, N., Salles, J.-M., Settele, J. & Vaissière, B. E. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics* **68**, 810-821 (2009).

2 Klein, D., Vaissiere, B. E., Cane, J. H., Stefan-Dewenter, I., Cunningham, S. A., Kremen, C., Tscharntke, T. Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B-Biological Sciences* **274**, 303-313, doi:10.1098/rspb.2006.3721 (2007).

3 Southwick, E. E. & Southwick, L. Estimating the economic value of honey bees (Hymenoptera: Apidae) as agricultural pollinators in the United States. *Journal of Economic Entomology* **85**, 621-633 (1992).

4 Brodschneider, R. & Crailsheim, K. Nutrition and health in honey bees. *Apidologie* **41**, 278-294; 10.1051/apido/2010012 (2010).

5 Smart, M., Pettis, J., Rice, N., Browning, Z. & Spivak, M. Linking measures of colony and individual honey bee health to survival among apiaries exposed to varying agricultural land use. *Plos One* **11**, e0152685; 10.1371/journal.pone.0152685 (2016).

6 Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L. P., Decourtye, A., Kretzschmar, A., Suchail, S., Brunet, J.-L., Cedric, A. Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? *Plos One* **8**, e72016; 10.1371/journal.pone.0072016 (2013).

7 Simone-Finstrom, M., Li-Byarlay, H., Huang, M. H., Strand, M. K., Rueppel, O., Tarpy, D. R. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. *Scientific Reports* **6**, 32023 (2016).

8 de Vere, N., Jones, L. E., Gilmore, T., Moscrop, J., Lowe, A., Smith, D., Hegarty, M. J., Creer, S., Ford, C. R. Using DNA metabarcoding to investigate honey bee foraging reveals limited flower use despite high floral availability. *Scientific Reports* **7**, 42838; 10.1038/srep42838 (2017).

9 Ahn, K., Xie, X., Riddle, J., Pettis, J. & Huang, Z. Y. Effects of long distance transportation on honey bee physiology. *Psyche: A Journal of Entomology* **2012**, 1-9 (2012).

10 Shaw, D. E. Bees and fungi, with special reference to certain plant pathogens. *Australasian Plant Pathology* **28**, 269-282; 10.1071/ap99044 (1999).

11 Parker, R. L. The collection and utilization of pollen by the honeybee. *Memoir: Cornell University Agricultural experiment Station* **98**, 1-55 (1926).

12 Doull, K. Analysis of bee behavior as it relates to pollination in The indispensable pollinators: a report of the ninth conference 5-18 (1970).

13 Inouye, D. W., Gill, D. E., Dudash, M. R. & Fenster, C. B. A model and lexicon for pollen fate. *American Journal of Botany* **81**, 1517-1530 (1994).

14 Westerkamp, C. Pollen in bee-flower relations some considerations on melittophily. *Botanica Acta* **109**, 325-332 (1996).

15 Thorp, R. W. The collection of pollen by bees. *Plant Systematics and Evolution* **222**, 211-223 (2000).

16 Portman, Z. M. & Tepedino, V. J. Convergent evolution of pollen transport mode in two distantly related bee genera (Hymenoptera: Andrenidae and Melittidae). *Apidologie* **48**, 461-472 (2017).

17 Shaw, D. E. The incidental collection of fungal spores by bees and the collection of spores in lieu of pollen. *Bee World* **71**, 158-176 (1990).

18 Schmidt, J. O., Thoenes, S. C. & Levin, M. D. Survival of honey bees, *Apis mellifera* (Hymenoptera: Apidae), fed various pollen sources. *Annals of the Entomological Society of America* **80**, 176-183 (1987).
19 Renzi, M. T. *et al*. Combined effect of pollen quality and thiamethoxam on hypopharyngeal gland development and protein content in *Apis mellifera*. *Apidologie* **47**, 779-788 (2016).
20 Hrassnigg, N. & Crailsheim, K. Adaptation of hypopharyngeal gland development to the brood status of honeybee (*Apis mellifera* L.) colonies. *Journal of Insect Physiology* **44**, 929-939 (1998).
21 Heylen, K., Gobin, B., Arckens, L., Huybrechts, R. & Billen, J. The effects of four crop protection products on the morphology and ultrastructure of the hypopharyngeal gland of the European honeybee, *Apis mellifera*. *Apidologie* **42**, 103-116 (2011).
22 Hoover, S. E., Higo, H. A. & Winston, M. L. Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition. *Journal of Comparative Physiology B* **176**, 55-63 (2006).
23 Brouwers, E. Measurement of hypopharyngeal gland activity in the honeybee. *Journal of Apicultural Research* **21**, 193-198 (1982).
24 Pernal, S. F. & Currie, R. W. Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (*Apis mellifera* L.). *Apidologie* **31**, 387-409; 10.1051/apido:2000130 (2000).
25 Bartnicki-Garcia, S. Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annual Reviews in Microbiology* **22**, 87-108 (1968).
26 Page Jr, R. E. & Peng, C. Y.-S. Aging and development in social insects with emphasis on the honey bee, Apis mellifera L. *Experimental gerontology* **36**, 695-711 (2001).
27 Schmidt, L. S., Schmidt, J. O., Rao, H., Wang, W. & Xu, L. Feeding preference and survival of young worker honey bees (Hymenoptera: Apidae) fed rape, sesame, and sunflower pollen. *Journal of Economic Entomology* **88**, 1591-1595 (1995).
28 Goulson, D., Nicholls, E., Botias, C. & Rotheray, E. L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**, 1255957; 10.1126/science.1255957 (2015).
29 Kleinschmidt, G., Kondos, A., Harden, J. & Turner, J. Colony management for eucalypt honey flows. *The Australasian Beekeeper* **75**, 261-264 (1974).
30 Anke, S., Niemüller, D., Moll, S., Hänsch, R. & Ober, D. Polyphyletic origin of pyrrolizidine alkaloids within the Asteraceae. Evidence from differential tissue expression of homospermidine synthase. *Plant Physiology* **136**, 4037-4047 (2004).
31 Boppré, M., Colegate, S. M. & Edgar, J. A. Pyrrolizidine alkaloids of *Echium vulgare* honey found in pure pollen. *Journal of Agricultural and Food Chemistry* **53**, 594-600 (2005).
32 Hartmann, T. & Ober, D. Biosynthesis and metabolism of pyrrolizidine alkaloids in plants and specialized insect herbivores in *Topics in current chemistry: Biosynthesis - Aromatic Polyketides, Isoprenoids, Alkaloids* (ed Vederas J.C. Leeper F.J.) 207-243 (Springer, 2000).
33 Praz, C. J., Müller, A. & Dorn, S. Specialized bees fail to develop on non-host pollen: do plants chemically protect their pollen. *Ecology* **89**, 795-804 (2008).
34 Modro, A. F. H., Silva, I. C., Message, D. & Luz, C. F. P. Saprophytic fungus collection by africanized bees in Brazil. *Neotropical Entomology* **38**, 434-436 (2009).
35 San-Blas, G., Guanipa, O., Moreno, B., Pekerar, S. & San-Blas, F. *Cladosporium carrionii* and *Hormoconis resinae* (*C. resinae*): cell wall and melanin studies. *Current Microbiology* **32**, 11-16 (1996).

36 Szaniszlo, P., Cooper, B. & Voges, H. S. Chemical compositions of the hyphal walls of three chromomycosis agents. *Sabouraudia: Journal of Medical and Veterinary Mycology* **10**, 94-102 (1972).
37 Soltanian, S., Stuyven, E., Cox, E., Sorgeloos, P. & Bossier, P. Beta-glucans as immunostimulant in vertebrates and invertebrates. *Critical Reviews in Microbiology* **35**, 109-138 (2009).
38 Mazzei, M. *et al*. Effect of 1, 3-1, 6 β-glucan on natural and experimental deformed wing virus infection in newly emerged honeybees (*Apis mellifera ligustica*). *Plos One* **11**, e0166297 (2016).
39 Stevanovic, J. *et al*. The effect of *Agaricus brasiliensis* extract supplementation on honey bee colonies. *Anais da Academia Brasileira de Ciências* **90**, 219-229 (2018).
40 Invernizzi, C. *et al*. *Epormenis cestri* secretions in *Sebastiania schottiana* trees cause mass death of honey bee *Apis mellifera* larvae in Uruguay. *Plos One* **13**, e0190697 (2018).
41 Calixto, J. B., Miguel, O. G., Rosendo, A., Yunes, R.S. & Rae, G. A. Action of 2-Hydroxy-4,6-dimethoxyacetophenone isolated from *Sebastiania schottiana*. *Planta Medica* **56**, 31–35 (1990).
42 Filho, V. C., Miguel, O. G., Nunes, R. J., Calixto, J. B. & Yunes, R. A. Antispasmodic activity of Xanthoxyline derivatives: structure-activity relationships', *Journal of Pharmaceutical Sciences*. **84**, 473–475 (1995).
43 Erdtman, G. *Handbook of palynology: morphology, taxonomy, ecology*. (Copenhagen, Munksgaard; 1969).
44 Kearns, C. A. & Inouye, D. W. *Techniques for pollination biologists*. (University Press of Colorado, 1993).
45 APSA. *Australasian Pollen and Spore Atlas*, <http://apsa.anu.edu.au/> (2007).
46 Graystock, P. *et al*. Hygienic food to reduce pathogen risk to bumblebees. *Journal of Invertebrate Pathology* **136**, 68-73; 10.1016/j.jip.2016.03.007 (2016).
47 Meeus, I., Mosalanejad, H., Niu, J., de Graaf, D. C., Wackers, F., Smagghe, G. Gamma irradiation of pollen and eradication of Israeli acute paralysis virus. *Journal of Invertebrate Pathology* **121**, 74-77; 10.1016/j.jip.2014.06.012 (2014).
48 Bradstreet, R. B. Kjeldahl method for organic nitrogen. *Analytical Chemistry* **26**, 185-187 (1954).
49 Bensch, K., Groenewald, J., Meijer, M., Dijksterhuis, J., Jurjević, Z., Andersen, B., Houbraken, J., Crous, P. W., Samson, R. A. *Cladosporium* species in indoor environments. *Studies in Mycology* **89**, 177-301 (2018).
50 National Center for Biotechnology Information (NCBI)[Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] – [October 2019]. Available from: https://www.ncbi.nlm.nih.gov/
51 Scheuerell, S. J. & Mahaffee, W. F. Variability associated with suppression of gray mold (*Botrytis cinerea*) on geranium by foliar applications of nonaerated and aerated compost teas. *Plant Disease* **90**, 1201-1208 (2006).
52 Preston, F. W. The volume of an egg. *American Ornithological Society* **91**, 132-138; 10.2307/4084667 (1974).
53 R Core Team. R: A Language and Environment for Statistical Computing. v. 1.2.1335 (Vienna, Austria; 2019).
54 Quinn, G. P. & Keough, M. J. *Experimental design and data analysis for biologists*. (Cambridge University Press, 2002).

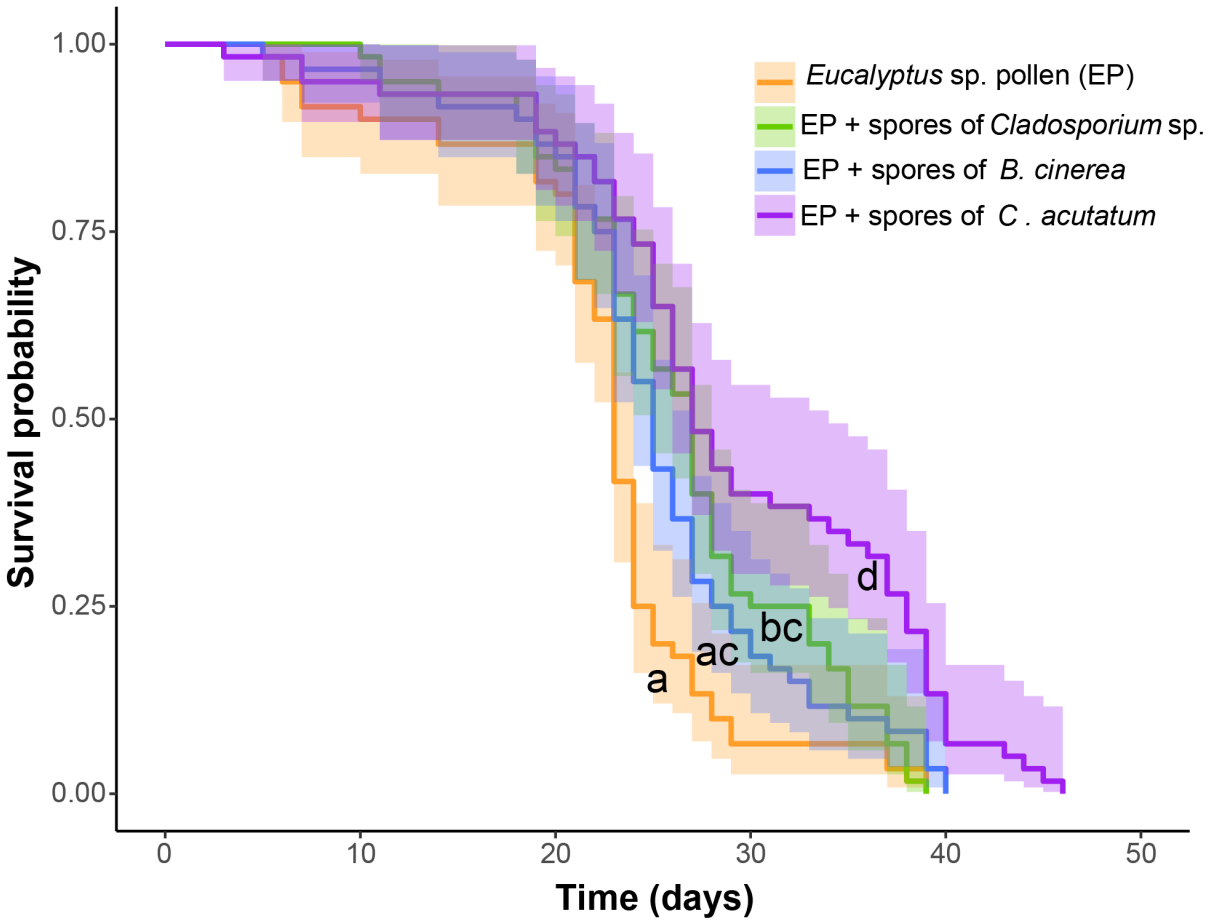

Figure 1. Survival probability of honey bee workers fed *Eucalyptus* sp. pollen alone or supplemented with spores of *Botrytis cinerea*, *Cladosporium* sp. or *Colletotrichum acutatum* at a mass ratio of 20:1 (Mean age at death ± standard error for the treatments in the above order were: 22.43 ± 0.90, 25.31 ± 0.92, 26.5 ± 0.89 and 29.1 ± 1.21). Shaded areas represent 95% confidence intervals. Lower case letters indicate significant differences among the diets (Kaplan-Meier method, log rank test P<0.01 and Bonferroni correction).

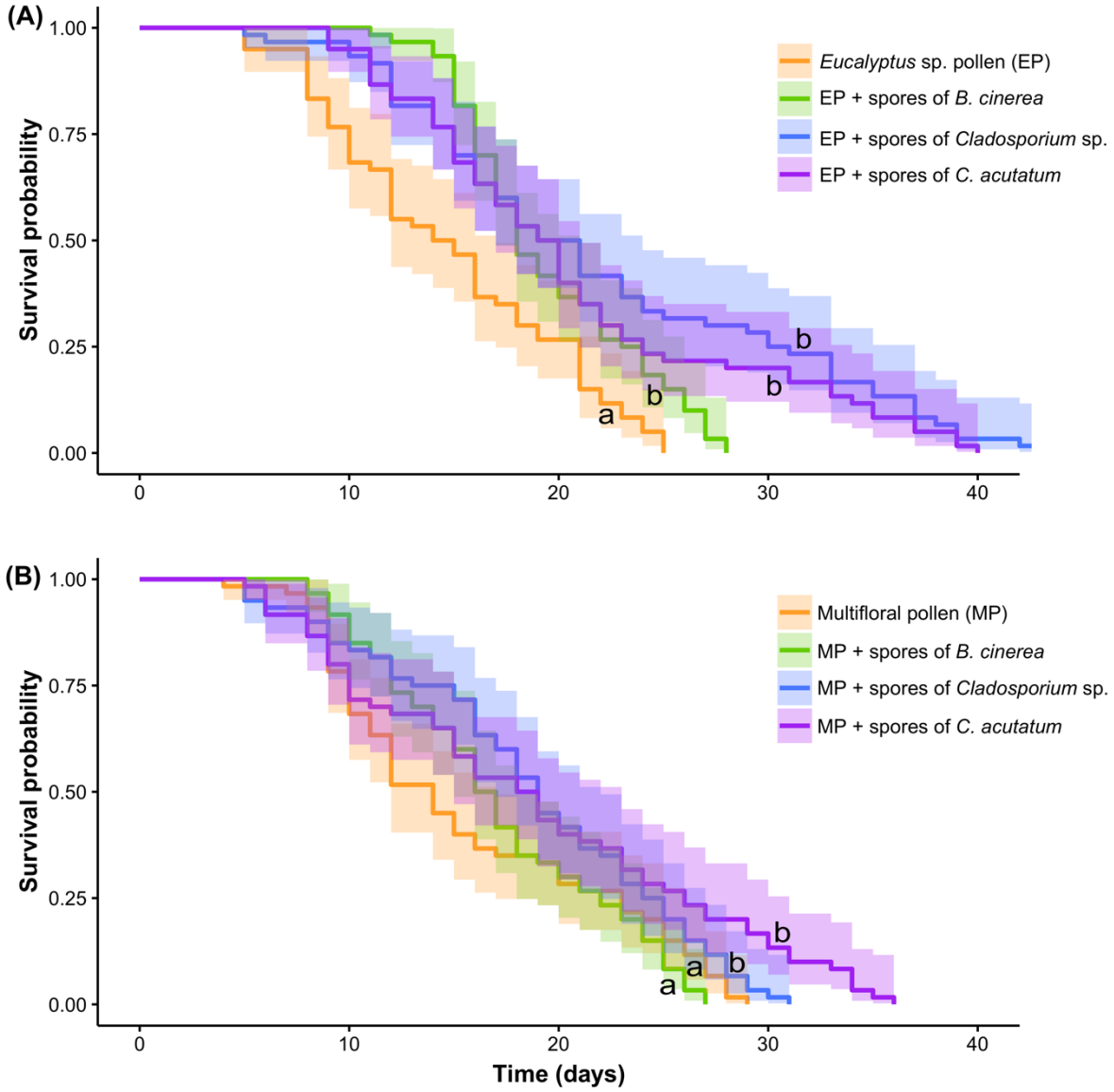

Figure 2. Survival of honey bee workers fed on pollen of *Eucalyptus* sp. (A) or multifloral sources (B) with or without spores of *Botrytis cinerea*, *Cladosporium* sp. or *Colletotrichum acutatum* at the mass ration of 20:1 (Mean age at death ± standard error for the treatments in the above order were: 14.80 ± 0.74, 19.55 ± 0.56, 22.2 ± 1.25 and 20.8 ± 1.09, 15.81 ± 0.89, 17.1 ± 0.71, 18.66 ± 0.90, 18.78 ± 1.15). Shaded areas represent 95% confidence intervals. Lower case letters indicate significant differences between the diets (Kaplan-Meier method, log rank test $P<0.01$ and

Table 1: P values of the survival analysis for both experiments by the Kaplan-Meier method, log rank test and Bonferroni correction.

	EP	EP + spores of *B. cinerea*	EP + spores of *Cladosporium* sp.	EP + spores of *C. acutatum*	MP	MP + spores of *B. cinerea*	MP + spores of *Cladosporium* sp.
			***Eucalyptus* sp. pollen only**				
EP + spores of *B. cinerea*	n.s.	-	-	-	-	-	-
EP + spores of *Cladosporium* sp.	0.02	n.s.	-	-	-	-	-
EP + spores of *C. acutatum*	2.5×10^{-6}	0.01	0.02	-	-	-	-
			***Eucalyptus* sp. and multifloral pollen**				
EP + spores of *B. cinerea*	0.001	-	-	-	-	-	-
EP + spores of *Cladosporium* sp.	4.2×10^{-5}	n.s.	-	-	-	-	-
EP + spores of *C. acutatum*	0.002	n.s.	n.s.	-	-	-	-
MP	n.s.	n.s.	0.0005	0.03	-	-	-
MP + spores of *B. cinerea*	n.s.	n.s.	0.004	n.s.	n.s.	-	-
MP + spores of *Cladosporium* sp.	0.005	n.s.	n.s.	n.s.	n.s.	n.s.	-
MP + spores of *C. acutatum*	0.01	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

EP = *Eucalyptus* sp. pollen, MP= Multifloral pollen, n.s. – Not significant

Table 2: Nutritional composition and amino acid profile of the pollen samples (mean ± standard deviation).

Composition (g/100g)	Pollen diet	
	Eucalyptus sp.	Multifloral
Ash	2.55 ± 0.13	1.91 ± 0.10
Carbohydrates	61.20 ± 3.06	64.40 ± 3.22
Fat	5.30 ± 0.33	6.80 ± 0.42
Protein	28.60 ± 0.92	20.70 ± 0.66
Sodium	14.00 ± 0.35	14.00 ± 0.35
Sugars	39.00 ± 4.29	38.00 ± 4.18
Alanine	1.11 ± 0.00	0.81 ± 0.06
Arginine	1.80 ± 0.04	0.85 ± 0.05
Aspartic acid	2.25 ± 0.00	1.84 ± 0.13
Glutamic acid	2.72 ± 0.02	1.82 ± 0.12
Glycine	1.03 ± 0.00	0.71 ± 0.05
Histidine	0.71 ± 0.01	0.51 ± 0.04
Isoleucine	1.02 ± 0.00	0.73 ± 0.05
Leucine	1.74 ± 0.00	1.25 ± 0.09
Lysine	1.39 ± 0.01	1.05 ± 0.08
Methionine	0.45 ± 0.01	0.33 ± 0.02
Phenylalanine	1.06 ± 0.01	0.74 ± 0.05
Proline	3.20 ± 0.01	1.85 ± 0.13
Serine	1.05 ± 0.01	0.78 ± 0.06
Threonine	0.89 ± 0.00	0.66 ± 0.05
Tyrosine	0.53 ± 0.03	0.39 ± 0.03
Valine	1.22 ± 0.00	0.85 ± 0.05

Supplementary Information

Table S1. Daily consumption of the different diets (mean ± standard error) at a mass ratio of 20:1 of pollen and fungal spores during both experiments (Generalized Linear Model).

Diets	Diet consumption (mg/bee/day)	P-value
***Eucalyptus* sp. pollen**		
Eucalyptus sp. pollen (EP)	2.43 ± 0.17	0.07
EP + spores of *B. cinerea*	2.22 ± 0.16	
EP + spores of *Cladosporium* sp.	2.16 ± 0.16	
EP + spores of *C. acutatum*	2.19 ± 0.15	
***Eucalyptus* sp. and multifloral pollen**		
EP	2.65 ± 0.23	0.49
EP + spores of *B. cinerea*	2.39 ± 0.26	
EP + spores of *Cladosporium* sp.	2.17 ± 0.38	
EP + spores of *C. acutatum*	2.18 ± 0.24	
Multifloral pollen (MP)	2.86 ± 0.27	
MP + spores of *B. cinerea*	3.01 ± 0.31	
MP + spores of *Cladosporium* sp.	2.86 ± 0.29	
MP + spores of *C. acutatum*	2.50 ± 0.29	

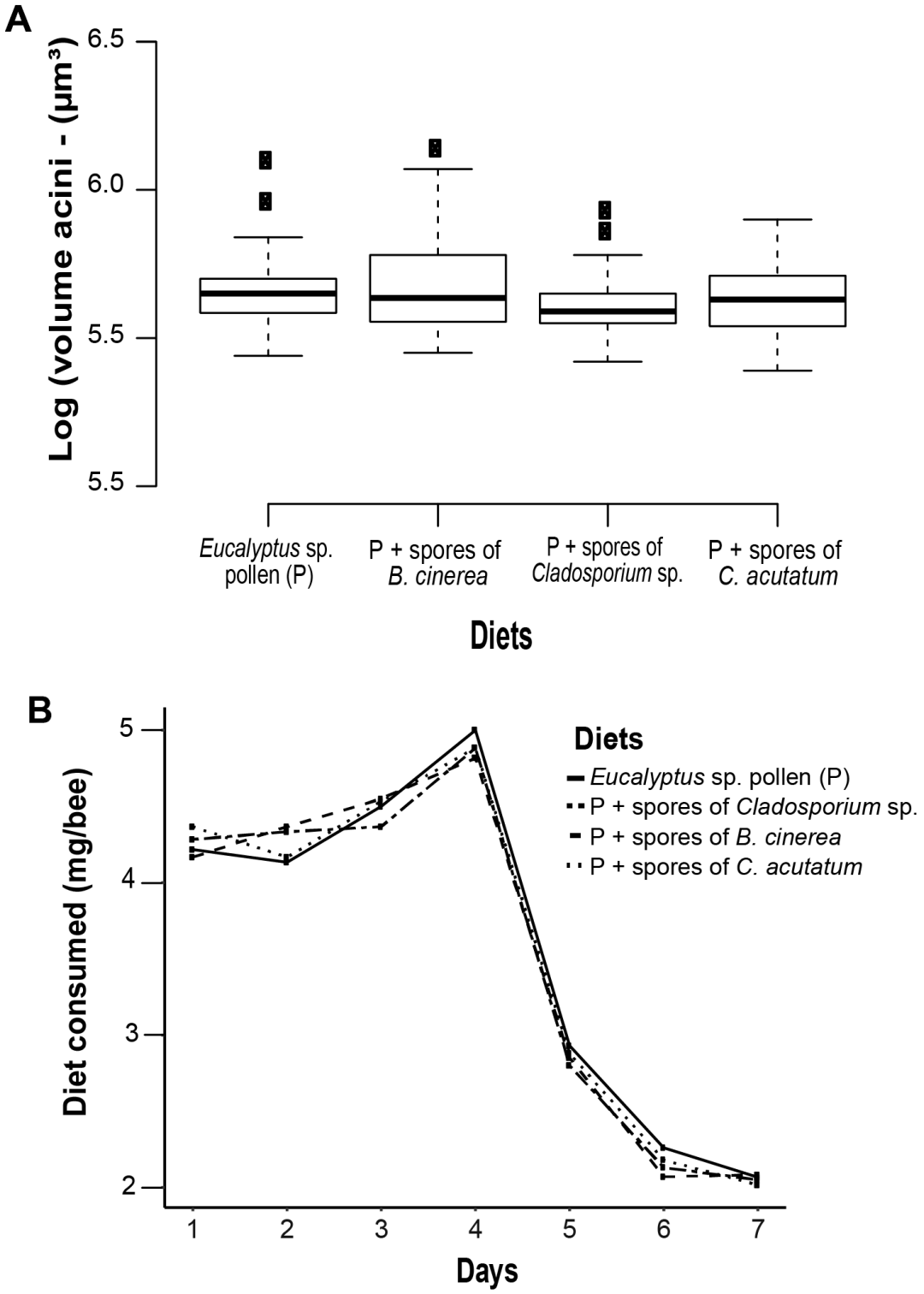

Figure S1. Average volume of the acini of the hypopharyngeal gland (A) (ANOVA, P>0.05) and (B) diet consumption of workers fed with *Eucalyptus* sp. pollen alone and *Eucalyptus* sp. pollen supplemented with spores of *Botrytis cinerea*, *Cladosporium* sp. or *Colletotrichum acutatum* at the mass ratio of 20:1 (ANOVA, P>0.05).

