DOCTOR OF PHILOSOPHY

Investigating floral resource use by pollinators using pollen DNA metabarcoding

Lowe, Abigail

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Abigail Lowe

A thesis submitted to the School of Natural Sciences in fulfilment for the degree of Doctor of Philosophy from Bangor University

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Summary

Plant-pollinator interactions are vital ecological relationships which underpin global biodiversity and provide key ecosystem services. Despite the importance of pollinators, evidence of species decline is increasing. Declines are caused by multiple interacting factors, however, a reduction in floral resources due to anthropogenic changes in land use is thought to be a major driver. For this reason, there is a requirement for increased knowledge of how plant use is structured within plant-pollinator networks. This information can then be used to ensure sufficient floral resources are provided throughout the year and that pollinator populations are appropriately supported. This thesis begins with a review of the literature surrounding the use of DNA metabarcoding for the identification of floral visitation by pollinators, including a detailed description of the methodological approach and guidance for users of this technique. The following three empirical chapters utilise DNA metabarcoding to identify the most frequently used floral resources by bumblebees, honeybees, non-corbiculate bees and hoverflies throughout the year in a diverse horticultural and agricultural landscape, using pollen from the bodies of insects (chapters three and four) and honey (chapter five). Native and near-native plants were found to be used most often throughout the year. However, horticultural plants offer an alternative resource at the end of the flowering season when native floral availability is reduced. Chapter three identified key seasonal differences in resource use between pollinator orders and functional groups (bumblebees, honeybees, non-corbiculate bees and hoverflies), allowing an evidence-based recommendation list of pollinator-friendly plants to be produced. To further explore floral resource use by pollinators, the levels of dietary specialisation and generalisation were investigated at varying hierarchical levels in chapter four. Whilst generalisation was common at the order, group and species level, individuals were found to be highly specialised both in relation to the number of resources used in a foraging trip and in their dietary niche within a species. In chapter five, the seasonal patterns of specialisation of honeybee colonies revealed periods of resource limitation, although floral surveys identified a higher availability of floral resources throughout the year. The phenomenon of resource limitation is likely due to the reliance of honeybees on mass-flowering resources such as woody trees e.g., *Prunus* spp. and *Salix* spp., and bramble *Rubus* spp. as major resources, of which there is a phenological gap in peak flowering between spring and summer. This thesis deploys novel pollen metabarcoding approaches to provide a temporally explicit evidence base that broadens our understanding of resource use by pollinators. Consequently, we are now in a position to provide informed recommendations to gardeners, landowners, and policy makers to determine which plants can be used for supplemental planting in urban and agricultural habitats and to highlight the importance of conserving semi-natural habitats.

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Chapter One

Introduction

1.1. General introduction

Pollinators are essential for the maintenance of ecosystem function and food security due to their role in the reproduction of flowering plants (Potts et al., 2016; Klein et al., 2007; Ollerton et al., 2011). Although some species are expanding their range, increased evidence demonstrates both regional (Powney et al., 2019; Fox et al., 2021) and global (Wepprich et al., 2019; Zattara & Aizen, 2021) declines in pollinator numbers, threatening both floristic biodiversity (Lundgren et al., 2016) and human health through the loss of key crops (Smith et al., 2015). Whilst the cause of pollinator decline is complex and is driven by multiple interacting factors, those related to the loss of habitat and floral resources are thought to be the principal cause (Dicks et al., 2021; Goulson et al., 2015).

Declines in pollinator numbers may be mitigated by increasing floral resources in the landscape (Durant & Otto, 2019). This is often achieved through targeted supplemental planting, particularly in urban (Blackmore & Goulson, 2014) and agricultural (Carvell et al., 2015) environments, where resource availability may be low. A wealth of information exists on which plants should be provided; however, these lists are subject to criticisms surrounding their evidence base and resultant accuracy (Garbuzov et al., 2017; Garbuzov & Ratnieks, 2014). To allow effective conservation of pollinator populations, there is a clear need for increased knowledge of which plants are used by pollinators throughout the season (Dicks et al., 2013).

An ecological network is defined as "a representation of species interactions in an ecosystem, in which species are connected by pairwise interactions" (Evans & Kitson, 2020). The interactions between pollinators and plants form mutualistic interspecific bipartite networks that have a nested structure (Bascompte & Jordano, 2006). These intricate networks can be used to study a broad range of ecological

questions relating to the dynamics of plant-pollinator interactions (Byers et al., 2017). For example, by comparing the structure of networks over time, the temporal changes in network properties such as specialisation can be assessed (Petanidou et al., 2008). In addition, simulations can be used to estimate the response of established networks to events such as phenological shifts (Memmott et al., 2007) and species losses (Rezende et al. 2007).

Previous research into floral use by pollinators has used observations of plants (Blackmore & Goulson, 2014) or microscopic analysis of pollen (Wood et al., 2016). DNA metabarcoding, a process involving identifying large-scale identification of unknown taxa within a mixed sample using DNA barcode markers and high-throughput sequencing (Cristescu, 2014; Evans and Kitson, 2020), may also be used to identify floral visitation (Leidenfrost et al., 2020). DNA metabarcoding has provided a unique opportunity to further understand our knowledge of plant use at a fine scale by expanding the spatiotemporal scope of information which can be gained from observational studies (Pornon et al., 2017), whilst increasing the taxonomic resolution achieved through microscopy (Brennan et al., 2019). DNA metabarcoding has been used to characterise plant-pollinator interactions by identifying plant material such as pollen or leaves from nest provisions (Gresty et al., 2018), insect bodies (Lucas et al., 2018) and honey (Jones et al., 2021a). Whilst DNA metabarcoding is a powerful tool, it is fundamental to note that the accuracy and reliability of identifications are dependent on the quality of the reference library used (Jones et al. 2021b).

As DNA metabarcoding can yield information from an individual's entire foraging trip, the use of this technique advances our understanding of the relationships between pollinators and plants (Pornon et al., 2019). DNA metabarcoding has been used to reveal short-term specialisation of individuals within generalised species and networks, enhancing our knowledge of how plants are shared at intra- and

interspecific levels within a community, whilst providing an opportunity to further explore any changes in response to spatiotemporal fluctuations in resource availability (Lucas et al., 2018; Pornon et al., 2019; Klečka et al., 2021). As this is still a developing field, more research is required to understand floral resource partitioning within networks, particularly across a range of pollinator groups.

