Native Bees and Pollen Collection: How Flexible Are They?

by

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Candidate's Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of the author’s knowledge, it contains no material previously published or written by another person, except where due reference is made in the text.

Helen Burke Date: 18/07/20

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Abstract

Aim: Globally, populations of the honey bee (*Apis mellifera*) are declining, threatening pollination services and thus the reproduction of crops and wild plants. Native bees may be a free source of pollination services and act as 'insurance' against potential honey bee losses in Australia. This study aims is to determine whether native bees collect pollen mainly from native plants, with which they have a shared evolutionary history, or whether they have diversified and also collect pollen from introduced plant species. To this end, we investigated whether native bees collected more or less pollen from native plants or introduced species, and whether there are differences in the patterns of pollen collection between the different bee genera studied.

Methods: This study collected different species of native bees off flowering plants from agricultural and remnant forest sites in the Yarra Valley, Victoria. Pollen was transferred from the bees and taken through acetolysis before being mounted on microscope slides. With the assistance of the Specicount tool, pollen on these slides was identified and counted using a microscope to determine what pollen the bees carried. Pollen loads and pollen load composition were calculated based on this analysis.

Results: Rather than all bees collected carrying pollen, for the most part only those with visible pollen on the bee at the time of collection or processing ended up with enough pollen on the microscope slide to analyse. The different taxa of native bees had statistically different pollen load compositions in both the agricultural and forest sites. There was also a correlation between bee body size and the overall amount of pollen carried. All the bees studied carried pollen from both native and non-native plant sources to some extent, ranging from 27% non-native pollen in *Exonuera* to 78% in *Homalictus*. When the data set was divided into agricultural sites, different trends emerged, with bees more likely to collect pollen from crops and weeds in agricultural sites. Site type was found as the primary driver of inter-species pollen load composition variation.

Main Conclusions: The finding that mostly only bees with visible pollen at the time of collection resulted in visible pollen on the slide will inform the processing of the remaining bee samples in subsequent studies. The pollen load composition of the native bees in this study is not limited to native plants. Location, rather than plant origin, determined the makeup of the pollen load carried by the bees. The native bees in the study have potential as crop pollinators as given the opportunity they will collect pollen from introduced species including crops.

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IPBES: Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services

Chapter 1: Introduction

1.1 Overview

The majority of studies investigating which bees visit which flowers use floral visitation surveys. There have been fewer studies that used pollen analysis to discover this. More recent studies suggest that a combination of the two methods may be ideal to get a full picture of native bee foraging behaviour. My study uses pollen analysis of pollen collected off the bodies of bees to determine which plants they have visited. It complements previous work done in the region based on floral visitation surveys.

In this introduction, I provide general information about bees, their pollination services, and terms used to describe their behaviour. I then provide some information about native Australian bees. Finally, I discuss methods of pollen analysis and articulate the research questions that I answer using my pollen data.

1.2 The Importance of Bee Pollination

Flowering plants (angiosperms) require pollination for their reproductive success. Pollination is the process by which animals remove pollen grains from the male (anther) part of a flower to the female part of the flower (stigma), which results in the fertilisation of the plant, which in turn produces fruit and seeds. While some plants are self-pollinating or wind-pollinated, the majority require the assistance of animal pollinators- an estimated 87.5% of wild flowering plants rely on pollination by insects and other animals (IPBES, 2016). Wilcox *et al.* (2017) put this figure as somewhere between 78 and 95 percent. Pollinators also play a significant role in pollinating more than 75% of the world's important agricultural plant species (Ferreira *et al.*, 2013; Willcox *et al.*, 2017). According to Kearns *et al.* (1998), one-third of the food that humans eat is directly or indirectly dependent on animal pollination.

Although some pollination is done by vertebrates and other insects, bees, both managed and wild, make up the majority of pollinators (Brown and Paxton, 2009) and are the most important group of pollinators worldwide (Lentini *et al.*, 2012). Wild bees are important in maintaining biodiversity and ecosystem processes (Carrié *et al.*, 2017), and wild bees and managed bees are important to crop production. Without bee pollination, food diversity, food quality and human nutrition would be adversely affected (Klein *et al.*, 2007).

Honey bees (*Apis mellifera*) are naturalised in most parts of the world. They provide essential pollination services to native and introduced plants, and commercial and feral honey bees form an important part of agricultural production. A significant threat to honey bee populations is

Varroa mite (*Varroa destructor* and *Varroa jacobsoni*). Not only do Varroa mites cause physical and physiological damage to honey bees themselves, they also act as vectors for viruses that cause significant damage to bees and bee colonies (Queensland Government Department of Agriculture and Fisheries, 2019). Varroa mites have not yet taken hold in Australia, and our honey bee populations are some of the healthiest in the world. Nevertheless, experts say that it is only a matter of time before Varroa mites take hold in Australia and there are already plans to deal with this (CSIRO, 2007). While there is limited research, Hafi, *et al.* (2012) report that 'it is considered highly unlikely that Australian native bees would be adversely affected by Varroa'. According to Wood *et al.* (2018), native bees could act as insurance against honey bee losses. The idea is that native bees could supplement honey bees in agricultural pollination during the honey bees' inevitable decline.

Globally, populations of the honey bee and other wild bee populations are declining, threatening their provision of pollination services, and thus the reproduction of crops and wild plants (IPBES, 2016; Biesmeijer *et al.*, 2006). With the decline of both wild and managed honey bee populations due to disease and other causes such as habitat loss; habitat fragmentation; invasive species; pesticides; and climate change, (IPBES, 2016; Brown and Paxton, 2009), researchers and producers are looking to native bee populations to mitigate pollination loss by filling the gap left by declining honey bees and therefore acting as insurance against honey bee losses (Wood *et al.,* 2018, Lentini *et al.*, 2012). The Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services' Assessment Report on Pollinators, Pollination and Food Production (2016) describes wild bees acting as a buffer against adverse changes (IPBES, 2016; Wood *et al.*, 2018a). Wild bee populations have been underestimated as contributors to the pollination of crop plants (Potts *et al.*, 2010) and can enhance the production of some crops (Cunningham *et al.*, 2013; Klein *et al.*, 2007). Garibaldi *et al.* (2013) highlighted the importance of wild bee pollinators as flower visitors to crops, increasing fruit set and potentially improving pollination services provided by honey bees through competition. Batley and Hoogendorn (2009) also consider that Australian native bees could potentially play an important role as crop pollinators. Moreover, diverse pollinator communities are thought to provide more stable pollination service as they may have "different food preferences, foraging behaviour and activity patterns" (Kleijn *et al.*, 2015).

According to Ritchie *et al.* (2016), native bees provide essential pollination services to both wild and cultivated plants. The functional diversity of pollination networks contributes to the maintenance of diversity in plant communities, with different groups of pollinators being complementary in their pollination services and diverse groups of plants being complementary in their roles as food plants for pollinators (Biesmeijer *et al.,* 2006).

Despite their importance to the pollination of wild plants, and their potential as crop pollinators, there is a paucity of research on wild bee species and their ecology. As Wood *et al.*, (2018b) reports at both the individual species and community levels, there is a poor understanding of the types of plants needed to support wild bee populations. This research is necessary if these wild bee populations are to be maintained or increased. (Lentini *et al.*, 2012) highlights that this type of research should be a priority because the decline of honey bees will increase the importance of alternative wild pollinators. Batley and Hoogendorn (2009) also advocate for the investigation of native bee species as alternatives to honey bees in crop pollination.

1.3 Pollen Collection Patterns of Bees

Different bee species have different foraging preferences; that is, species, genera or families of plants that they exclusively visit. These preferences are described as degrees of specialisation and generalization. While the terms specialization and generalisation seem to suggest a binary definition, they in fact represent two extremes of a spectrum of levels of specialization and generalization, also known as diet breadth (Roulston and Goodell, 2011).

Specialisation refers to foraging on, or a preference for a subset of the available flowering plants in an environment (Wood *et al.*, 2018a). Specialisation can be present in 15% to 60% of bee species depending on the biogeographic region (Roulston and Goodell, 2011). Generalisation refers to foraging that uses most, if not all, of the available flowering plants in a given habitat. Bee species that exhibit generalization appear to develop better on a mixed pollen diet (Russo and Danforth, 2017). How selective they are determines their level of specialization and generalization.

The most recent definitions for levels of specialisation and generalisation come from Müller and Kuhlmann (2008) and are visually represented below (Figure 1.1).

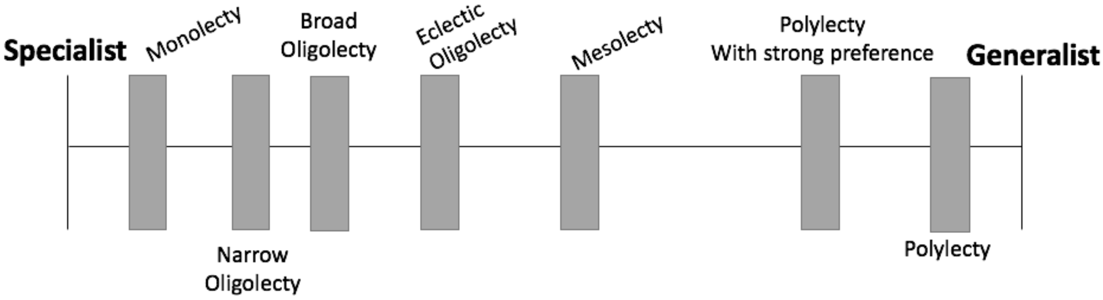


Figure 1-1

Visual Representation of the pollen collection classifications following Müller and Kuhlmann (2008)

Monolecty refers to bee species that forage on a single plant species. Oligolecty refers to a preference for a narrow range of host plants such as a few species or specific genus of plant. Polylectly refers to species that opportunistically visit a wide range of host plants from many families for nectar or pollen. Mesolecty refers to behaviour that is somewhere between Oligolecty and Polylecty.

Relationships between pollinators and plants can be codified in a pollination network or web. A pollination network is an ecological network that represents the interactions between plants and pollinators in a system. They incorporate information about which plant species and which pollinator species interact with each other and the strength of these interactions. The information contained in a pollination web can determine whether pollinators and plants are specialists or generalists (IPBES, 2016). Pollination webs are relatively richly connected, and they can shift in time and space (Kearns *et al.*, 1998). They are often asymmetrical, with specialist pollinators often relying on generalist plants and specialist plants on generalist pollinators. They also tend to be nested, which means that there is a core group that interacts within itself and other pollinators and plants interact with this core group of generalists rather than each other (specialists). The core group of generalist species has key roles in the pollination web. (Carrié *et al.*, 2017; IPBES, 2016; Ferreira *et al.*, 2013; Potts *et al.*, 2010).

1.4 Native Australian Bees

Native bees play an important role in crop and native plant pollination worldwide (Ritchie *et al.*, 2016) and Australia has an extensive native bee fauna (Batley and Hogendoorn, 2009). According to Lentini *et al.* (2012), there are around 1600 species of native bees in Australia, however Horskins and Turner (1999) state that there are an estimated 3000 species. Finding an accurate number of bee species is further confounded by the fact that many species remain undescribed (Batley and Hogendoorn, 2009; Cunningham *et al.*, 2013).

The Australian bee fauna is characterized by having no native *Apis* or *Bombus* species. Solitary bee species make up the majority of bee species in Australia, and there are few eusocial species. Solitary bees do not share a communal nest like honey bees, instead they nest in soil (e.g. *Homalictus*), hollow stems (e.g. *Exoneura*), or woody debris (e.g. *Xylocopa*) and leave their young to develop without any parental care (IPBES, 2016; Cunningham *et al.*, 2013; Lentini *et al.*, 2012; Horskins and Turner, 1999).

More than half of the described species of Australian bees belong to the Colletidae family. (Danforth and Ji, 2001) and the families Colletidae, Stenotritidae and Halictidae make up more than three-quarters of known species (Batley and Hogendoorn, 2009). The subfamily Euryglossinae is also endemic to Australia.

The majority of Australian species are thought to be polylectic, with only 100 species suspected or known to be oligolectic. However, according to Batley and Hoogendorn (2009), there may be a bias towards describing polylectic species as until the nature of their specialisation is found, oligolectic species are often overlooked. Therefore, of the 300-400 species that remain undescribed, many of them may be oligolectic.

An example of an oligolectic relationship between an Australian bee species and a plant genus was found in a review of the biogeography of some Australian bee species. A relationship was found between a small *Lasioglossum* species, and Goodeniaceae, based on another data review that found that all records of *Lasioglossum megacephalum* were collected foraging on it (Coombes, 2019; Danforth and Ji, 2001).