Table S2: Concentrations of pesticides detected in pollen samples.

Pesticides	*Eucalyptus* sp.	Multifloral
2-phenylphenol (mg/kg)	<0.010	<0.010
Abamectin (mg/kg)	<0.010	<0.010
Acephate (mg/kg)	<0.010	<0.010
Aldrin (mg/kg)	<0.010	<0.010
Atrazine (mg/kg)	<0.010	<0.010
Azinphos methyl (mg/kg)	<0.010	<0.010
Azoxystrobin (mg/kg)	<0.010	<0.010
Benalaxyl (mg/kg)	<0.010	<0.010
BHC alpha (mg/kg)	<0.010	<0.010
BHC beta (mg/kg)	<0.010	<0.010
BHC delta (mg/kg)	<0.010	<0.010
BHC gamma (Lindane) (mg/kg)	<0.010	<0.010
BHC Total (mg/kg)	<0.010	<0.010
Bifenazate (mg/kg)	<0.010	<0.010
Bifenthrin (mg/kg)	<0.010	<0.010
Bioresmethrin (mg/kg)	<0.010	<0.010
Bitertanol (mg/kg)	<0.010	<0.010
Boscalid (mg/kg)	<0.010	<0.010
Buprofezin (mg/kg)	<0.010	<0.010
Captan (mg/kg)	<0.050	<0.050
Carbaryl (mg/kg)	<0.010	<0.010
Carbendazim / Benomyl (mg/kg)	<0.010	<0.010
Chlorantraniliprole (mg/kg)	<0.010	<0.010
Chlordane (mg/kg)	<0.010	<0.010
Chlorfenapyr (mg/kg)	<0.010	<0.010
Chlorfenvinphos (mg/kg)	<0.010	<0.010
Chlorothalonil (mg/kg)	<0.050	<0.050
Chlorpyrifos (mg/kg)	<0.010	<0.010
Chlorpyrifos methyl (mg/kg)	<0.010	<0.010
Chlorthal dimethyl (mg/kg)	<0.010	<0.010
Clofentezine (mg/kg)	<0.010	<0.010
Cyfluthrin (mg/kg)	<0.010	<0.010
Cyfluthrin beta (mg/kg)	<0.010	<0.010
Cyhalothrin (mg/kg)	<0.010	<0.010
Cyhalothrin lambda (mg/kg)	<0.010	<0.010
Cypermethrin (mg/kg)	<0.010	<0.010
Cypermethrin alpha (mg/kg)	<0.010	<0.010
Cyproconazole (mg/kg)	<0.010	<0.010
Cyprodinil (mg/kg)	<0.010	<0.010
DDD p,p (mg/kg)	<0.010	<0.010

Table S2 (continued): Concentrations of pesticides detected in pollen samples.

DDE p,p (mg/kg)	<0.010	<0.010
DDT p,p (mg/kg)	<0.010	<0.010
DDT Total (mg/kg)	<0.010	<0.010
Deltamethrin (mg/kg)	<0.010	<0.010
Diazinon (mg/kg)	<0.010	<0.010
Dichlorvos (mg/kg)	<0.010	<0.010
Dicloran (mg/kg)	<0.010	<0.010
Dicofol (mg/kg)	<0.010	<0.010
Dieldrin (mg/kg)	<0.010	<0.010
Difenoconazole (mg/kg)	<0.010	<0.010
Dimethoate (mg/kg)	<0.010	<0.010
Dimethoate (Total) (mg/kg)	<0.010	<0.010
Dimethomorph (mg/kg)	<0.010	<0.010
Diphenylamine (mg/kg)	<0.010	<0.010
Disulfoton (mg/kg)	<0.010	<0.010
Dithianon (mg/kg)	<0.1	<0.1
Diuron (mg/kg)	<0.010	<0.010
Endosulphan alpha (mg/kg)	<0.010	<0.010
Endosulphan beta (mg/kg)	<0.010	<0.010
Endosulphan sulphate (mg/kg)	<0.010	<0.010
Endosulphan Total (mg/kg)	<0.010	<0.010
Endrin Total (mg/kg)	<0.010	<0.010
Esfenvalerate (mg/kg)	<0.010	<0.010
Ethoprofos (mg/kg)	<0.010	<0.010
Etoxazole (mg/kg)	<0.010	<0.010
Fenamiphos (mg/kg)	<0.010	<0.010
Fenarimol (mg/kg)	<0.010	<0.010
Fenhexamid (mg/kg)	<0.010	<0.010
Fenitrothion (mg/kg)	<0.010	<0.010
Fenoxycarb (mg/kg)	<0.010	<0.010
Fenpyroximate (mg/kg)	<0.010	<0.010
Fenthion (mg/kg)	<0.010	<0.010
Fenvalerate (mg/kg)	<0.010	<0.010
Fenvalerate (Total) (mg/kg)	<0.010	<0.010
Fipronil (mg/kg)	<0.010	<0.010
Flubendiamide (mg/kg)	<0.010	<0.010
Flucythrinate (mg/kg)	<0.010	<0.010
Fludioxonil (mg/kg)	<0.010	<0.010
Flumethrin (mg/kg)	<0.010	<0.010
Flusilazole (mg/kg)	<0.010	<0.010
Fluvalinate (mg/kg)	<0.010	<0.010

Table S2 (continued): Concentrations of pesticides detected in pollen samples.

Fluvalinate tau (mg/kg)	<0.010	<0.010
HCB (mg/kg)	<0.010	<0.010
Heptachlor (mg/kg)	<0.010	<0.010
Heptachlor epoxide (mg/kg)	<0.010	<0.010
Hexaconazole (mg/kg)	<0.010	<0.010
Hexythiazox (mg/kg)	<0.010	<0.010
Imazalil (mg/kg)	<0.010	<0.010
Imidacloprid (mg/kg)	<0.010	<0.010
Indoxacarb (mg/kg)	<0.010	<0.010
Iprodione (mg/kg)	<0.010	<0.010
Kresoxim methyl (mg/kg)	<0.010	<0.010
Linuron (mg/kg)	<0.010	<0.010
Malathion (mg/kg)	<0.010	<0.010
Metalaxyl (mg/kg)	<0.010	<0.010
Methamidophos (mg/kg)	<0.010	<0.010
Methidathion (mg/kg)	<0.010	<0.010
Methomyl (mg/kg)	<0.010	<0.010
Methomyl Oxime (mg/kg)	<0.010	<0.010
Metribuzin (mg/kg)	<0.010	<0.010
Mevinphos (mg/kg)	<0.010	<0.010
Monocrotophos (mg/kg)	<0.010	<0.010
Myclobutanil (mg/kg)	<0.010	<0.010
Omethoate (mg/kg)	<0.010	<0.010
Oxyfluorfen (mg/kg)	<0.010	<0.010
Paclobutrazol (mg/kg)	<0.010	<0.010
Parathion ethyl (mg/kg)	<0.010	<0.010
Parathion methyl (mg/kg)	<0.010	<0.010
Penconazole (mg/kg)	<0.010	<0.010
Pendimethalin (mg/kg)	<0.010	<0.010
Penthiopyrad (mg/kg)	<0.010	<0.010
Permethrin (mg/kg)	<0.010	<0.010
Phenothrin (mg/kg)	<0.010	<0.010
Phorate (mg/kg)	<0.010	<0.010
Phosmet (mg/kg)	<0.010	<0.010
Piperonyl butoxide (mg/kg)	<0.010	<0.010
Pirimicarb (mg/kg)	<0.010	<0.010
Pirimiphos methyl (mg/kg)	<0.010	<0.010
Prochloraz (mg/kg)	<0.010	<0.010
Procymidone (mg/kg)	<0.010	<0.010
Profenofos (mg/kg)	<0.010	<0.010
Propargite (mg/kg)	<0.010	<0.010

Table S2 (continued): Concentrations of pesticides detected in pollen samples.

Propiconazole (mg/kg)	<0.010	<0.010
Prothiofos (mg/kg)	<0.010	<0.010
Pymetrozine (mg/kg)	<0.010	<0.010
Pyraclostrobin (mg/kg)	<0.010	<0.010
Pyrethrins (mg/kg)	<0.010	<0.010
Pyrimethanil (mg/kg)	<0.010	<0.010
Pyriproxyfen (mg/kg)	<0.010	<0.010
Quintozene (mg/kg)	<0.010	<0.010
Spinetoram (mg/kg)	<0.010	<0.010
Spinosad (mg/kg)	<0.010	<0.010
Spirotetramat (mg/kg)	<0.010	<0.010
Sulfoxaflor (mg/kg)	<0.010	<0.010
Tebuconazole (mg/kg)	<0.010	<0.010
Tebufenozide (mg/kg)	<0.010	<0.010
Tebufenpyrad (mg/kg)	<0.010	<0.010
Terbufos (mg/kg)	<0.010	<0.010
Tetradifon (mg/kg)	<0.010	<0.010
Thiabendazole (mg/kg)	<0.010	<0.010
Thiacloprid (mg/kg)	<0.010	<0.010
Thiamethoxam (mg/kg)	<0.010	<0.010
Tolclofos methyl (mg/kg)	<0.010	<0.010
Triadimefon (mg/kg)	<0.010	<0.010
Triadimenol (mg/kg)	<0.010	<0.010
Trichlorfon (mg/kg)	<0.010	<0.010
Trifloxystrobin (mg/kg)	<0.010	<0.010
Vinclozolin (mg/kg)	<0.010	<0.010

Chapter 5

Fungal and bacterial communities associated with honey bee corbicular pollen in almond orchard landscapes, with special reference to plant pathogens

Manuscript prepared for submission to Plos One, except that minor adjustments were made to be consistent with the format used elsewhere in this thesis.

Statement of Authorship

Title of Paper	Fungal and bacterial communities associated with honey bee corbicular pollen in almond orchard landscapes, with special reference to plant pathogens
Publication Status	Published ☐ Submitted for Publication ☐ Accepted for Publication ☐ Unpublished and unsubmitted work written in manuscript style **X**
Publication Details	Jorgiane B. Parish, Kelly Hill, Eileen S. Scott, Nathan S. Watson-Haigh, Raymond Correll, Katja Hogendoorn

Principal author

Name of Principal Author (Candidate)	Jorgiane Benevenute Parish		
Contribution to the Paper	Designed and performed the experiment, analysed the data, and wrote the manuscript.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/11/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kelly Hill		
Contribution to the Paper	Contributed to research ideas, design and data analysis; provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	11/11/2019

Name of Co-Author	Eileen S. Scott		
Contribution to the Paper	Contributed to research ideas and design, provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	12/11/2019

Name of Co-Author	Nathan S. Watson-Haigh		
Contribution to the Paper	Contributed to data analysis and writing of the manuscript		
Signature		Date	11/11/2019

Name of Co-Author	Raymond Correll		
Contribution to the Paper	Contributed to data analysis and writing of the manuscript		
Signature		Date	11/11/2019

Name of Co-Author	Katja Hogendoorn		
Contribution to the Paper	Contributed to research ideas, design and sample collection; provide technical assistance and support of experiments, and writing of the manuscript		
Signature		Date	12/11/2019

Chapter 5

Fungal and bacterial communities associated with honey bee corbicular pollen in almond orchard landscapes, with special reference to plant pathogens

Jorgiane B. Parish[1], Kelly Hill[1,2], Eileen S. Scott[1], Nathan S. Watson-Haigh[1], Raymond Corell[3], Katja Hogendoorn[1]

[1]School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, South Australia 5005, Australia; [2]South Australian Research and Development Institute (SARDI), Adelaide, South Australia 5000, Australia; [3] Rho Environmetrics, Highgate, South Australia 5063, Australia

Abstract

Honey bees, *Apis mellifera*, collect, consume and transport microbes that are associated with floral resources. Despite the importance of pollinators and food crops such as almonds, the diversity and richness of fungal and bacterial communities, their potential transfer by pollinators and impact in almond orchard landscapes are as yet poorly understood. Here, we investigate the fungal and bacterial communities present in corbicular pollen of honey bees foraging on a range of plant species in and around flowering almond orchards. The Illumina MiSeq platform was used to generate sequences of the ITS region and 16S rRNA gene. Fungal and bacterial alpha diversity differed significantly among pollen species. Pollen species and locations were significantly dissimilar for beta diversity of fungal, but not of bacterial, communities. Plant pathogens were identified in pollen of almonds and other plant species, with stronger associations at the end of almond bloom. This study provides fundamental

knowledge of microbial ecology of pollen species in almond orchards.

Keywords: *Apis mellifera*, Illumina MiSeq, pollen, fungal and bacterial microbiomes, ITS and 16S rRNA

Introduction

Plant microbiomes have received considerable attention in recent years due to their importance in plant health and productivity [1]. These microbiomes can be influenced by plant species, genotype, organ and health status as well as by environmental factors such as management and climate [1, 2]. However, communities of microorganisms associated with pollen and nectar of multiple plant species in agricultural systems and their transfer by pollinators are not well documented.

Honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), are considered the most valuable pollinator of crops worldwide [3, 4] and pollination by bees from managed hives contributes to improved yield and fruit quality in a range of crops, including almonds (*Prunus dulcis*) [5]. However, honey bee workers can collect plant pathogens that are present on the flowers and transfer them to the hive or to other flowers [6]. This can contribute to the spread of diseases among crops and native vegetation [7]. Although honey bees have been implicated as potential vectors of plant pathogens and infected pollen in crops [8-11], the associations among foragers, pollen species and communities of plant pathogenic fungi and bacteria in almond orchards have not been investigated. Information about these associations may help to reduce the risk of transmission of plant pathogens and, consequently, improve the efficiency of crop production as well as assist conservation of native plant communities [12, 13].

Almond crops, which require pollination by honey bees for nut set [14, 15], contribute approximately US$11 billion and AU$429 million to the USA and Australian economies, respectively [16, 17]. The Australian almond industry is currently undergoing a large expansion

in investment. This rapid growth raises concerns about the capacity of the agricultural and ecological environments to support adequate pollination services [18] and the availability of plant diversity in almond orchards that can provide complementary food sources for pollinators.

Sustainable orchard management practices of preserving populations of weeds, wildflowers and remnants of native vegetation in and around almond orchards benefit pollinators [19], soil fertility, pest control and productivity [20-22]. However, such plants could also serve as hosts of microbiomes that are potentially transferred between plant species by pollinators. Additionally, management practices such as the use of fungicides to protect crops from disease can affect pollinator health [23] and the composition of microorganisms in floral resources [24, 25]. While there is evidence that alternative host plantings benefit bees in agricultural systems [22], little is known about microbial populations of weeds and native vegetation in almond orchards.

Metabarcoding has been shown to be useful in ecology for determining the diversity of microorganisms in environmental samples [25-27]. While this approach has been used to study bacteria associated with pollen collected by honey bees [28, 29], it has not, to our knowledge, been applied to examine both plant pathogenic fungi and bacteria of honey bee corbicular pollen. This implies that the potential of honey bees as vectors, and of plant species as hosts, of plant pathogens has not been thoroughly investigated. Thus, this study aimed to determine the diversity profile of fungal and bacterial communities associated with corbicular pollen collected from almonds, weeds and native vegetation in and surrounding almond orchards, where possible, during the almond flowering season, with particular attention to plant pathogens.

Materials and Methods

Study sites and pollen collection

Foraging worker honey bees that carried large pollen loads in their corbiculae were randomly collected from flowers of: *Acacia* sp. B. R. Maslin (golden wattle), *Arctotheca calendula* L. Levyns (capeweed), *Asphodelus fistulosus* L. (onion weed), *Brassica napus* L. (canola), *Brassica rapa* L. (mustard), *Eucalyptus* sp. L'Her. (white mallee), *Oxalis pes-caprae* L. (soursob), *Prunus dulcis* (Mill.) D. A. Webb (almond), *Raphanus raphanistrum* L. (wild radish), *Raphanus tournefortii* L. (wild turnip), *Viburnum tinus* L. (laurestine) and *Zygophyllum apiculatum* F. Muell (thick twinleaf) in five almond orchards (designated A to E) located in Adelaide and in the Riverland of South Australia, Australia (Table 1). Pollen foragers were collected from plants surrounding the orchard at orchard A only. Collection occurred during the early (08-11 August), middle (14-19 August) and late (23-30 August) period of the almond bloom of 2017 for orchard A and at all other locations during 2018. Individual pollen foragers were captured in sterile Falcon tubes, kept on ice and subsequently stored at -80^{o}C.

The almond orchards were planted with a range of varieties in alternating rows and were surrounded by vineyards, citrus and stone fruit orchards, grain crops and native vegetation. In orchards A, B, D and E the fungicide Luna Sensation® (fluopyram/trifloxystrobin) was applied to almond trees during bloom to minimize damage caused by pathogens [30]. In orchard C, fungicide was not applied during bloom.

The pollen loads of workers collected on the same host plant, date and orchard were removed from their corbiculae in a laminar flow cabinet using a sterile needle and combined in a sterile Eppendorf tube (see Table 1 for number of foragers per sample). Composite pollen collections were used to obtain the best estimate of the microbial community of each pollen species [31].

DNA extraction, amplification and sequencing

Genomic DNA was extracted from combined pollen samples using the Machery-Nagel NucleoSpin Food Kit (Duren, Germany). DNA extractions were performed according to the

manufacturer's specifications. During the extraction process, two negative controls (sterile water and reagents only in place of a pollen sample) were used to monitor for atmospheric or cross-contamination among the samples. Two positive controls were used for the fungal DNA extractions (pollen plus mycelia of *Botrytis cinerea* scraped from about 2 cm^2 of pure cultures on potato dextrose agar and pollen plus mycelia of *Colletotrichum acutatum* prepared in the same way). The choice of fungi used as positive controls reflected their importance as pathogens of almond [32, 33].

Table 1. Number of pollen foragers collected per plant species at each of five locations in South Australia.

Collection times	Orchard	Location	Host	Number of foragers
Early	A	Paringa	*Prunus dulcis*	22
	A	Paringa	*P. dulcis*	19
	A	Paringa	*Raphanus tournefortii*	37
	A	Paringa	*R. tournefortii*	10
	E	McLaren Vale	*P. dulcis*	40
	E	McLaren Vale	*R. raphanistrum*	29
Middle	A	Paringa	*P. dulcis*	26
	A	Paringa	*R. tournefortii*	41
	A (surroundings)	Paringa	*Acacia* sp.	28
	A (surroundings)	Paringa	*Asphodelus fistulosus*	28
	A (surroundings)	Paringa	*A. fistulosus*	28
	A (surroundings)	Paringa	*Eucalyptus* sp.	34
	A (surroundings)	Paringa	*Zygophyllum apiculatum*	30
	B	Lyrup	*Brassica napus*	30
	B	Lyrup	*Oxalis pes-caprae*	30
	B	Lyrup	*P. dulcis*	30
	D	Adelaide	*P. dulcis*	30
Late	A	Paringa	*Arctotheca calendula*	34
	A	Paringa	*B. rapa*	45
	A	Paringa	*P. dulcis*	45
	A	Paringa	*P. dulcis*	46
	A	Paringa	*R. tournefortii*	39
	A (surroundings)	Paringa	*Acacia* sp.	20
	A (surroundings)	Paringa	*A. calendula*	25
	A (surroundings)	Paringa	*Eucalyptus* sp.	8
	A (surroundings)	Paringa	*Eucalyptus* sp.	24
	A (surroundings)	Paringa	*Viburnum tinus*	48
	A (surroundings)	Paringa	*Z. apiculatum*	30
	A (surroundings)	Paringa	*Z. apiculatum*	30
	B	Lyrup	*B. napus*	30
	B	Lyrup	*B. napus*	30
	B	Lyrup	*P. dulcis*	30
	C	Paringa	*P. dulcis*	45

Repeat entries for the same period, host and orchard indicate samples collected on different days.

DNA amplification for Illumina amplicon library preparation was carried out using a two-step PCR protocol, initially to target the gene region of interest and subsequently adding barcodes and adaptors for sequencing. The first PCR targeted the fungal ITS rRNA gene region using primer pair ITS1F (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CTT GGT CAT TTA GAG GAA GTA A) and ITS2R (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G GCT GCG TTC TT CAT CGA TGC) [34] combined with an overhanging target region (underlined) for the barcoded adaptor primers in the second PCR. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using primer pair 341F (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG) and 785R (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C) [35], combined with overhangs as described above. HPLC-purified primers were purchased from Integrated DNA Technologies, Inc (Singapore). Each reaction contained: 2 µL DNA (1-15 ng/µL), 8.6 µL nuclease-free water, 1.6 µL of each primer, 0.4 µL dNTPs, 0.4 µL Q5 High-Fidelity DNA Polymerase (New England, Biolabs) and 4 µL Deoxynucleotide Solution Mix (New England, Biolabs) in a total volume 20 µL. Amplification cycle conditions were as follows: initial denaturation at 98°C for 30 s, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 55°C (ITS) or 50^{o}C (16S) for 40 s and elongation at 72°C for 20 s, and a final elongation at 72°C for 2 min, carried out using an SC 300 thermal cycler (Kyratec, Australia). Amplicon size and purity were confirmed by electrophoresis at 110 V for 30 min in a 2% agarose gel containing 0.003% nucleic acid staining solution (GelRed, Intron Biotechnology) in 1 x Tris acetate EDTA buffer and viewed under UV light. These amplicons constituted the template for the second PCR.

The second PCR was carried out with primers containing a 6 bp barcode sequence and Illumina adapters (Nextera combinatorial dual indexes) and each reaction contained 4 µL DNA, 11.25 µL nuclease-free water, 2 µL of each primer, 0.5 µL dNTPs, 0.25 µL Q5 High-Fidelity DNA

Polymerase (New England, Biolabs) and 5 µL Deoxynucleotide Solution Mix (New England, Biolabs). Amplification was as described above, with the exception that 12 cycles were used for the second PCR. Amplicons were purified using carboxyl-coated magnetic beads (SPRI beads, Agencourt AMPure XP, Agencourt, Beverly, MA, USA) and quantified using the Qubit dsDNA HS Assay kit (Life Technologies). Samples were diluted to a final concentration of 10 ng/µL and DNA size spectra were visualized on a 2% agarose gel as described above.

Duplicate libraries were prepared for each sample and all libraries were pooled using equimolar amounts. The combined library was diluted to 10 nM using 10 mM Tris pH 8.5. The final preparation was sequenced using the MiSeq 2 x 300 bp paired-end run protocol at the Australian Genome Research Facility. Machine-processed sequencing output has been deposited in GenBank under BioProject PRJNA548419.