Complementary to identifying which plants are used most often by pollinators is a need to develop an understanding of whether these resources are provided at suitable levels within the landscape. Periods of resource shortage have been identified in diverse habitats, increasing pressures on populations (Timberlake et al., 2019; Couvillon et al., 2014). It is imperative that any periods of resource limitation are identified, as this can have a direct effect on pollinator health (Requier et al., 2020), and may cause exploitative competition between species (Wignall et al., 2020). Understanding how resources are partitioned in a pollinator community could help to identify periods of resource limitation, as individuals are assumed to favour specialisation when resource availability is low to minimise competition (Bolnick et al., 2003; Pornon et al., 2019).

1.2. Thesis aims and objectives

The overall aim of this thesis is to extend our understanding of the relationships between pollinators (at the level of individuals, species, and functional groups) and plants by using pollen DNA metabarcoding to increase the spatiotemporal scope and taxonomic resolution of current knowledge.

Chapter Two

To thoroughly review, for the first time, the use of DNA barcoding and related approaches for identifying floral visitation, whilst providing guidance on the pollen metabarcoding workflow for researchers interested in using these techniques.

Chapter Three

To identify the plants used by pollinating insects in an extensive, well characterised, and complex horticultural and agricultural landscape, using a multi-locus (*rbcL* and ITS2) pollen DNA metabarcoding approach. We specifically answer the following questions:

- Do Diptera (hoverflies) and Hymenoptera (bees) have distinct foraging preferences?
- How does foraging differ between broad pollinator groups (bumblebees, honeybees, non-corbiculate bees, and hoverflies)?
- Do ecological functional categories within these groups (related to tongue length in bumblebees, body size in non-corbiculate bees and larval requirements in hoverflies) affect the plant taxa used?
- How does foraging change over the flowering season and year?
- Do pollinators prefer native or non-native plants?

The results are used to present novel recommendations for gardeners, landowners and conservation organisations based on time resolved, empirical data, to support pollinator populations and ensure effective conservation.

Chapter Four

To use DNA metabarcoding to understand how plant use is structured within an insect community at different hierarchical levels. We specifically address the following questions:

1. Within a plant-pollinator network, do taxonomic orders (Diptera and Hymenoptera) and groups (bumblebees, hoverflies, honeybees, and non-corbiculate bees) show floral specialisation?
2. What are the patterns of specialisation and generalisation between species?
3. Do individuals exhibit floral fidelity?
4. How do the foraging choices of individuals reflect the overall species patterns?

Chapter Five

To investigate floral resource use in a social central-placed forager, the honeybee, *Apis mellifera*. Specifically, we ask the following questions:

- What are the seasonal changes in honeybee foraging in a diverse area of agricultural habitat and horticultural planting?
- Can periods of resource limitation be identified by assessing the level of diet specialisation between honeybee colonies throughout the year?
- Can preference analysis identify plants which are used more or less than expected by chance given their relative abundance in the landscape?
- Is the use of plants by honeybees proportional to their relative abundance in the landscape with regard to their growth form (tree/shrub/herb) or native status?

1.3. Thesis outline

Chapter One provides a general introduction, followed by the thesis aims and objectives and thesis outline.

Chapter Two reviews the use of DNA metabarcoding to study plant-pollinator interactions, along with their opportunities and challenges. The ecological questions relating to floral resource use by pollinators that can be investigated using DNA metabarcoding techniques are explored, along with key considerations and guidance throughout the metabarcoding workflow, providing an opportunity for future researchers to consider adopting these techniques.

Chapter Three is the first empirical data chapter which uses DNA metabarcoding to identify the plants visited by bumblebees, honeybees, non-corbiculate bees and hoverflies in a horticultural and agricultural landscape. This chapter explores how floral choice differs between pollinators and provides recommendations as to how pollinator friendly plant lists can be improved for effective conservation of species.

Chapter Four explores how plant-pollinator networks are structured at varying hierarchical levels. Specifically, it uses DNA metabarcoding to investigate the level of specialisation and generalisation of pollinator groups on floral resources from the community to the individual level.

Chapter Five investigates honeybee foraging throughout the year using DNA metabarcoding of honey samples. It explores whether patterns in resource partitioning can be used to identify periods of resource shortage, and if floral resource use is linked to the relative abundance of plants in the landscape.

Chapter Six is a general synthesis of the previous four chapters, revisiting the main findings and providing an overarching discussion of the use of DNA metabarcoding to advance both pollinator ecology and wider ecological processes.

The appendices include a paper on honeybee foraging that I co-authored during my PhD and a list of conference presentations.

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Chapter Two

Using DNA metabarcoding to identify floral visitation by pollinators

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2.1. Abstract

The identification of floral visitation by pollinators provides an opportunity to improve our understanding of the fine-scale ecological interactions between animals and plants, contributing to biodiversity conservation and ecosystem health. In this review we outline the various methods which can be used to identify floral visitation, providing a comparison between molecular and non-molecular methods. We describe thoroughly, for the first time, the ways in which DNA metabarcoding has been used to answer ecological questions related to plant use by pollinators and discuss their findings. We present detailed methodological considerations throughout each step of the metabarcoding workflow, from sampling through to amplification and finally bioinformatic analysis, whilst simultaneously providing guidance to researchers for utilisation of these techniques. The future opportunities and directions of using molecular methods to analyse plant-pollinator interactions are discussed, with the hope of improving reliability of results along with standardisation of methods.

2.2. Background

Understanding the relationship between plants and pollinators is vital for biodiversity conservation, food security, and ecosystem function (Klein et al., 2007; Potts et al., 2016). Worldwide, there are approximately 350,000 animal pollinators, of which insects contribute a significant proportion (Ollerton et al., 2017). Despite the importance of pollinators, evidence of declines in abundance is increasing across the globe (Powney et al., 2019; Wepprich et al., 2019). A global analysis of pollinator decline has found that the most significant drivers of decline are land use change, pesticides, climate change and pests and pathogens (Dicks et al., 2021).

DNA metabarcoding provides a powerful tool for ecosystem monitoring, characterising biodiversity within air, soil, and water where morphological approaches are limited (Bohmann et al., 2014; Deiner et al., 2017). A multitude of ecological questions may be answered through the use of DNA metabarcoding (Creer et al., 2016). Species richness and diversity of environmental samples can be assessed quickly and efficiently to complement other survey techniques (Deiner et al., 2017), dietary diversity can be characterised through a range of environmental samples including faeces, gut contents, and pollen (Fahimee et al., 2021; Pompanon et al., 2012), and ecological information can be gained from historical samples (Niemeyer et al., 2017).