According to Lentini *et al.* (2012), the ecology of solitary wild bees in Australia is poorly understood, and assertions about which species of bees are common and which are rare cannot be made due to how little we know about them. In Batley and Hogendoorn's (2009) overview of the biodiversity of bees in Australia, they comment that many species remain undescribed and more than half of the taxa which are described need revision.

1.5 Pollen Load Analysis to Determine Bee Diet

Analysis of pollen on the bodies of bees can be undertaken in order to understand what flowers bees have visited, as pollen from different plant genera have distinctive features that separate it from other plants' pollen (see Figure 2.2) Previously, information about foraging behaviour has been based on floral visitation data. However, pollen analysis can be used in addition to determine the 'full spectrum of floral use by a pollinator' (Ritchie *et al.*, 2016). For Russo and Danforth (2017), the benefits of pollen analysis were that they could determine which species of bees had a preference for their target species (apple) and which alternative plant species could be different hosts for bees that did not visit apple blossom exclusively.

Pollen analysis can be conducted on freshly trapped bees or preserved museum specimens (IPBES, 2016 ; Burkle *et al.*, 2013; Wood *et al.*, 2018b). Museum samples can be used to determine changes in diet composition over time (Burkle *et al.*, 2013). Trapped samples can be used to identify pollen diet niches at bee species level and for analysing the relative densities of different pollen types (Russo and Danforth, 2017; Wood *et al.*, 2018b).

There are some variations in methodology, but the core steps remain the same. To analyse pollen of unknown provenance from a bee sample, the first step is to create or use an existing reference collection. A reference collection consists of samples of known pollen types from the relevant site, which are processed and then generally photographed multiple times using light microscopy. These known pollen types can then be compared with the unknown pollen taken off the bee sample, to determine the kind of plants the bee was visiting (Ritchie *et al.*, 2016; Russo and Danforth, 2017; Wood *et al.*, 2018b).

To undertake pollen analysis of bee samples, the pollen must be removed from the bee and transferred onto a microscope slide so that it can be examined. In some research, the pollen load present on the bee has been visually estimated (Wood *et al.*, 2018a; Wood *et al.*, 2018b). Some studies started with soaking the bee samples in ethanol, then vortexing the samples and scraping any pollen left on the bees (Horskins and Turner, 1999; Ritchie *et al.*, 2016), while in other studies pollen was transferred straight from the bee onto a slide (Russo and Danforth, 2017; Wood *et al.*, 2018a). In most of the papers that used manual methods of pollen identification (i.e. identification by a human using a light microscope) fuchsin dye was added to stain the pollen sample to make it easier to visualize under the microscope (Russo and Danforth, 2017; Horskins and Turner, 1999; Wood *et al.*, 2018a; Wood *et al.*, 2018b). Vianna *et al.* (2015) took their pollen samples through acetolysis, and Holt and Bebbington (2014) used acetolysis to prepare slides as the process produces optimum images for the Classifynder, an automated pollen identification and counting machine (see Appendix 1). In some papers, glycerin was added to the pollen sample on the microscope slide (Horskins and Turner, 1999; Wood *et al.*, 2018a; Wood *et al.*, 2018b). Despite these different methods the end result is the same; that is a pollen sample on a microscope slide ready to be analysed under a microscope.

The number of bees and the amount of pollen varied in different studies. In some, all the specimens collected and all the pollen removed were analysed (Ritchie *et al.*, 2016; Wood *et al.*, 2018b). In Russo and Danfoth (2017), only 10 specimens from each of the most common apid genera in their study region were analysed, while in Wood *et al.* (2018a), all the bee samples collected were analysed, however the pollen on the slide was only analysed along three transects on the coverslip. This is common in slide-based pollen analysis as it is thought to be representative of the pollen makeup of the whole slide. In Horskins and Turner (1999) pollen from the corbiculae of the bee specimens was removed, and the remaining pollen was analysed by counting the grains under a microscope.

1.6 Research Questions

Pollination is essential to the reproduction and persistence of flowering plants, both for native plant species and introduced plant species, including agricultural crops. Bees are one of the most important providers of pollination services worldwide but are declining globally, including the commercially important honey bee (*Apis mellifera*). Native bees could provide pollination support to the declining honey bee. To achieve this, more needs to be known about what crops native bees collect pollen from and what other plants they collect pollen from. Once we know these foraging behaviours we can better support native bees both in general and as potential crop pollinators.

In general, there is a need for more research on plant-pollinator networks. Waser (1996) stated that community-wide studies of pollination would be invaluable to 'strengthen the empirical foundation on which we base conclusions about the fundamental nature of pollination systems.' Wilcox *et al.* (2017) also identified plant-pollinator community interactions as important research areas. One of the reasons for this, according to the IPBES report (2016), is to be able to predict the impact of drivers such as climate change on the ecosystems and interaction networks of pollinators. Another reason is to aid the conservation of bees and the environments that they interact with. In the literature, Brown and Paxton (2009) highlight the need for more pollination studies outside of well-known bee taxa to aid in conservation efforts. Furthermore, the foraging patterns of native bees are poorly understood (Ritchie *et al.*, 2016; Russo and Danforth, 2017). Examining foraging behaviour of native bees may provide insight into what plants should be conserved to maintain bee biodiversity and thus the provision of ecosystem services (Wood *et al.*, 2018a). In determining the foraging behaviour of native bees in my study site, the importance of different plant groups for the maintenance of native bee populations is highlighted. Hingston (2005) conducted a study into the floral preferences of the introduced bumblebee *Bombus terrestris* in Australia by studying whether they preferred flowers of introduced or native plants. This is similar to my research and shows that there is a precedent for this kind of work.

With this in mind, my research aims to answer the following questions;

1. Do native bees collect pollen mainly from native plants, with which they have a shared evolutionary history, or do they also collect pollen from introduced plant species?
2. Are there differences in the patterns of pollen collection between the different bee taxa in my study?

Chapter 2: Methods

2.1 Study sites and Sample Collection

The study was conducted at thirteen farms and seven remnant forest locations, all within the Yarra Valley region, east of the city of Melbourne, Victoria in Australia (Figure 2.1).

Figure 2-1

Study site location relative to Melbourne and types (legend in lower left)

Samples were collected from seven remnant native forest sites, seven berry farms (Blueberry (*Vaccinium sp.*)) and six orchards (Apple (*Malus domestica*), Pear (*Pyrus sp.*), Cherry (*Prunus sp.*), Raspberry and Blackberry (*Rubus sp.*)

Prior to my commencement of my project, vegetation surveys were conducted at all sites, in order to estimate the number of flowers within the sample sites during each visit. Also prior to the commencement, bees were collected from flowers using a specimen jar to prevent cross-contamination between the specimens. Information recorded at the time of collection included the plant the bee was collected from and whether there was any visible pollen on the bee. Samples were collected in Spring 2017 (September to November) and 2018 (October to November). Overall, 717 bee samples were collected.

2.2 Bee Processing

Each bee sample was soaked in a vial of 70% ethanol for 24 hours. After this, the bee vials were placed in a Vortex machine and vibrated for 10 minutes at low speed. Both the bee and vial were rinsed with ethanol into a test tube. If there was any visible pollen left on the bee this was scraped off with tweezers and washed into the test tube with ethanol. The tweezers were washed and sonicated between each bee sample to ensure no cross-contamination. The bees were returned to the original vials and were later identified to genus or subgenus level. Limited lab access during the Covid-19 crisis prevented taking the level of identification lower than the subgenus. The test tubes were placed in a centrifuge for 3 minutes at 3000 rpm. After spinning, the supernatant was carefully removed using separate plastic pipettes for each sample (to avoid cross contamination), leaving approximately 1ml of liquid per tube. The samples were then processed using the acetolysis protocol outlined below.

Alongside the bee samples a pollen reference collection was also created. Flowers were collected from the study sites and the parent plant identified to the lowest possible classification. The anthers were removed and put through the same acetolysis procedure as the bee samples (outlined below), before being mounted on microscope slides.

2.3 Pollen Processing Protocol

The pollen recovered from the bee samples as well as anthers from the pollen reference samples were processed using standard processing techniques (Bennett and Willis, 2001).

Processing Steps:

Steps 1 to 4 for pollen reference material only.

1. 10% KOH – 5ml was added to each test tube and the uncapped tubes placed in a 65-70 degree water bath for 20 mins. The KOH loosens the pollen from the anthers.

2. Sieving – samples were then sieved at 125 microns to remove all large and non-pollen organic fragments.

3. The <125um fraction was then transferred back to the 15ml test tubes, centrifuged for 3 minutes at 3000 rpm and the supernatant removed.

4. Water washes – 5ml of water was added to each test tube which were centrifuged for 3 minutes at 3000rpm and supernatant removed. This process was repeated twice or until the supernatant was clear to remove KOH and humic acids.

Steps 5 to 8 for pollen reference and bee pollen samples.

5. Glacial Acetic Acid - 5 ml of glacial acetic acid was added to each tube, centrifuged for 3 minutes at 3000rpm and supernatant removed. This step was conducted twice to remove all water from the samples as the acetolysis solution is highly reactive with water.

6. Acetolysis solution- acetic anhydride and concentrated sulfuric acid were combined in a 9:1 ratio under a fume hood. 5 ml of the acetolysis solution was added to uncapped test tubes, which were placed in a 65-70°C water bath for 10 minutes.

7. Acetolysis- the tubes were centrifuged for 3 minutes at 3000 rpm and the supernatant was removed. 5 ml glacial acetic acid was added to each test tube, which were then centrifuged for 3 mins at 3000 rpm. The supernatant was removed, and the process was completed once more with acetic acid, then three times with water. Acetolysis removes polysaccharides from the pollen and renders the ornamentation of the pollen wall visible enabling more confident identification

8. After processing the samples were observed visually and a note was made as to whether there was: no visible pollen, a smear of visible pollen, or a pellet of visible pollen. Where a pellet was observed the height of the pellet in the tube was measured using callipers.

2.4 Slide Mounting

Three different slide mounting methods were used;

In the first method, used for samples 1-8, 1-2 drops of sample were mixed with glycerol on a microscope slide, covered with a 22x22mm coverslip, then sealed with nail varnish. This was repeated on the same slide until the whole slide area was covered, approximately 3 coverslips per slide. This process was repeated on multiple slides until the entire sample was depleted. This resulted in low pollen concentrations across the slides making analysis difficult. Therefore, a different method was used going forward

For the second method, a drop of glycerol was placed onto the slide and heated to 60 degrees using a heat plate. The entire pollen sample was pipetted onto the slide and mixed with the glycerol. The water was allowed to evaporate and then a coverslip was applied, and the sample was sealed with nail varnish. Occasionally, if the sample contained a large pollen load it was mounted on two slides.

The third method used glycerol jelly (a mixture of glycerol and gelatine) as the mounting medium to improve image capture. When mounting the samples using this method, observations were made of the amount of pollen in the tube before mounting. Where a significant amount of pollen was visible, callipers were used to estimate the depth of the pollen pellet and this was also recorded. A small amount of liquid glycerol jelly was then added to the slide, and approximately

10µl was taken from the bottom of the sample tube using a pipette and mixed with the jelly on the slides. A square 18mmx18mm coverslip was then lowered on to the sample.

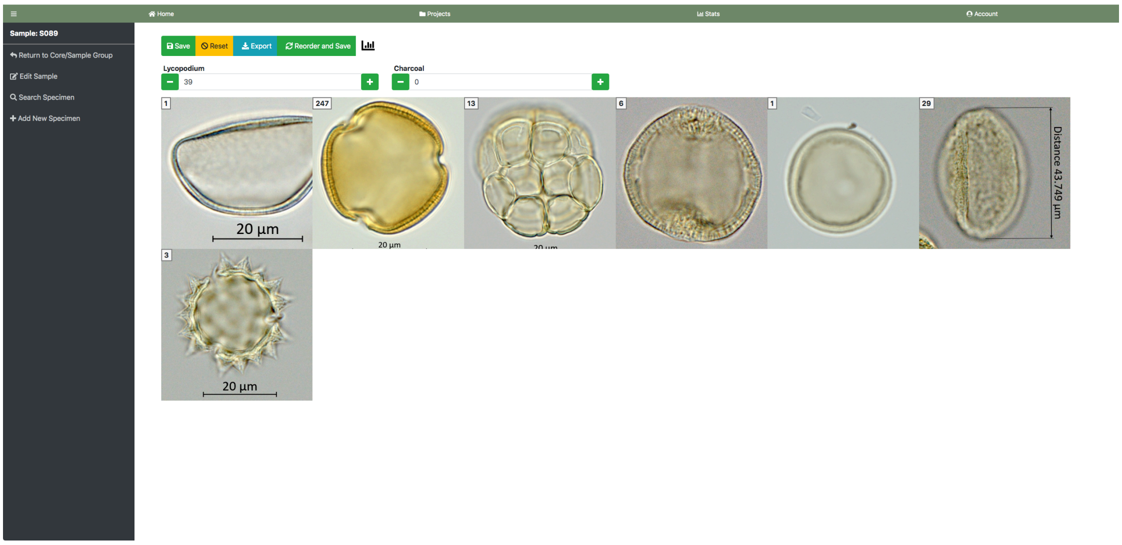
2.5 Pollen Identification and Counting

Originally, a semi-automated pollen identification and counting system, called the Classifynder, was intended to be used for pollen identification and counting. However, due to numerous problems with this system (for more details see Appendix 1), I could not be confident that the Classifynder analysis would be able to be completed in the given timeframe. For this reason, I reverted to standard microscope technique.