Bioinformatics and statistics

QIIME2-2017.6 was used to process the ITS and 16S rRNA sequences [36]. Sequences were first trimmed to remove primers and low quality sequence (based on a Phred quality score cut-off of Q35). DADA2 was used to identify amplicon sequence variants (ASVs), remove chimeric sequences and to filter the data according to quality [37]. The Naive Bayes classifier was selected and pre-trained against the dynamic reference UNITE QIIME release for Fungi 2 (version 18.11.2018 accessed February 2019) [38] and the Greengenes 16S rRNA database based on 97-99% and 99% sequence similarity (version gg_13_8 99 accessed February 2019) respectively [39]. These trained classifiers were then used for taxonomic assignment of ITS and 16S sequences, respectively. Sequences identified as chloroplast DNA were manually removed. Alpha diversity indices (Chao-1, Dominance, Evenness, Shannon and Simpson) were calculated using the PAST software [40] and statistically compared between pollen species and orchard locations by Kruskal-Wallis and Mann–Whitney–Wilcoxon test in R statistical software version 3.3.2 [41]. The Shannon index, which is sensitive to rare species, was used to

extrapolate the total richness from the observed ASVs; the Simpson index was used to quantify the diversity; Dominance and Evenness assessed the distribution of the taxa in the samples and Chao-1 estimated species richness and rare ASVs. Beta diversity analysis of Bray-Curtis Unifrac distances was used to assess dissimilarities of the fungal and bacterial communities among the pollen species, orchards, time of collection and the interaction between pollen species and time of collection by Permanova at $P<0.01$. The ASVs shared by the pollen species are shown as the number of ASVs in the UpSet plots generated using UpSetR [42]. Network analyses were constructed using the "bipartite" package [43] for orchard A only by matching pollen species and ASVs of plant pathogenic bacteria and fungi. Orchard A was chosen because it had the highest plant diversity. The relative incidences of the agriculturally relevant fungi and bacteria were calculated based on the percentage of detected ASVs found in each pollen species, collection time or location.

Results

Sequencing quality control and taxa generated

A total of 10,724,489 reads was generated across the 144 libraries (i.e. ITS and 16S libraries, duplicates and controls) and 2,404,531 and 3,376,615 reads remained after the initial quality trimming for the ITS and 16S rRNA gene sequences, respectively (Table 2). ITS and 16S ASVs were clustered into four and 22 phyla, respectively. Overall, 987 and 1,996 fungal and bacterial ASVs were resolved across the samples, at 97-99% and 99% similarity, respectively.

Table 2. Summary of ITS and 16S rRNA gene sequence processing.

Time of collection	Orchard	Plant species	Total libraries	ITS			16S		
				N initial reads	N after quality trimming	% chimeras	N initial reads	N after quality trimming	% chimeras
Early	A	*Prunus dulcis*	4	165,308	88,920	3.13	197,333	151,288	7.3
	A	*Raphanus tournefortii*	4	68,169	24,000	0.61	321,568	179,856	14.23
	E	*Prunus dulcis*	2	65,147	38,148	11.39	187,288	142,942	8.22
	E	*Raphanus raphanistrum*	2	90,229	54,717	0.96	194,882	138,476	8.25
Middle	A	*Acacia* sp.	2	96,222	62,871	0.73	178,586	78171	4.22
	A	*Asphodelus fistulosus*	4	179,099	86,513	4.84	263,117	193,811	11.46
	A	*Eucalyptus* sp.	2	79,967	37,593	1.03	158,521	97,651	10.86
	A	*Prunus dulcis*	2	94,226	48,838	4.27	109,186	83,688	8.61
	A	*Raphanus tournefortii*	2	73,973	34,992	0	43,505	26,747	9.32
	A	*Zygophyllum apiculatum*	2	175,446	122,100	2.65	275,055	185,916	14.48
	B	*Brassica napus*	2	96,093	58,559	16.72	230,055	175,993	18.38
	B	*Oxalis pes-caprae* L.	2	91,969	54,004	5.77	322,572	244,395	28.38
	B	*Prunus dulcis*	2	126,913	77,839	6.74	471,230	358,400	10.9
	D	*Prunus dulcis*	2	57,468	29,725	3.62	48,404	37,829	7.38
Late	A	*Acacia* sp.	2	106,326	58,855	1.11	200,315	89,466	4.33
	A	*Arctotheca calendula*	4	168,467	103,167	3.96	308,599	208,642	9.15
	A	*Brassica rapa*	2	114,915	58,584	15.65	188,672	97,659	20.77
	A	*Eucalyptus* sp.	4	156,415	72,006	2.71	488,467	334,261	8.22
	A	*Prunus dulcis*	4	240,658	131,680	6.75	242,099	184,866	9.37
	A	*Raphanus tournefortii*	2	90,631	57,661	1.36	201,983	143,761	10.57
	A	*Viburnum tinus*	2	1,155,236	722,053	5.1	110,595	83,576	10.85
	A	*Zygophyllum apiculatum*	4	307,818	219,022	4.37	475,393	307,240	15.5
	B	*Brassica napus*	4	271,388	179,851	15.95	271,574	202,611	20.68
	B	*Prunus dulcis*	2	172,318	106,258	6.35	185,356	125,233	11.82
	C	*Prunus dulcis*	2	124,321	72,219	6.11	271,379	196,165	8.84
Controls	-	*Negative*	2	22,108	222	0	86,281	44,397	0.43
	-	Pollen + *Botrytis cinerea*	2	50,435	30,593	0	-	-	-
	-	Pollen + *Colletotrichum acutatum*	2	36,246	19,555	0.03	-	-	-
	-	Water	2	18,218	423	0	8,201	504	0

Fungal and bacterial community composition

Of the four fungal phyla identified, Ascomycota and Basidiomycota were the most abundant. In total, 33 distinct fungal genus identities were assigned to ASVs and the most common genus in terms of sequence reads was *Alternaria*. Of the 22 bacterial phyla identified, Cyanobacteria, Proteobacteria and Actinobacteria were the most abundant. A total of 201 distinct bacterial genera were assigned to ASVs and the most common genus in terms of sequence reads was *Hymenobacter* (Supporting Information Fig. S1). The composition and proportion of ASVs in duplicates were generally similar (Fig. 1).

The almond pathogen, *Aspergillus flavus*, was detected during early almond bloom in pollen of *P. dulcis* in orchards A and E, in the middle of bloom in pollen of *Acacia* sp. in the surroundings of orchard A, and at the end of bloom in pollen of *P. dulcis* in orchards A, B and C, *B. napus* in orchard B and *Acacia* and *Eucalyptus* spp., *V. tinus* and *Z. apiculatum* in the surroundings of orchard A. Another almond pathogen, *Colletotrichum acutatum,* was identified during mid-bloom in pollen of *R. tournefortii* and *Eucalyptus* sp. in and around orchard A, and at the end of bloom in pollen of *P. dulcis* and *B. napus* in orchard B, and *A. calendula*, *B. rapa* and *Z. apiculatum* in and around orchard A (Fig. 1).

Additional fungi capable of causing plant diseases were identified in and in the surroundings of the orchards. These were; *Alternaria carthami*, *A. niger*, *Ceratobasidium cereale*, *Clypeophysalospora latitans*, *C. asianum*, *Itersonilia perplexans* and species of the genera *Cladosporium*, *Fusarium*, *Phoma* and *Pyrenophora*, which include pathogens. In addition, there were sequences of fungi that could either not be assigned to a genus or

have no known agricultural relevance. These were grouped as “Others” (Fig. 1).

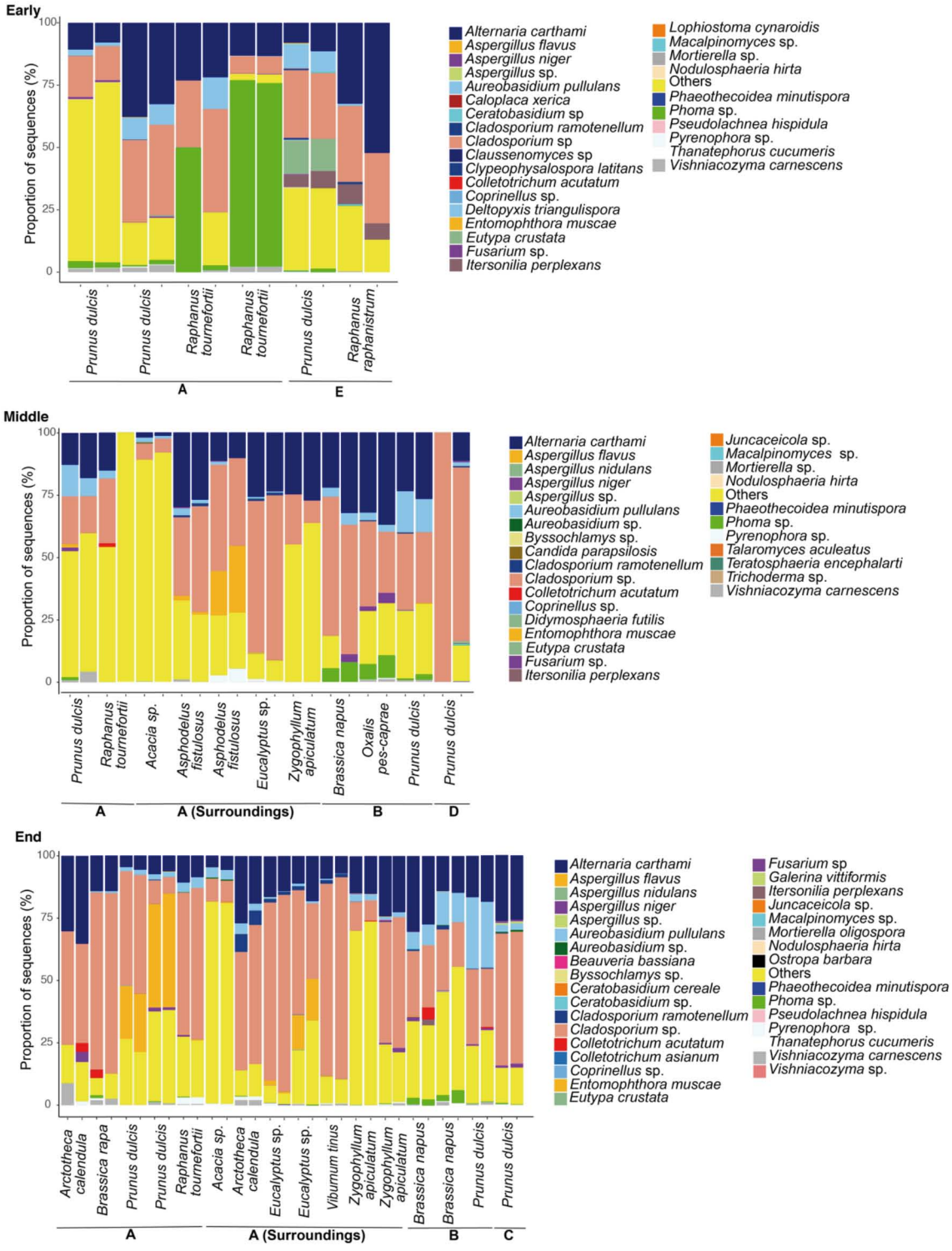

Figure 1. Relative abundance (proportion of sequences) of agriculturally relevant fungi in the corbicular pollen of *Apis mellifera* foragers. Bars indicate plants from which pollen foragers were collected and each colour represents amplicon sequence variants of fungal genera or species during almond bloom in five orchards (A-E). Each bar is followed by its duplicate. Repeat entries for the same host plant within the season period indicate samples collected on different days.

Two genera that include bacteria pathogenic to almond trees, *Agrococcus* and *Pseudomonas*, were identified in pollen of *R. tournefortii* in orchard A and of *P. dulcis* and *R. raphanistrum* in orchard E early in almond bloom. In the middle of almond bloom, *Agrococcus* sp. was found in pollen of *B. napus* in orchard B and of *Eucalyptus* sp. in the surroundings of orchard A; and *Pseudomonas* sp. was associated with pollen of *Eucalyptus* sp. in the surroundings of orchard A. At the end of almond bloom, *Pseudomonas* sp. was found in pollen of *Acacia* sp. in the surroundings of orchard A (Fig. 2). Other bacteria potentially pathogenic to plants other than almond were identified in pollen from plants surrounding orchard A. These were; *Acidovorax avenae*, *Rhodococcus fascians* and species of the genera *Agrobacterium*, *Clavibacter* and *Rickettsia*, which include several plant pathogens (Fig. 2). Sequences grouped as "Others" (Fig. 2) include unassigned bacterial sequences which were not classified to genus and some bacteria with no known agricultural relevance.

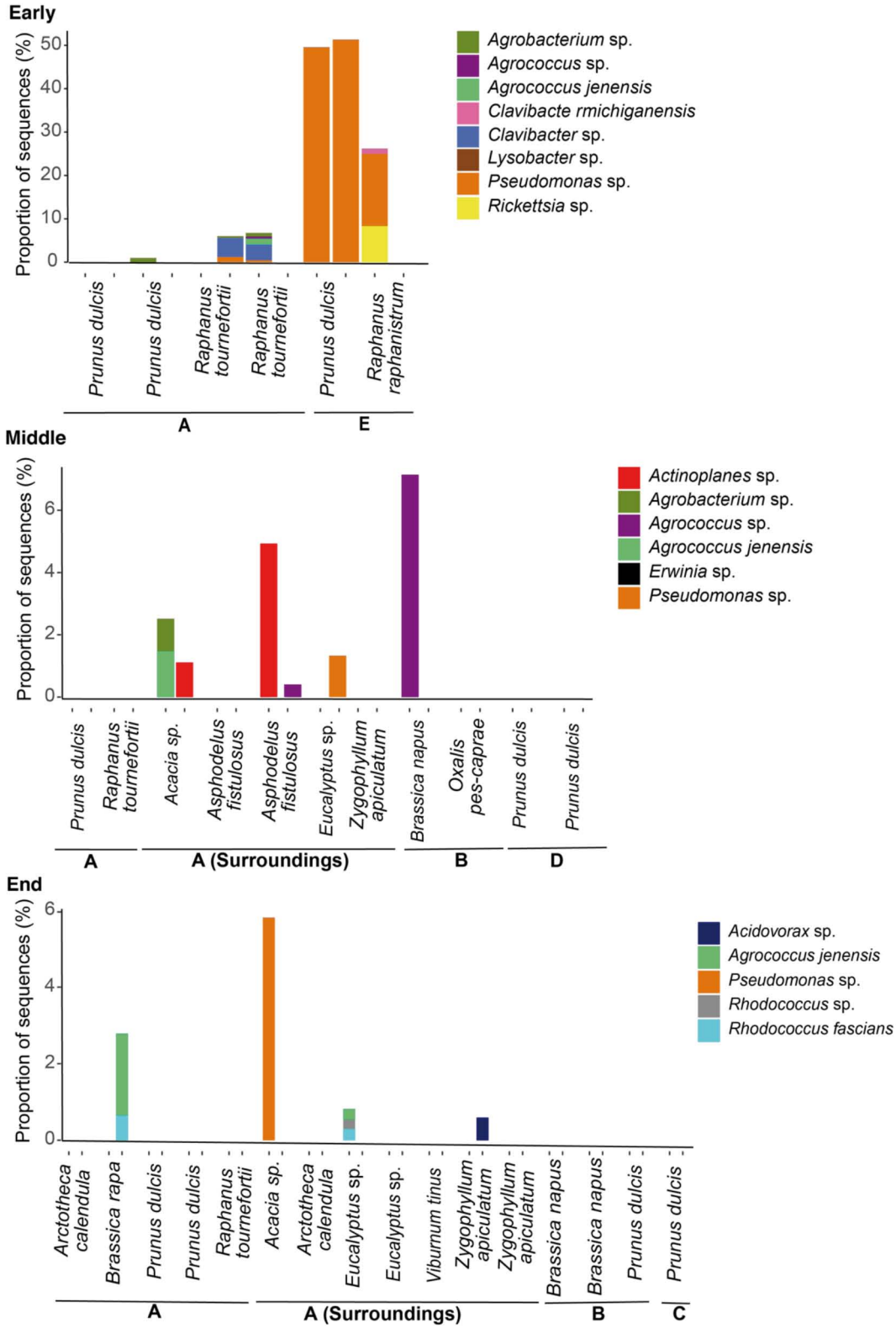

Figure 2. Relative abundance (proportion of sequences) of agriculturally relevant bacteria in the corbicular pollen of *Apis mellifera* foragers. Bars indicate plants from which pollen foragers were collected and each colour represents amplicon sequence variants groups of bacterial genera or species in the as described in Fig. 1

Bacterial and fungal diversity

For all five indices of alpha richness and diversity, except Evenness, significant differences were found in the microbial composition of the pollen species (Supplementary Information Tables S1 and S2). The indices Dominance, Shannon and Simpson differed significantly between orchards A and B for fungal communities (Kruskal-Wallis, $P<0.05$; Table S3). There were no significant differences in the bacterial composition among the almond pollen samples collected from the five locations (Kruskal-Wallis, $P>0.05$).

Bray-Curtis beta diversity analysis showed significant clusters among the pollen species for fungal community diversity but there were no clusters of dissimilar bacterial composition (ITS - Adonis $R^2=0.35$, $P<0.001$; 16S - $R^2=0.2$, $P=0.79$; Figure 3). There were no significant clusters for location (ITS – Adonis $R^2=0.063$, $P=0.16$; 16S – $R^2=0.06$, $P=0.63$), time of collection (ITS – Adonis $R^2=0.026$, $P=0.24$; 16S – $R^2=0.031$, $P=0.35$) or for the interaction between pollen species and collection time (ITS – Adonis $R^2=0.06$, $P=0.68$; 16S – $R^2=0.08$, $P=0.509$).

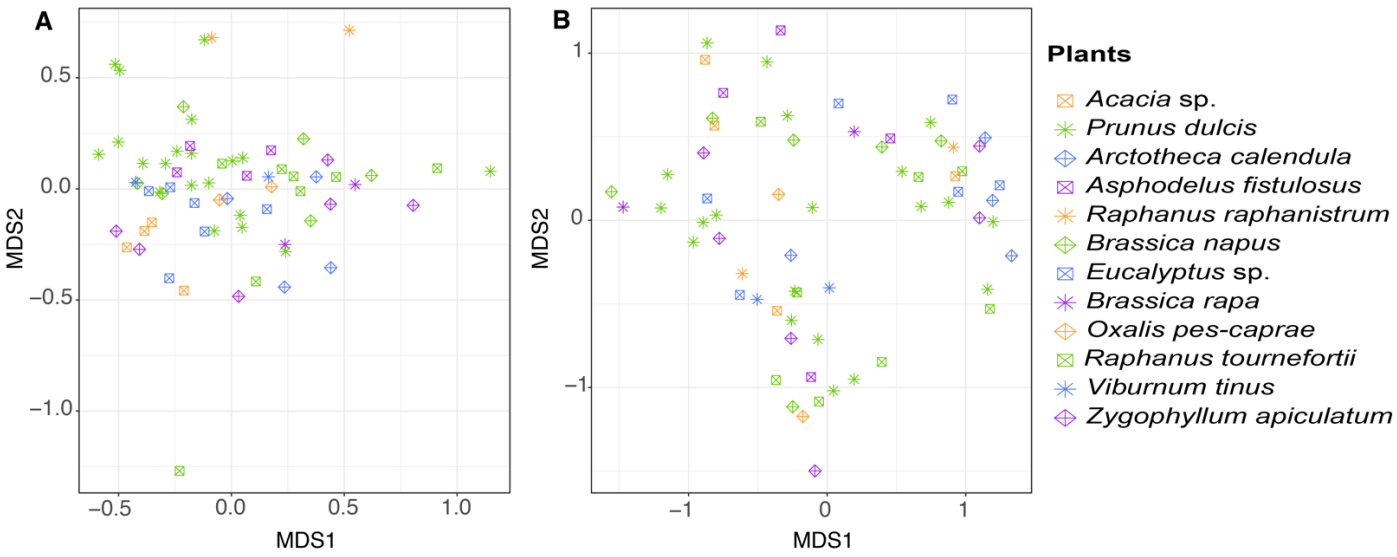

Figure 3. Differences in composition of (A) fungal and (B) bacterial communities in pollen of plants in and around almond orchards. Non-metric multidimensional scaling plots are based on Bray-Curtis dissimilarities for fungal and bacterial amplicon sequence variants, respectively.

ASVs shared among pollen species

UpSet plots were constructed to determine the number of fungal and bacterial ASVs shared among the pollen species at each sample collection time for orchards A, B and E, where pollen foragers were collected from almonds, weeds or native vegetation. Results show multiple interactions, with greater complexity at the last collection time (Figs 4-7) but, overall, few fungal and bacterial ASVs were shared among the pollen species across all three collection times and locations.

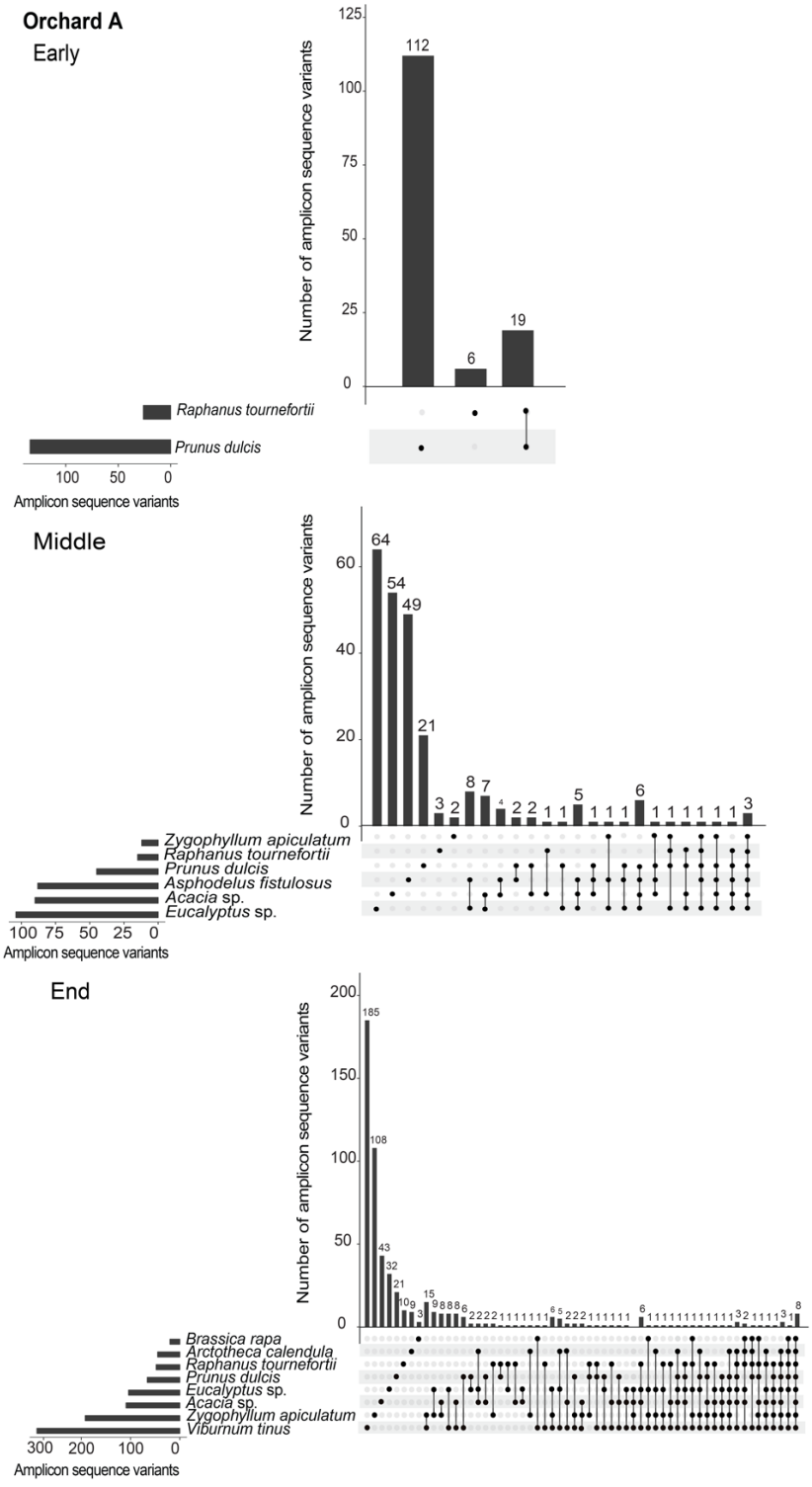

Figure 4. Fungal amplicon sequence variants shared among pollen species in orchard A. UpSet plots represent sample collection times (i.e. early, middle and late) during almond bloom.

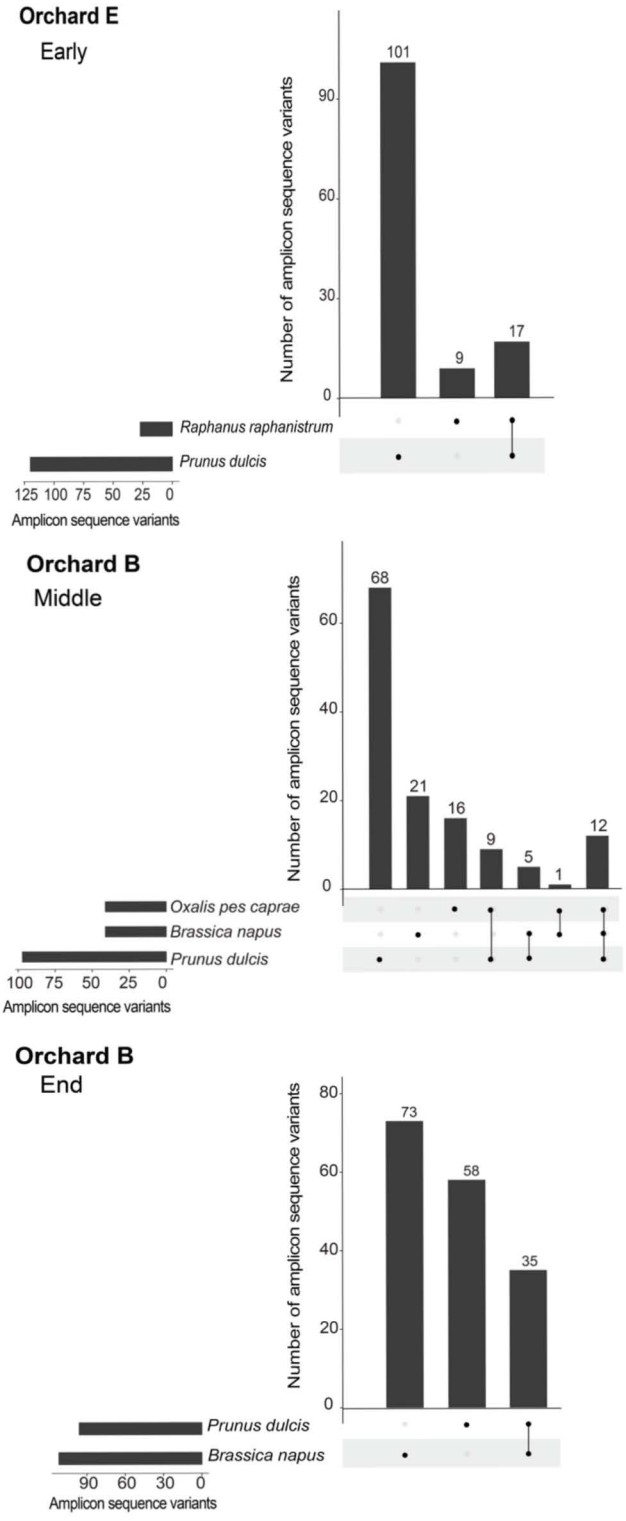

Figure 5. Fungal amplicon sequence variants shared among pollen species in orchards B and E. UpSet plots represent sample collection times (i.e. early, middle and late) during almond bloom.