Identifying floral visitation provides an insight into the resources used by insects and the pollination services they deliver (Ballantyne et al., 2015). Whilst visitation is not synonymous with true pollination, we use the term pollinators as a general term to refer to flower-visiting insects. The ability to characterise pollinator foraging preferences through DNA metabarcoding is enabling molecular methods to become a standard part of the ecologist's toolkit.

The aim of this review is to describe the range of approaches and methods available to identify floral visitation through DNA metabarcoding, along with their opportunities and challenges. For the first time, we thoroughly explore the ecological questions that can be answered from identifying floral visitation across a range of species and habitats and present a summary of findings from the literature. The entire pollen metabarcoding workflow is described along with considerations and guidance for each step, in order to inspire more researchers to adopt these techniques.

2.3. Methods for identifying floral visitation by pollinators

Floral visitation studies may be plant- or insect- focussed. Historically, the diet of pollinators has been studied by observing insects visiting plants. This involves recording which pollinators visit plants, either within established habitats (Goulson et al., 2008; Hanley et al., 2014; Klečka et al., 2018) or in experimental conditions (Sutherland et al., 1999). Alternatively, insects themselves may be observed and flower visitation tracked by methods such as mark recapture using paint, plastic tags (Heinrich, 1976) or harmonic radar (Osborne et al., 1999), although electronic tags require prior knowledge of the location of floral resources. In honeybees, the location of resources is communicated to the colony using waggle dances (Seeley, 1995) which can be de-coded to elucidate forage preferences and behaviour (Balfour et al., 2015).

Floral visitation may also be investigated by identifying the pollen collected by pollinators. Pollen microscopy has been widely utilised for diet characterisation by identifying pollen grains obtained from the bodies of individuals (Eckhardt et al., 2014; Wood et al., 2016), honey (Ebenezer and Olugbenga, 2010; Ponnuchamy et al., 2014) or nest provisions (Lawson et al., 2016; Williams and Kremen, 2007). As

identification to species level is difficult or not possible for some taxonomic groups using microscopy (Bell et al., 2016), development of automated machine learning systems to identify pollen from images has attracted significant attention in recent years, showing great promise (Holt and Bennett, 2014; Polling et al., 2021; Sevillano and Aznarte, 2018).

Alternatively, pollen may be identified by DNA metabarcoding: a process involving identifying large-scale identification of unknown taxa within a mixed sample using DNA barcode markers and high-throughput sequencing (Cristescu, 2014; Evans and Kitson, 2020; Leidenfrost et al., 2020). DNA metabarcoding has been used to successfully identify pollen from provisions within nests (Eeraerts et al., 2021; Gresty et al., 2018; Vaudo et al., 2020), honey (de Vere et al., 2017; Jones et al., 2021a; Lucek et al., 2019), proboscises (Chang et al., 2018; Macgregor et al., 2019), guts (Mayr et al., 2021; Wilson et al., 2010), and the legs or bodies of insects (Fahimee et al., 2021; Lucas et al., 2018b; Potter et al., 2019) (Table S2.1). Whilst the majority of DNA metabarcoding studies utilise pollen, some have identified raw plant material from within nests to identify the leaf preferences of solitary bees (MacIvor, 2016; Müller et al., 2019; Müller and Richter, 2018).

Shotgun metagenomics are an alternative tool which can be used to identify taxonomic diversity within a mixed sample using untargeted sequencing of genomic fragments mapped to whole genomes or barcode regions (Bell et al., 2021; Creer et al., 2016). By mapping genome-skims to a constructed reference library of plastid genomes, Lang et al., (2019) demonstrated quantitative identification of >97% taxa in mixed pollen samples. The advantages of metagenomic methods are the option of PCR-free processes which reduce possible amplification biases, long read lengths, and increasing taxonomic resolution compared to targeted sequencing of specific regions (Bell et al., 2021; Peel et al., 2019). The main limitation facing whole-genome studies is that currently, few whole plant genomes

are available, resulting in difficulties assembling reference material (Bell et al., 2021). A further promising approach is the use of reverse metagenomics to map long reads produced by the MinION to genomic skims, a method which has produced semi-quantitative identification of plant species in mixed pollen loads (Peel et al., 2019).

An example of a more novel approach to elucidating floral visitation is through the method of obtaining residual insect DNA from plants themselves (Thomsen and Sigsgaard, 2019). Similarly, the identification of 'microbial signatures' specific to pollinators within nectar can also be used to elucidate visitation (Aizenberg-Gershtein et al., 2013; Ushio et al., 2015).

2.4. Comparison of molecular methods with non-molecular methods

Each method of identifying floral visitation has advantages and disadvantages which must be considered (Table 2.1). Observational methods allow plant-pollinator interactions and networks to be identified and constructed quickly and cheaply, however, as the period of observation is often limited both spatially and temporally, this leads to missed interactions (Olesen et al., 2011). As a result, sampling effort is a major limiting factor of the number of links which are recorded. Some pollinators are thought to visit many plants in one foraging trip (Beil et al., 2008), therefore by observing plants rather than pollinators, incomplete networks can be formed, and key forage species may be missed. By analysing pollen loads of bumblebees, Carvell et al. (2006) found that the dominant plant in pollen loads was not always the plant the bee had been caught on, demonstrating that observation of floral networks does not reveal all interactions with visitors.

Table 2.1: Methods of detecting floral visitation by pollinators along with their advantages and disadvantages.

Method	Advantages	Disadvantages
Observations of plants or pollinators	• Easy to set up and conduct surveys • Cheap • The type of resource used can be identified (e.g., pollen, nectar, resin) • Can track individuals using mark recapture methods	• Some interactions are unseen due to spatiotemporal restrictions of both plants and pollinators • Sampling effort is labour intensive and has a direct effect on number of links • Limited by field identification of pollinators and plants • Often qualitative data only
Waggle dance analysis	• Can track foraging location of individuals over short and long distances	• Only suitable for honeybees • Need prior knowledge of location of resources to infer visitation • Landscape-level analysis rather than specific resource use
Harmonic radar	• Can track insects up to 700m • Can track individuals	• Need prior knowledge of location of resources to infer visitation • Physical obstacles e.g., hedges affect signal • Weight of tracker limits which insects can be monitored
Pollen microscopy	• Can give information on an individual's entire foraging trip • Pollinator specimens can be retained for morphological identification • Can identify pollen from historic specimens • Automated processes for identifying images are being developed • Some measure of abundance is achievable	• Need expert palynologists for identification • Requires phenological and local knowledge of plant taxa for verification • Identification in some taxa is difficult beyond family or genus • Quantitative but a small subset is often analysed • Time-consuming with not much ability to scale up unless automated processes are used • Rare taxa are difficult to detect • Temporal limit is unknown – can range from flight period of minutes to hours • Cannot distinguish if plants were visited for pollen, nectar, or resin