I conducted the pollen analysis using light microscopy under 40x magnification (Zeiss AxioimagerZ2) with colour images captured using an Axiocam 512 colour camera attachment. Pollen counts were recorded in Specicount (https://specicount.anu.edu.au/) for the recording of pollen data

Specicount is a web application for digitising the counting and analysis of palaeoecological data. It is a collaboration between Palaeoworks members and the ANU College of Engineering and Computer Science and it allows for the digital counting of pollen. Images of reference pollen were captured using the Axiocam 512 colour camera attachment to the Zeiss Imager.Z2m microscope at 40x magnification, then cropped to single pollen grains and converted to jpeg format. These images were uploaded to Specicount along with information about the characteristics of the pollen type such as dimensions, shape, number, and arrangement of apertures and surface ornamentation to create a reference library

The pollen on the sample slide was identified by comparison to the reference library in Specicount and counted in the app (Figure 2.3). In the case where there was more than one identification candidate for an unknown pollen type, if the flower that the bee was captured on (as recorded in the field) matched one of the candidates this was selected as the most likely pollen source. Pollen grains that could not be identified were photographed, given a label and counted, to be identified at a later date. Priority was given to bee samples with visible pollen.



Sample: S089

Lycopodium 39

Charcoal 0

Figure 2-2

Example of pollen counting in Specicount. This is a screenshot from Specicount, showing photos of the pollen types identified in the sample, with their counts in the top left corner of the image.

Different counting techniques were used depending on how the samples were mounted, with the overall aim to capture the diversity of pollen on the slide as well as a measure of pollen concentration.

All slides were first analysed by taking a transect across the centre of the coverslip at 10x magnification. The slides were then identified as either barren (no pollen visible), sparse (some pollen but low concentration), and abundant (high concentration of pollen). No further analysis was done on the barren slides. For the remaining slides different counting techniques were applied depending on how the samples were mounted and whether they were sparse or abundant.

Of samples 1-8, 31 slides were barren, 11 were sparse and 7 were abundant. Barren slides were not analysed. For sparse slides, a continuous transect was taken across the middle of the slide from left to right at 40x magnification and all pollen encountered was counted. For abundant slides, three random fields of view were taken at 40x magnification and all pollen in those views counted.

From the remaining 143 slides; 25 were barren, 24 were sparse and 94 were abundant.

Due to time constraints, only two sparse slides were analysed, using the same method as for samples 1-8. Abundant slides were analysed at 40x magnification by counting and identifying all the pollen grains in one field of view of the microscope, starting from the left side centre of cover slip, and recording this information in Specicount. This was repeated moving from left to right until a minimum of 100 pollen grains were counted. Pollen grains continued to be counted until the species accumulation chart in Specicount became saturated (Figure 2.4). If this was not achieved in the first transect then a second transect (above or below first transect, right to left) was conducted, and more transects were done as necessary to reach 100 grains, or species accumulation chart saturation. For both sparse and abundant slides the area analysed was recorded to determine pollen concentration (details below).

2.6 Initial Calculations

The output from Specicount consisted of a .csv file with the pollen counts for each sample listed by the identified pollen type. The total pollen count was also listed as well as the number of fields of view counted. The field data and bee identifications were added to this data. One sample was excluded from the analysis as it was the single representative of the genus *Leioproctus* (Family Colletidae) and it was not possible to draw firm conclusions about this genus due to only one pollen carrying specimen.

The frequency with which each pollen type occurred in the sample set was calculated.

The total area analysed was calculated by multiplying the area of the 40x field of view by the number of fields of views analysed. This was subsequently used to calculate the concentration of total pollen and each pollen type in the sample by dividing the pollen count by the area.

2.6.1 Pollen Load

The total pollen load on each bee sample was calculated as follows;

Equation 2-1
Pollen load calculation

$$\lfloor \text{Pollen concentration } (\text{mm}^2) \times \text{Area of Coverslip } (\text{mm}^2) \rfloor \times \text{ Sample volume} \approx \text{total pollen grains on bee}$$

Where coverslip area includes the total area of coverslips when the sample was spread over multiple slides.

At the end of the processing stage observations were made of whether there was visible pollen present and how much. For samples that were recorded as 1 (nothing visible) and 2 (smear) (11), the assumption was made that all the pollen in the sample was transferred to the slide and sample volume = 1. For samples that were recorded as 3 (pellet visible) and the depth of the pellet was recorded (15), it could not be assumed that all of the sample made it on to the slide. 0.1mm depth was estimated to correspond to 10µl of sample, approximately the amount of sample on the slide. Therefore, for a sample depth greater than 0.1mm the slide pollen load was multiplied to get the equivalent bee load. For example, for a sample depth of 0.4mm the pollen load was multiplied by 4;

Equation 2-2
Pollen Load Calculation example

$$0.1\ \text{mm x } 4\ = 0.4\ \text{mm}\ \approx\ 40\ \mu\text{l}\ \approx\ \text{approximate volume of sample in tube}$$

$$\lfloor Pollen\ concentration\ (mm^2) \times Area\ of\ Coverslip\ (mm^2) \rfloor \times\ 4$$
$$\approx total\ pollen\ grains\ on\ bee$$

These bee pollen load estimates were then summarised in box and whisker plots.

2.7 Data Analysis

2.7.1 Pie Charts

To reveal different patterns in pollen load composition, I generated pie charts for each bee taxon based on plant genus (represented by the pollen), plant family, and plant origin (native or non-native) to give an overview of the diet of each bee genus or subgenus, both overall and in forest and agricultural settings.

2.7.2 Network Analysis

The package *bipartite* in R (Dormann *et al.*, 2019) was used to construct two network diagrams based on the proportions of the sums of each bee taxon put into a matrix configuration. This was done to illustrate the pattern of pollen collection that links different taxon of bees to different plants. A network diagram in this case is a visual representation of the interactions between pollinators (our bees) and flowers. A separate network was created for the bees from agricultural sites and the bees from forest sites to demonstrate whether the interactions between the bees and plants changed depending on site type, considering that the plant makeup of the two site types differs.

2.7.3 Redundancy Analysis

The package *vegan* in R (Oksanen et al., 2013) was used to conduct a redundancy analysis on the agricultural and forest sites separately. The data input to this analysis was the pollen load proportion data for each individual (pollen carrying) bee specimen. Each replicate sample of the same bee taxon was used to assess statistically whether there were differences in the pollen collected by the different taxon, and whether that varied between forest and agricultural sites.

The graphical output of the redundancy analysis demonstrates how similar or dissimilar the different bee taxon are in terms of their pollen load composition, as well as the association between bees and the pollen types that make their pollen load composition distinctive.

Chapter 3: Results

In total, pollen samples from 104 bees were analysed (Table 3.1). Bees were identified to genus or sub genus level with 18 from the Apidae family and 86 from the Halictidae family. Of the Apidae family only one genus, *Exonuera* (18) was represented. Genera from the Halictidae family included *Homalictus* (10), *Lasioglossum* (73) and *Lipotriches* (3). In the genus *Lasioglossum* the sub genera *Chilalictus* (58) and *Parasphecodes* (15) were recognised. The bees analysed came from 19 different sites; 6 forest and 13 agricultural (Table 3.1). Of the 104 bee samples analysed, 72 were from agricultural sites and 32 were from forest sites.

Table 3-1
Breakdown of specimen origins.

			Apidae	**Halictidae**			
Site	**Site Type**	**# of specimens**	***Exoneura***	***Homalictus***	***Lasioglossum***		***Lipotriches***
					Chilalictus	*Parasphecodes*	
		Size	8mm	up to 8mm	5-12mm	5.5-11mm	10-12 mm
Avonsleigh	Berry farm	4	2	2			
Big berry	Berry farm	1			1		
Chapman	Berry farm	6	1		4	1	
Folly Farm	Berry farm	1				1	
Gembrook	Berry farm	4	3		1		
Jay Berry	Berry farm	1		1			
Monbulk	Berry farm	4	1	2	1		
Brad Finger	orchard	12		1	8	2	1
Burgi	orchard	8			8		
David Finger North	orchard	12	1	3	7		1
David Finger South	orchard	3			2	1	
Maroondah	orchard	9		1	8		
Montague	orchard	7			5	2	
Bulak Willam	forest	8	2		4	2	
Kirth Kiln North	forest	6	2		4		
Kirth Kiln South	forest	4	2		2		
Olinda Creek	forest	2	1		1		
Sherbrooke	forest	3			2	1	
Waramate Hills	forest	9	3			5	1
Total		**104**	**18**	**10**	**58**	**15**	**3**

When the project was conceived it was assumed that most of the bees collected would have pollen on them, however, when the slides began to be analysed it was found that this was not the case. The best predictors for having enough pollen on the microscope slide to be analysed were when a field note was made that there was visible pollen on the bee, or a lab note was made that there was a visible pellet of pollen after the acetolysis process. Just under 30% of bees that had no visible pollen before or after processing had enough pollen on the slide to analyse. The 104 samples analysed were therefore a subset of the collection as a whole (717 bees). This sampling may reflect the abundance of each type of bee at each site carrying pollen.

Sixty-five different pollen types were identified from the bee specimens; 36 were identified while 29 remain unknown. Five of the pollen types identified came from crop plants; *Malus* (Apple), *Prunus* (Cherry), *Rubus* (Blackberry and Raspberry), *Pyrus* (Pear) and *Vaccinium* (Blueberry). Of the unknown pollen types 14 made up >5% of any samples, the other 15 were combined into a single category; pooled rare unknowns. The relative frequency of each pollen type in the study set i.e. how many individual bees it was present on, is shown in Figure 3.1. The pollen taxa have been organised by whether the plant they derive from is native, non-native or unknown (if the pollen could not be identified). The most common non-native pollen encountered belonged to the Asteraceae (*Arthotheca*), Brassicaceae (*Brassica*), Rosaceae (*Malus* and *Prunus*) and the most common native pollen to the Goodeniaceae (*Goodenia*), Fabaceae (*Acacia*), *Myrtaceae* (Melaleuca) and Asteraceae (*Olearia*). Non-native pollen occurred more than native pollen, however this could be due to the fact that more bees with pollen were found in agricultural sites (which have a higher proportion of non-native plants) than forest sites.

The distribution of the total pollen loads of the different bee taxon sampled (Figure 3.2) shows that *Exoneura* and *Homalictus* have similar median values (5185 vs 3795). *Lasioglossum (Chilalictus)*, *Lasioglossum (Parasphecodes)* and *Lipotriches* have higher median values that are within the same range (9793, 13683, 16517 respectively). This corresponds broadly with bee body size (Figure 3.2). *Lasioglossum (Parasphecodes)* has the broadest range of pollen loads, including some of the highest pollen loads of the sample set. This could be due to the large range of body sizes indicated for this bee taxon in the literature.