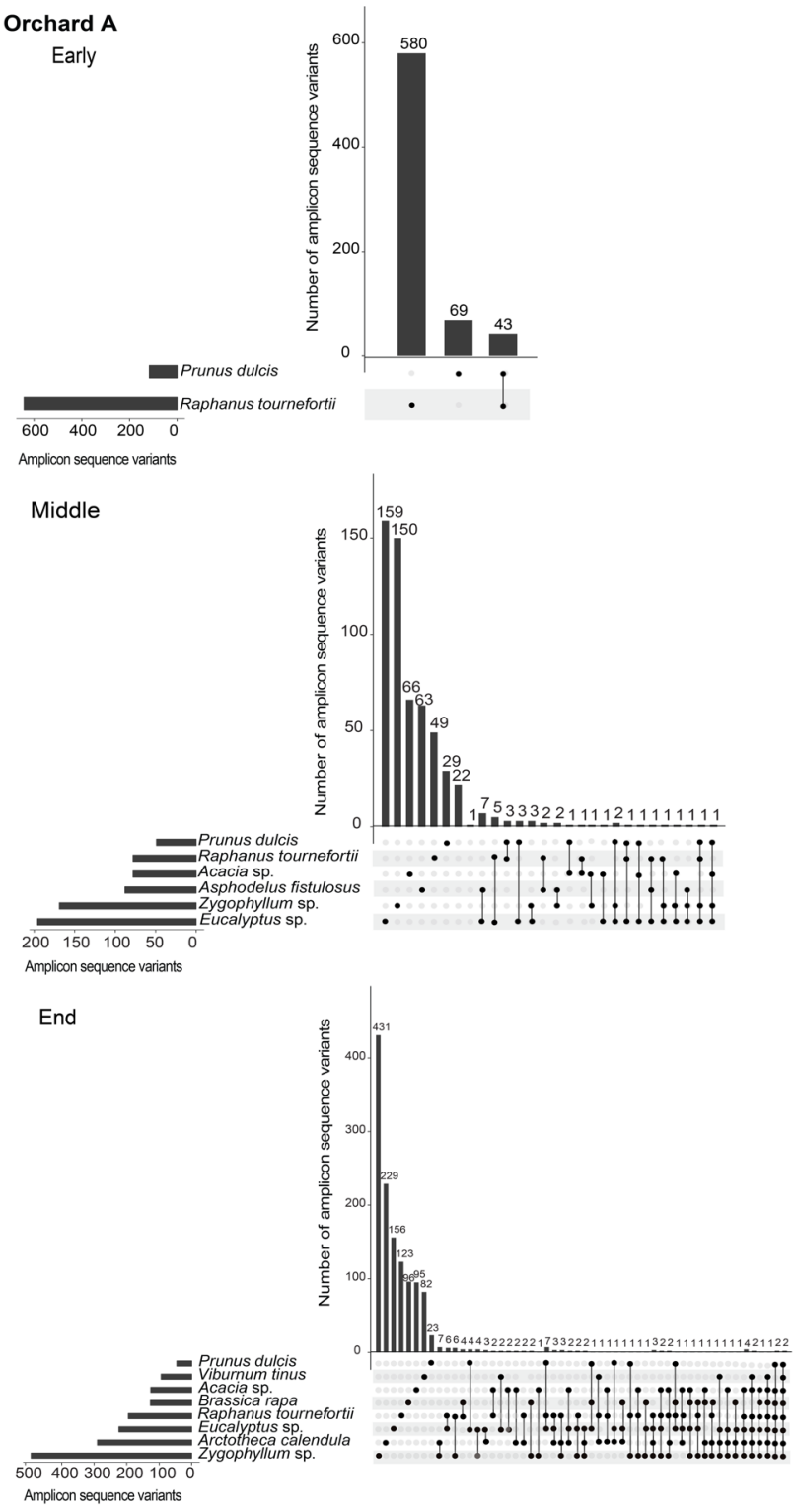

Figure 6. Bacterial amplicon sequence variants shared among pollen species in orchard A. UpSet plots represent sample collection times (i.e. early, middle and late) during almond bloom.

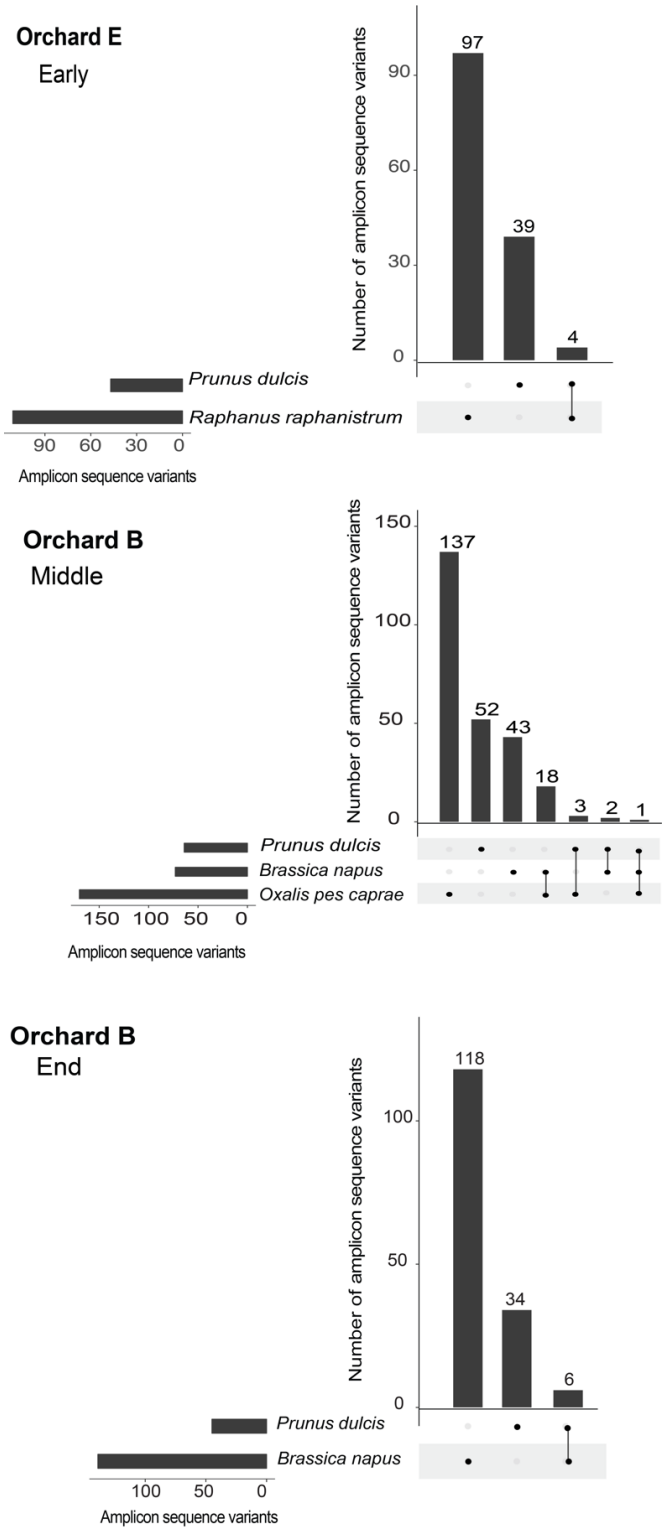

Figure 7. Bacterial amplicon sequence variants shared among pollen species in orchards B and E. UpSet plots represent sample collection times (i.e. early, mid and late) during almond bloom.

Network structure of pollen species and plant pathogens

Associations between generalist plant pathogenic fungi and bacteria and pollen of nine and six species of plants, respectively are shown in the network plots (Table 3, Fig. 8). Nestedness values were similar between the webs for plant species and fungi or bacteria. Overall connectance, mean number of links per plant species and robustness HL values (plant pathogens) were higher for the interactions between the plant species and phytopathogenic fungi than bacteria, with the exception of the robustness LL (plant species), which was higher for bacteria. The greatest number of interactions was found for the fungus *Aspergillus flavus* and the bacterial genus *Pseudomonas*, which were found in six and three pollen species present in orchard A, respectively, where pollen foragers were sampled on nine plant species.

Table 3. Network metrics for the bipartite analysis of pollen species – plant pathogenic fungi and bacteria networks for orchard A from *networklevel* function. Nestedness values range from 0 (maximum nestedness) to 100 (no nestedness). Connectance values indicate proportion of possible ecological interactions among the plant species and phytopathogens. Links per species indicate the mean number of links per plant species. High robustness (HL and LL) indicates resilience of the web to the removal of species.

Network level index	Fungi	Bacteria
Nestedness	31.28	31.88
Connectance	0.48	0.3
Links per species	1.08	1
Robustness HL	0.76	0.63
Robustness LL	0.59	0.65

Figure 8. Bipartite network of pollen species and plant pathogenic fungi and bacteria for almond orchard A. Links between pollen species and plant pathogenic fungi and bacteria are represented by lines. The width of the nodes represents relative abundance based on sequence read counts.

Relative incidences of agriculturally relevant fungi and bacteria

The relative incidences of the agriculturally relevant fungi varied across the 12 pollen species, five orchards and three collection times, and are presented in Tables S4-S6. Overall, the fungi *A. carthami, Aureobasidium pullulans* and *Cladosporium* sp. had moderate to high incidences (i.e. >50%) in all pollen species, sample collection times and orchards. The relative incidence of agriculturally relevant bacteria was low to moderate across all pollen species, orchard and collection times, with the exception of *Pseudomonas* sp., which had a high incidence value in orchard E (Tables S7-S9).

Positive and negative controls

No PCR product was visible following agarose gel electrophoresis for both negative controls. From the 16S rRNA sequencing, the 'water' negative control yielded, between the duplicates, 59-263 bacterial ASVs identified as *Propionibacterium* sp. and *Mycobacterium* sp. and the 'reagents only' negative control yielded, between the duplicates, 620-2123 bacterial ASVs, which included the genera *Candidatus*, *Cellulomonas*, *Curtobacterium*, *Devosia*, *Micrococcus*, *Micronulatus*, *Paracoccus*, *Propionibacterium*, *Pseudonocardia*, *Ralstonia*, *Sphingomonas*, *Thermus* and unassigned sequences.

From the ITS sequencing, the 'water' negative control yielded, between the duplicates, 15-195 fungal ASVs identified as *A. carthami* and unassigned sequences and the 'reagents only' negative control yielded, between the duplicates, 6-27 unassigned sequences. The positive control which comprised pollen plus mycelia of *B. cinerea* yielded, between the duplicates, 12,861-17,691 fungal ASVs, which were identified as *B. cinerea*, *C. acutatum, Cladosporium sp.* and unassigned sequences and the positive control which comprised pollen plus mycelia of *C. acutatum* yielded, between the

duplicates, 8,189-10,771 fungal ASVs identified as *C. acutatum* and *Cladosporium* sp..

Discussion

Despite the economic value of almond orchards, to the best of our knowledge, this is the first study to provide fundamental knowledge about the diversity and richness of fungal and bacterial communities associated with pollen of plant species in and around almond orchards during bloom. Pollen was shown to be a reservoir of plant pathogens and, therefore, pathogens may be transferred by pollinators between plants of the same and different species, insects [44], neighbouring crops and even to different locations due to migratory beekeeping practices. While it has long been established that invertebrates such as honey bees are vectors of plant pathogens, notably *Erwinia amylovora* [45], the UpSet plots revealed a number of ASVs shared between pollen species which may, in part, provide evidence of a broader range of microorganisms potentially vectored by bees.

We assumed that the microbiome found in the corbiculae was associated with the pollen from the plant on which the bee was collected. However, it is possible that a small amount of pollen from other host plants was accidentally acquired in a given load due to contact of foragers with pollen deposited by other pollinators, wind or physical contact between flowers of different species (22). Our study did not investigate this possibility.

Plant pathogenic fungi were identified in pollen collected from almonds in orchards A, B, C and E and genera of potentially plant pathogenic bacteria were identified in almond pollen in orchards E only. There was no indication of the presence of plant pathogens on weeds or native vegetation during the early period of almond bloom nor of their potential transfer to almond plants during the middle or late period of sample collection. Disease incidence was not expressly monitored at the orchards during the sampling period, however, diseased flowers were not observed and fungicide applications at four of the

orchards would serve to minimise the risk of disease.

For fungal communities, the diversity indices showed that the pollen samples were composed of many taxa with moderate diversity and evenness, and relatively high species richness. For bacterial communities, the pollen samples had high bacterial diversity and richness and the ASVs were not evenly distributed among most samples. Additionally, as determined by the beta diversity analysis, clusters of communities of fungi differed significantly among the pollen samples and locations but were similar for time of collection, which may reflect the short duration of bloom. This short period of time would limit microbial succession and resulting changes in bacterial and fungal diversity and richness in the pollen species over the sampling period [12]. Also, the low connectance level between the plant species and the plant pathogenic bacterial ASVs in the network analysis revealed a much higher diversity of bacterial sequences between plant species than for the fungal ASVs. The occurrence, abundance and adhesion of bacterial and fungal communities to pollen are likely to be influenced by electrostatic charges, the structure of the outer layer of pollen grains of each genus or species [46-48] and management approaches, such as the use of fungicides. Although this study was not designed to investigate differences in the microbial composition of pollen species due to the use of chemical control of plant pathogens, there was no evidence that spraying or withholding fungicides during bloom affected the microbial communities in orchards A, B, D, E (treated) compared with C (untreated).

Among the bacterial phyla identified, Actinobacteria represent a reservoir of potential biocontrol agents and members of this phylum are well known for their ability to produce secondary metabolites that function as plant growth promoters and disease resistance inducers [45]. Microbes exploited as biocontrol agents in agriculture identified in this

study were *Lactobacillus plantarum* (50), *Lysobacter* sp. [51] and *Trichoderma* sp., which are used against plant pathogens that infect flowers [52], and *Beauveria bassiana*, which is used in the control of insect pests [53]. Although these microbes did not co-occur in single pollen samples in this study, the possibility that these microbes occur in consortia in the environment may warrant future research into formulating mixtures for the effective management of plant pathogens [54].

Other groups of agriculturally relevant bacteria and fungi identified in the pollen samples include bacteria commonly found in the gut of honey bees, hive environments [28] and pollen [55], namely *Acinetobacter, Enterobacter, Lactobacillus, Massilia, Saccharibacter* and *Sphingomonas* spp., and *Snodgrassella alvi* [56-58]. Some of these bacteria are considered to be commensals within the honey bee gut and, although their specific functions have not yet been identified [59, 60], it is believed that *S. alvi* participates in the metabolism of carbohydrates [61] and may have a role in protecting bees from opportunistic infections [62], whereas some *Enterobacter* species exert antibiotic activity against honey bee pathogens [63].

Although the fungi *A. carthami* and *Cladosporium* sp. were found in our controls, we cannot exclude the possibility that they originated from our pollen samples. The small number of sequences identified in the negative controls for both fungal and bacterial ASVs and the presence of *C. acutatum* in the ITS positive control 'pollen and *B. cinerea*', is likely to reflect small amounts of atmospheric DNA within the laboratory and cross-contamination, but this had minimal influence on the outcomes of this research. The *B. cinerea* ASV was not included in data analysis because the reference used (UNITE QIIME release for Fungi 2) excluded it on the bases of matches with other fungi (e.g. *Sclerotinia sclerotiorum*). The *B. cinerea* ASV was present in pollen of *P. dulcis* in

orchards A and E and of *R. raphanistrum* in orchard E during early almond bloom. In the middle of the bloom period it was present in pollen of *Acacia* sp., *A. calendula* and *A. fistulosus* in orchard A and of *P. dulcis* in orchard D. At the end of bloom it was present in pollen of *A. calendula*, *B. rapa*, *Eucalyptus* sp., *P. dulcis* and *Z. apiculatum* in and in the surroundings of orchard A and in pollen of *B. napus* and *P. dulcis* in orchard B. These results highlight that a single gene region or primer pair is unlikely to cover all important pathogenic fungal taxa equally in a metabarcoding study, and investigation of additional primer pairs that would increase this coverage is warranted.

It is likely that the relative incidences of agriculturally relevant fungi and bacteria have been influenced by the number of plant species available at each collection time and the characteristics of the orchards. Due to the cool temperatures during almond bloom (i.e. approximately 5 ^{o}C–18 ^{o}C) and the variation in availability of pollen on the flowers with time of day, there was a short period of time (i.e. <5 h) in which workers carrying full loads of pollen could be collected individually from the flowers of different plants. Despite that, our results highlight the complexity of the pollen microbiota as well as the potential for further investigation of the microbial composition of pollen and nectar in orchards employing different management practices and in a range of ecological habitats. The microbial associations identified in this study, especially the presence of plant pathogens in collections of several pollen species, add to our understanding of microbial ecology and serve as a foundation for future research to explore experimental approaches, modelling and the manipulation of plant microbiomes in order to improve crop productivity [13] and maintain sustainable environments. Further investigations are also warranted of the storage of plant pathogens with pollen and or nectar in honey bee hives, especially in pollination events where the deployment of large numbers of hives may

increase the risk of transmission of plant diseases among crops and agricultural areas due to migratory beekeeping practices [11, 64]. In addition, the identification of functional attributes of microorganisms that inhabit floral resources is necessary to develop an understanding of the interactions between pollinators and microbiomes.

Acknowledgements

The first author was supported by the Adelaide Scholarships International (ASI) scheme. We thank the School of Agriculture, Food and Wine, The University of Adelaide, for operating support. We are grateful to almond growers from Amaroo, Aroona, Omega and Waite orchards and to G & P Giles who permitted sampling.

Author Contributions

JBParish: designed and performed experiments, sample collection, data analyses, and wrote the paper; KHogendoorn: experimental design, sample collection, technical assistance, and manuscript editing; KHill: experiment design, technical assistance, data analyses and manuscript editing; ESScott: experiment design, technical assistance and manuscript editing; NSWatson-Haigh and RCorell: data analyses and manuscript editing. All authors read and approved the final manuscript.

References

1. Purahong W, Orrù L, Donati I, Perpetuini G, Cellini A, Lamontanara A, Michelotti V, Tacconi G, Spinelli F. Plant microbiome and its link to plant health: tree species, organs and Pseudomonas syringae pv. actinidiae infection shaping bacterial phyllosphere communities of kiwifruit plants. Front Plant Sci. 2018; 9: 1563.
2. Bringel F, Couée I. Pivotal roles of phyllosphere microorganisms at the interface between plant functioning and atmospheric trace gas dynamics. Front Microbiol. 2015; 6: 486.
3. McGregor SE. Insect pollination of cultivated crop plants: Agricultural Research Service, US Department of Agriculture; 1976.
4. Watanabe ME. Pollination worries rise as honey bees decline. Science. 1994; 265: 1170.

5. Keogh R, Robinson A, Mullins IJ. Pollination Aware: The real value of pollination in Australia: Rural Industries Reasearch and Development Corporation; 2010.
6. Shaw DE. Bees and fungi, with special reference to certain plant pathogens. Australas Plant Pathol. 1999; 28:269-282.
7. Corbet SA. A typology of pollination systems: implications for crop management and the conservation of wild plants. In: Waser MN, Ollerton J, editors. Plant–pollinator interactions: from specialization to generalization. University of Chicago; 2006. pp. 315-340.
8. Johnson KB, Stockwell VO, Burgett DM, Sugar D, Loper JE 1993, Dispersal of Erwinia amylovora and Pseudomonas fluorescens by honey bees from hives to apple and pear blossoms. Phytopathology. 1993; 83:478-484.
9. Pattemore DE, Goodwin RM, McBrydie HM, Hoyte SM, Vanneste, JL. Evidence of the role of honey bees (Apis mellifera) as vectors of the bacterial plant pathogen *Pseudomonas syringae*, Australas Plant Pathol. 2014; 43: 571-575.
10. Gasparoto MCG, Lourenco SA, Tanaka FAO, Sposito MB, Marchini LC, Silva GJ, Amorim L. Honeybees can spread Colletotrichum acutatum and C. gloeosporioides among citrus plants. Plant Pathol, 2017; 66: 777-782.
11. Parish JB, Scott ES, Hogendoorn K. Survival and probability of transmission of plant pathogenic fungi through the digestive tract of honey bee workers. Apidologie. 2019; 50: 871.
12. Aleklett K, Hart M, Shade A. The microbial ecology of flowers: an emerging frontier in phyllosphere research. Botany. 2014; 92(4): 253-66.
13. Mueller UG, Sachs JL. Engineering microbiomes to improve plant and animal health. Trends Microbiol. 2015; 23(10): 606-17.
14. Cunningham SA, FitzGibbon F, Heard TA. The future of pollinators for Australian agriculture. Aust J Agric Res. 2002; 53(8): 893-900.
15. Hill S, Stephenson D, Taylor B. Almond pollination studies: pollen production and viability, flower emergence and cross-pollination tests. Aust J Exp Agric. 1985; 25(3): 697-704.
16. Almond Board of Australia. Almond Insights 2017/2018: Almond Board of Australia Inc.; 2019 [Available from: https://industry.australianalmonds.com.au/almond-board/almond-insights/.
17. Sumner DA, Matthews WA, Medellín-Azuara J, Bradley A. The economic impacts of the California almond industry. Online publication. https://aic.ucdavis.edu/almonds/Economic Impacts of California Almond Industry_Full Report_FinalPDF_v2.pdf; 2014.
18. Aizen MA, Harder LD. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr Biol. 2009;19(11):915-8.
19. Saunders ME, Luck GW, Mayfield MM. Almond orchards with living ground cover host more wild insect pollinators. J Insect Conserv. 2013; 17(5): 1011-25.
20. Altieri MA. Agroecology: the science of sustainable agriculture. Boulder. Westview Press Part three: Development, climate and rights. 1995; 238: 12052-57.
21. Reintjes C, Haverkort B, Waters-Bayer A. Farming for the future: An introduction to low-external input and sustainable agriculture. London: Macmillan 1992.

22. Altieri MA. Linking ecologists and traditional farmers in the search for sustainable agriculture. Front Ecol Environ. 2004; 2(1): 35-42.
23. Fisher A, Coleman C, Hoffmann C, Fritz B, Rangel J. The synergistic effects of almond protection fungicides on honey bee (Hymenoptera: Apidae) forager survival. J Econ Entomol. 2017; 110(3): 802-8.
24. Chauzat M-P, Faucon J-P, Martel A-C, Lachaize J, Cougoule N, Aubert M. A survey of pesticide residues in pollen loads collected by honey bees in France. J Econ Entomol. 2006; 99(2): 253-62.
25. Schaeffer RN, Vannette RL, Brittain C, Williams NM, Fukami T. Non-target effects of fungicides on nectar-inhabiting fungi of almond flowers. Environ Microbiol Rep. 2017; 9(2): 79-84.
26. Cornman RS, Otto CRV, Iwanowicz D, Pettis JS. Taxonomic characterization of honey bee (Apis mellifera) pollen foraging based on non-overlapping paired-end sequencing of nuclear ribosomal loci. Plos One. 2015; 10(12): e0145365.
27. Schmidt PA, Balint M, Greshake B, Bandow C, Rombke J, Schmitt I. Illumina metabarcoding of a soil fungal community. Soil Biol Biochem. 2013; 65: 128-32.
28. Anderson KE, Sheehan TH, Mott BM, Maes P, Snyder L, Schwan MR, Walton A, Jones BM, Corby-Harris V. Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apis mellifera). PloS one. 2013; 8: e83125.
29. Corby-Harris V, Maes P, Anderson KE. The bacterial communities associated with honey bee (Apis mellifera) foragers. PloS one. 2014; 9: e95056.
30. Adaskaveg JE, Gubler W, Michailides TJ, Holtz BA. Efficacy and timing of fungicides, bactericides, and biologicals for deciduous tree fruit, nut, strawberry, and vine crops. University of California Online Statewide IPM Program Available: http://cemaderaucdavisedu/files/63413pdf). 2011.
31. Ushio M, Yamasaki E, Takasu H, Nagano AJ, Fujinaga S, Honjo MN, Ikemoto M, Sakai S, Kudoh H. Microbial communities on flower surfaces act as signatures of pollinator visitation. Scientific Reports. 2015; 5: 8695.
32. McKay SF, Shtienberg D, Sedgley M, Scott ES. Anthracnose on almond in Australia:disease progress and inoculum sources of Colletotrichum acutatum. Eur J Plant Pathol. 2014; 139: 773-83.
33. Elmer PA, Michailides TJ. Epidemiology of Botrytis cinerea in orchard and vine crops. In: Elad Y, Williamson B, Tudzynski P, Delen N, editors. Botrytis: Biology, pathology and control: Dordrecht, Springer; 2004. pp. 243-72.
34. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: a guide to methods and applications. 1990; 18: 315-22.
35. Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J. 2011; 5(10): 1571-1579.
36. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA, Alexander H, Alm EJ, Arumugan M, Asnicar F, Bai Y. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. PeerJ Preprints; 2018; v27295v2.

37. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods. 2016; 13(7): 581-583.
38. UNITE Community. UNITE QIIME release for Fungi 2. Version 18.11.2018: UNITE Community; 2019.
39. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006; 72(7): 5069-72.
40. Hammer Ø, Harper DA, Ryan PD. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron. 2001; 4(1): 9.
41. R-Project. R Core Team. R: A Language and Environment for Statistical Computing. 1.2.1335 ed. Vienna, Austria; 2019.
42. Conway JR, Lex A, Gehlenborg N. UpSetR: an R package for the visualization of intersecting sets and their properties. Bioinformatics. 2017; 33(18): 2938-40.
43. Dormann CF, Fründ J, Blüthgen N, Gruber B. Indices, graphs and null models: analyzing bipartite ecological networks. Open Ecol J. 2009; 2(1): 7-24.
44. Fürnkranz M, Lukesch B, Müller H, Huss H, Grube M, Berg G. Microbial diversity inside pumpkins: microhabitat-specific communities display a high antagonistic potential against phytopathogens. Microb Ecol. 2012; 63(2): 418-28.
45. Agrios GN. Plant Pathology. 5th edition, Elsevier Academic Press, Burlington MA 2005.
46. Kosenko V. Contributions to the pollen morphology and taxonomy of the Liliaceae. Grana. 1999; 38(1): 20-30.
47. Punt W, Hoen P, Blackmore S, Nilsson S, Le Thomas A. Glossary of pollen and spore terminology. Rev Palaeobot Palyno. 2007; 143(1-2): 1-81.
48. Marques JPR, Amorim L, Sposito MB, Marin D, Appezzato-da-Gloria B. Infection of citrus pollen grains by Colletotrichum acutatum. Eur J Plant Pathol. 2013; 136: 35-40.
49. Himaman W, Thamchaipenet A, Pathom-aree W, Duangmal K. Actinomycetes from Eucalyptus and their biological activities for controlling Eucalyptus leaf and shoot blight. Microbiol Res. 2016; 188: 42-52.
50. Völksch B, May R. Biological control of Pseudomonas syringae pv. glycinea by epiphytic bacteria under field conditions. Microb Ecol. 2001; 41(2): 132-9.
51. Folman LB, Postma J, van Veen JA. Characterisation of Lysobacter enzymogenes (Christensen and Cook 1978) strain 3.1 T8, a powerful antagonist of fungal diseases of cucumber. Microbiol Res. 2003; 158(2): 107-15.
52. Verma M, Brar SK, Tyagi RD, Surampalli RY, Valero JR. Antagonistic fungi, Trichoderma spp.: Panoply of biological control. Biochem Eng J. 2007; 37(1): 1-20.
53. Feng MG, Poprawski TJ, Khachatourians GC. Production, formulation and application of the entomopathogenic fungus Beauveria bassiana for insect control: current status. Biocontrol Sci Technol. 1994; 4: 3-34.
54. Sarma BK, Yadav SK, Singh S, Singh HB. Microbial consortium-mediated plant defense against phytopathogens: readdressing for enhancing efficacy. Soil Biol Biochem. 2015; 87: 25-33.
55. Ambika Manirajan B, Ratering S, Rusch V, Schwiertz A, Geissler-Plaum R, Cardinale M, et al. Bacterial microbiota associated with flower pollen is influenced by

pollination type, and shows a high degree of diversity and species-specificity. Environ Microbiol. 2016; 18(12): 5161-74.
56. Kaznowski A, Szymas B, Jazdzinska E, Kazimierczak M, Paetz H, Mokracka J. The effects of probiotic supplementation on the content of intestinal microflora and chemical composition of worker honey bees (Apis mellifera). J Apic Res. 2005; 44(1): 10-4.
57. Powell JE, Martinson VG, Urban-Mead K, Moran NA. Routes of acquisition of the gut microbiota of *Apis mellifera*. Appl Environ Microbiol. 2014; 80: 7378-87.
58. Vásquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L, et al. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PloS one. 2012; 7(3): e33188.
59. Babendreier D, Joller D, Romeis J, Bigler F, Widmer F. Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. FEMS Microbiol Ecol. 2007; 59(3): 600-10.
60. Jeyaprakash A, Hoy MA, Allsopp MH. Bacterial diversity in worker adults of *Apis mellifera capensis* and Apis mellifera scutellata (Insecta: Hymenoptera) assessed using 16S rRNA sequences. J Invertebr Pathol. 2003; 84(2): 96-103.
61. Lee FJ, Rusch DB, Stewart FJ, Mattila HR, Newton IL. Saccharide breakdown and fermentation by the honey bee gut microbiome. Environ Microbiol. 2015; 17: 796-815.
62. Maes PW, Rodrigues PA, Oliver R, Mott BM, Anderson KE. Diet-related gut bacterial dysbiosis correlates with impaired development, increased mortality and Nosema disease in the honeybee (Apis mellifera). Mol Ecol. 2016; 25: 5439-50.
63. Kaltenpoth M, Engl T. Defensive microbial symbionts in Hymenoptera. Funct Ecol. 2014;28(2):315-27.
64. Benecke FS. Commercial beekeeping in Australia, A report for the Rural Industries Research and Development Coorporation. RIRDC Publication, ACT, Australia, 2003. no. 07/059.

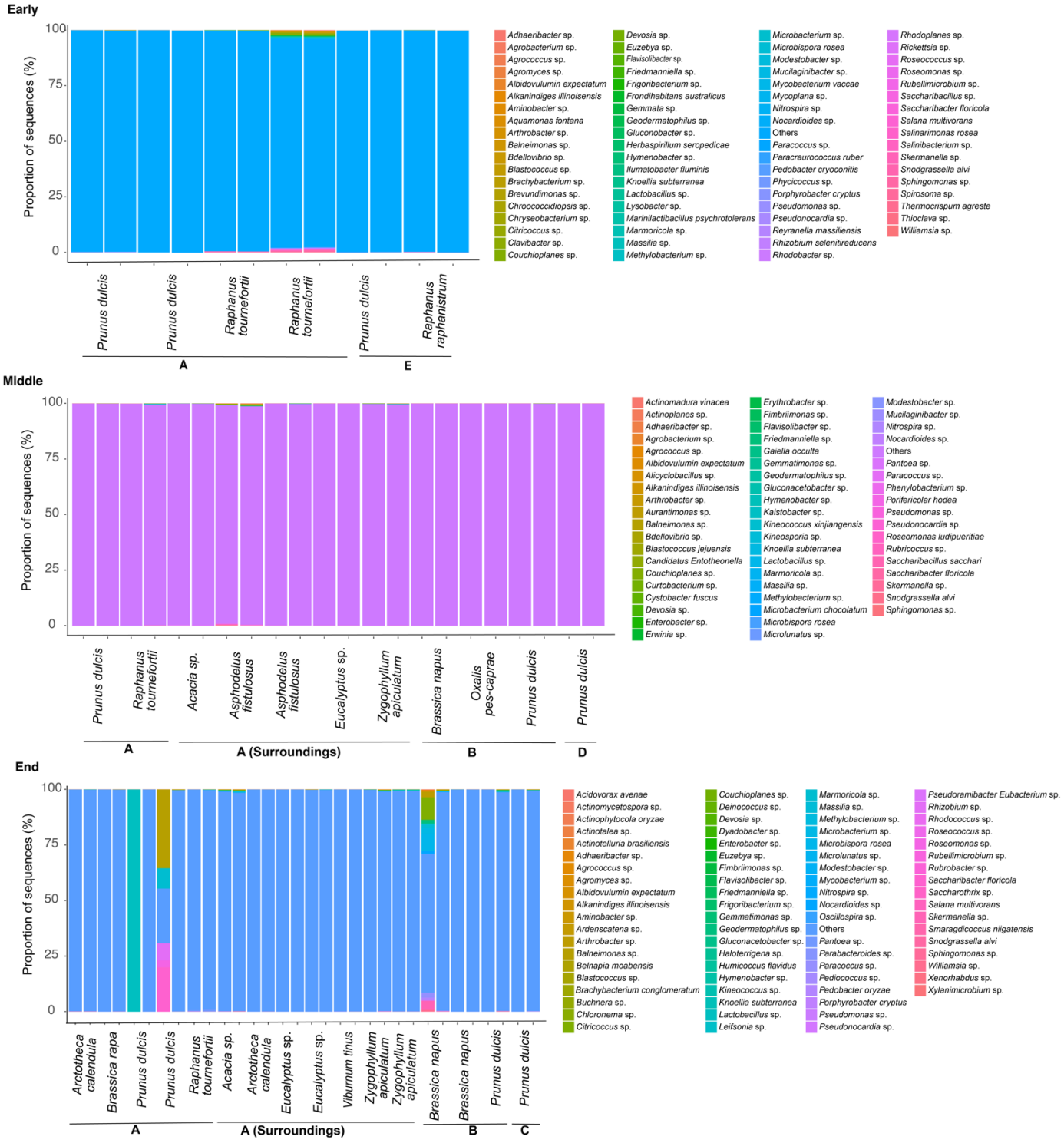

Figure S1. Relative abundance (proportion of sequences) of bacteria in the corbicular pollen of *Apis mellifera* foragers. Bars indicate plants from which pollen foragers were collected and each colour represents groups of amplicon sequence variants of bacterial genera or species during almond bloom in five orchards (A-E). Each bar is followed by its duplicate.

Table S1: Alpha diversity indices based on the fungal ASVs (mean ± standard deviation) of the different pollen species compared by Kruskal-Wallis and Mann–Whitney–Wilcoxon test at P<0.05. Dominance: calculated as $D = sum\left(\left(\frac{n_i}{n}\right)^2\right)$ where n_i is number of individuals of taxon i. It ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely); **Simpson**: calculated as 1-Dominance. It measures the probability that two individuals randomly selected from a sample will belong to the same species and ranges from 0 (infinite diversity) to 1 (no diversity). **Shannon**: calculated as $H = -sum\left(\left(\frac{n_i}{n}\right) ln\left(\frac{n_i}{n}\right)\right)$. It accounts for both abundance and evenness of the species present and ranges from 0 for communities with only a single taxon to high values for communities with many taxa; **Evenness**: expresses how evenly the microbial genera/species in a community are distributed among the pollen species; **Chao-1**: Estimate of diversity from abundance of microbial sequences in pollen.

ITS								
Collections time	Orchard	Plant species	Total libraries	Dominance	Simpson	Shannon	Evenness	Chao-1
Early	A	*Prunus dulcis*	4	0.27 ± 0.10 a	0.73 ± 0.10 a	2.00 ± 0.34	0.18 ± 0.09	51.75 ± 24.62 a
	A	*Raphanus tournefortii*	4	0.44 ± 0.14 a	0.56 ± 0.14 a	1.19 ± 0.29 a	0.43 ± 0.33	13.50 ± 7.40 b
	E	*Prunus dulcis*	2	0.08 ± 0.00	0.92 ± 0.00	3.06 ± 0.25	0.41 ± 0.16	70.00 ± 41.00
	E	*Raphanus raphanistrum*	2	0.20 ± 0.08	0.80 ± 0.08	1.98 ± 0.57	0.65 ± 0.17	16.00 ± 11.00
Middle	A	*Acacia* sp.	2	0.32 ± 0.01	0.68 ± 0.01	1.67 ± 0.09	0.10 ± 0.03	58.50 ± 22.50
	A	*Asphodelus fistulosus*	4	0.15 ± 0.02 b	0.85 ± 0.02 b	2.35 ± 0.25 b	0.40 ± 0.15	32.00 ± 14.92
	A	*Eucalyptus* sp.	2	0.31 ± 0.02	0.69 ± 0.02	1.85 ± 0.10	0.08 ± 0.00	75.00 ± 4.00
	A	*Prunus dulcis*	2	0.18 ± 0.00	0.82 ± 0.00	2.29 ± 0.06	0.37 ± 0.11	29.50 ± 10.50
	A	*Raphanus tournefortii*	2	0.59 ± 0.41	0.41 ± 0.41	1.05 ± 1.05	0.77 ± 0.23	8.00 ± 7.00
	A	*Zygophyllum* sp.	2	0.37 ± 0.12	0.63 ± 0.12	1.28 ± 0.42	0.62 ± 0.16	7.50 ± 4.50
	B	*Brassica napus*	2	0.23 ± 0.02	0.77 ± 0.02	1.76 ± 0.24	0.47 ± 0.29	23.00 ± 17.00
	B	*Oxalis pes-caprae*	2	0.17 ± 0.00	0.83 ± 0.00	2.30 ± 0.04	0.36 ± 0.11	30.50 ± 10.50
	B	*Prunus dulcis*	2	0.12 ± 0.01	0.88 ± 0.01	2.60 ± 0.15	0.30 ± 0.14	61.00 ± 35.00
	D	*Prunus dulcis*	2	0.69 ± 0.31	0.31 ± 0.31	1.00 ± 1.00	0.55 ± 0.45	34.50 ± 33.50
Late	A	*Acacia* sp.	2	0.21 ± 0.00	0.79 ± 0.00	2.33 ± 0.03	0.13 ± 0.00	81.50 ± 1.50
	A	*Arctotheca calendula*	4	0.20 ± 0.02 a	0.80 ± 0.02 a	1.95 ± 0.18 b	0.46 ± 0.14	18.75 ± 11.30
	A	*Brassica rapa*	2	0.44 ± 0.03	0.56 ± 0.03	1.32 ± 0.15	0.32 ± 0.09	13.50 ± 5.50
	A	*Eucalyptus* sp.	4	0.26 ± 0.10	0.74 ± 0.10	1.96 ± 0.39	0.23 ± 0.13	40.00 ± 16.36 a
	A	*Prunus dulcis*	4	0.24 ± 0.03 ac	0.76 ± 0.03 ac	1.94 ± 0.22	0.29 ± 0.09	28.00 ± 13.06
	A	*Raphanus tournefortii*	2	0.29 ± 0.03	0.71 ± 0.03	1.88 ± 0.17	0.26 ± 0.09	31.50 ± 15.50
	A	*Viburnum tinus*	2	0.41 ± 0.03	0.59 ± 0.03	1.64 ± 0.18	0.07 ± 0.05	163.00 ± 125.00
	A	*Zygophyllum* sp.	4	0.24 ± 0.05	0.76 ± 0.05	2.00 ± 0.32	0.22 ± 0.18	66.00 ± 46.56
	B	*Brassica napus*	4	0.13 ± 0.03 a	0.87 ± 0.03 a	2.42 ± 0.35 b	0.46 ± 0.22	38.25 ± 28.78
	B	*Prunus dulcis*	2	0.15 ± 0.00	0.85 ± 0.00	2.34 ± 0.00	0.17 ± 0.04	65.50 ± 16.50
	C	*Prunus dulcis*	2	0.20 ± 0.00	0.80 ± 0.00	2.35 ± 0.10	0.13 ± 0.04	89.50 ± 32.50

Different letters indicate statistically significant differences between means across pollen species within diversity indices (Kruskal-Wallis and Mann–Whitney–Wilcoxon test, $P<0.05$).

Table S2: Alpha diversity indices based on the bacterial ASVs (mean ± standard deviation) of the different pollen species compared by Kruskal-Wallis and Mann–Whitney–Wilcoxon test at P<0.05. Dominance: calculated as $D = sum\left(\left(\frac{n_i}{n}\right)^2\right)$ where n_i is number of individuals of taxon *i*. It ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely); **Simpson**: calculated as 1-Dominance. It measures the probability that two individuals randomly selected from a sample will belong to the same species and ranges from 0 (infinite diversity) to 1 (no diversity). **Shannon**: calculated as $H = -sum\left(\left(\frac{n_i}{n}\right) ln\left(\frac{n_i}{n}\right)\right)$. It accounts for both abundance and evenness of the species present and ranges from 0 for communities with only a single taxon to high values for communities with many taxa; **Evenness**: expresses how evenly the microbial genera/species in a community are distributed among the pollen species; **Chao-1**: Estimate of diversity from abundance of microbial sequences in pollen.

16S rRNA gene								
Collection time	Orchard	Plant species	Total libraries	Dominance	Simpson	Shannon	Evenness	Chao-1
Early	A	*Prunus dulcis*	4	0.90 ± 0.07 a	0.10 ± 0.07 a	0.29 ± 0.14 a	0.06 ± 0.02	34.5 ± 25.99
	A	*Raphanus tournefortii*	4	0.26 ± 0.05 b	0.74 ± 0.05 b	2.41 ± 0.25 b	0.08 ± 0.05	209.43 ± 140.93
	E	*Prunus dulcis*	2	0.98 ± 0.00	0.02 ± 0.00	0.10 ± 0.02	0.05 ± 0.03	28.00 ± 14.00
	E	*Raphanus raphanistrum*	2	0.39 ± 0.01	0.61 ± 0.01	1.35 ± 0.07	0.07 ± 0.03	67.0 ± 31.00
Mid	A	*Acacia* sp.	2	0.84 ± 0.01	0.15 ± 0.01	0.54 ± 0.06	0.03 ± 0.00	52.50 ± 1.50
	A	*Asphodelus fistulosus*	4	0.84 ± 0.12 a	0.16 ± 0.12 a	0.46 ± 0.32 a	0.06 ± 0.02	31.75 ± 9.58
	A	*Eucalyptus* sp.	2	0.67 ± 0.00	0.33 ± 0.00	1.13 ± 0.00	0.03 ± 0.00	117.00 ± 18.00
	A	*Prunus dulcis*	2	0.96 ± 0.00	0.04 ± 0.00	0.16 ± 0.00	0.06 ± 0.04	27.50 ± 15.50
	A	*Raphanus tournefortii*	2	0.39 ± 0.23	0.61 ± 0.23	1.48 ± 0.78	0.40 ± 0.27	39.67 ± 36.67
	A	*Zygophyllum apiculatum*	2	0.56 ± 0.02	0.44 ± 0.02	1.26 ± 0.02	0.06 ± 0.04	95.05 ± 57.05
	B	*Brassica napus*	2	0.39 ± 0.01	0.61 ± 0.01	1.44 ± 0.01	0.11 ± 0.04	47.50 ± 18.50
	B	*Oxalis pes-caprae*	2	0.19 ± 0.00	0.81 ± 0.00	1.86 ± 0.06	0.22 ± 0.18	91.35 ± 76.35
	B	*Prunus dulcis*	2	0.97 ± 0.00	0.03 ± 0.00	0.10 ± 0.03	0.12 ± 0.10	34.50 ± 29.50
	D	*Prunus dulcis*	2	0.96 ± 0.02	0.04 ± 0.02	0.12 ± 0.06	0.16 ± 0.05	8.00 ± 3.00
Late	A	*Acacia* sp.	2	0.82 ± 0.02	0.37 ± 0.02	1.26 ± 0.08	0.05 ± 0.01	141.01 ± 28.10
	A	*Arctotheca calendula*	4	0.75 ± 0.06 a	0.25 ± 0.06 a	0.91 ± 0.25 c	0.02 ± 0.00	107.13 ± 24.46
	A	*Brassica rapa*	2	0.27 ± 0.02	0.73 ± 0.02	2.20 ± 0.07	0.10 ± 0.01	87.88 ± 0.88
	A	*Eucalyptus* sp.	4	0.84 ± 0.14 a	0.16 ± 0.14 a	0.55 ± 0.45 a	0.04 ± 0.02	65.86 ± 49.78
	A	*Prunus dulcis*	4	0.89 ± 0.12 a	0.11 ± 0.12 a	0.23 ± 0.18 a	0.09 ± 0.06	17.00 ± 4.95
	A	*Raphanus tournefortii*	2	0.76 ± 0.04	0.24 ± 0.04	0.87 ± 0.16	0.02 ± 0.00	119.10 ± 21.10
	A	*Viburnum tinus*	2	0.79 ± 0.10	0.21 ± 0.10	0.54 ± 0.07	0.24 ± 0.22	47.50 ± 43.50
	A	*Zygophyllum apiculatum*	4	0.57 ± 0.08 c	0.43 ± 0.08 c	1.20 ± 0.44 d	0.27 ± 0.37	137.20 ± 168.72
	B	*Brassica napus*	4	0.39 ± 0.02 d	0.61 ± 0.02 d	1.63 ± 0.10 d	0.12 ± 0.05	55.13 ± 25.30
	B	*Prunus dulcis*	2	0.95 ± 0.01	0.05 ± 0.01	0.20 ± 0.04	0.06 ± 0.03	29.00 ± 15.00
	C	*Prunus dulcis*	2	0.96 ± 0.01	0.04 ± 0.01	0.16 ± 0.06	0.05 ± 0.03	53.50 ± 39.50

Letters indicate statistically significant differences between means across pollen species within diversity indices (Kruskal-Wallis and Mann–Whitney–Wilcoxon test, $P<0.05$).

Table S3: Statistical significances of the alpha diversity indices for fungi among the different orchards by the Kruskal-Wallis and Mann–Whitney–Wilcoxon test at P<0.05.

ITS Dominance					
Orchards	**A**	**B**	**E**	**A (surroundings)**	**C**
B	7.10E-06*	-	-	-	-
E	0.01*	0.26	-	-	-
A (surroundings)	0.12	0.02*	0.08	-	-
C	0.32	0.20	0.53	1.00	-
D	0.09	0.02*	0.13	0.10	0.33
ITS Simpson					
B	7.10E-06	-	-	-	-
E	0.01*	0.26	-	-	-
A (surroundings)	0.12	0.02*	0.08	-	-
C	0.32	0.20	0.53	1.00	-
D	0.09	0.02*	0.13	0.10	0.33
ITS Shannon					
B	0.00*	-	-	-	-
E	0.06	0.30	-	-	-
A (surroundings)	0.30	0.05*	0.16	-	-
C	0.06	1.00	0.53	0.27	-
D	0.42	0.12	0.27	0.42	0.3333

*Indicates statistical significance.

Table S4: **Relative incidence of fungi per pollen species.**

Fungi	A	B	C	D	E	F	G	H	I	J	K	L
Alternaria carthami	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.88	1.00	1.00
Aspergillus flavus	0.50	0.00	0.25	0.17	0.00	0.17	0.00	0.30	0.00	0.00	0.50	0.50
Aspergillus nidulans	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.10	0.00	0.00	0.00	0.17
Aspergillus niger	0.00	0.00	0.50	0.17	0.00	0.00	0.00	0.25	0.00	0.00	0.50	0.33
Aspergillus sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.50	0.00
Aureobasidium pullulans	1.00	0.50	0.75	1.00	1.00	1.00	1.00	0.95	0.50	0.75	1.00	0.67
Aureobasidium sp.	0.75	0.00	0.00	0.17	0.00	0.33	0.00	0.10	0.00	0.13	0.50	0.00
Beauveria bassiana	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17
Byssochlamys sp.	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.15	0.00	0.00	0.50	0.00
Caloplaca xerica	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Candida parapsilosis	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ceratobasidium cereale	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17
Ceratobasidium sp.	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.05	0.00	0.00	0.50	0.00
Cladosporium ramotenellum	0.25	0.50	0.50	0.33	0.00	0.83	0.50	0.45	0.00	0.00	1.00	0.33
Cladosporium sp.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.88	1.00	1.00
Claussenomyces sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Clypeophysalospora latitans	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.50	0.00	0.00	0.00
Colletotrichum acutatum	0.25	0.25	0.00	0.17	0.50	0.17	0.00	0.05	0.00	0.13	0.00	0.17
Colletotrichum asianum	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.17
Coprinellus sp.	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.15	0.00	0.00	0.50	0.00
Deltopyxis triangulispora	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Didymosphaeria futilis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Entomophthora muscae	0.75	0.00	1.00	0.17	0.00	1.00	0.00	0.60	0.00	0.38	1.00	0.00

Table S4 (continued): **Relative incidence of fungi per pollen species.**

Fungi	A	B	C	D	E	F	G	H	I	J	K	L
Eutypa crustata	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.25	0.00	0.25	0.50	0.00
Fusarium sp.	0.00	0.25	0.00	0.50	0.00	0.33	1.00	0.65	0.00	0.38	0.50	0.33
Galerina vittiformis	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.50	0.00
Itersonilia perplexans	0.25	0.00	0.00	0.33	0.00	0.00	0.00	0.10	1.00	0.00	1.00	0.00
Lophiostoma cynaroidis	0.75	0.00	0.00	0.00	0.00	0.17	0.00	0.20	0.00	0.13	0.50	0.33
Macalpinomyces sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.50	0.00	0.50	0.00
Mortierella oligospora	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mortierella sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00
Nodulosphaeria hirta	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.00	0.50	0.00
Ostropa barbara	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17
Phaeothecoidea minutispora	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.10	0.00	0.00	0.00	0.00
Phoma sp.	0.50	0.25	0.00	1.00	0.50	0.17	1.00	0.70	0.00	0.63	0.50	0.33
Pseudolachnea hispidula	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.50	0.00
Pyrenophora sp.	0.75	0.75	0.50	0.00	0.50	0.83	1.00	0.25	0.00	0.50	1.00	0.67
Thanatephorus cucumeris	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.13	0.50	0.00
Talaromyces aculeatus	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00
Teratosphaeria encephalarti	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00
Trichoderma sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Vishniacozyma carnescens	0.25	0.75	0.25	0.33	1.00	0.67	1.00	0.70	0.50	0.63	1.00	0.50
Vishniacozyma sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00
Other	1.00	1.00	1.00	0.83	1.00	1.00	1.00	0.95	1.00	0.88	1.00	1.00

A= *Acacia* sp., **B**= *Arctotheca calendula*, **C**= *Asphodelus fistulosus*, **D**= *Brassica napus*, **E**= *Brassica rapa*, **F**= *Eucalyptus* sp., **G**= *Oxalis pes-caprae*, **H**= *Prunus dulcis*, **I**= *Raphanus raphanistrum*, **J**= *Raphanus tournefortii*, **K**== *Virburnum tinus*, **L**= *Zygophyllum apiculatum*.