Pollen metabarcoding	• Can give information on an individual's entire foraging trip • Can be scaled up easily • Pollinator specimens can be retained for morphological identification • Eliminates the need for expert palynologists • Can identify the entire pollen assemblage of an individual • Can distinguish taxa which are difficult to identify morphologically • Can identify pollen from historic specimens • Semi-quantitative data can be obtained	• Identification in some taxa is difficult beyond family or genus • Expensive, high start-up costs • Rare taxa are difficult to detect • Requires phenological and local knowledge of plant taxa for verification • Temporal limit is unknown – can range from flight period of minutes to hours • May not be fully quantitative • Cannot distinguish if plants were visited for pollen, nectar, or resin • Biases during DNA extraction, PCR and sequencing may reduce how quantitative the data can be.
Metagenomics	• Can give information on an individual's entire foraging trip • Can be scaled up easily • Pollinator specimens can be retained for morphological identification • Eliminates the need for expert palynologists • Can identify the entire pollen assemblage of an individual • Can distinguish taxa which are difficult to identify morphologically • Can identify pollen from historic specimens • PCR-free processes reduce possible amplification biases • Long read lengths • Potential to obtain quantitative data	• Identification in some taxa is difficult beyond family or genus • Expensive • Reference libraries for identification require whole genome or genome skims. High workload to achieve this at scale for many plant species. • Rare taxa are difficult to detect • Requires phenological and local knowledge of plant taxa for verification • Temporal limit is unknown – can range from flight period of minutes to hours • Cannot distinguish if plants were visited for pollen, nectar, or resin

Insect-focussed methods of identifying floral visitation such as DNA metabarcoding and pollen microscopy can reveal interactions which are unseen using observational methods (de Manincor et al., 2020; Galliot et al., 2017; Pornon et al., 2017, 2016; Wilson et al., 2010; Zhao et al., 2019). These methods are free from the spatial limitations of observations which come as a result of visual bias e.g., height (Wood et al., 2016) and tag ranges (Osborne et al., 1999), as they provide a record of any resources which have been accessed by the individual which may be up to several kilometres away (Beekman and Ratnieks, 2000). On the contrary, during observations the time spent foraging can be recorded, however, it is currently not possible to distinguish the temporal range of pollen found on an insect's body.

Arstingstall et al. (2021) found that when comparing plant-pollinator networks characterised by DNA metabarcoding of pollen to those constructed from observations of foraging bees, networks constructed from molecular analysis had increased species richness and reduced specialisation. However, observed interactions can be undetected using DNA metabarcoding and pollen microscopy, owing to their rarity (Pornon et al., 2016), size of plants (Arstingstall et al., 2021; Galliot et al., 2017), or use for nectar with limited or no pollen production (Potter et al., 2019; Wilson et al., 2010). These factors reduce the amount of pollen transferred to the insect and therefore identified. The key advantage of using observations over pollen identification is the ability to identify which resource (pollen, nectar, or resin) is being collected when plants are visited (Goulson et al., 2005), a vital component of pollinator ecology.

The identification of many pollinator species is difficult in the field (Falk and Lewington, 2015), therefore observational studies are restricted by taxonomic expertise, leading to some studies operating at genus level (Robinson et al., 2018).

Retaining insect specimens for pollen analysis through microscopy or metabarcoding allows careful identification either through traditional morphology or DNA barcoding (Hebert et al., 2003), avoiding limitations of identifications in the field.

Interactions between plants and pollinators using both plant and insect-focussed observations are usually characterised at the species level (Klečka et al., 2018) due to difficulties tracking individuals (Heinrich, 1976). The identification of pollen from the body of an insect using DNA metabarcoding or pollen microscopy allows valuable individual foraging preferences to be characterised more easily than in visual surveys (Pornon et al., 2019). In pollen microscopy, a small sub-sample is fully identified and used to estimate the composition of the total pollen load (Bosch et al., 2009), whereas molecular analysis can sample the entire pollen assemblage on the body of an insect (Lucas et al., 2018a). Although there is some congruence between the taxa which are difficult to identify using microscopy and those which are indistinguishable using DNA e.g., some taxa within the rose family Rosaceae (Jones et al., 2021a), both methods may detect additional taxa when compared to the other (Richardson et al., 2015a; Smart et al., 2017). In comparing pollen microscopy and DNA metabarcoding, several authors have found higher taxonomic resolution of plant taxa identified (Keller et al., 2015; Macgregor et al., 2019) and a greater number of species detected (Hawkins et al., 2015; Keller et al., 2015; Richardson et al., 2015a; Smart et al., 2017) using DNA metabarcoding. Both methods, however, are subject to the stochasticity of detecting rare taxa (Hawkins et al., 2015; Richardson et al., 2015b).

Whilst both pollen microscopy and DNA metabarcoding yield valuable individual-level information on foraging, identification of plant taxa using DNA eliminates the need for expert palynologists for microscopy. Although also time-consuming and initially expensive (Bell et al., 2017a), molecular processes may be

easily scaled up (Sickel et al., 2015; Smart et al., 2017). The use of semi-quantitative data (discussed in detail within the methodological considerations) also allows plant-pollinator relationships to be measured and can provide a more sensitive representation of diet compared to frequency of occurrence (Deagle et al., 2019).

2.5. Using DNA metabarcoding to answer questions about pollinator foraging preferences

The use of DNA metabarcoding to answer ecological questions about pollinator foraging preferences has increased rapidly over recent years alongside key methodological developments (Table S2.1). The questions addressed can be broadly grouped into four topics: (1) How does foraging change throughout time and space? (2) How is foraging affected by resource availability? (3) How are resources partitioned between species and individuals in a plant-pollinator network? (4) What is the relationship between plant use and pollinator health?