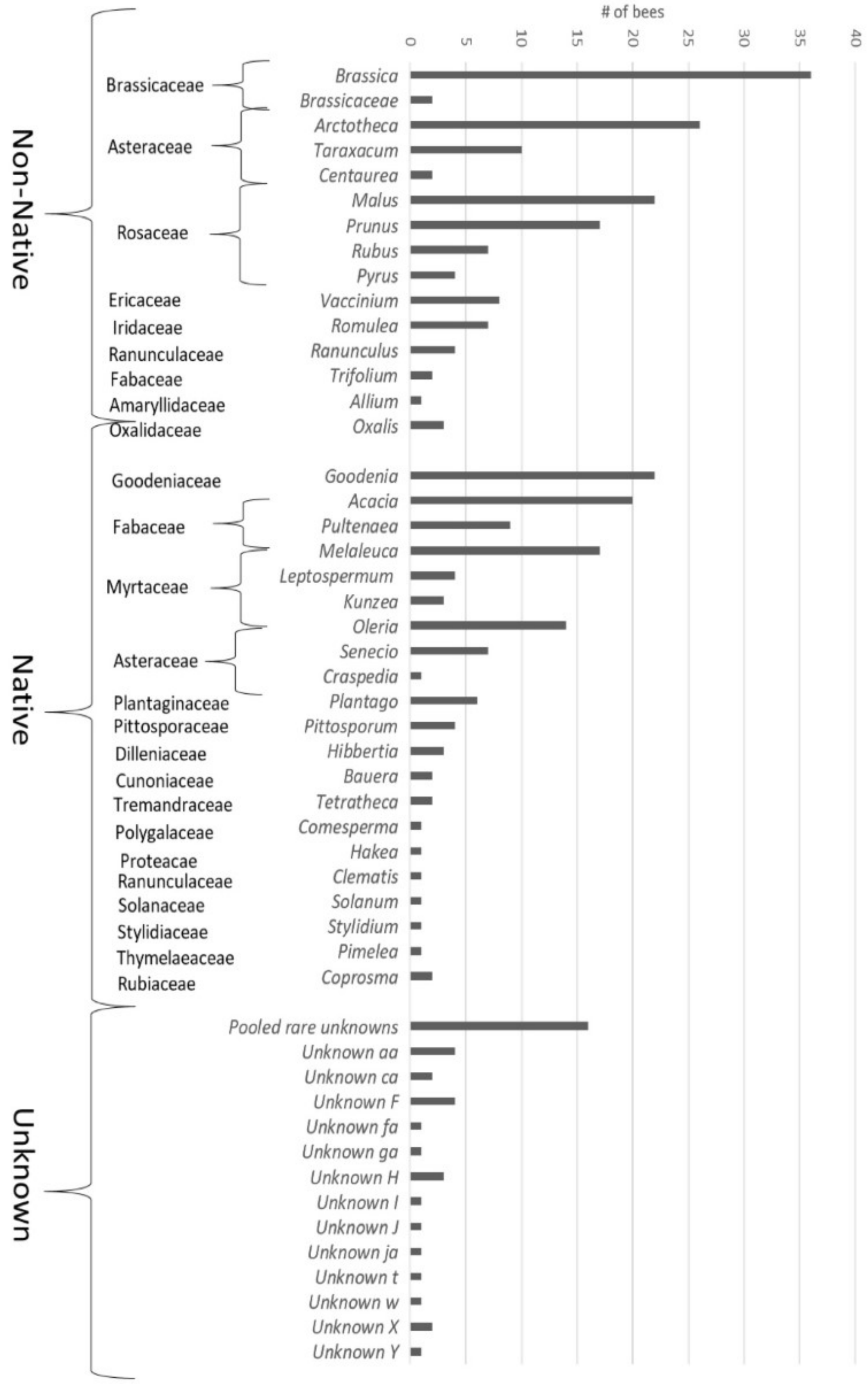


Figure 3-1

Provenance and frequency of different pollen types. The x axis identifies the different pollen types that were discovered in our analysis at family and genus level, and are separated into whether they are native, introduced or of unknown origin. The y-axis represents how many bees that pollen type was present on.

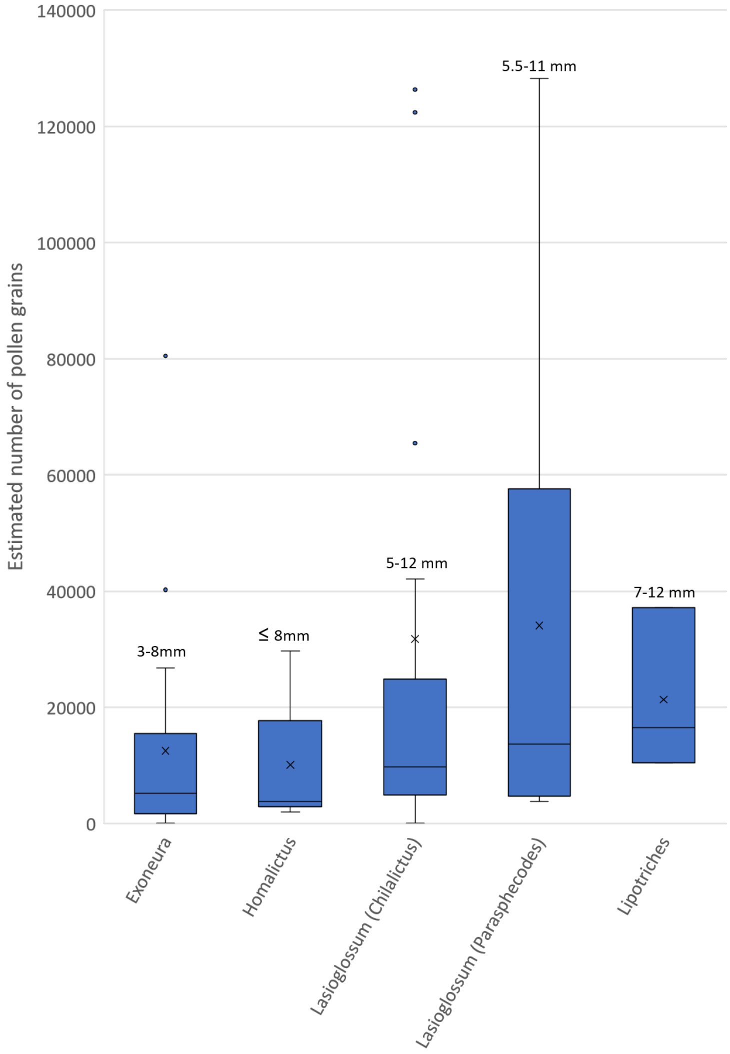


Figure 3-2

Distribution of total pollen load by bee taxa. The box represents the range of values between the first quartile and the third quartile. The x is the mean value, the line through the box is the median value, the whiskers represent the minimum and maximum values respectively and the dots represent outlying values. The numbers above the boxes are the size ranges for each bee taxon.

While pollen varied between bee species, the pollen loads for all the bees, when split between native and non-native source plants, were similar (Figure 3.3). Overall, the load of native and non-native pollen on the bees has a comparable distribution. This means on average the bees carry analogous amounts of pollen from native and non-native plants.

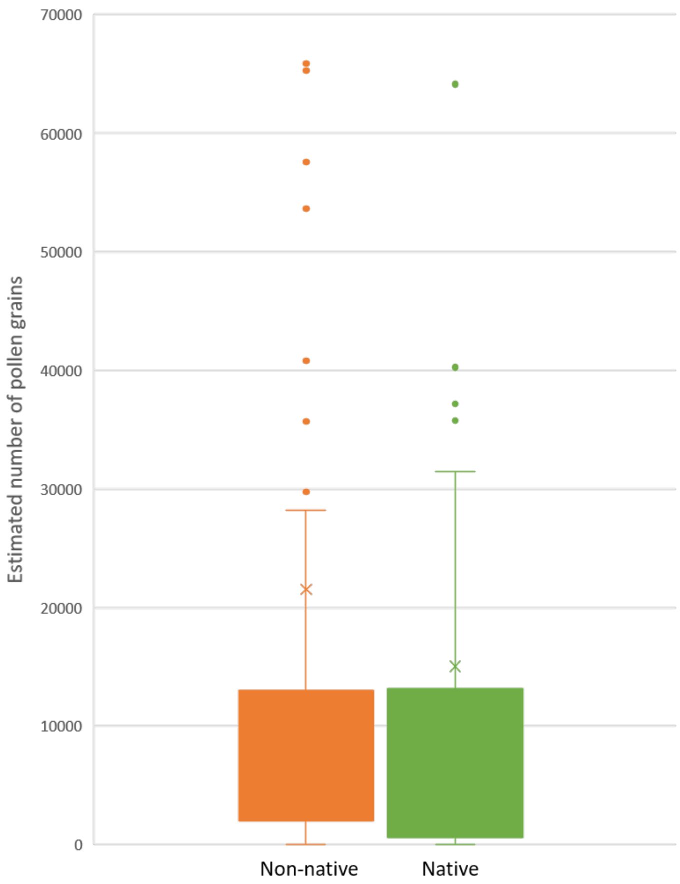


Figure 3-3

Distribution of total pollen load by native or non-native status of plants. The box represents the range of values between the first quartile and the third quartile. The x is the mean value, the line through the box is the median value, the whiskers represent the minimum and maximum values respectively and the dots represent outlying values.

Figure 3.4 supports this finding as it shows the proportions of each bee taxon's pollen load that is made up of native and non-native plants. It shows that all bees in the study carried pollen from non-native plants to some extent, ranging from 27% non-native in *Exonuera* to 78% in *Homalictus*. *Lasioglossum (Chilalictus)* and *Homalictus* had a higher proportion of non-native pollen compared to native pollen, whereas *Exoneura, Lasioglossum (Parasphecodes)* and *Lipotriches* carried more native pollen then non-native pollen. However, these trends could be driven by the site type that bee samples were collected from; *Homalictus* and *Lasioglossum (Chilalictus)* had more samples collected from agricultural sites than forest sites. The *Lipotriches* data were collected from just 3 specimens and thus should be treated with caution.

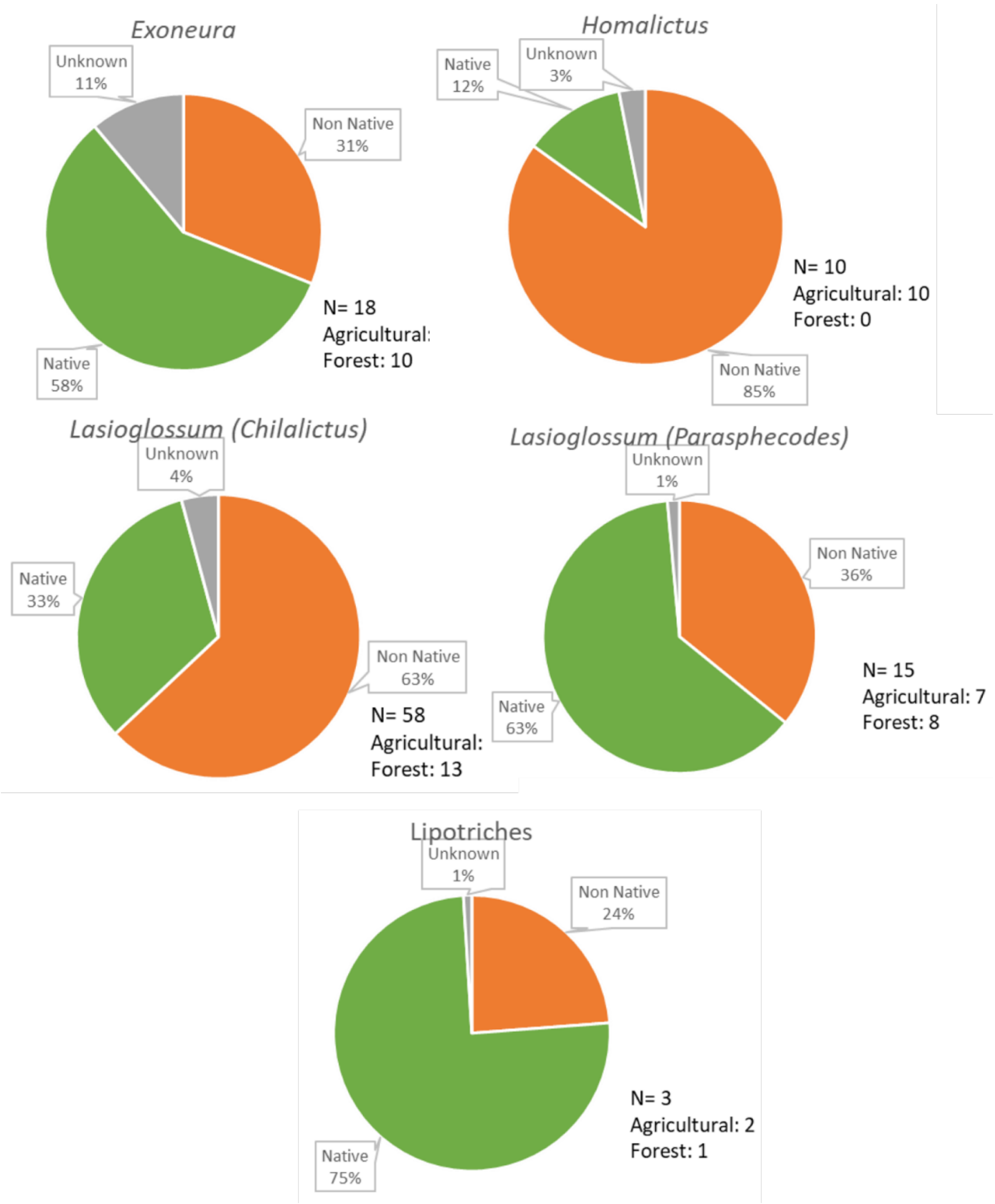


Figure 3-4
Proportions of pollen load composition that come from native and non-native plants.