Table S5: **Relative incidence of fungi per pollen species in five almond orchards.**

Fungi	A	A (surroundings)	B	C	D	E
Alternaria carthami	0.97	1.00	1.00	1.00	0.50	1.00
Aspergillus flavus	0.19	0.36	0.17	0.50	0.00	0.25
Aspergillus nidulans	0.00	0.07	0.08	0.50	0.50	0.00
Aspergillus niger	0.00	0.36	0.17	1.00	0.50	0.25
Aspergillus sp.	0.00	0.07	0.00	1.00	0.00	0.25
Aureobasidium pullulans	0.88	0.79	1.00	1.00	0.50	0.75
Aureobasidium sp.	0.19	0.07	0.08	1.00	0.00	0.00
Beauveria bassiana	0.03	0.07	0.00	0.00	0.00	0.00
Byssochlamys sp.	0.03	0.07	0.08	0.50	0.50	0.00
Caloplaca xerica	0.00	0.00	0.00	0.00	0.00	0.25
Candida parapsilosis	0.03	0.00	0.00	0.00	0.00	0.00
Ceratobasidium cereale	0.00	0.07	0.00	0.00	0.00	0.00
Ceratobasidium sp.	0.00	0.07	0.08	0.00	0.00	0.25
Cladosporium ramotenellum	0.28	0.57	0.50	0.50	0.50	0.25
Cladosporium sp.	0.97	1.00	1.00	1.00	1.00	1.00
Claussenomyces sp.	0.00	0.00	0.00	0.00	0.00	0.25
Clypeophysalospora latitans	0.00	0.00	0.00	0.00	0.00	0.50
Colletotrichum acutatum	0.16	0.07	0.17	0.00	0.00	0.00
Colletotrichum asianum	0.03	0.07	0.00	0.00	0.00	0.00
Coprinellus sp.	0.06	0.07	0.00	0.00	0.50	0.25
Deltopyxis triangulispora	0.00	0.00	0.00	0.00	0.00	0.25

Table S5 (continued): **Relative incidence of fungi per pollen species in five almond orchards.**

Fungi	A	A (surroundings)	B	C	D	E
Didymosphaeria futilis	0.00	0.00	0.00	0.00	0.50	0.00
Entomophthora muscae	0.59	0.43	0.25	0.50	0.50	0.25
Eutypa crustata	0.19	0.07	0.08	0.00	0.50	0.50
Fusarium sp.	0.41	0.21	0.67	1.00	0.00	0.25
Galerina vittiformis	0.03	0.07	0.00	0.00	0.50	0.00
Itersonilia perplexans	0.03	0.14	0.17	0.00	0.00	1.00
Lophiostoma cynaroidis	0.16	0.21	0.17	0.50	0.00	0.25
Macalpinomyces sp.	0.00	0.07	0.08	0.00	0.50	0.50
Mortierella oligospora	0.00	0.00	0.08	0.00	0.00	0.00
Mortierella sp.	0.03	0.00	0.08	0.00	0.00	0.00
Nodulosphaeria hirta	0.09	0.07	0.17	1.00	0.00	0.25
Ostropa barbara	0.00	0.07	0.00	0.00	0.00	0.00
Phaeothecoidea minutispora	0.06	0.00	0.00	0.00	0.50	0.25
Phoma sp.	0.47	0.29	1.00	1.00	0.00	0.50
Pseudolachnea hispidula	0.03	0.07	0.00	0.00	0.00	0.00
Pyrenophora sp.	0.59	0.71	0.17	0.00	0.00	0.00
Thanatephorus cucumeris	0.03	0.07	0.17	0.00	0.00	0.00
Talaromyces aculeatus	0.03	0.00	0.00	0.00	0.00	0.00
Teratosphaeria encephalarti	0.03	0.00	0.00	0.00	0.00	0.00
Trichoderma sp.	0.00	0.00	0.08	0.00	0.00	0.00
Vishniacozyma carnescens	0.66	0.57	0.67	0.50	0.00	0.50
Vishniacozyma sp.	0.00	0.07	0.00	0.00	0.00	0.00
Others	0.97	1.00	0.92	1.00	0.50	1.00

Table S6: **Relative incidence of fungi during sample collection times.**

Fungi	Early	Mid	Late
Alternaria carthami	0.86	0.83	1.00
Aspergillus flavus	0.21	0.08	0.31
Aspergillus nidulans	0.00	0.08	0.06
Aspergillus niger	0.07	0.17	0.19
Aspergillus sp.	0.07	0.00	0.09
Aureobasidium pullulans	0.71	0.71	0.94
Aureobasidium sp.	0.00	0.08	0.25
Beauveria bassiana	0.00	0.00	0.06
Byssochlamys sp.	0.00	0.08	0.09
Caloplaca xerica	0.07	0.00	0.00
Candida parapsilosis	0.00	0.04	0.00
Ceratobasidium cereale	0.00	0.00	0.03
Ceratobasidium sp.	0.07	0.00	0.06
Cladosporium ramotenellum	0.21	0.33	0.47
Cladosporium sp.	0.86	0.88	1.00
Claussenomyces sp.	0.07	0.00	0.00
Clypeophysalospora latitans	0.14	0.00	0.00
Colletotrichum acutatum	0.07	0.13	0.19
Colletotrichum asianum	0.00	0.00	0.06
Coprinellus sp.	0.07	0.08	0.06
Deltopyxis triangulispora	0.07	0.00	0.00
Didymosphaeria futilis	0.00	0.04	0.00
Entomophthora muscae	0.29	0.46	0.50

Table S6 (continued): **Relative incidence of fungi during sample collection times.**

Fungi	Early	Mid	Late
Eutypa crustata	0.29	0.17	0.09
Fusarium sp.	0.50	0.25	0.44
Galerina vittiformis	0.00	0.08	0.03
Itersonilia perplexans	0.29	0.00	0.16
Lophiostoma cynaroidis	0.07	0.13	0.25
Macalpinomyces sp.	0.14	0.08	0.03
Mortierella oligospora	0.00	0.00	0.03
Mortierella sp.	0.07	0.04	0.00
Nodulosphaeria hirta	0.29	0.04	0.13
Ostropa barbara	0.00	0.00	0.03
Phaeothecoidea minutispora	0.07	0.04	0.06
Phoma sp.	0.71	0.33	0.53
Pseudolachnea hispidula	0.07	0.00	0.03
Pyrenophora sp.	0.43	0.29	0.56
Thanatephorus cucumeris	0.07	0.00	0.09
Talaromyces aculeatus	0.00	0.04	0.00
Teratosphaeria encephalarti	0.00	0.04	0.00
Trichoderma sp.	0.00	0.04	0.00
Vishniacozyma carnescens	0.64	0.42	0.66
Vishniacozyma sp.	0.00	0.00	0.03
Others	0.86	0.88	1.00

Table S7: **Relative incidence of bacteria per pollen species.**

Bacteria	A	B	C	D	E	F	G	H	I	J	K	L
Acidovorax sp.	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinoplanes sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.14
Agrobacterium sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.05	0.00	0.25	0.00	0.00
Agrococcus sp.	0.00	0.00	0.00	0.14	0.00	0.20	0.00	0.00	0.00	0.13	0.00	0.00
Agrococcus jenensis	0.00	0.33	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.13	0.00	0.14
Clavibacter michiganensis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00
Clavibacter sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00
Erwinia sp.	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lysobacter sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00
Pseudomonas sp.	0.25	0.00	0.00	0.00	0.00	0.20	0.00	0.10	0.50	0.25	0.00	0.00
Rhodococcus fascians	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14
Rhodococcus sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14
Rickettsia sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00
Others	0.50	0.67	0.00	0.14	0.00	0.40	0.50	0.20	1.00	0.25	0.00	0.29

A= *Acacia* sp., **B**= *Arctotheca calendula*, **C**= *Asphodelus fistulosus*, **D**= *Brassica napus*, **E**= *Brassica rapa*, **F**= *Eucalyptus* sp., **G**= *Oxalis pes-caprae*, **H**= *Prunus dulcis*, **I**= *Raphanus raphanistrum*, **J**= *Raphanus tournefortii*, **K**== *Virburnum tinus*, **L**= *Zygophyllum apiculatum*.

Table S8: **Relative incidence of bacteria per pollen species in five almond orchards.**

Bacteria	A	A (surroundings)	B	C	D	E
Acidovorax sp.	0.03	0.00	0.00	0.00	0.00	0.00
Agrobacterium sp.	0.08	0.00	0.07	0.00	0.00	0.00
Agrococcus sp.	0.06	0.00	0.07	0.00	0.00	0.00
Agrococcus jenensis	0.06	0.07	0.07	0.00	0.00	0.00
Pseudomonas sp.	0.11	0.00	0.00	0.00	0.00	0.75
Rickettsia sp.	0.00	0.00	0.00	0.00	0.00	0.25
Lysobacter sp.	0.03	0.00	0.00	0.00	0.00	0.00
Erwinia sp.	0.03	0.00	0.00	0.00	0.00	0.00
Rhodococcus fascians	0.03	0.07	0.00	0.00	0.00	0.00
Rhodococcus sp.	0.00	0.07	0.00	0.00	0.00	0.00
Clavibacter sp.	0.06	0.00	0.00	0.00	0.00	0.00
Clavibacter michiganensis	0.00	0.00	0.00	0.00	0.00	0.25
Actinoplanes sp.	0.03	0.07	0.00	0.00	0.00	0.00
Others	0.28	0.14	0.14	0.00	0.00	1.00

Table S9: **Relative incidence of bacteria during sample collection times.**

Bacteria	Early	Mid	Late
Acidovorax sp.	0.00	0.00	0.03
Agrobacterium sp.	0.19	0.04	0.00
Agrococcus sp.	0.06	0.08	0.00
Agrococcus jenensis	0.06	0.04	0.06
Pseudomonas sp.	0.31	0.04	0.03
Rickettsia sp.	0.06	0.00	0.00
Lysobacter sp.	0.06	0.00	0.00
Erwinia sp.	0.00	0.04	0.00
Rhodococcus fascians	0.00	0.00	0.06
Rhodococcus sp.	0.00	0.00	0.03
Clavibacter sp.	0.13	0.00	0.00
Clavibacter michiganensis	0.06	0.00	0.00
Actinoplanes sp.	0.00	0.08	0.00
Others	0.44	0.27	0.11

Chapter 6

General Discussion

Chapter 6

General Discussion

This research has provided new information about:

i) the role of honey bee workers as potential vectors of the plant pathogenic fungi, *Botrytis cinerea* and *Colletotrichum acutatum;* by combining several microbiological techniques it was demonstrated that spores of these fungi can survive through the digestive tract of workers;

ii) the potential of spores of *B. cinerea, C. acutatum* and *Cladosporium* sp. to contribute to the health and longevity of workers;

iii) the fungal and bacterial communities associated with pollen of almonds, weeds and native vegetation in commercial South Australian almond orchards, with special reference to plant pathogens.

This study shows that transport of hives can pose a high risk of transmission of plant pathogens between crops and regions, as spores of *B. cinerea* and *C. acutatum* can remain viable in the digestive tract of honey bee workers for at least 24 h after initial contact with a contaminated food source (Figure 6.1). While previous research has relied mostly on molecular techniques to assess microbial composition of the digestive tract of honey bees (Martinson, Moy & Moran 2012; Sabree, Hansen & Moran 2012; Vojvodic, Rehan & Anderson 2013), the present research has provided useful information for agricultural and ecological management using microbiological culture methods. The advantage of this approach is that, in addition to the assessment of presence, it allows quantification of abundance and viability of inoculum. In addition, this research has laid the foundation for future work, for example, to explore for how long after ingestion plant pathogens will remain viable in the digestive tract of honey bee workers and the proportion of spores that are capable of infecting plants and causing disease after defecation.

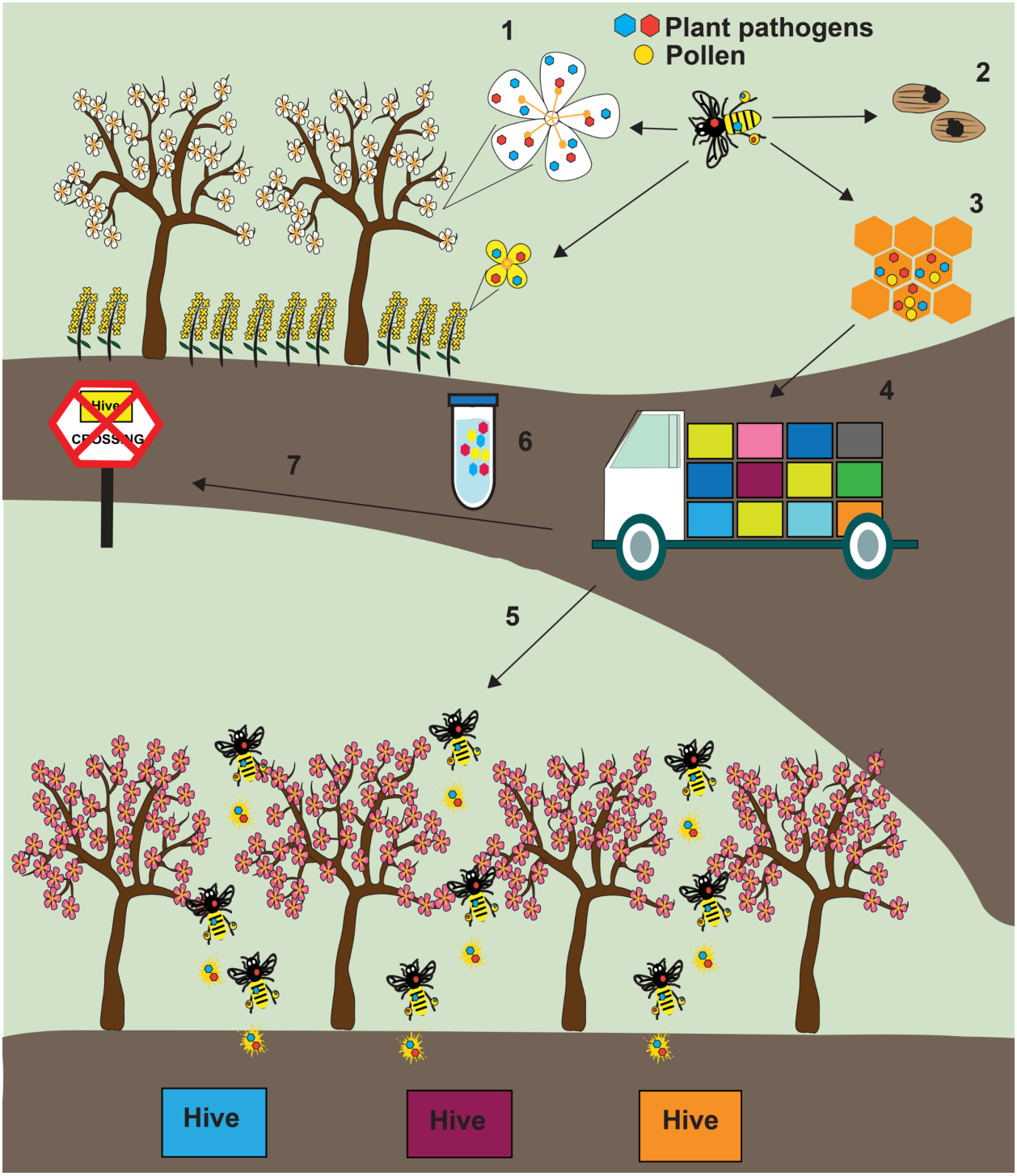

Figure 6.1. Illustration of the potential routes of transmission of plant pathogens by honey bees in and around almond orchards. 1: Honey bee workers collect almond pollen contaminated with plant pathogens; 2: Fungal pathogens sporulate on almond nuts or mummified fruit; 3: Plant pathogens are stored in the hive; 4: Hives are transported to other locations; 5: Workers transport plant pathogens on their bodies and through their digestive tracts to a different orchard or to other areas; 6: Development of a rapid test for plant pathogen content of the hive; 7: Imposition of quarantine restrictions to limit the transport of plant pathogens.

As adults in healthy honey bee colonies defecate outside the hive when the weather permits (Winston 1991), future research could focus on establishing the distances over which plant pathogenic spores could be distributed during defecation flights in field situations. This could be done, for instance, by adding fluorescent dyes to hive diets, and studying the consequent distribution of bees' faeces in the landscape over space and time. Combined with data about the period over which the spores remain viable and infective in the digestive tract of workers, this could inform modelling studies about the probability of transmission of plant pathogens through honey bee faeces. These insights could then be used to reduce risks, for example, by testing hives for known biosecurity risks prior to transport and regulating the transport of hives and thereby protecting crops, producers and native plant ecosystems (Figure 6.1).

Further investigation is also required to assess the survival of plant pathogens through the digestive tract of older workers, as these contain a mature gut microbiota (Martinson, Moy & Moran 2012) that may interfere with the survival of introduced microorganisms. However, challenges would include the need to keep the faeces free from any environmental microbial contamination that may also interfere with the survival of propagules of the plant pathogens of interest through the digestive tract of workers and growth of contaminant microorganisms on culture media used to assess viability.

Investigations should be undertaken to assess whether viable spores in the faeces of honey bee foragers can cause disease on plants and for how long plant pathogens can retain viability in the faeces suspensions. The latter could be done, for example, by placing suspensions of contaminated faeces on flowers and/or by exposing suspensions of honey bee faeces infected with plant pathogens to sunlight, as UV radiation and desiccation can affect the survival of microbes (Schmid-Hempel et al. 1999). Estimates of the risk of transmission of plant pathogenic fungi by honey bees could benefit from assessment of the abundance of various

species of fungi collected either in sole loads, or with pollen, nectar and other possible honey bee collectables, such as sawdust or honey dew.

In addition, further experiments are required to investigate the duration of survival of spores of a range of plant pathogens stored in honey bee hives and the effects of consumption of spores, possibly mixed with pollen, on the health of the colonies. Pattemore et al. (2018) showed that spores of the myrtle rust fungus can remain viable in honey bee hives and on the bodies of workers for at least 9 days after they had been experimentally inoculated, but this is not necessarily the same for all plant pathogenic fungal spores. In the early stages of this research, efforts were made to investigate the survival of spores of the beneficial fungus, *Trichoderma harzianum,* stored with pollen in honey bee hives and the effects of its consumption on the health and longevity of workers. In a technology called entomovectoring (see section 1.7), spores of biocontrol agents such as *T. harzianum* are transported to flowers by pollinators to control plant pathogens (Hokkanen & Menzler-Hokkanen 2007) and could also be stored with pollen in the hives. Unfortunately, due to the poor viability or dormancy of the spores of *T. harzianum* in the commercial product, it was not possible to estimate with any confidence the amount of colony forming units that were stored in the hives. Therefore, these experiments were discontinued (data not shown).

Future research could develop rapid and reliable tests for the detection of multiple plant pathogens in hive using DNA based methods. The development of low-cost tests, that are user friendly, provide results within hours and are non-destructive would greatly support the assessments of hives that are due to be transported from agricultural areas contaminated with plant pathogens (Figure 6.1).

Our observations that honey bee workers maintained in cages failed to consume diets consisting solely of fungal spores concur with Schmidt, Thoenes and Levin (1987). However,

it is unlikely that nurse bees would encounter bee bread that contains fungal spores only, as bees mix pollen from different sources during storage. Although the avoidance behaviour in respect of diets consisting solely of fungal spores in cage experiments has not been thoroughly investigated, this may be influenced by differences in the cellular components of spores, which vary among species (Bartnicki-Garcia 1968).

We observed honey bee workers collecting loads entirely of spores of *Podosphaera xanthii* from zucchini leaves (Chapter 3). It has been suggested that such active collection of fungal spores could be a response to deficiencies in pollen availability and quality within the forage range of a colony (Modro et al. 2009; Shaw 1999). However, this has not been experimentally demonstrated, and we were unable to do so. Testing this would require documenting foraging choices of bees in experimental confinenment, offering spores of a range of species of fungi in circunstances of high and low pollen abundance and quality.

In addition, the components of fungal spores that are attractive to bees could be identified to better understand the behavioural preferences of workers. Honey bees can discriminate shape, colours and odours, fast and reliably (Beker et al. 1989; Masson et al. 1993; Pham-Delegue et al. 1989). The spores and spore-bearing structures of several species of fungi may contain a range of carotenoids, which account for pigmentation (Davoli & Weber 2002; Irvine, Golubchuk & Anderson 1954; Shaw 1990). Furthermore, spores and mycelia may produce various volatile organic compounds (Paul & Park 2013), which may be attractive to foraging honey bees (Shaw 1990). Thus, future studies might explore the attractiveness of the colour and volatile compounds of spores of a range of fungal species to honey bees.

In addition to volatile attractants, honey bees can also recognize several essential and non-essential amino acids, either by palatability (Hendriksma, Oxman & Shafir 2014; Hendriksma & Shafir 2016; Kim & Smith 2000) or odour (Linander, Hempel de Ibarra & Laska 2012). So,

it is possible that they are simply attracted to the spores if they can recognise them as a source of protein when food is scarce. The findings in this study that spores of *B. cinerea*, *C. acutatum* and *Cladosporium* sp. in association with either of two pollen sources can extend the survival of honey bees workers represent, to the best of our knowledge, the first experimental evidence in support of the early hypothesis by Shaw (1990) that honey bees might obtain nutritional benefits from the consumption of fungal spores. However, additional investigations are necessary to identify the potential nutritional contribution of selected species of fungi to the learning, memory and health of honey bees, including resilience to pathogens.

Consumption of spores of the *Cladosporium* sp. from bee bread was beneficial in the present study. However, the genus *Cladosporium* includes fungi which have been associated with negative effects on the hive, such as causing diseases or imbalances in the hive (Calderón et al 2004). Modro et al. (2009) reported the collection of spores of *Cladosporium* sp. by honey bees during scarcity of pollen and nectar in Brazil. Also, *Cladosporium* sp. was one of numerous fungi identified in nectar samples that caused mass death of honey bees in Uruguay (Invernizzi et al. 2018). However, neither Modro et al. (2009) nor Invernizzi et al. (2018) provided details of how they identified their isolates as belonging to the genus *Cladosporium*, nor did they provide accession numbers, which confounds further comparisons of the identity and possible role of these fungi. The *Cladosporium* sp. used in this study was not identified to species level but has 100% of similarity with four species of *Cladosporium* and only 55% of similarity to the almond scab pathogen, *V. carpophila* (Appendix 1).

Another possible explanation for the collection of fungal spores is self-medication (Tihelka 2018). Honey bees use organic substances such as propolis, i.e. plant resins used within the hive, for self-medication (Simone-Finstrom et al. 2017; Simone-Finstrom & Spivak 2012; Tihelka 2018). Propolis possesses many antimicrobial properties and bee colonies that had the

inside surfaces of their nests experimentally treated with propolis invested less energy into the expression of two immune system-related genes than untreated controls (Simone, Evans & Spivak 2009). In addition, self-medicating behaviour has been observed in response to infection by the entomopathogenic fungus *Ascosphaera apis*, such that a greater number of bees foraged on resin than in the non-inoculated (or non-infected) control (Simone-Finstrom & Spivak 2012). Extracts of the mushroom species *Fomes fomentarius*, *Ganoderma applanatum* and *G. resinaceum* fed to workers in sucrose solution were able to reduce viral infection in adult honey bees (Stamets 2017; Stamets 2018; Stamets et al. 2018). However, to verify self-medication requires an experimental approach (Simone-Finstrom & Spivak 2012). Future research could explore if workers collect and consume fungal spores in their diets for self-medication purposes.

Several studies have investigated the importance of the honey bee gut microbiota in the metabolism of carbohydrates and protection of bees against opportunistic infections (Bonilla-Rosso & Engel 2018; Engel, Martinson & Moran 2012; Guo et al. 2015; Jones et al. 2018; Kesnerova et al. 2017; Khan et al. 2017; Lee et al. 2015; Maes et al. 2016; Martinson, Moy & Moran 2012). In addition, the results presented in this thesis demonstrated that the consumption of some species of fungi can improve the survival of workers. In addition, it is likely that the consumption of floral microbiomes also affects the health of honey bees. However, the quantification of these dynamics is complicated because the functionality of the majority of microorganisms in communities has not been elucidated (Busby et al. 2017). Therefore, experimental research is needed to evaluate the impact of floral microbiomes on bee fitness and to improve the understanding of interactions among plants, pollinators and microbiomes.