2.5.1. How does foraging change throughout time and space?

DNA metabarcoding provides a useful method for monitoring plant use across wide spatiotemporal scales, such as multiple countries or regions (Lu et al., 2021) and time periods as long as decades (Gous et al., 2019; Jones et al., 2021a). The reproducibility of DNA metabarcoding allows continued sampling of foraging across a species' entire flight period, allowing an understanding of plant selection at specific time points. Danner et al (2017) and Park and Nieh (2017) sampled pollen from honeybee hives at regular intervals throughout the year and identified the plant species used using DNA metabarcoding. Both studies found that the amount and diversity of pollen collected was strongly influenced by season, most likely influenced by the phenology of surrounding plants (Danner et al., 2017; Park and Nieh, 2017). As well as tracking present foraging habits, DNA metabarcoding has been shown to be a useful tool for analysing pollen from historical specimens

(Gous et al., 2021, 2019; Simanonok et al., 2021). By sequencing pollen obtained from museum specimens, Simanonok et al. (2021) successfully identified the plants used by an endangered bumblebee species over 100 years, vastly improving current knowledge of resource use and mechanisms of decline. Similarly, analysing the pollen DNA within UK honey and comparing the plant diversity to samples characterised 65 years prior using microscopy revealed landscape-scale shifts in foraging habits due to changes in agricultural intensification, crop use, and the spread of invasive species (Jones et al., 2021a). Long-range movements can be tracked by identifying pollen on migrating insects (Chang et al., 2018; Suchan et al., 2018). Suchan et al (2018) detected plant species endemic to Africa on butterflies using DNA metabarcoding, significantly improving the understanding of migration patterns which were previously limited when using traditional techniques. As well as increasing the spatial scale of studies, pollen metabarcoding has highlighted the importance of trees and woody species to pollinators, taxa with flowers which are often visually restricted and therefore may be missed during observational surveys (de Vere et al., 2017; Kratschmer et al., 2020). Whilst most of these spatial assessments of foraging focus on geographic differences, only one study has specifically demonstrated the ability of pollen metabarcoding to elucidate changes in resource use across elevational gradients to better understand how physiological changes in the environment impact foraging (Mayr et al., 2021).

2.5.2. How is foraging affected by resource availability?

A key area of research in pollinator foraging ecology is understanding why specific plants are used and whether this is driven by true preferences relating to characteristics of the plant e.g., nectar quality (Hicks et al., 2016), or an artefact of resource availability (Hegland and Boeke, 2006). By conducting floral surveys and comparing the flowering plants available to the plants identified in honey using DNA metabarcoding, de Vere et al. (2017) found that honeybees only used 11% of genera available. Park & Nieh (2017) also used a metabarcoding method along

with herbarium records to illustrate that honeybees used between 2.7-10% of flowering species available over three seasons.

Insect visitation can be influenced by the abundance of floral resources in a landscape (Fowler et al., 2016), which is itself affected both temporally by plant phenology (Timberlake et al., 2019) and spatially by habitat type (Richardson et al., 2021). Timberlake (2019) utilised a null model method and DNA metabarcoding of pollen samples collected from bumblebees within farmland to illustrate that floral choice was not always driven by the abundance of plant species nor their nectar availability. By identifying plants which are visited more than expected compared to their abundance, management recommendations can be given for effective conservation of bumblebees on farmland (Timberlake, 2019). Likewise, Jones (2020) found no significant correlation between the abundance of plant taxa in the landscape and the abundance of plants found in honey samples each month. However, Nürnberger et al. (2019) found that the number of plant genera in pollen loads of honeybees identified by metabarcoding was lower when floral availability was reduced. Recent work by Quinlan et al. (2021) suggests that whilst honeybees may sometimes preferentially select plants found in high abundance, this is dependent on the time of year and nutritional demand.

DNA metabarcoding can be used to monitor how spatiotemporal changes in resource availability across landscapes affect the diet of pollinators (Bontšutšnaja et al., 2021; Casanelles-Abella et al., 2021; Lu et al., 2021; Richardson et al., 2021). By assessing honeybee diet across gradients of land use, multiple authors have found that the richness and diversity of pollen collected is not strongly linked to the composition of surrounding landscapes (Lucek et al., 2019; Smart et al., 2017; Tommasi et al., 2021a). Instead, seasonality of resources appears to be the greatest driver of diet, irrespective of land use (Danner et al., 2017; Wilson et al., 2021).

2.5.3. How are resources partitioned between species and individuals in a plant-pollinator network?

The use of DNA-based methods for identifying species interactions allows complex networks to be constructed and analysed (Evans et al., 2016; Macgregor et al., 2019). Constructing accurate networks is important to help fully understand their structure, as the level of specialisation and generalisation of networks, species, or individuals within can affect their robustness against environmental change (Biesmeijer et al., 2006; Memmott et al., 2004).

A number of authors have used molecular approaches to assess resource partitioning within large plant-pollinator networks (Lucas et al., 2018a; Macgregor et al., 2019; Pornon et al., 2019). Elliott et al. (2021) used DNA metabarcoding to construct an interaction network between honeybees, native bees and the floral resources used to identify resource overlap. The number of known floral hosts of many species were increased compared to the previous literature based on observational studies, improving the understanding of how wild and introduced bees co-exist in a landscape (Elliott et al., 2021).

The ability to identify an individual's entire pollen assemblage results in the valuable characterisation of interactions at varying hierarchical levels throughout a plant-pollinator community (Brosi, 2016). Of the studies that have identified resource partitioning within plant-pollinator networks using DNA metabarcoding, all have found that generalised networks or species are made up of specialised individuals (Klečka et al., 2021; Lucas et al., 2018b, 2018a; Pornon et al., 2019). This presents a promising area of research to further investigate the levels of specialisation and generalisation exhibited by pollinators.

2.5.4. What is the relationship between plant use and pollinator health?

Floral resources vary in the quality of their nectar and pollen rewards (Hicks et al., 2016), and as a result the diversity of resources used has been found to impact pollinator fitness (Kaluza et al., 2018). Insights into the nutritional ecology of pollinators can be unearthed using DNA metabarcoding, by quantifying the relationship between plant taxa found in provisions and their protein, carbohydrate, lipid, and amino acid content (Donkersley et al., 2017; Trinkl et al., 2020). For example, Donkersley et al. (2017) found that the protein content of bee bread (honeybee pollen stores) was positively correlated with the presence of dandelion *Taraxacum* spp. pollen and negatively correlated with pollen from cherries and plums *Prunus* spp., improving our understanding of how floral resources meet the dietary requirements of pollinators.