The different bee taxon had distinctive compositions of collected pollen and overall, they collected pollen from a wide variety of plant species (Figure 3.5). The pollen load composition also varies significantly between each bee taxon and could be an indication of recent foraging. Separating the agricultural from the forest sites reveals different patterns in the bees' pollen collecting. The two network diagrams (Figures 3.6-3.7) capture the diets of all the bee taxa in the dataset and demonstrates the difference between the bees collected from agricultural and forest sites.

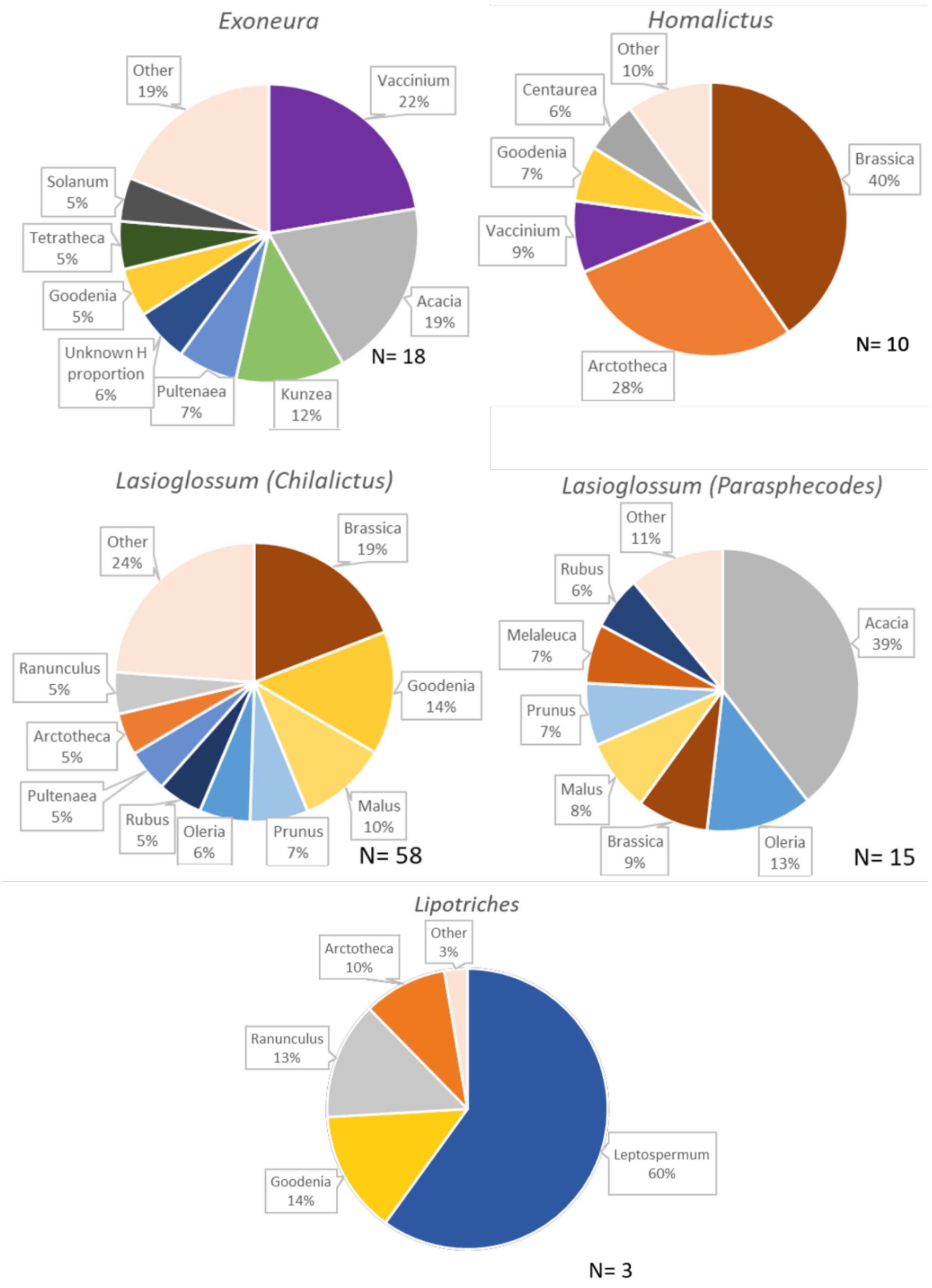


Figure 3-5

Average pollen load composition for each bee taxon. N represents the number of specimens of each bee taxon.

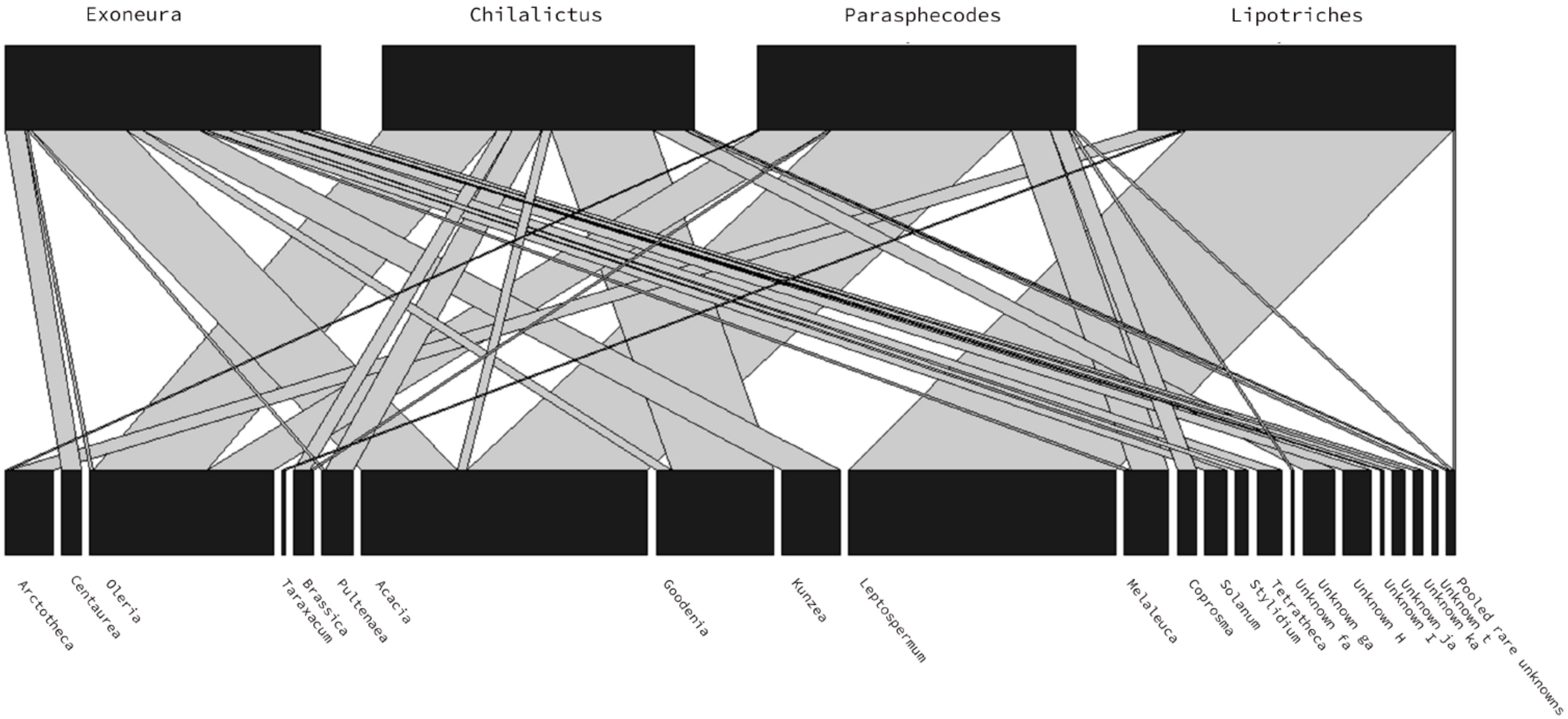


Figure 3-6

Network diagram derived from network analysis conducted on forest sites. The upper bars represent the bee taxa, the bottom bars represent the plant genera. The size of each block is proportional to the pollen count.

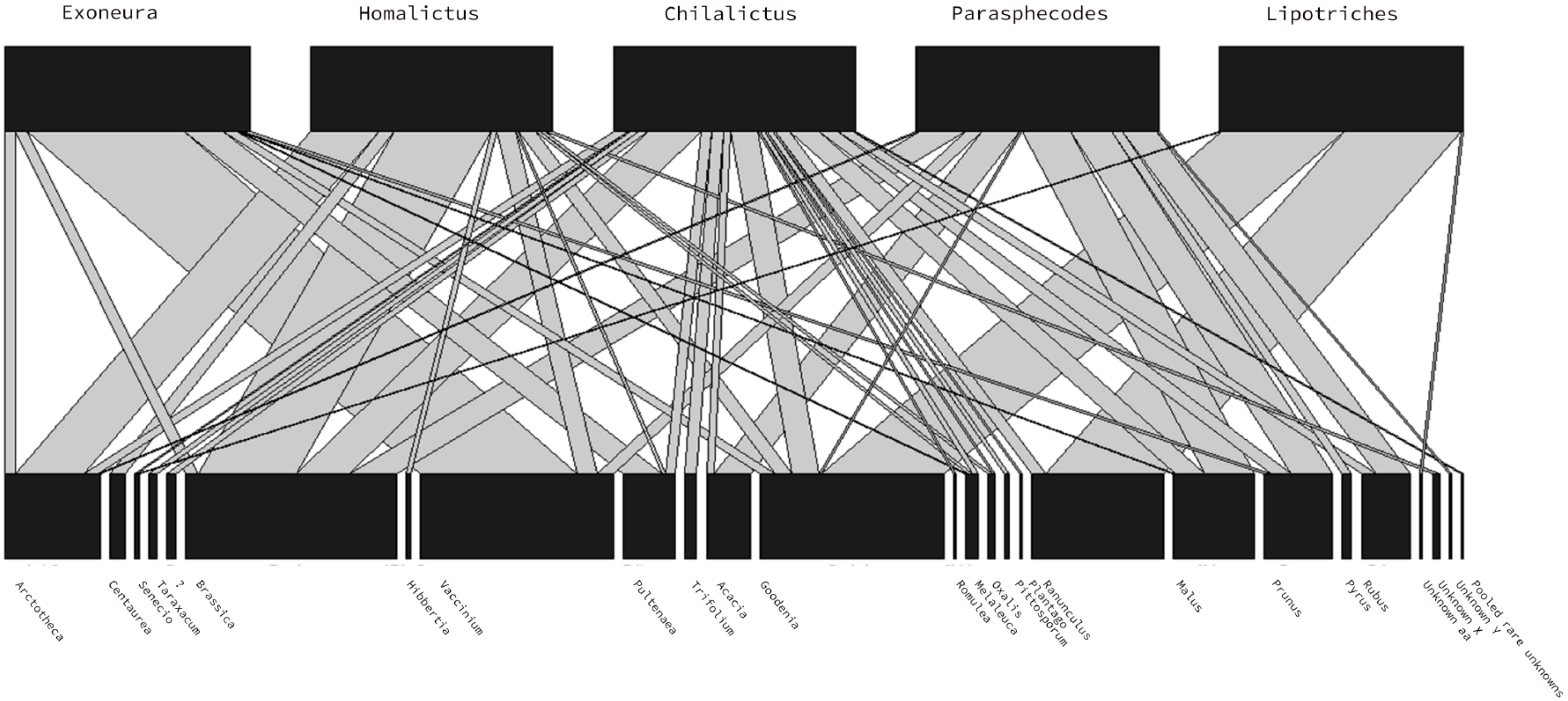


Figure 3-7
Network diagram derived from network analysis conducted on agricultural sites. The upper bars represent the bee taxa, the bottom bars represent the plant genera. The size of each block is proportional to the pollen count.