The fact that some fungal spores are able to extend the survival of honey bee workers may be linked to the presence of 1,3 -1,6 ß-glucans in their cell walls (San-Blas et al. 1996; Szaniszlo,

Cooper & Voges 1972). ß-glucans initiate immune responses against pathogenic bacteria, fungi and viruses in many vertebrates and invertebrates (Soltanian et al. 2009). Recent findings showed that the consumption of 1,3 -1,6 ß-glucan produced by the yeast *Saccharomyces cerevisiae* could extend the survival of honey bee workers, and reduce the number of workers naturally affected by Deformed Wing Virus and the virus load they carried (Mazzei et al. 2016). However, the potential effect of ß-glucan against honey bee bacterial pathogens such as *Paenibacillus larvae*, the causal agent of American foulbrood, a serious disease of honey bee larvae (Antunez et al. 2008) has not been explored. Additional research is required to investigate whether the consumption of β-glucans could represent a possible strategy to cure or reduce infection by various pathogens in honey bee colonies. This could open up possibilities to develop a safe, easy to use and cost-effective commercial product for application into hives to increase tolerance to diseases and hive strength (i.e. increase brood production, adult bee population and honey and pollen reserves).

To date, few publications have addressed the interactions between honey bees and spores of plant pathogenic fungi. This is likely to be associated with the challenges of producing large amounts of pure spores, which is very labour intensive, expensive, due to the requirement for large amounts of culture medium and incubation facilities with controlled light, photoperiod and temperature, and presents difficulties when spores need to be stored for periods of time without changing their characteristics. The methods described in this research allowed the production of about 10 to 20 mg of spores from three Petri dishes (9 cm diameter). Large-scale production of inoculum often involves growing fungi on cereal grains (Khonga & Sutton 1988), which are then milled for use in the commercial production of some biological control agents. However, this could interfere with the attractiveness and palatability of spores to honey bees as spores are not separated completely from the substrate and may not mimic natural field situations. Further development of in vitro systems for large-scale production of pure spores is

necessary for further research on interactions between honey bees and fungal spores. For example, adaptation of the cyclone separator device used by Evans et al. (1996) to harvest spores of *Erysiphe necator* (formerly *Uncinula necator*) from infected detached grapevine leaves may facilitate the harvesting of large quantities of spores without contamination by the growth substrate.

This research provided the first identification of fungal and bacterial communities associated with honey bee corbicular pollen collected from almonds, weeds and native plants in South Australian commercial almond orchards. While fungal spores occur on surfaces, such as flowers, because they are commonly dispersed by wind and wind-blown rain, this research has also provided evidence for the potential role of honey bees in vectoring pathogens of almonds, such as *C. acutatum*. Future research with a larger sample size of bee pollen collections per location and over time, and using a variety of reference databases, is required to improve understanding of the interactions between honey bees, plant species and plant pathogens in agricultural systems. Additionally, future studies should investigate how the management of almond orchards, such as bare-ground monoculture or with a diverse ground-cover as well as comparison of strategies for crop protection such as the use of conventional fungicides, bactericides and insecticides and biological control agents might affect the incidence of propagules of plant pathogens on the flowers of the crop.

In addition to plant pathogens, microorganisms that may be used as biocontrol agents of plant pathogens and insect pests were identified in association with the pollen samples. Further research could investigate the co-functional attributes of consortia of beneficial microorganisms naturally occurring in agricultural systems (Sarma et al. 2015). This could be done, for example, by mixing pure propagules of microorganisms that co-occurred naturally in pollen samples and testing their ability to control plant diseases in vitro and in field situations.

This information may allow formulation of mixtures of biocontrol products, support management approaches and, consequently, improve the efficiency of crop production and assist in the maintenance of sustainable environments.

Overall, this study has shown that spores of some plant pathogenic fungi can survive through the digestive tract of honey bee workers, serve as a food supplement for bees and be present on flowers of a number of plant species in an orchard setting. The suggested benefits of the consumption of fungal spores by honey bee workers, if confirmed by independent, large scale experiments, would warrant future research focused on application to improve hive health. Furthermore, new evidence for the role of honey bees as vectors of plant pathogens has implications for biosecurity associated with hive transport among crops and regions.

Appendix

Appendix

Appendix 1: Sequence similarity comparison between the *Cladosporium* isolate (MK402112) used in Chapter 4 and other species in Bench et al. 2018 and National Center for Biotechnology Information (NCBI).

Species	**Similarity**	**Accession number**	**Reference**
Cladosporium halotolerans	100%	MF473069.1	Bensch et al. 2018
C. sphaerospermum	100%	MH482916.1	NCBI
C. parahaloterans	100%	MF473151.1	Bensch et al. 2018
C. cladosporioides	100%	MK262923.1	NCBI
Venturia carpophila	55%	MH857426.1	Vu et al. 2018

References

(Literature Review & General Discussion)

References

Adaskaveg, JE & Förster, H 2000, 'Occurrence and management of anthracnose epidemics caused by *Colletotrichum* species on tree fruit crops in California', in D Prusky, Freeman, S & Dickman, M (eds), *Colletotrichum: Host specificity, pathology, and host-pathogen interaction*, The American Phytopathological Society, Saint Paul, MN.

Adaskaveg, JE & Hartin, RJ 1997, 'Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California', *Phytopathology*, vol. 87, pp. 979-987.

Agostini, JP, Gottwald, T & Timmer, LW 1993, 'Temporal and spatial dynamics of postbloom fruit drop of citrus in Florida', *Phytopathology*, vol. 83, pp. 485-490.

Agrios, G 2005, *Plant pathology*, 5th edn, Elsevier Academic Press, Burlington, MA.

Ahmad, R & Ahmad, M 1986, 'Introduction to *Apis mellfera* (L.) and factors affecting its establishment in Pakistan', *Apiacta*, vol. 21, pp. 9-14.

Aleklett, K, Hart, M & Shade, A 2014, 'The microbial ecology of flowers: an emerging frontier in phyllosphere research', *Botany*, vol. 92, pp. 253-266.

Alexandrova, M, Cimini, B, Bazzi, C, Carpana, E, Massi, S & Sabatini, AG 2002a, 'The role of honeybees in spreading *Erwinia amylovora*', *Acta Horticulturae*, vol. 590, pp. 55-60.

Alexandrova, M, Porrini, C, Bazzi, C, Carpana, E, Bigliardi, M & Sabatini, AG 2002b, '*Erwinia amylovora* longevity in beehives, beehive products and honeybees', *Acta Horticulturae*, vol. 590, pp. 201-205.

Altieri, MA 1995, 'Agroecology: the science of sustainable agriculture'. *Boulder CO: Westview Press. Part three: development, climate and rights*, vol. 238, pp. 12052-12057.

Altieri, MA 2004, 'Linking ecologists and traditional farmers in the search for sustainable agriculture', *Frontiers in Ecology and the Environment*, vol. 2, pp. 35-42.

Almond Board of Australia 2019, Almond Insights 2017/2018, Almond Board of Australia Inc., acessed October 2019, <https://industry.australianalmonds.com.au/almond-board/almond-insights/>.

Ambika Manirajan, B, Ratering, S, Rusch, V, Schwiertz, A, Geissler-Plaum, R, et al. 2016, 'Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity', *Environmental Microbiology*, vol. 18, pp. 5161-5174.

Anderson KE, Sheehan, TH, Mott, BM, Maes, P, Snyder, L, Schwan, MR, Walton, A, Jones, BM, Corby-Harris, V 2013, 'Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (*Apis mellifera*)', *Plos One*, vol. 8, p. e83125.

Antunez, K, Harriet, J, Gende, L, Maggi, M, Zunino, P 2008, 'Efficacy of natural propolis extract in the control of American Foulbrood', *Veterinary Microbiology*, vol. 131, pp.324-331.

Asadollahi, M, Fekete, E, Karaffa, L, Flipphi, M, Árnyasi, M, Esmaeili, M, Váczy, KZ & Sándor, E 2013, 'Comparison of *Botrytis cinerea* populations isolated from two open-field cultivated host plants', *Microbiological Research*, vol. 168, pp. 379-388.

Australian Pesticides and Veterinary Medicine Authority (APVMA), Public Chemical Registration Information System - PUBCRIS. [Internet] Canberra: Commonwealth of Australia; [November 2019], <http://services.apvma.gov.au>.

Bartnicki-Garcia, S 1968, 'Cell wall chemistry, morphogenesis, and taxonomy of fungi', *Annual Reviews in Microbiology*, vol. 22, pp. 87-108.

Batra, L, Batra, S & Bohart, G 1973, 'The mycoflora of domesticated and wild bees (Apoidea)', *Mycopathologia et Mycologia Applicata*, vol. 49, pp. 13-44.

Bee Aware 2019, Australia, acessed October 2019 <https://beeaware.org.au/>.

Beekman, M & Ratnieks, FLW 2000, 'Long-range foraging by the honey-bee, *Apis mellifera* L', *Functional Ecology*, vol. 14, pp. 490-496.

Beker, R, Dafni, A, Eisikowitch, D & Ravid, U 1989, 'Volatiles of two chemotypes of *Majorana syriaca* L. (Labiatae) as olfactory cues for the honeybee', *Oecologia*, vol. 79, pp. 446-451.

Benecke, FS 2003, Commercial beekeeping in Australia, A report for the Rural Industries Research and Development Coorporation. RIRDC Publication, ACT, Australia, no. 07/059.

Bennie, RB 1942, 'Pollen substitute for bees', *New Zealand Beekeeper*, Vol. 4, pp. 17-18.

Bensch, K, Braun, U, Groenewald, JZ, Crous, PW 2012, 'The genus *Cladosporium*', *Studies in Mycology*, vol. 72, pp. 1-401.

Bensch, K, Groenewald, J, Meijer, M, Dijksterhuis, J, Jurjević, Z, Andersen, B, Houbraken, J, Crous, PW, Samson, RA 2018 '*Cladosporium* species in indoor environments', *Studies in Mycology*, vol. 89, pp. 177-301.

Bernstein, B, Zehr, EI, Dean, RA & Shabi, E 1995, 'Characteristics of *Colletotrichum* from peach, apple, pecan and other hosts ', *Plant Disease*, vol. 79, pp. 478-482.

Berthon, K, Esperon-Rodriguez, M, Beaumont, L, Carnegie, A & Leishman, M 2018, 'Assessment and prioritisation of plant species at risk from myrtle rust (*Austropuccinia psidii*) under current and future climates in Australia', *Biological Conservation*, vol. 218, pp. 154-162.

Betts, AD 1912, 'The fungi of the bee-hive', Journal of Economic Biology, vol. 7, pp. 129-162.

Bock, CH, Hotchkiss, MW, Okie, WR & Wood, BW 2011, 'The distribution of peach scab lesions on the surface of diseased peaches', *European Journal of Plant Pathology*, vol. 130, pp. 393-402.

Bonilla-Rosso, G & Engel, P 2018, 'Functional roles and metabolic niches in the honey bee gut microbiota', *Current Opinion in Microbiology*, vol. 43, pp. 69-76.

Braganca, CAD, Damm, U, Baroncelli, R, Massola, NS & Crous, PW 2016, 'Species of the *Colletotrichum acutatum* complex associated with anthracnose diseases of fruit in Brazil', *Fungal Biology*, vol. 120, pp. 547-561.

Brodschneider, R & Crailsheim, K 2010, 'Nutrition and health in honey bees', *Apidologie*, vol. 41, pp. 278-294.

Busby, PE, Soman, C, Wagner, MR, Friesen, ML, Kremer, J, Bennett, A, Morsy, M, Eisen, JA, Leach, JE & Dangl, JL 2017, 'Research priorities for harnessing plant microbiomes in sustainable agriculture', *Plos Biology*, vol. 15, p. e2001793.

Calderón, RA, Rivera, G, Sánchez, LA, Zamora, LG 2004, 'Chalkbrood (*Ascophaera apis*) and some other fungi associated with africanized honey bees (*Apis mellifera*) in Costa Rica. J*ournal of Apiculture Research*, vol. 43, pp. 187-188.

Calixto, JB, Miguel, OG, Rosendo, A, Yunes, RS, Rae, GA 1990, 'Action of 2-Hydroxy-4,6-dimethoxyacetophenone isolated from *Sebastiania schottiana*', *Planta Medica*; vol. 56: pp. 31–35.

Card, S, Pearson, M & Clover, G 2007, 'Plant pathogens transmitted by pollen', *Australasian Plant Pathology*, vol. 36, pp. 455-461.

Carnegie, AJ, Kathuria, A, Pegg, GS, Entwistle, P, Nagel, M & Giblin, FR 2016, 'Impact of the invasive rust *Puccinia psidii* (myrtle rust) on native Myrtaceae in natural ecosystems in Australia', *Biological Invasions*, vol. 18, pp. 127-144.

Carnegie, AJ, Lidbetter, JR, Walker, J, Horwood, MA, Tesoriero, L, Glen, M & Priest, MJ 2010, '*Uredo rangelii*, a taxon in the guava rust complex, newly recorded on Myrtaceae in Australia', *Australasian Plant Pathology*, vol. 39, pp. 463-466.

Carroll, MJ, Brown, N, Goodall, C, Downs, AM, Sheenan, TH & Anderson, KE 2017, 'Honey bees preferentially consume freshly-stored pollen', *Plos One*, vol. 12, p. e0175933

Chapman, G 1964, 'Urediospore collections by honey bees from *Puccinia psidii*', *Annals of the Entomological Society of America*, vol. 57, p. 264.

Chen, Y, Evans, JD, Smith, IB & Pettis, JS 2008, '*Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States', *Journal of Invertebrate Pathology*, vol. 97, pp. 186-188.

Chen, Y, Pettis, JS, Collins, A & Feldlaufer, MF 2006, 'Prevalence and transmission of honeybee viruses', *Applied and Environmental Microbiology*, vol. 72, pp. 606-611.

Chen, C, Bock, CH & Bruce, W 2017, 'Draft genome sequence of *Venturia carpophila*, the causal agent of peach scab', *Standards in Genomic Sciences*, vol. 12, p. 68

Connell, JH 2000, 'Pollination of almonds: practices and problems', *HortTechnology*, vol. 10, pp. 116-119.

Cook, AJ 1 885, -'Fungus spores for bee-bread', *Gleanings in Bee Culture*, vol. 12, pp. 455-456.

Corby-Harris, V, Maes, P & Anderson, KE 2014, 'The bacterial communities associated with honey bee (*Apis mellifera*) foragers', *Plos One*, vol. 9, p. e95056.

Crailsheim, K, Schneider, L, Hrassnigg, N, Bühlmann, G, Brosch, U, Gmeinbauer, R & Schöffmann, B 1992, 'Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): dependence on individual age and function', *Journal of Insect Physiology*, vol. 38, pp. 409-419.

Cunningham, SA, FitzGibbon, F & Heard, TA 2002, 'The future of pollinators for Australian agriculture', *Australian Journal of Agricultural Research*, vol. 53, pp. 893-900.

Davoli, P & Weber, RW 2002, 'Identification and quantification of carotenoid pigments in aeciospores of the daisy rust fungus, *Puccinia distincta*', *Phytochemistry*, vol. 60, pp. 309-313.

De Groot, AP 1953, 'Protein and amino acid requirements of the honeybee (*Apis mellifica* L.)', *Physiologia Comparata et Oecologia*, vol. 3, pp. 197-285.

Dedej, S, Delaphane, KS & Scherm, H 2004, 'Effectiveness of honey bees in delivering the biocontrol agent *Bacillus subtilis* to blueberry flowers to suppress mummy berry disease', *Biological Control*, vol. 31, pp. 422-427.

Dedej, S & Delaplane, KS 2003, 'Honey bee (Hymenoptera : Apidae) pollination of rabbiteye blueberry *Vaccinium ashei* var. 'Climax' is pollinator density-dependent', *Journal of Economic Entomology*, vol. 96, pp. 1215-1220.

DeGrandi-Hoffman, G, Chen, Y, Huang, E & Huang, MH 2010, 'The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.)', *Journal of Insect Physiology*, vol. 56, pp. 1184-1191.

Delaplane, KS, Mayer, DR & Mayer, DF 2000, *Crop pollination by bees*, CABI Publishing, New York, NY.

Dell, B 1977, 'The collection of poplar rust spores by honey bees', *Western Australian Naturalist*, vol. 13, pp. 199-201.

Deodikar, GP, Thakar, CV, Shah, PN, Salvia, SR, Chitale,PS 1958, 'Foraging of honeybees on fungal rust spores (*Cytopsora oleae*) on *Olea dioica*', *Bee World*, vol.39, pp.120-121.

Di Pasquale, G, Salignon, M, Le Conte, Y, Belzunces, LP, Decourtye, A, Kretzschmar, A, Suchail, S, Brunet, J-L & Alaux, C 2013, 'Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter?', *Plos One*, vol. 8, p. e72016.

Dimou, M, & Thrasyvoulou, A 2007, 'Collection of wax scale (*Ceroplastes* sp.) by the honey bee *Apis mellifera*', *Journal of Apicultural Research*, vol. 46, p. 129.

Donkersley, P, Rhodes, G, Pickup, RW, Jones, KC, Wilson, K 2018, 'Bacterial communities associated with honeybee food stores are correlated with land use', *Ecology and Evolution*, vol. 8, pp. 4743-4756.

Doull, K 1971, 'Analysis of bee behavior as it relates to pollination', *The American Bee Journal*, vol. 111, pp. 266-341.

Droby, A & Lichter, A 2004, 'Post-harvest *Botrytis* infection: etiology, development and management ' in Elad, Y, Williamson, B, Tudzynski, P, Delen, N (eds), *Botrytis: biology, pathology and control*, The Netherlands: Kluwer Academic Press, Dordrecht, pp. 349–367.

Dugan, FM, Schubert, K, Braun, U 2004, 'Check-list of *Cladosporium* names', *Schlechtendalia*, vol. 11, pp. 1–103.

Egorova, A 1971, 'Preservative microflora in stored pollen', *Veterinariya*, vol. 8, pp. 40-41.

Ellis, AM & Hayes Jr, G 2009, 'An evaluation of fresh versus fermented diets for honey bees (*Apis mellifera*)', *Journal of Apicultural Research*, vol. 48, pp. 215-216.

Engel, P, Martinson, VG & Moran, NA 2012, 'Functional diversity within the simple gut microbiota of the honey bee', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, pp. 11002-11007.

Evans , JD & Schwarz, RS 2011 'Bees brought to their knees', *Trends in Microbiology,* vol. 19, pp. 614-620.

Evans, KJ, Whisson, DL, Scott, ES 1996, 'An experimental system for characterizing isolates of *Uncinula necator*', *Mycological Research*, vol. 100, pp. 675-680.

Filipiak, M, Kuszewska, K, Asselman, M, Denisow, B, Stawiarz, E, Woyciechowski, M & Weiner, J 2017, 'Ecological stoichiometry of the honeybee: Pollen diversity and adequate species composition are needed to mitigate limitations imposed on the growth and development of bees by pollen quality', *Plos One*, vol. 12, p. e0183236.

Filho, VC, Miguel, OG, Nunes, RJ, Calixto, JB, Yunes, RA 1995, 'Antispasmodic activity of Xanthoxyline derivatives: structure-activity relationships', *Journal of Pharmaceutical Sciences*, vol. 84, pp. 473–475.

Fisher, EE 1961, *'Venturia carpophila* sp. nov., the ascigerous state of the apricot freckle fungus', *Transactions of the British Mycological Society*, vol. 44, pp. 337-342.

Franck, P, Garnery, L, Loiseau, A, Oldroyd, B, Hepburn, H, Solignac, M & Cornuet, J-M 2001, 'Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data', *Heredity*, vol. 86, pp. 420-430.

Free, J 1963, 'The flower constancy of honeybees', *The Journal of Animal Ecology*, vol. 32, pp. 119-131.

Gallelli, A, Talocci, S, L'aurora, A & Loreti, S 2012, 'Detection of *Pseudomonas syringae* pv. *actinidiae*, causal agent of bacterial canker of kiwifruit, from symptomless fruits and twigs, and from pollen', *Phytopathologia Mediterranea*, vol. 50, pp. 462-472.

Gasparoto, MCG, Lourenco, SA, Tanaka, FAO, Sposito, MB, Marchini, LC, Silva, GJ & Amorim, L 2017, 'Honeybees can spread *Colletotrichum acutatum* and *C. gloeosporioides* among citrus plants', *Plant Pathology*, vol. 66, pp. 777-782.

Gilliam, M, Prest, D & Lorenz, B 1989, 'Microbiology of pollen and bee bread: taxonomy and enzymology of molds', *Apidologie*, vol. 20, pp. 53-68.

González-Domínguez, E, Armengol, J & Rossi, V 2017, 'Biology and epidemiology of *Venturia* species affecting fruit crops: A review', *Frontiers in Plant Science*, vol. 8, p. 1496.

Grant, V 1950, 'The flower constancy of bees', *The Botanical Review*, vol. 16, pp. 379-398.

Guo, J, Wu, J, Chen, YP, Evans, JD, Dai, RG, Luo, WH & Li, JL 2015, 'Characterization of gut bacteria at different developmental stages of Asian honey bees, *Apis cerana*', *Journal of Invertebrate Pathology*, vol. 127, pp. 110-114.

Hendricks, L 1996, 'Orchard planning, design, and development', *Almond Production Manual*, pp. 47-51.

Hendriksma, HP, Oxman, KL & Shafir, S 2014, 'Amino acid and carbohydrate tradeoffs by honey bee nectar foragers and their implications for plant–pollinator interactions', *Journal of Insect Physiology*, vol. 69, pp. 56-64.

Hendriksma, HP & Shafir, S 2016, 'Honey bee foragers balance colony nutritional deficiencies', *Behavioral Ecology and Sociobiology*, vol. 70, pp. 509-517.

Hill, S, Stephenson, D & Taylor, B 1985, 'Almond pollination studies: pollen production and viability, flower emergence and cross-pollination tests', *Australian Journal of Experimental Agriculture*, vol. 25, pp. 697-704.

Hokkanen, H & Menzler-Hokkanen, I 2007, 'Use of honeybees in the biological control of plant diseases', *International Congress of Insect Biotechnology & Industry (ICBI)*, vol. 37, pp. A62-A63, Seoul, South Korea.

Holliday, P 2001, *A Dictionary of Plant Pathology*, 2nd edn, Cambridge University Press.

Hong, C, Michailides, TJ & Holtz, BA 1998, 'Effects of wounding, inoculum density, and biological control agents on postharvest brown rot of stone fruits', *Plant Disease*, vol. 82, pp. 1210-1216.

Hoover, SE, Higo, HA & Winston, ML 2006, 'Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition', *Journal of Comparative Physiology B*, vol. 176, pp. 55-63.

Horsfield, A, Wicks, T & Wilson, D 2010, 'Field evaluation of fungicides for the control of rust, brown rot, shot hole and scab in almonds', *Australasian Plant Pathology*, vol. 39, pp. 112-119.

Hua L, Yong, C, Zhanquan, Z, Boqiang, L, Guozheng, Q, Shiping, T 2018, 'Pathogenic mechanisms and control strategies of *Botrytis cinerea* causing post-harvest decay in fruits and vegetables', *Food Quality and Safety*, vol. 2, pp. 111-119.

Inouye, DW, Gill, DE, Dudash, MR & Fenster, CB 1994, 'A model and lexicon for pollen fate', *American Journal of Botany*, vol. 81, pp. 1517-1530.

Invernizzi, C, Nogueira, E, Juri, P, Santos, E, Arredondo, D, Branchiccela, B, Mendoza, Y & Antunez, K 2018, '*Epormenis cestri* secretions in *Sebastiania schottiana* trees cause mass death of honey bee *Apis mellifera* larvae in Uruguay', *Plos One*, vol. 13, p. e0190697.

Irvine, G, Golubchuk, M & Anderson, J 1954, 'The carotenoid pigments of the uredospores of rust fungi', *Canadian Journal of Agricultural Science*, vol. 34, pp. 234-239.

Johannsmeier, MF 1981, 'Corbicular loads of the african honeybee', *South African Bee Journal*, vol. 53, pp. 3-6.

Johnson, BR 2008, 'Within-nest temporal polyethism in the honey bee', *Behavioral Ecology and Sociobiology*, vol. 62, pp. 777-784.

Johnson, KB, Stockwell, VO, Burgett, DM, Sugar, D & Loper, JE 1993, 'Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by honey bees from hives to apple and pear blossoms', *Phytopathology*, vol. 83, pp. 478-484.

Jones, JC, Fruciano, C, Marchant, J, Hildebrand, F, Forslund, S, Bork, P, Engel, P & Hughes, WOH 2018, 'The gut microbiome is associated with behavioural task in honey bees', *Insectes Sociaux*, vol. 65, pp. 419-429.

Kapongo, JP, Shipp, L, Kevan, P & Sutton, JC 2008, 'Co-vectoring of *Beauveria bassiana* and *Clonostachys rosea* by bumble bees (*Bombus impatiens*) for control of insect pests and suppression of grey mould in greenhouse tomato and sweet pepper', *Biological Control*, vol. 46, pp. 508-514.

Kesnerova, L, Mars, RAT, Ellegaard, KM, Troilo, M, Sauer, U & Engel, P 2017, 'Disentangling metabolic functions of bacteria in the honey bee gut', *Plos Biology*, vol. 15, p. e2003467.

Kevan, PG, Kapongo, J-P, Al-mazra'awi, M & Shipp, L 2008, Honey bees, bumble bees, and biocontrol: new alliances between old friends*,* in James, RR & Pitts-Singer, TL (eds), *Bee pollination in agricultural ecosystems*, Oxford University Press, UK.