As well as affecting the nutritional quality of provisions, the plant species visited by pollinators may also influence the bacteria present in the nest (Dew et al., 2020). DNA metabarcoding allows plant-microbe relationships to be explored, increasing the understanding of plant-pollinator interactions throughout an insect's lifecycle. The relationship between the diversity of pollen species collected and the diversity of the microbiome appears complex, however, both positive and negative associations have been found between particular pollen types and bacteria (McFrederick and Rehan, 2016, 2019; Voulgari-Kokota et al., 2019). For example, Voulgari-Kokota et al. (2019) found that the presence of *Acinetobacteria* in pollen provisions of a solitary bee was positively associated with the presence of some taxa such as European goldenrod *Solidago virgaurea,* oxeye daisy *Leucanthemum vulgare* and yarrow *Achillea millefolium,* but negatively associated with spear thistle *Cirsium vulgare,* red poppy *Papaver rhoeas* and sycamore *Acer pseudoplatanus*.

The identification of pollen in nests has also been used to investigate the relationship between mass-flowering crops and the prevalence of parasites,

finding that increased abundance of resources may help to reduce transmission by diluting parasite transmission through reducing visitation frequency per flower (Piot et al., 2021).

2.6. Key methodological considerations for using DNA approaches and their challenges

2.6.1 Study design and sampling

Careful considerations are required for every stage of the molecular approach, from the initial stages of study design to the resultant bioinformatic analysis (Table. 2.2). Firstly, the nature of the study system must be considered in order to understand the information which will be produced. For example, sampling pollen from a single bee which is actively foraging will yield different results to pollen collected through pollen traps or honey as the latter methods represent the foraging efforts of multiple bees over multiple trips (de Vere et al., 2017). Pollen may be transferred from plants visited solely for nectar (Goulson et al., 2005), and some plants do not produce nectar at all (Stout et al., 2002). In addition, nectar can itself be contaminated with pollen as a result of plant visitors (Willmer, 1980). Therefore, molecular analysis of pollen generates information on which plants have been visited for both pollen and nectar collection. Another important consideration is that the presence of pollen on insects does not assume pollination has occurred (Ballantyne et al., 2015), therefore the identification of pollen represents floral visitation only. It is also important to consider that when identifying plant material within nest provisions, contamination may occur from multiple sources of plant DNA such as pollen provisions, leaf or soil material used to build nests (Keller et al., 2015).

Capturing methods such as on transect walks or during observations will also influence the number and diversity of insects caught and therefore the resulting

sampling universe. The flight times of insects and phenology of plants must be considered due to their influence on foraging, for example, sampling one species across its entire flight period will give a global picture of resources used whilst studies undertaken within a shorter time period will have limited information on the total resources used.

The nature of pollen sampling results in a high risk of cross-contamination occurring in the field therefore samples should be collected using a combination of nets and sterile tubes, with nets changed regularly and sterilised between surveys (Lucas et al., 2018b). Airborne pollen may also contaminate samples (Pornon et al., 2017), leading some authors to use thresholds to exclude rare taxa (reviewed in Tommasi et al., 2021b) or removing all wind-pollinated species from analysis (Tanaka et al., 2020). However, it should be noted that rare taxa may include real interactions, and some pollinators are known to visit wind-pollinated plants (Bertrand et al., 2019; Rotheray and Gilbert, 2011). Further work to quantify the prevalence of residual pollen left on plants by insect visitors would be useful to infer thresholds for removal (Arstingstall et al., 2021). The method of preserving samples may also affect the success of the study (Liu et al., 2019). Whilst successful sequencing of pollen from historical specimens is possible (Gous et al., 2019), samples should be preserved quickly to avoid degradation of DNA. Most pollen metabarcoding studies have preserved samples by freezing at –20 °C, however, recent work by Quaresma et al., (2021) suggests that the use of silica gel for preserving pollen should not be overlooked, particularly when samples are collected by citizen scientists.

2.6.2. DNA extraction

Numerous DNA isolation methods exist which can influence the quality of the DNA template (Abdel-Latif and Osman, 2017; Swenson and Gemeinholzer, 2021). Membrane-based isolation techniques are most commonly used for pollen

metabarcoding studies, providing a fast and simple way of yielding DNA, although they are costly (Abdel-Latif and Osman, 2017). Regardless of the technique used, standard principles are followed: first the pollen cell wall (exine) is lysed to enable access to genomic material whilst preventing DNA degradation. Methods for pollen exine rupture can be chemical or mechanical, e.g., bead beating (the most common method) (Swenson and Gemeinholzer, 2021). This lysis step is followed by degradation of the cell membrane, removal of contaminants, and finally precipitation of DNA from protein. Prior to amplification, additional purification steps may be required to remove PCR inhibitors, a common step when using honey as a source of pollen (Jones et al., 2021a).

2.6.3. Amplification

The choice of barcode marker is regarded as one of the most important considerations of DNA barcoding studies and its applications, ultimately affecting the number of taxa recovered and the level of species discrimination gained (Jones et al., 2021b). DNA regions require high universality so that a large proportion of species in a sample are amplified, but also low intra-specific and high intra-specific variation for species discrimination (Hollingsworth et al., 2011). Short markers allow amplification of environmental DNA which is often degraded (Taberlet et al., 2012), however, come with a caveat of reduced taxonomic resolution (Richardson et al. 2019).

There is no one marker which meets the ideal requirements for a plant barcode, however, the standard markers are *rbcL* and *matK*, with *trnH-psbA* and ITS2 being used as additional markers for increased species discrimination (Hollingsworth et al., 2011). For pollen metabarcoding, five regions are commonly used: *rbcL*, ITS2, *matK*, *trnL* and *trnH-psbA* (Table S2.1, Supporting Information). A multi-locus approach is recommended to ensure the greatest number of taxa are identified (Bell et al., 2016; Jones et al., 2021a; Richardson et al., 2019). The length of *matK* (800

bp), restricts its use in metabarcoding due to limitations in read length on standard sequencing platforms (Jones et al., 2021b). Therefore, it is recommended that *rbcL* and ITS2 are used for pollen metabarcoding, due to their ability to identify the same taxa at varying taxonomic levels, or additional taxa unique to one marker which provides accurate identification of plant species within mixed pollen samples (Arstingstall et al., 2021; Jones et al., 2021a; Lowe et al., 2022).

Contamination may also occur in the laboratory; therefore, stringent cleaning procedures are required to minimise these risks. The use of controls (negative in extraction, positive and negative in PCR) helps identify sources of contamination. If sequences occur in negative controls, the number of reads of each taxon should be removed from all samples (Bell et al., 2017a).