The output of the redundancy analysis (RDA) is plotted in Figure 3.8. Whereas network analysis (Figures 3.6-3.7) and pie charts (Figures 3.4-3.5) summarise pooled data per bee taxon, the RDA was conducted with each bee as an independent data point. The angles and distance between the pollen types and bee taxon show the level of correlation between the variables i.e. the closer pollen species are to the bee genera the stronger the association between them. The patterns of association in the RDA between bees and pollen (Figure 3.8) were consistent with the data in Figures 3.5 and 3.6. The results of permutation tests run on this redundancy analysis (Table 3.2) illustrate that the there was a statistically significant effect of site type on pollen composition on bees. When the forest and agriculture data sets were separated (Table 3.3 and 3.4), there is a statistically significant effect of bee genus on pollen composition for both site types.

Table 3-2

Results of permutation test conducted on RDA

	Df	Variance	F	P-value
Ag vs Forest	1	4.640	5.8118	0.001
Bee taxon	4	7.444	2.331	0.001
Residual	94	75.045		

Table 3-3

Results of permutation test conducted on RDA in (forest sites only)

	Df	Variance	F	P-value
Bee taxon	3	18.412	2.331	0.001
Residual	25	63.114		

Table 3-4

Results of permutation test conducted on RDA (agricultural sites only)

	Df	Variance	F	P-Value
Bee taxon	4	10.474	2.3489	0.001
Residual	66	73.578		

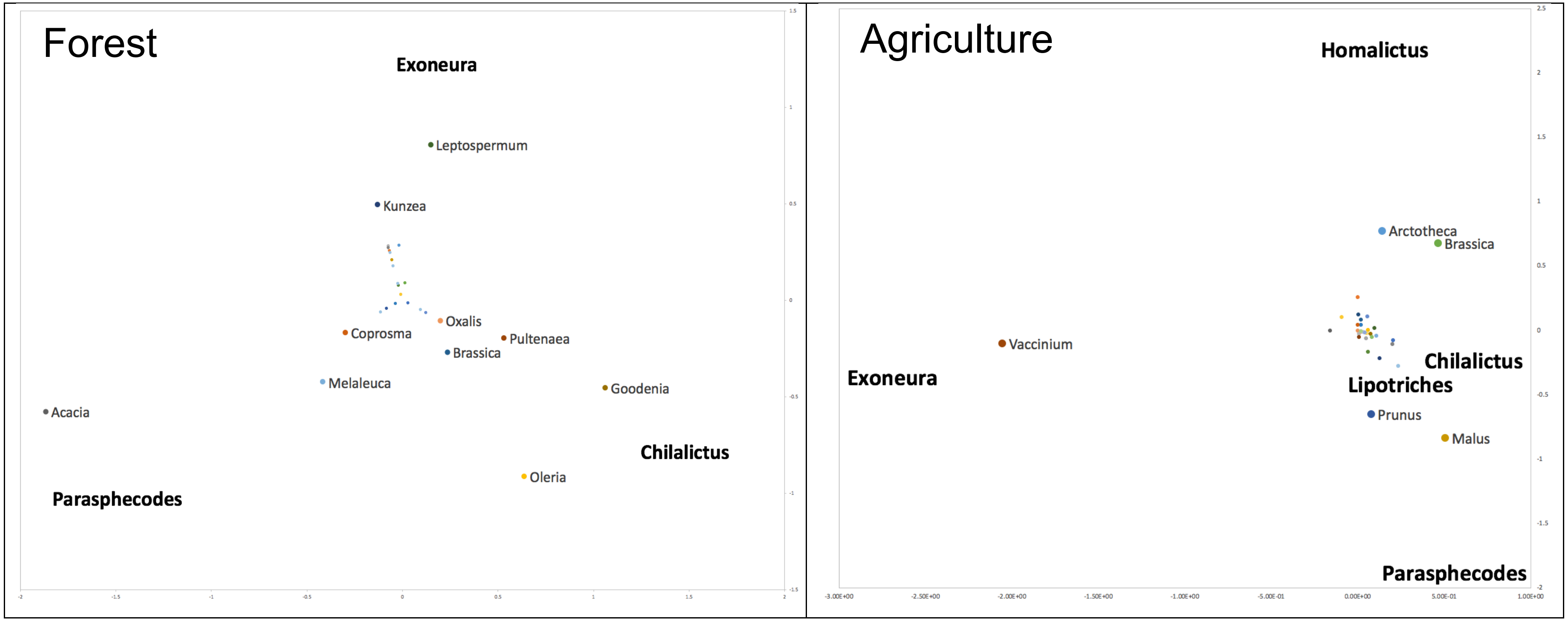


Figure 3-8

Graphical representations of the results of redundancy analysis conducted on bee samples from Forest (left) and Agricultural (right) sites. The points in the middle of the graph represent plant genera. The point representing a pollen type is closer to the point representing the bee taxon when it is more influential in explaining differences among bees' pollen load composition.

Below I summarise the pollen composition of samples from each of the major groups of bees in this study, with reference to network diagrams (Figure 3.6 and 3.7) and pollen composition pie charts (Figures 3.4-3.5) and RDA (Figure 3.8).

Of the bees that were analysed, *Homalictus* samples were collected only from agricultural sites. The majority of pollen load from these specimens was composed of non-native weeds, with remaining load coming from *Vaccinium* (Blueberry) and small amounts of native pollen (Figure 3.5). Pollen came from 8 different plant families, with two examples from Asteraceae (*Arctotheca* and *Centaurea*), and from both herb and shrub plant types. The pollen load composition was dominated by *Brassica* and *Arctotheca*, as evidenced by the network analysis (Figure 3.6 and 3.7) and the redundancy analysis (Figure 3.8), which showed that the variation of the pollen load composition between the different bee taxon was driven by these two pollen types. The rest of its pollen load composition was made up of *Goodenia*, a native groundcover, *Centaurea* which has native and introduced species in the region and a small proportion of *Vaccinium* (Blueberry). Its total pollen load was the second smallest of the bee taxon which corresponded to its small size.

Chilalictus was the most frequently sampled bee that carried pollen. It occurred in both forest and agricultural sites, however in our data set was more commonly found in agricultural sites. As would be expected from this, they collected more pollen from introduced plant species (Figures 3.4 and 3.5). The *Lasioglossum (Chilalictus)* in agriculture showed a much greater diversity of pollen types than in the forest, with 31 distinct pollen types in the agricultural sites compared to 15 in the forest sites (Figure 3.6 and 3.7). *Goodenia* and *Olearia* dominated in forest sites as demonstrated by the redundancy analysis (Figure 3.8) and network diagram (Figure 3.6) and pollen was mostly collected from shrubs. In agricultural sites there was a greater diversity of pollen types and the majority of this pollen came from non-native plants. The families Asteraceae, Brassicaceae and Fabaceae were represented in both site types and all the examples of the Rosaceae family (Apple, Cherry, Pear, Blackberry) in the dataset were represented in the *Lasioglossum (Chilalictus)* from agricultural sites (Figure 3.6 and 3.7). The agricultural *Lasioglossum (Chilalictus)* had a mix of non-native and native vegetation represented in the pollen load, whereas the only non-native pollen in the forest samples is a small proportion of Brassica (Figure 3.6).

Parasphecodes and *Exoneura* were sampled carrying pollen at similar frequency in forest and agricultural sites, and both favoured native pollen over introduced. There was a smaller disparity in the diversity of pollen collected in the agricultural and forest sites by *Lasioglossum*

(Parasphecodes), with 14 different pollen types in the agricultural sites and 11 in the forest sites (Figure 3.6 and 3.7). *Parasphecodes* carried *Acacia* in both the forest and agricultural sites. In the forest sites *Acacia* represented over half of the pollen load composition. In the agricultural sites *Parasphecodes* carried significant proportions of pollen from introduced species including all of the crop plants present in the region. While *Lasioglossum (Parasphecodes)* in agricultural sites collected from both native and non-native pollen, over half of the pollen was from the crop plants in the Rosaceae family (Apple, Cherry, Pear and Blackberry). *Vaccinium* (Blueberry) was also represented but to a much lesser extent than on the *Exoneura* from agricultural sites (Figure 3.6). In the *Lasioglossum (Parasphecodes)* from forest sites, the pollen collected was overwhelmingly from native plant species. Samples did, however, share some pollen types with the agricultural samples, namely two Asteraceae (*Arctotheca* and *Oleria*) and the ubiquitous *Brassica* (Figure 3.6 and 3.7).

The pollen load composition of *Exoneura* in the forest sites showed greater diversity than in the agricultural sites, with 19 distinct pollen types in forest sites and 10 in agricultural sites (Figure 3.6). *Exoneura* favoured introduced pollen in the agricultural sites and native pollen in the forest sites. *Leptospermum* and *Kunzea* were significant in the forest sites and while there was a small proportion of non-native pollen present in the forest pollen loads, the composition for the agricultural sites was dominated by non-native plants, the greatest proportion coming from *Vaccinium* (Blueberry) (Figure 3.6 and 3.7). While different genera were present at each site (Figure 3.6), there were plant families that spanned both sites. This includes Fabaceae (*Acacia* in forest, *Pultenaea* in agriculture), Asteraceae (*Centaurea* in forest, *Arctotheca* in agriculture) and Myrtacae (*Kunzea* in forest, *Melalueca* in agriculture). Most of the plants represented by pollen across both site type were shrubs, with some herbs in the Asteraceae.

Lipotriches was represented by only three samples (Figure 3.5), two agricultural and one forest, therefore its results should be interpreted with caution. However, looking at the data it was significantly different from the other bee taxa in a few ways. The forest samples were more diverse than the agricultural samples (Figure 3.6 and 3.7), and just over half of the pollen from the agricultural samples came from native sources. Overall these samples tended to be biased towards native pollen, even when only looking at the agricultural sites. Pollen from plants from the Asteraceae family were present across both site types, but surprisingly the Asteraceae in the forest sites were non-native and the Asteraceae in the agricultural sites were native (Figure 3.6 and 3.7). The vegetation type varied between herbaceous and shrub over both sites.

Chapter 4: Discussion

The primary goal of the study was to determine whether native bees collected pollen mostly from native plants that they have a shared evolutionary history with, or whether they can also collect from introduced plants. My finding that the common bee taxa were flexibly collecting from introduced species is consistent with some of the literature globally. Russo and Danforth (2017) found that native bees pollinated introduced Apple crops and other *Prunus sp.* crops in orchards in New York. In Australia, native *Amegilla* spp. bees have been used successfully in greenhouse tomato pollination (Hogendoorn *et al.*, 2006) and previous studies in the Yarra Valley showed that native bees visit native and introduced plant flowers (Brown *et al.*, 2020). However, in Danforth and Ji (2001) two species from the sub genus *Lasioglossum (Chilalictus)* (also present in my study) were said to specialise on native plant genera. My study therefore provides some further insight into the foraging behaviour of native bees in this region and their potential as crop pollinators.

I also investigated whether there were differences in pollen collection patterns between the bee taxa in my study. I discovered that there were statistically significant differences in the pollen load composition of the different bee genera in my study. From these results, it is clear, that while all the bees in my study collected pollen from native and introduced plants they are not doing so in identical ways. The data also indicate that the pollen composition on bees depends significantly on whether they were collected from agricultural or forest sites.

4.1 Native and Non-Native Plants

Native bees in this study do carry pollen from non-native plants. The calculated pollen load for all the bee samples (Figure 3.3) shows that overall the bees carried similar amounts of native and non-native pollen. The pollen load composition of each bee taxon based on the plant origin (Figure 3.4) confirms that each bee type carried some pollen from introduced plant species. The results of network analysis (Figure 3.6) reveal that native bees collect from non-native pollen in agricultural sites, and to a lesser extent in forest sites. This finding can most likely be attributed to the vegetation types present in each site type and it provides further evidence for native bees flexibly collecting from introduced plants when given the opportunity.

4.2 Foraging Behaviour

Müller and Khulmann (2008) provide definitions that describe bee foraging behaviour. Based on pollen load composition, all my bees can be classified as polylectic, as they collect pollen from various plant genera belonging to several plant families. Based on the *Lipotriches* data, *Lipotriches* could be classified as polylectic with strong preference for Myrtaceae. However, this finding must be treated with caution as the *Lipotriches* data derives from just 3 samples.

Contrary to previous studies, I did not find that *Lasioglossum* was oligolectic, that is, they did not collect pollen only from one plant genus. Specifically, in Danforth and Ji (2001) *Lasioglossum (Chilalictus) megacephalum* was found to be oligolectic to the genus *Goodeniacae* and *Lasioglossum (Chilalictus) frankenia* to the genus *Frankeniaceae*. Since I was not able to obtain species level identification for the *Lasioglossum* samples in my data set it cannot be determined if my findings directly contradict that of the existing literature.