Kevan, PG, Sutton, J, Tam, L, Boland, G, Broadbent, B, Thomson, S & Brewer, G 2003, 'Using pollinators to deliver biological control agents against crop pests', in *Pesticide formulations and delivery systems: meeting the challenges of the current crop protection industry,* American Society for Testing and Materials International.

Khan, KA, Ansari, MJ, Al-Ghamdi, A, Nuru, A, Harakeh, S & Iqbal, J 2017, 'Investigation of gut microbial communities associated with indigenous honey bee (*Apis mellifera jemenitica*) from two different eco-regions of Saudi Arabia', *Saudi Journal of Biological Sciences*, vol. 24, pp. 1061-1068.

Khonga, E & Sutton, J 1988, 'Inoculum production and survival of *Gibberella zeae* in maize and wheat residues', *Canadian Journal of Plant Pathology*, vol. 10, pp. 232-239.

Kim, YS & Smith, BH 2000, 'Effect of an amino acid on feeding preferences and learning behavior in the honey bee, *Apis mellifera*', *Journal of Insect Physiology*, vol. 46, pp. 793-801.

Klein, D, Vaissiere, BE, Cane, JH, Steffan-Dewenter, I, Cunningham, SA, Kremen, C & Tscharntke, T 2007, 'Importance of pollinators in changing landscapes for world crops', *Proceedings of the Royal Society B-Biological Sciences*, vol. 274, pp. 303-313.

Kleinschmidt, G, Kondos, A, Harden, J & Turner, J 1974, 'Colony management for eucalypt honey flows', *The Australasian Beekeeper*, vol. 75, pp. 261-264.

Kovach, J, Petzoldt, R & Harman, GE 2000, 'Use of honey bees and bumble bees to disseminate *Trichoderma harzianum* 1295-22 to strawberries for *Botrytis* control', *Biological Control*, vol. 18, pp. 235-242.

Korpelainen, H, Pietiläinen, M & Huotari, T 2016, 'Effective detection of indoor fungi by metabarcoding', *Annals of Microbiology*, vol. 66, pp. 495-498.

Kraus, PX 1920, '(White apple mildew as bee bread)', *Der deutsche Imker aus Bohemen*, vol. 33, pp. 120-122.

Lan, Z & Scherm, H 2003, 'Moisture sources in relation to conidial dissemination and infection by *Cladosporium carpophilum* within peach canopies', *Phytopathology*, vol. 93, pp. 1581-1586.

Lang, WHJr, 1901, 'Fungus spores as bee bread', *Plant World,* vol. 4, pp. 49-51.

Lee, FJ, Rusch, DB, Stewart, FJ, Mattila, HR & Newton, IL 2015, 'Saccharide breakdown and fermentation by the honey bee gut microbiome', *Environmental Microbiology*, vol. 17, pp. 796-815.

Leroux, P 2004, *Chemical control of Botrytis and its resistance to chemical fungicides*, In: *Botrytis*: Biology, Pathology and Control. Elad, Y, Williamson, B, Tudzynski, P & Delen, N (eds), pp. 195-222, Dordrecht, The Netherlands: Kluwer Academic Press.

Linander, N, Hempel de Ibarra, N & Laska, M 2012, 'Olfactory detectability of L-amino acids in the European honeybee (*Apis mellifera*)', *Chemical Senses*, vol. 37, pp. 631-638.

Lindauer, M 1952, 'Ein beitrag zur frage der arbeitsteilung im bienenstaat', *Zeitschrift für vergleichende Physiologie*, vol. 34, pp. 299-345.

Maas, JL & Howard, CM 1985, 'Variation of several anthracnose fungi in virulence to strawberry and apple ', *Plant Disease*, vol. 69, pp. 164-166.

Maccagnani, B, Bazzi, C, Biondi, E, Tesoriero, D, Maini, S 2006, Potential of *Osmia cornuta* as a carrier of antagonist bacteria in biological control of fire blight: a comparison with *Apis mellifera*, in: Bazzi, C. & Mazzucchi, U (eds.), Proceedings of the Xth International Workshop on Fire Blight, pp. 379-386.

Maes, PW, Rodrigues, PA, Oliver, R, Mott, BM & Anderson, KE 2016, 'Diet-related gut bacterial dysbiosis correlates with impaired development, increased mortality and Nosema disease in the honeybee (*Apis mellifera*)', *Molecular Ecology*, vol. 25, pp. 5439-5450.

Marques, JPR, Amorim, L, Sposito, MB, Marin, D & Appezzato-da-Gloria, B 2013, 'Infection of citrus pollen grains by *Colletotrichum acutatum*', *European Journal of Plant Pathology*, vol. 136, pp. 35-40.

Martinson, VG, Moy, J & Moran, NA 2012, 'Establishment of characteristic gut bacteria during development of the honeybee worker', *Applied and Environmental Microbiology*, vol. 78, pp. 2830-2840.

Masson, C, Pham-Delegue, M, Fonta, C, Gascuel, J, Arnold, G, Nicolas, G & Kerszberg, M 1993, 'Recent advances in the concept of adaptation to natural odour signals in the honeybee, *Apis mellifera* L', *Apidologie*, vol. 24, pp. 169-194.

Maurizio, A & Hodges, FED 1950, 'The influence of pollen feeding and brood rearing on the length of life and physiological condition of the honeybee preliminary report', *Bee World*, vol. 31, pp. 9-12.

Mazzei, M, Fronte, B, Sagona, S, Carrozza, ML, Forzan, M, Pizzurro, F, Bibbiani, C, Miragliotta, V, Abramo, F & Millanta, F 2016, 'Effect of 1, 3-1, 6 β -glucan on natural and experimental deformed wing virus infection in newly emerged honeybees (*Apis mellifera ligustica*)', *Plos One*, vol. 11, p. e0166297.

McGregor, SE 1976, *Insect pollination of cultivated crop plants*, vol. 496, Agricultural Research Service, US Department of Agriculture.

McKay, SF, Shtienberg, D, Sedgley, M & Scott, ES 2014, 'Anthracnose on almond in Australia: disease progress and inoculum sources of *Colletotrichum acutatum*', *European Journal of Plant Pathology*, vol. 139, pp. 773-783.

Modro, AFH, Silva, IC, Message, D & Luz, CFP 2009, 'Saprophytic fungus collection by africanized bees in Brazil', *Neotropical Entomology*, vol. 38, pp. 434-436.

Mommaerts, V, Platteau, G, Boulet, J, Sterk, G & Smagghe, G 2008, '*Trichoderma*-based biological control agents are compatible with the pollinator *Bombus terrestris*: A laboratory study', *Biological Control*, vol. 46, pp. 463-466.

Mommaerts, V & Smagghe, G 2011, 'Entomovectoring in plant protection', *Arthropod-Plant Interactions*, vol. 5, pp. 81-95.

Mommaerts, V, Sterk, G, Hoffmann, L & Smagghe, G 2009, 'A laboratory evaluation to determine the compatibility of microbiological control agents with the pollinator *Bombus terrestris*', *Pest Management Science*, vol. 65, pp. 949-955.

Mommaerts, V, Jans, J & Smagghe, G 2010, 'Side effects of commercial *Bacillus thuringiensis* insecticides on micro-colonies of *Bombus terrestris*', *Pest Management Science*, vol. 66, pp. 68-69.

National Center for Biotechnology Information (NCBI)[Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] – [October 2019]. Available from: https://www.ncbi.nlm.nih.gov/

Nuclo, RL, Johnson, KB, Stockwell, VO & Sugar, D 1998, 'Secondary colonization of pear blossoms by two bacterial antagonists of the fire blight pathogen', *Plant Disease*, vol. 82, pp. 661-668.

Ogawa, JM & English, H 1991, *Diseases of temperate zone tree fruit and nut crops*, University of California Agriculture and Natural Resources, Oakland, California: Publication 3345.

Oswald B, McKay, S, Edwards, J, Sosnowski, M, 2018, Lower limb dieback research update, Horticulture Inovation Australia. Acessed October 2019. Available from://<https://industry.australianalmonds.com.au/growing/lld-research-update/>

Page, RE & Robinson, GE 1991, 'The genetics of division of labour in honey bee colonies', in *Advances in Insect Physiology*, vol. 23, pp. 117-169.

Pain, J & Maugenet, J 1966, 'Recherches biochimiques et physiologiques sur le pollen emmagasiné par les abeilles', *Les Annales de l'Abeille*, vol. 9, pp. 209-236.

Parker, RL 1926, 'The collection and utilization of pollen by the honeybee', *Memoir Cornell University, Agricultural Experiment Station*.

Pattemore, DE, Bateson, M, Buxton, M, Pegg, G & Hauxwell, C 2018, 'Assessment of the risks of transmission of myrtle rust (*Austropuccinia psidii*) spores by honey bees (*Apis mellifera*)', A Plant & Food Research report prepared for: Ministry for Primary Industries, New Zealand.

Pattemore, DE, Goodwin, RM, McBrydie, HM, Hoyte, SM & Vanneste, JL 2014, 'Evidence of the role of honey bees (*Apis mellifera*) as vectors of the bacterial plant pathogen *Pseudomonas syringae*', *Australasian Plant Pathology*, vol. 43, pp. 571-575.

Paul, D & Park, KS 2013, 'Identification of volatiles produced by *Cladosporium cladosporioides* CL-1, a fungal biocontrol agent that promotes plant growth', *Sensors*, vol. 13, pp. 13969-13977.

Peres, NAR, Kuramae, EE, Dias, MSC & de Souza, NL 2002, 'Identification and characterization of *Colletotrichum* spp. affecting fruit after harvest in Brazil', *Journal of Phytopathology-Phytopathologische Zeitschrift*, vol. 150, pp. 128-134.

Pertot, I, Giovannini, O, Benanchi, M, Caffi, T, Rossi, V, Mugnai, L 2017, 'Combining biocontrol agents with different mechanisms of action in a strategy to control *Botrytis cinerea* on grapevine', *Crop Protection*, vol. 97, pp.85-93.

Pham-Delegue, M, Etievant, P, Guichard, E & Masson, C 1989, 'Sunflower volatiles involved in honeybee discrimination among genotypes and flowering stages', *Journal of Chemical Ecology*, vol. 15, pp. 329-343.

Portman, ZM & Tepedino, VJ 2017, 'Convergent evolution of pollen transport mode in two distantly related bee genera (Hymenoptera: Andrenidae and Melittidae)', *Apidologie*, vol. 48, pp. 461-472.

Prasil, O, Urajova, P, Kristufek, V & Frydrychova, R 2016, 'Vcely a rasy.', *Modernı vcelar*, vol. 13, pp. 23-24.

Purahong, W, Orrù, L, Donati, I, Perpetuini, G, Cellini, A et al. 2018, 'Plant microbiome and its link to plant health: tree species, organs and *Pseudomonas syringae* pv. *actinidiae* infection shaping bacterial phyllosphere communities of kiwifruit plants', *Frontiers in Plant Science* vol. 9, pp. 1563.

Ram, V & Bhardwaj, L, 2004, 'Stone fruit diseases and their management', in Naqvi, SAMH (ed), *Diseases of Fruits and Vegetables. Diagnosis and management*, Kluwer, Dordrecht, The Netherlands.

Rathé, A, Pilkington, L, Gurr, G, Daugherty, M 2012, 'Potential for persistence and within-plant movement of *Xylella fastidiosa* in Australian native plants', *Australasian Plant Pathology*, vol. 41, pp. 405-412.

Reintjes, C, Haverkort, B & Waters-Bayer, A 1992, '*Farming for the future: An introduction to low-external input and sustainable agriculture*', London: Macmillan.

Ribière, M, Lallemand, P, Iscache, AL, Schurr, F, Celle, O, Blanchard, P, Olivier, V & Faucon, JP 2007, 'Spread of infectious chronic bee paralysis virus by honeybee (*Apis mellifera* L.) feces', *Applied and Environmental Microbiology*, vol. 73, pp. 7711-7716.

Renzi, MT, Rodríguez-Gasol, N, Medrzycki, P, Porrini, C, Martini, A, Burgio, G, Maini, S & Sgolastra, F 2016, 'Combined effect of pollen quality and thiamethoxam on hypopharyngeal gland development and protein content in *Apis mellifera*', *Apidologie*, vol. 47, pp. 779-788.

Roco, A & Perez, LM 2001, 'In vitro biocontrol activity of *Trichoderma harzianum* on *Alternaria alternata* in the presence of growth regulators', *Electronic Journal of Biotechnology*, vol. 4, pp. 68–73.

Rösch, GA 1925, 'Untersuchungen über die Arbeitsteilung im Bienenstaat', *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, vol. 2, pp. 571-631.

Rose, E 1996, 'Vectoring of *Botrytis cinerea* (Persoon: Freis) to kiwifruit (*Actinidia deliciosa*) flowers by honey bees (*Apis mellifera Linnaeus*)', PhD Thesis, Lincoln University, New Zealand.

Roulston, TH, Cane, JH & Buchmann, SL 2000, 'What governs protein content of pollen: pollinator preferences, pollen–pistil interactions, or phylogeny?', *Ecological Monographs*, vol. 70, pp. 617-643.

Ruttner, F 1988, *Biogeography and taxonomy of honeybees*, Springer-Verlag, Berlin, Germany.

Sabree, ZL, Hansen, AK & Moran, NA 2012, 'Independent studies using deep sequencing resolve the same set of core bacterial species dominating gut communities of honey bees', *Plos One*, vol. 7, p. e41250.

Sakagami, S 1953, 'Untersuchungen uber die Arbeitsteilung in einen Zwergvolk der Honigbienen. Beitrage zur Biologie des Bienenvolkes, *Apis mellifera* L., I', *Japanese Journal of Applied Entomology and Zoology*, vol. 11, pp. 117-185.

Sampson, BJ & Cane, JH 2000, 'Pollination efficiencies of three bee (Hymenoptera : Apoidea) species visiting rabbiteye blueberry', *Journal of Economic Entomology*, vol. 93, pp. 1726-1731.

San-Blas, G, Guanipa, O, Moreno, B, Pekerar, S & San-Blas, F 1996, '*Cladosporium carrionii* and *Hormoconis resinae* (*C. resinae*): cell wall and melanin studies', *Current Microbiology*, vol. 32, pp. 11-16.

Santas, LA, 1983, 'Insects producing honeydew exploited by bees in Greece', *Apidologie*, vol. 14, pp. 93-103.

Sarma, BK, Yadav, SK, Singh, S & Singh, HB 2015, 'Microbial consortium-mediated plant defense against phytopathogens: readdressing for enhancing efficacy', *Soil Biology and Biochemistry*, vol. 87, pp. 25-33.

Saunders, ME, Luck, GW & Mayfield, MM 2013, 'Almond orchards with living ground cover host more wild insect pollinators', *Journal of Insect Conservation*, vol. 17, pp. 1011-1025.

Scherm, H, Savelle, A, Boozer, R & Foshee, W 2008, 'Seasonal dynamics of conidial production potential of *Fusicladium carpophilum* on twig lesions in southeastern peach orchards', *Plant Disease*, vol. 92, pp. 47-50.

Schmid-Hempel, P, Puhr, K, Kruger, N, Reber, C & Schmid-Hempel, R 1999, 'Dynamic and genetic consequences of variation in horizontal transmission for a microparasitic infection', *Evolution*, vol. 53, pp. 426-434.

Schmidt, JO, Thoenes, SC & Levin, MD 1987, 'Survival of honey bees, *Apis mellifera* (Hymenoptera: Apidae), fed various pollen sources', *Annals of the Entomological Society of America*, vol. 80, pp. 176-183.

Schubert, K 2005, 'Taxonomic revision of the genus *Cladosporium* s. lat. 3. A revision of *Cladosporium* species described by J.J. Davis and H.C. Greene (WIS)', *Mycotaxon* vol. 92, pp. 55–76.

Schaeffer, RN, Vannette, RL, Brittain, C, Williams, NM, Fukami, T 2017, 'Non-target effects of fungicides on nectar-inhabiting fungi of almond flowers', *Environmental Microbiology Reports*, vol. 9, pp. 79-84.

Seeley, TD 2014, '*Honeybee ecology: a study of adaptation in social life*', Princeton University Press.

Shade, A, McManus, PS, Handelsman, P 2013, 'Unexpected diversity during community succession in the apple flower microbiome', *mBio, vol.* 4, pp. e00602-00612.

Shafir, S, Dag, A, Bilu, A, Abu-Toamy, M & Elad, Y 2006, 'Honey bee dispersal of the biocontrol agent *Trichoderma harzianum* T39: effectiveness in suppressing *Botrytis cinerea* on strawberry under field conditions', *European Journal of Plant Pathology*, vol. 116, pp. 119-128.

Shaw, DE 1990, 'The incidental collection of fungal spores by bees and the collection of spores in lieu of pollen.', *Bee World*, vol. 71, pp. 158-176.

Shaw, DE 1999, 'Bees and fungi, with special reference to certain plant pathogens', *Australasian Plant Pathology*, vol. 28, pp. 269-282.

Sheppard, WS, Arias, MC, Grech, A & Meixner, MD 1997, '*Apis mellifera ruttneri*, a new honey bee subspecies from Malta', *Apidologie*, vol. 28, pp. 287-293.

Sheppard, WS & Meixner, MD 2003, '*Apis mellifera pomonella*, a new honey bee subspecies from Central Asia', *Apidologie*, vol. 34, pp. 367-375.

Silva-Junior, G, Spósito, M, Marin, D, Ribeiro-Junior, P & Amorim, L 2014, 'Spatiotemporal characterization of citrus postbloom fruit drop in Brazil and its relationship to pathogen dispersal', *Plant Pathology*, vol. 63, pp. 519-529.

Simone-Finstrom, M & Spivak, M 2010, 'Propolis and bee health: the natural history and significance of resin use by honey bees', *Apidologie*, vol. 41, pp. 295-311.

Simone-Finstrom, MD & Spivak, M 2012, 'Increased resin collection after parasite challenge: a case of self-medication in honey bees?', *Plos One*, vol. 7, p. e34601.

Simone-Finstrom, M, Borba, R, Wilson, M & Spivak, M 2017, 'Propolis counteracts some threats to honey bee health', *Insects*, vol. 8, p. 46.

Simone, M, Evans, JD & Spivak, M 2009, 'Resin collection and social immunity in honey bees', *Evolution: International Journal of Organic Evolution*, vol. 63, pp. 3016-3022.

Soltanian, S, Stuyven, E, Cox, E, Sorgeloos, P & Bossier, P 2009, 'Beta-glucans as immunostimulant in vertebrates and invertebrates', *Critical Reviews in Microbiology*, vol. 35, pp. 109-138.

Somerville, D 2005, Fat Bees Skinny Bees: A Manual on Honey Bee Nutrition for Beekeepers: a Report for the Rural Industries Research and Development Corporation, Rural Industries Research and Development Corporation.

Southwick, EE & Southwick, L 1992, 'Estimating the economic value of honey bees (Hymenoptera: Apidae) as agricultural pollinators in the United States', *Journal of Economic Entomology*, vol. 85, pp. 621-633.

Spencer-Booth, Y 1960, 'Feeding pollen, pollen substitutes and pollen supplements to honeybees', *Bee World*, vol. 41, pp. 253-263.

Stamets, PE 2017, 'Integrative fungal solutions for protecting bees', U. S. Patent Application Publication No. 15/332,803.

Stamets, PE 2018, 'Antiviral activity from medicinal mushrooms and their active constituents', U.S. Patent Application Publication No. 15/918,082.

Stamets, PE, Naeger, NL, Evans, JD, Han, JO, Hopkins, BK, Lopez, D, Moershel, HM, Nally, R, Sumerlin, D & Taylor, AW 2018, 'Extracts of polypore mushroom mycelia reduce viruses in honey bees', *Scientific Reports*, vol. 8, p. 13936.

Stefani, E 2010, Economic significance and control of bacterial spot/canker of stone fruits caused by *Xanthomonas arboricola* pv. *pruni*, *Journal of Plant Pathology*, vol. 92, pp.99-103.

Stevanovic, J, Stanimirovic, Z, Simeunovic, P, Lakic, N, Radovic, I, Sokovic, M & Griensven, LJV 2018, 'The effect of *Agaricus brasiliensis* extract supplementation on honey bee colonies', *Anais da Academia Brasileira de Ciências*, vol. 90, pp. 219-229.

Stokland, JN, Siitonen, J & Jonsson, BG 2012, *Biodiversity in dead wood*, Cambridge University Press, Cambridge, UK.

Sun, G, Yang, Q, Zhang, A, Guo, J, Liu, X, Wang, Y, Ma, Q 2018, 'Synergistic effect of the combined bio-fungicides ε-poly-l-lysine and chitooligosaccharide in controlling grey mould (*Botrytis cinerea*) in tomatoes', *International Journal of Food Microbiology*, vol. 276, pp. 46-53.

Szaniszlo, P, Cooper, B & Voges, HS 1972, 'Chemical compositions of the hyphal walls of three chromomycosis agents', *Sabouraudia: Journal of Medical and Veterinary Mycology*, vol. 10, pp. 94-102.

Thorp, RW 2000, 'The collection of pollen by bees', in *Pollen and pollination*, Springer, pp. 211-223.

Tihelka, E 2018, 'The immunological dependence of plant-feeding animals on their host's medical properties may explain part of honey bee colony losses', *Arthropod-Plant Interactions*, vol. 12, pp. 57-64.

Trujillo Flores, FJ & Peña Garcia, LE 1989, 'Collection and storage of spores of *Cronartium conigenum* Hedc. et Hunt, by *Apis mellifera* L.', *Apicultura Moderna*, vol. 1, pp. 15-16.

Vandenberg, JD & Shimanuki, H 1986, 'Two commercial preparations of the β exotoxin of *Bacillus thuringiensis* influence the mortality of caged adult honey bees, *Apis mellifera* (Hymenoptera: Apidae)', *Environmental Entomology*, vol. 15, pp. 166-169.

vanEngelsdorp, D, Evans, JD, Saegerman, C, Mullin, C, Haubruge, E, Nguyen, BK, Frazier, M, Frazier, J, Cox-Foster, D, Chen, YP, Underwood, R, Tarpy, DR & Pettis, JS 2009, 'Colony Collapse Disorder: A Descriptive Study', *Plos One*, vol. 4, p. e6481.

vanEngelsdorp, D & Meixner, MD 2010, 'A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them', *Journal of Invertebrate Pathology*, vol. 103, pp. 80-95.

Vanneste, J, Giovanardi, D, Yu, J, Cornish, D, Kay, C, Spinelli, F & Stefani, E 2011, 'Detection of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit pollen samples', *New Zealand Plant Protection*, vol. 64, pp. 246-251.

Vasquez, A & Olofsson, TC 2009, 'The lactic acid bacteria involved in the production of bee pollen and bee bread', *Journal of Apicultural Research*, vol. 48, pp. 189-195.

Verma, M, Brar, SK, Tyagi, RD, Surampalli, RY & Valero, JR 2007, 'Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control', *Biochemical Engineering Journal*, vol. 37, pp. 1-20.

Vojvodic, S, Rehan, SM & Anderson, KE 2013, 'Microbial gut diversity of africanized and european honey bee larval instars', *Plos One*, vol. 8, p. e72106.

Walker, J, Hartman, D, & Bertus, AL, 1974, Poplar rusts in Australia with comments on potential conifer rusts', *European Journal of Forest Pathology*, vol. 5, pp. 100-118.

Watanabe, ME 1994, 'Pollination worries rise as honey bees decline', *Science*, vol. 265, p. 1170.
Westaway, JO 2016, 'The pathogen Myrtle Rust ('*Puccinia psidii*') in the Northern Territory: First detection, new host and potential impacts', *Northern Territory Naturalist*, vol. 27, pp. 13-28.

Westerkamp, C 1996, 'Pollen in bee-flower relations some considerations on melittophily', *Botanica Acta*, vol. 109, pp. 325-332.

Williams, SC & Tomlinson, JT 1985, 'Gathering of aecial spores of willow rust by the honey bee, *Apis mellifera* (Hymenoptera:Apinae)', *Pan-Pacific Entomologist*, vol. 61, p. 345.

Wilkinson, J 2012, *Almonds in Australia: From pioneer planting to prime production*, Almond Board of Australia, Berri, Australia.

Willey, J, Sherwood, L, Woolverton, C 2017, *Prescott's Microbiology*, McGraw-Hill, New York NY.

Williamson, B, Tudzynsk, B, Tudzynski, P & van Kan, JAL 2007, '*Botrytis cinerea*: the cause of grey mould disease', *Molecular Plant Pathology*, vol. 8, pp. 561-580.

Wingfield, MJ, van Wyk, PS & Vivers, M, 1989, 'Rust spores, bees and pollen', *The Mycologist*, vol. 3, pp. 31-32.

Winston, ML 1991, *The biology of the honey bee*, Harvard University Press, Cambridge MA.

Wolfenbarger, DO 1977, 'Honey bees forage rust spores of *Oxalis* sp. leaves', *American Bee Journal*, vol. 117, p. 441.

Yoder, JA, Jajack, AJ, Rosselot, AE, Smith, TJ, Yerke, MC & Sammataro, D 2013, 'Fungicide contamination reduces beneficial fungi in bee bread based on an area-wide field study in honey bee, *Apis mellifera*, colonies', *Journal of Toxicology and Environmental Health-Part a-Current Issues*, vol. 76, pp. 587-600.

Zabriskie, JL 1875, 'Remarkable forage for bees', *Beekeepers Magazine*, vol. 3, pp. 186-187.