2.6.4. Multiplexing and library preparation

The ability to scale up metabarcoding studies relies on the use of sample-specific labels in the form of unique sequences of nucleotides which are attached to amplicons. These unique identifiers allow hundreds or thousands of samples to be pooled for sequencing (multiplexing), significantly increasing the capacity of one sequencing run. Three methods exist for indexing of samples, occurring either during the initial PCR through nucleotide additions to amplicons or through a secondary PCR along with adapters to allow successful sequencing (library indices) (reviewed in Bohmann et al., 2021). Each of the three methods comes with trade-offs between many factors, mainly the risk of cross-contamination, efficiency of PCR and overall cost (Bohmann et al., 2021). The two-step PCR approach is most widely used in pollen metabarcoding studies (Table S2.1, Supporting Information), allowing a cost-effective approach to sample labelling whilst allowing effective detection of cross-contamination, but comes with the caveat of increased risk of biases due to an additional amplification stage (Bohmann et al., 2021).

2.6.5. Sequencing

Following amplification of DNA, the sequencing strategy used is dependent on a variety of factors including the choice of marker, with most studies thus far utilising Illumina MiSeq. Although concerns are raised over the read length of Illumina platforms (Evans and Kitson, 2020; Suchan et al., 2018), multiple studies have demonstrated successful sequencing of longer markers such as *rbcL* (~500 bp) along with additional adapters and primers (Potter et al., 2019). Newer sequencing technologies such as the MinION (Oxford Nanopore Technologies) and SMRT platform (PACBIO, Pacific Biosciences) produce longer read lengths, but they generate less reads than Illumina (Evans and Kitson, 2020). The development of ultra-deep short read sequencing technologies such as Illumina NovaSeq provide an opportunity to increase sequencing depth and improve the detection rate of taxa. The requirement for high quality and quantity of input DNA may be a limiting factor for some applications of these technologies (Peel et al., 2019).

2.6.6. Reference library

The accuracy of DNA barcoding is reliant on a comprehensive reference library (Geiger et al., 2016; Jones et al., 2021b). The creation of large-scale, national DNA barcode reference library for plants has been achieved in the UK (de Vere et al., 2012; Jones et al., 2021b) and Canada (Kuzmina et al., 2017) using a multi-locus approach, allowing reliable species identification in subsequent pollen metabarcoding studies (de Vere et al., 2017; Jones et al., 2021a). If a regional reference database is not available (Kress, 2017) then authors are encouraged to compile custom, relevant reference libraries using the sequences available in GenBank. Curation of these libraries is required however to identify incorrect sequences (Arstingstall et al., 2021; Biella et al., 2019; Elliott et al., 2021; Tommasi et al., 2021a). Nevertheless, it is important to understand the coverage of the reference library being used compared to the plant taxa that could be detected (Jones et al., 2021b).

2.6.7. Bioinformatic analysis

The quantity of data produced from DNA metabarcoding studies requires automated processes for curation of sequences, including steps for quality control. The main purpose of this process is to remove any additional nucleotide sequences (index tags and adapter tags) and to separate each sample for subsequent analysis (demultiplexing). Within the bioinformatic pipeline are standard steps, and packages such as DADA2 (Callahan et al., 2016) can be used for increased reproducibility. However, often, bespoke pipelines are required for different points in the workflow depending on the taxonomic group being studied and the questions addressed (Ford & Jones, 2020). The method used to demultiplex samples is dependent on the indices and sequencing platform used (Bohmann et al., 2021), however all require identification of the sample tag and amplicon primer for removal. The reduction of the need for expert taxonomists to identify pollen grains is often cited as one of the major advantages of molecular methods over pollen microscopy (Gous et al., 2019). However, few authors highlight the importance of knowledge of the taxonomic group in question (i.e., plants in pollen metabarcoding), including their distribution and phenology for accurate species identification (Cornman et al., 2015; de Vere et al., 2017; Jones et al., 2021a; Smart et al., 2017). Misidentifications may occur during the bioinformatic process due to low interspecific variance (Jones et al., 2021b) or incorrectly identified sequences in GenBank (Harris, 2003), therefore with a manual verification step in the assignment process, known spatiotemporal mismatches of species may be detected.

2.6.8. Towards standardisation of methods

Although each step of the pollen metabarcoding process has a range of different approaches, only certain elements of the entire pollen metabarcoding workflow have been reviewed (Swenson 2021; Bohmann 2021; Tommassi 2021), leaving a large proportion of the study design to the author's discretion. Without a

standardised approach to these methods, comparison of results across multiple studies must be interpreted with caution. Until each stage has been critically reviewed and a robust, standardised approach is established, we encourage researchers to carefully assess the considerations outlined in Table 2.2 for guidance prior to conducting a pollen metabarcoding study. Further, we call upon authors to be transparent in reporting every aspect of their molecular methods to ensure studies are reproducible, utilising supporting information where word limits are restricting.

2.6.9. How quantitative is DNA metabarcoding?

Finally, there is continued considerable debate over whether DNA metabarcoding may characterise pollen samples in a quantitative manner, with mixed results across studies (Bell et al., 2019; Keller et al., 2015; Polling et al., 2022; Pornon et al., 2016; Richardson et al., 2015a). Quantification has been found to be affected by a combination of marker and primer used, pollen type, mixture characteristics and PCR conditions (Baksay et al., 2020; Lamb et al., 2019; Piñol et al., 2019; Richardson et al., 2015b). It is likely that relationships between the proportion of DNA reads and pollen counts are more likely for the most abundant taxa within a sample (Bänsch et al., 2020; Smart et al., 2017). Rare taxa are difficult to detect, however, this is also the case for microscopy and for most questions asked, the most frequently used resources are most important (Hawkins et al., 2015). For this reason, along with the potential biases which could occur, DNA metabarcoding should be considered as semi-quantitative with the abundance of DNA reads treated as estimates of relative abundance (Deagle et al., 2019). We do not recommend the use of a presence/absence method due to rare taxa being overstated and abundant taxa devalued (Deagle et al., 2019).

Table 2.2: Key considerations required for each step of the pollen metabarcoding workflow.

Step	Description of method	Consideration	Notes
Sampling	Plant DNA can be captured through a number of sampling methods: 1. Pollen obtained from individuals collected on transects or within observational plots 2. Pollen obtained from within nest provisions 3. Leaf material obtained from within nest provisions 4. Pollen obtained from honey samples	Source of pollen influences information obtained	
		Capture methods influence the number and diversity of insects caught	
		Contamination may occur	Collect insects in sterile pots and replace nets if any pollen transfer is suspected
		Sampling period limits the knowledge which can be gained	
Sample preservation	Avoidance of DNA degradation	Preservation method may affect downstream success	Freeze samples as soon as possible to limit degradation of DNA
DNA extraction	Extraction of DNA from plant cells within pollen	Quantity of DNA obtained is affected by extraction method	
		Success of DNA extraction may depend on pollen type	
		Contamination may occur	Stringent cleaning procedures are required using 10% bleach solution before and after each process Use of filter tips Use of negative controls
		Membrane-based commercial kits offer a fast and simple way of yielding DNA, although are costly.	