Coombes (2019) found that small *Lasioglossum* (mostly *Chilalictus* and *Parasphecodes*) were oligolectic in a study also conducted in the Yarra Valley, which is not supported by my results. Her finding was based on floral visitation data, where almost 60% of total visits were to Hop goodenia (*Goodenia ovata*). She therefore concluded that small *Lasioglossum* had an oligolectic relationship with Hop goodenia. Pollen load composition in this study indicates that Goodenia makes up only a small proportion for both *Lasioglossum Chilalictus* and *Lasioglossum Parasphecodes* (14% for *Chilalictus* and <1% for *Parasphecodes*). Coombes' (2019) study was conducted in agricultural sites only, however removing the forest sites from my data, results in Goodenia comprising 11% of *Chilalictus*, and 19% of *Parasphecodes* total pollen load composition, are not nearly as high as the 60% floral visitation rate from Coombes' (2019) study.

Homalictus was also present in Coombes' (2019) study and the results from this bee taxon were more consistent with the results from my study. In Coombes (2019), *Homalictus* visited 13 different flowers and showed a strong preference for introduced flora (>97% total recorded visits). In my study *Homalictus* collected pollen from 10 different plants and 85% of its pollen came from introduced plants. Eighty-six percent of *Homalictus* visits were to only 5 plant species, and in my study 84% of *Homalictus* pollen load composition came from 5 different pollen types, of which 3 were shared with plants in the floral visitation study.

Exoneura had some differences between Coombes (2019) and this study, however to a lesser extent than for *Lasioglossum*. Coombes (2019) reported that *Exoneura* was only found foraging on one-third of the recorded plant species, and the agricultural *Exoneura* from my study similarly collected from just 9 different pollen types (out of 65). *Exoneura* also visited 6 introduced plants in the Coombes (2019) study, and in my study *Exoneura* collected pollen from 7. On the other hand, a large proportion of the pollen load composition of *Exoneura* from my study came from *Vaccinium* (Blueberry), which was not observed at all in Coombes' study. Coombes also stated that the only tree species that *Exoneura* visited was *Malus* (Apple), whereas my pollen load analysis detected both *Malus* and *Prunus* (Cherry) pollen. Furthermore, *Malus* was reported to comprise over 50% of *Exoneura* visits, but it made up less than 1% of the pollen load composition in this study. A large proportion of floral visitation in Coombes (2019) was to Rosaceae, which made up a much smaller proportion in my study of pollen loads.

While there are important differences between the results of Coombes (2019) and my study, on a broad level they do support each other. The bees studied visited both native and non-native plants and visited a variety of plant families. At the finer level, not all of the plants that were identified in the floral visitation survey had pollen of that plant identified on the same bee taxon. By the same token there were plants identified from pollen that did not appear in the floral visitation survey of the same bee. This suggests that there are differences in the plants that are visited and the plants that pollen is collected from.

The differences between my results and Coombes (2019) could have a few explanations. My study was conducted at 13 agricultural sites as compared to 9 in Coombes' (2019) study. That there are more sites and the sites may not have all the same crops may account for some of the variation. Importantly, the studies used different methods for analysing foraging behaviour; floral visitation versus pollen analysis. It could simply be the case that bees visit some plants that they do not collect pollen from, or that the amount of pollen that they can collect from that plant is low compared to other plants that they may visit less often but collect more pollen from. It may be worth investigating the relative size of the pollen grains from the different plants that the bees visit, or which are present in the pollen load collected from the bees. Difference in bee morphology may also have an impact on how much, and what different types of pollen they can carry.

The above observations highlight the importance of conducting floral visitation studies and pollen analysis in tandem to get a complete picture of the pollen diet of bees, as suggested by Richie *et al.* (2016), and implemented successfully in a number of recent studies.

Zhao *et al.* (2019), found that not all flower visitors acted as pollinators and identified 'cheater' bees that visited flowers but did not collect pollen, using a combination of flower visitation data and pollen load analysis. Similarly, Oleques *et al.* (2020) used visitation frequency and pollen load analysis of pollen-and oil-collecting bees to determine which bees were effective pollinators and which were 'oil thieves' of floral oil producing species in South America. Jedrzejewska-Szmek and Zych (2013) used pollen load analysis to verify their flower visitation data and Diniz and Buschini (2015) stated that pollen analysis is a complementary tool to flower visitation studies and used it to assist in creating a more complete pollination network in Atlantic Forest areas in southern Brazil.

My study is not the first to find that bees which were originally thought to be specialist, turn out to have generalist qualities. In a study conducted on native bees in the central Texas prairie system, Ritchie *et al.* (2016) found that the pollen load diversity was greater in the supposed specialist species *Diadasia rinconis* than the generalist *Melissodes tepaneca*.

Of particular note are the findings of Vianna *et al.* (2013), who found that when pollen analysis was added to floral visitation data, generalisation of pollinators in the plant-pollinator network increased.

4.3 Crop Pollination?

My pollen analysis shows that native bees frequently collected pollen from crop species in agricultural sites, which suggests that they may be useful as crop pollinators. Prior research into native bees in the Yarra Valley shows that (1) native bees do visit flowers of crops in this region (Brown *et al.,* 2020) and (2) two native bee genera (*Exoneura* and *Lasioglossum*) pollinate *Rubus* flowers (Coates, 2020). In other parts of Australia, *Amegilla* spp. have been found to be a viable alternative to introduced bumblebees for greenhouse tomato pollination (Hogendoorn *et al.*, 2006). In North America, Russo and Danforth (2017) found that native bees pollinated apple crops and other *Prunus sp.* crops in orchards, as well as wild plant species in fragmented forest adjacent to them. Consequently, there is a precedent for native bees providing crop pollination services.

It is important to highlight that this study focuses on pollen load composition. The question remains whether carrying pollen from a certain plant means that the bee is effectively pollinating that plant. For that reason, it is still unclear from this study whether native bees could replace honey bees as pollinators for the crops in this area, were honey bee numbers to drop significantly. This question could be answered by determining whether a flower visit from a native bee can lead to fruit production, and if this is the case, analysing the fruit quality from flowers where it is known that they have been pollinated by a native bee. Fruit quality may also be improved by having a combination of honey bees and native bees pollinating the plants, and reduced without honey bee pollination.

Honey bees also have some practical advantages over native bees. Large numbers of honey bees can be transported in commercial hives to wherever their pollination services are needed. *Exoneura* is a semi social bee, and as such could potentially be transported to other locations if the pithy stems that they nest in could be mobilised. There have been experiments done where *Exonuera* successfully nested in canes bundled together and housed in PVC pipe, which could potentially be transportable (Port Phillip and Westernport Catchment Management Authority, 2019). However, even if this were possible, the number of bees that could be transported would be dwarfed by what is possible with the eusocial honey bee in commercial hives. *Lasioglossum*, *Homalictus* and *Lipotriches* are all ground nesting bees (Houston 2018) and as such it is not practical to transport them to other locations. In the event of a reduction in honey bee populations it would need to be determined if these native bees, in the same area, could fulfil this ecosystem service. If that is the case, it is also important to know whether these bee populations could be enhanced by different land management strategies (Brown and Paxton, 2009; Klein *et al.*, 2007; Wood *et al.*, 2018a). To that end, it is important to repeat the type of experiment conducted here in different environments in order to establish whether this is the case.

4.4 Pollen Collection Patterns

4.4.1 Common Features among Different Bees

Brassica, a non-native weed was common in the pollen loads of many bees in this study. All the bee taxa except *Lipotriches* carried *Brassica* in the agricultural sites and *Parasphacodes* and *Chilalictus* also carried *Brassica* in forest sites. *Exoneura* carried a small proportion of *Brassica* in the agricultural sites but none in the forest sites. These differences could be related to the different foraging ranges of each bee taxa as *Brassica* was not recorded at the forest sites.

Arctotheca pollen (Cape Weed) is present to some extent in all bee taxa, in both forest and agricultural sites. According to the vegetation surveys, *Arctotheca* was observed in all agricultural sites but very rarely, if at all, in the forest sites. The rarity of *Arctotheca* in the forest sites was reflected in the pollen load data; apart from *Lipotriches* (14% derived from only one specimen), the percentages of *Arctotheca* were negligible in the forest sites (<1%).

Brown el at (2020) found that, in the same region as my study, cleared grassy areas in or adjacent to agriculture supported abundant populations of both *Arctotheca* and *Brassica* species, so it is understandable then that the majority of bees in my study collected pollen from them. In Saunders *et al.* (2013), *Arctotheca* was one of the exotic groundcover species found in grassy Almond orchards. This study found that the almond orchards with this living groundcover hosted more wild insect pollinators than those without. This might suggest that supporting native bees in agriculture could involve keeping these weedy species in the environment.

Apart from these two observations, there were more general patterns shared amongst the different bee species. All bees carried native and non-native pollen to a lesser or greater extent. Excluding *Lipotriches*, which was rare in my dataset (3 samples), all bees collected some pollen from crop species. These patterns correspond broadly with the floral visitation data provided in

Brown *et al.* (2020). Dijk *et al.* (2016) further lists native bees as important to the pollination of crops in Australia.

4.4.2 Distinctive Features of Different Bees

Each bee taxon has different proportions of native to non-native pollen in their average pollen load composition. Within the non-native proportion the different bees had differences in pollen collecting patterns. For *Exoneura*, the majority of the non-native pollen was from *Vaccinium* (Blueberry) with only a small proportion made up of introduced weeds. In comparison, 90% of *Homalictus* non-native pollen came from introduced weeds and only 10% came from *Vaccinium*. For *Lasioglossum (Chilalictus)*, 35% of the non-native pollen came from crop species and in *Lasioglossum (Parasphecodes)* 72% came from crops.

While the bees in this study show versatility in their ability to collect pollen from both non-native and native plants, it does not mean they collected indiscriminately. Of the pollen from crop plants, not all crop pollens were represented in all bee taxa. *Vaccinium* was well represented in agricultural *Exoneura*, but not present at all in *Lasioglossum (Chilalictus)*. Fifty-five percent of the pollen in agricultural *Lasioglossum (Parasphecodes)* came from crops in the *Rosaceae* family (*Malus*, *Prunus*, *Pyrus* and *Rubus*), whereas *Homalictus* had no pollen from these genera.

Observing the differences in the pollen load composition comparing the same bee taxa at agricultural and forest sites (Figure 3.6-3.7), there are some plant families that were collected from in both kinds of sites even if from different plant genera. For example, Asteracae is present in different plant genera in agricultural and forest sites for all bee taxa, and Fabaceae is common to *Exoneura* and *Lasioglossum (Chilalictus)* at both site types. The permutation test run on the redundancy analysis (Tables 3.2-3.4) verified that there is a statistically significant relationship between pollen type and bee type. That is, the variation in pollen load composition between the different bee taxa is statistically significant.

Wood *et al.* (2018) stated that that ‘the importance of different plant groups to maintaining populations of wild bee pollinators is poorly understood’. The above results show the importance of the specified plant groups in maintaining the native bee populations in this area.

The network diagrams (Figure 3.6 and Figure 3.7) separate the bee samples into those collected at forest sites and those collected at agricultural sites. This illustrates that there are significant differences between the pollen load composition from these two site types. The results of the redundancy analysis (Figure 3.8) illuminate this with greater clarity as it shows which pollen types drive the variation between bee types. For example, *Goodenia* and *Olearia* are important to *Lasioglossum (Chilalictus)* in the forest sites, whereas *Malus*, *Brassica* and *Arctotheca* are important in the agricultural sites. *Vaccinium* is important to *Exoneura* in agricultural sites, whereas in the forest sites *Leptospermum* and *Kunzea* (both Myrtaceae) are the drivers. A permutation test conducted on this redundancy analysis (Table 3.2) found that there was a statistically significant difference in pollen load composition between forest and agricultural sites as well as genera. According to Ritchie, Ruppel and Jha (2016), the foraging patterns of native bees are poorly understood, therefore the above findings make a valuable contribution to existing research.

4.5 Methods and Innovations

A significant finding from this study was that contrary to expectations, many bees that were collected in the field had no pollen on them. The expectation was that most of the bees collected would have pollen on them, and that when this pollen was taken through acetolysis and mounted on a microscope slide that there would be enough to successfully analyse. This was a fair assumption to make as the bees were collected off flowering plants. However, once the slides were examined it became clear that only a subset of the bees that had been processed had enough pollen on them to be suitable for microscope analysis. In Vianna *et al.* (2013), they speculated that if a bee was collected as soon as it landed on the plant, there was no time for pollen collection and thus no pollen on the bee

What was found during this process was that the best indicators that a bee sample would have enough pollen on it to be analysed were: 1) that there was a field note that there was visible pollen on the bee at the time of collection, or 2) that there was a visible pellet of pollen after the bee sample had been taken through the acetolysis process.

In our sample set just under 30% of bee with no visible pollen (on the bee or after processing) were able to be analysed. Therefore, it is worth questioning whether it is worthwhile mounting and analysing slides from bees where there is no visible pollen after acetolysis.

To update the methodology in order to more efficiently analyse bees the following protocol could be applied:

1. At the time of collection note whether bees have visible pollen
2. Process all the bees with visible pollen
3. Process the remaining bees
4. Note after acetolysis which samples contain visible pollen
5. Mount these samples on microscope slides.

I assumed in analysing the data collected in this project that a higher load or proportion of pollen meant that the plant of origin was more important to the bee that was carrying it. However, my results show that the amount of pollen collected may not necessarily correspond to higher plant visitation. Perhaps the bees were drinking nectar, rather than collecting pollen, resulting in more visitations but less pollen load (Zhao *et al.*, 2018). It could be that a plant produces more pollen so a bee collects more pollen from that plant per visit (Diniz and Buschini, 2015), or that the size of the pollen grains of different plants has some bearing on how much of it a bee can carry (Wood, 2019). In order to correct for pollen productivity rates between plant species, pollen counting could be conducted on pollen collected directly off the plants, and size measurements could be taken of pollen in the reference collection to estimate the relative size of each pollen type.

4.6 Limitations and further research

A limitation in this study was the relative shortage of bees from forest sites. The bee specimens used in this study were collected as part of another research project and as such there was a higher proportion of samples from agricultural sites than forest sites (about 25% forest sites). Coupled with the fact that not all bees collected had pollen on them this meant that there ended up being more samples from agricultural sites than forest sites. It would be ideal to have a more even balance between forest and agricultural sites. It also meant that my data set contained fewer bee taxa than were in the dataset as a whole. One bee taxa was only represented at agricultural sites (*Homalictus*) and there was only one sample from the Colletidae family (*Leioproctus*, data not presented).

Also, due to technical and time constraints, only a subset of available bees was analysed (about 100 of approximately 700 bees). It is worth noting however that we did analyse every bee that had visible pollen on it at the time it was collected. While for a small number of samples that I analysed there was no visible pollen on the bee this was definitely the minority. Therefore, there is no guarantee that the remaining 600 or so bees would elicit 600 more samples that I could analyse. In addition, the ratio of agricultural to forest samples of the 100 or so samples that were analysed was comparable with that of the whole set and there were enough samples to draw conclusions from. Furthermore, the fact that more bees from agricultural sites had pollen on them than bees from forest sites, and some bee taxa were more likely to be carrying pollen than others, are interesting findings in themselves. Looking at the entire set of bee samples, we could determine overall which bee taxa carried pollen and where they were most likely to be collected carrying pollen.

If, in the larger dataset, there were samples from bee families that arrived in Australia at different times than the families that I sampled, I could ask questions about whether pollen load composition was related to biogeographic history. For example, Colletid bees have a longer history in Australia than Apidae or Halictidae but I only counted pollen off an individual Colletid bee (*Leioproctus*) and as such could not include it in my analysis.

The bees in this study were identified to genus or sub genus level. It would be ideal to have the bees identified to species level, which may promote a deeper level of understanding to the questions asked in this thesis and would also provide the opportunity to answer other questions, such as whether life history traits have an impact on the pollen load characteristics of different species, as life history traits can vary between species of the same genus (Houston, 2018).

My study was undertaken on bees from the Yarra Valley in Victoria but the methods that were developed over the course of my project could be used to replicate the study in other locations. A strength of these methods is that they provide more detail about pollinator-plant networks in conjunction with flower visitation surveys than flower visitation studies alone. Therefore, they could be applied in places where flower visitation studies have been conducted on their own in order to elucidate more detail about the plant pollinator networks in that region. This in turn could be used to find out the extent to which native pollinators interact with crop species, and what other plants can support native pollinator populations both in agricultural and forest settings.

An interesting further research question is whether the pollen collection for the bee species studied was different under different land use scenarios in the same region. This could potentially be achieved by repeating the analysis on historical bee samples from museums. Other pollen analysis studies have been conducted on museum specimens (IPBES, 2016; Burkle *et al.*, 2013; Ritchie *et al.*, 2016; Wood *et al.*, 2018b). For example, Burkle *et al.* (2013) used museum samples to determine the impact of habitat alteration and climate change on plant-pollinator interactions over time in a dataset that spanned 120 years. Wood *et al.,* (2019) used museum specimens to quantify the diet of 12 Bumble bee species in Michigan, USA, while Goldstein and Ascher (2016) used museum collections to construct historical records for some bee species in Massachusetts, USA. If the samples dated back far enough, I could see what the pollen load composition looked like before agricultural land use in the region, and potentially before introduced plants were prevalent.

Chapter 5: Conclusions

My study contained a number of significant findings, both as a result of the pollen analysis conducted and in developing the methodology.

In answer to the question of whether native bees in the Yarra Valley collect pollen from introduced plants, or just from native plants, I found that all the native bee taxa in my study did collect pollen from non-native plants to a greater or lesser extent and that when examining my whole dataset that native bees carry similar amounts of native and non-native pollen. This result indicates that bees do not only collect pollen from plants that they have a shared history with, but that they can expand into pollen from more recent introduced species.

Apart from *Lipotriches*, all the bee taxa that I studied carried some pollen from crop plants in the region. Further studies would need to be undertaken to determine whether native bees carrying pollen from a crop plant results in fruit production.

The different taxa of native bees had statistically significant differences in their pollen collection patterns. They each collected different proportions of native and non-native pollen and while they all collected pollen from a wide variety of plant species their pollen load composition varied significantly between each bee taxon.

The pollen load composition also varied within bee taxon, depending on whether they were collected from an agricultural site or forest site. While the plant genera (from which the pollen was collected) varied somewhat between the forest and agricultural sites, there were often plant families that bees collected pollen from in both kinds of sites.

According to the definitions of bee pollen foraging behaviour, all the bees that were analysed could be described as polylectic. This is different for some bee taxa than previous studies conducted in the same region, however I have discussed possible explanations for this disparity such as bees visiting plants but collecting no pollen, or that the amount of pollen they collect per visit is different for different plants.

I also found that contrary to my initial expectations, not all of the bees that were collected for analysis had pollen on them. The result was that there is a bias in my pollen analysis study towards bees from agricultural sites and certain bee taxa, however this bias tells us what bees are most likely to be collected with pollen on them and that is an interesting result within itself. A possible explanation for why some of the bee specimens had no pollen on them when they were collected is that the bees may not have the chance to collect pollen before they were captured. Finally, improvements to the methodology such as screening the bee samples for visible pollen were suggested in order to more efficiently analyse the bees.

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Appendix 1: The Classifynder

Pollen analysis is routinely conducted by a palynologist through the identification of pollen grains mounted on a microscope slide and using high resolution compound light microscopy. This is quite a labour-intensive task and for some time, there have been calls to automate the pollen identification process. The Classifynder system is one attempt to do so.

The Classifynder is a semi-automated pollen analysis system manufactured by Veritaxa. It is marketed as potentially a more efficient method of carrying out pollen analysis. The system consists of a set of two microscopes, one low resolution, and one high resolution, with attached cameras. These are connected to a computer and controlled robotically with specialist software that captures and analyses images. The Classifynder uses broadly the same steps as manual analysis (creating a reference library by photographing known pollen then comparing these to images captured from sample slides with 'unknown pollen') but uses software rather than a human to conduct the analysis. See https://www.veritaxa.com/ for a full description of the system.

The first step in any study using the Classifynder is the creation of a reference library. Reference pollen is pollen collected from a known parent plant either in the field or from herbarium collections. The reference library is then created by scanning slides of the reference pollen. To scan a pollen slide, pollen grains are first located with the low-resolution microscope in the area selected for scanning. With some input from the user, object parameters are adjusted so that the system captures images of pollen of a similar size, shape and texture, while ignoring other objects (often plant debris) on the slide. Each pollen grain either on the whole slide or as a subset of the slide is then scanned at various depths using the high-resolution microscope and camera to build up a 3D image of each grain. The user then goes through the images and excludes those that are not of the pollen of interest or in some cases multiple grains or debris to build up a 'clean' reference library of images for that pollen type. This process is repeated for all the pollen types relevant to the study. Decisions about which pollen types to initially include in the reference library are usually based on field assessments.

These individual libraries are then combined into a training set that is used to 'train' the Classifynder. This training involves using machine learning, specifically a neural network, to 'teach' the system how to classify the pollen grains. The neural network splits the training set in in half, training itself on one half of the set and testing its performance on the other half of the set. This allows the user to tweak the parameters so that over several runs the classification of the reference sample is improved.

To analyse a sample slide with ‘unknown’ pollen, the slide is scanned using the same process as above. The software then analyses the images and classifies them according to the reference library neural network, and also produces counts and percentage breakdowns of each pollen type present in the sample. It is important to recognise that the Classifynder uses a forced classification system, and so any image captured during the classification process is forced into one of several ‘known’ bins. There is no ‘unknown’ bin. In addition, an expert then needs to review and adjust the classification results, manually moving misidentified pollen into their correct bins or creating new bins for pollen which were not part of the reference library and neural network but are known to the user. This is why the Classifynder is referred to as a semi-automated system.

There are both strengths and limitations to this semi-automated approach to pollen analysis. One of the perceived advantages over a manual approach it that datasets are quicker and cheaper to obtain. Another is that more pollen grains are analysed overall, with the Classifynder counting 3-7 times more grains than a human analyst according to Lagerstrom *et al.* (2015). The automated approach also has the potential to expand the scope of pollen analysis as it can analyse a higher volume of material as it doesn’t experience fatigue as a human analyst would. In practice this means more pollen grains per samples can be analysed, more samples can be processed, and depending on the study, the potential to analyse samples from more sites. An automated approach is also thought to be more objective and consistent, as it minimizes intra and inter-analyst variation.

A perceived strength of automated systems is that they are more sensitive than the human eye to subtle morphological variations and as such could hypothetically be able to identify pollen to a higher taxonomic resolution. In practice however, this is yet to be realized as the human eye in combination with high resolution compound light microscopy seems to be better at seeing fine detail (like surface ornamentation and edges) than the Classifynder.

Another limitation of the Classifynder system in its present form is that the neural network does not provide a measure of error for the pollen grain classification (Lagerstrom *et al.*, 2015). The Classifynder still relies on a palynologist to manually sort the grain images, so although it can speed up the identification process it is not in fact fully automated.

The Classifynder proved to have some disadvantages over manual methods. These included the time it takes to scan the reference material and then the sample; depending on the density of the pollen grains and the size of the area to be analysed this could take anywhere from 30 minutes to 12 hours. There were also a limited set of parameters for slides that the Classifynder was able to analyse; slides had to be ‘thin’, that is, with a thin layer of glycerol (see Methods). When doing manual analysis, it can be useful to have a thicker layer of glycerol to be able to move pollen grains around in the mounting medium to see them from multiple angles however the Classifynder needs stationary grains in order to photograph good images. With thick glycerol slides the grains can also be on multiple planes. This is difficult for the machine to parse as it can only focus on one plane. These constraints are part of the reason that the slide mounting method was moved from liquid glycerol to glycerol jelly (see Methods).

The perceived benefits of the Classifynder system do not reflect the real-life experience working with it. Both the hardware and the software proved to be unreliable in different ways and attempts to rectify the problems that arose were unsuccessful. This included travelling to New Zealand to meet with the manufacturers in person to address these problems.

The Classifynder system uses robotics, controlled by the attached computer, to move the microscopes around the slide. This mechanism would often stall, requiring a hard reset, which in turn would require parts of the analysis to be repeated. This problem was brought to the attention of the manufacturers, however when the machine was returned to them, they could not replicate the same levels of stalling and therefore they dismissed it as not a major concern.

An additional problem was that the two cameras would periodically stop working; sometimes they would spontaneously start working again however there didn’t seem to be any method that could reliably fix this problem.

The part of the Pollen Release software that creates the neural network and identifies pollen (the main purpose of the machine), progressively stopped functioning. At first, the results of the pollen identification and counting process, which were meant to update live, did not update. Eventually, the software was also unable to create new neural networks. There did not seem to be any reason behind this degradation. Regardless, it made the system completely unusable. Again, these problems were brought to the attention of the manufacturers, who instructed us to work with ANU IT to fix them. However, as ANU IT was unfamiliar with the software they were of limited help and were unable to return the software to a functional state. It has been frustrating that the developers of the software were not willing to provide this essential support.