Amplification	PCR amplification of extracted DNA using primers which target specific region of interest	Choice of marker will influence which taxa are recovered and their taxonomic resolution	We recommend a multi-locus approach using *rbcL* and ITS2
		Contamination may occur	Stringent cleaning procedures are required using 10% bleach solution before and after each process Use of filter tips Use of positive and negative controls
		Biases may be introduced through primer specificity	Complete three rounds of PCR per sample and pool
Multiplexing and library preparation	Addition of nucleotide sequences to primers to allow for pooling of samples and compatibility with sequencing platforms	Each method has a trade-off between multiple factors including overall cost, risk of contamination and PCR efficiency Tag-jumping can occur causing misidentification	Index strategy used should be based on research question and experimental set-up A two-step PCR approach allows cost effective indexing
Sequencing	Identification of nucleotide sequences	Sequencing strategy is dependent on choice of marker	Illumina MiSeq (2 x 300bp) allows sequencing of *rbcL* and ITS2
Reference library	Comparison of DNA sequences to a reference library for identification	Identifications made through DNA metabarcoding will only be as good as the reference library	Create a reference library which is appropriate to the question being asked
Bioinformatic analysis	Automated processes used to curate sequences for analysis including quality control	Species may be incorrectly assigned during automated processes	Requires manual verification steps by someone with knowledge of relevant plant taxa
		Metabarcoding data is considered to be semi-quantitative	Treat proportion of sequences as relative read abundance for analysis

2.7. Opportunities and future directions

DNA metabarcoding provides another tool to investigate pollinator foraging, however, is not free from limitations. Overall, the biggest limitation is the cost and reproducibility of the molecular techniques (Deiner et al., 2017), which determine which methods are used. The use of DNA metabarcoding as a tool has allowed increased insight into the interactions between plants and pollinators, however, it is still a developing field. Whilst the interpretation of data remains semi-quantitative, future work may lead to the ability to accurately measure pollen abundance, significantly improving the application of this technique (Lamb et al., 2019; Piñol et al., 2019). Ultimately, future work will rely heavily on whole genomes, however, coverage of eukaryotic organisms in reference libraries still remains low, and as assembly is very costly, it is likely that DNA metabarcoding will remain the standard technique (Bell et al., 2021). Until then, genome-skimming techniques may hold promise to identify beyond the species level e.g., to population or individual, if the nuclear genome is retained (Bohmann et al., 2020).

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2.9. Supporting Information

Table S2.1: Details of studies which use plant DNA metabarcoding to identify floral resource use by pollinators or developed methods to support.

Author	Year	Title	Country and Continent	Plant DNA source	DNA Region	Type of study	Question	Aim of study specifically related to metabarcoding
Arstingstall et al.	2021	Capabilities and limitations of using DNA metabarcoding to study plant-pollinator interactions	USA, North America	pollen from whole body	*rbcL* and ITS2	Specific methodological question	How do the results from metabarcoding and observation compare?	To assess whether specialisation and species richness differs between metabarcoding and observational networks?
Baksay et al.	2020	Experimental quantification of pollen with DNA metabarcoding using ITS1 and trnL	N/A	mock pollen samples	tnrL, ITS1	Specific methodological question	Is DNA metabarcoding quantitative?	To investigate the relationship between the amount of pollen grains in mock solutions and the abundance of sequence reads
Bänsch et al.	2020	Using ITS2 metabarcoding and microscopy to analyze shifts in pollen diets of honey bees and bumble bees along a mass-flowering crop gradient	Germany, Europe	pollen from legs	ITS2	Specific ecological question	How is foraging affected by resource availability?	To assess how honeybee and bumblebee foraging is affected by a mass-flowering crop
Bell et al.	2017	Applying Pollen DNA Metabarcoding to the Study of Plant–Pollinator Interactions	USA, North America	pollen from whole body	*rbcL*, ITS2	Method development		To develop methods for characterising networks via metabarcoding
Bell et al.	2019	Quantitative and qualitative assessment of pollen DNA metabarcoding using constructed species mixtures	N/A	mock pollen samples	*rbcL*, ITS2	Specific methodological question	Is DNA metabarcoding quantitative?	To test the quantitative and qualitative robustness of metabarcoding in constructed pollen mixtures
Biella et al.	2019	Foraging strategies are maintained despite workforce reduction: A multidisciplinary survey on the pollen collected by a social pollinator	Czech Republic, Europe	pollen from legs	ITS2	Specific ecological question	What is the relationship between plant use and pollinator health?	To investigate the foraging of *Bombus terrestris* in a situation of workforce loss
Bontšutšnaja et al.	2021	Bumble Bee Foraged Pollen Analyses in Spring Time in Southern Estonia Shows Abundant Food Sources	Estonia, Europe	pollen from legs	ITS2	Specific ecological question	How is foraging affected by resource availability?	To investigate whether pollen use change over sites and landscape?

Casanelles-Abella et al.	2021	How wild bees find a way in European cities:Pollen metabarcoding unravels multiple feeding strategies and their effects on distribution patterns in four wild bee species	France, Switzerland, Poland, Belgium and Estonia, Europe	pollen from nests	ITS2	Specific ecological question	How is foraging affected by resource availability?	To assess how diet varies across urban areas and whether diet specialisation is linked to distribution?
Chang et al.	2018	Molecular-assisted pollen grain analysis reveals spatiotemporal origin of long-distance migrants of a noctuid moth	China, Asia	pollen from proboscis	ITS, *rbcL*	Specific ecological question	How does foraging change over time and space?	To illuminate the host relationship and geographic origin of *Agrotis segetum* moths
Cornman et al.	2015	Taxonomic characterization of honey bee (Apis mellifera) pollen foraging based on non-overlapping paired-end sequencing of nuclear ribosomal loci	USA, North America	pollen from legs	ITS1, ITS2	Identifying floral resource use		To investigate pollen foraging by honeybees
Danner et al.	2017	Honey bee foraging ecology: Season but not landscape diversity shapes the amount and diversity of collected pollen	Germany, Europe	pollen from legs	ITS2	Specific ecological question	How does foraging change over time and space?	To investigate pollen foraging by honeybees across the year
de Vere et al.	2017	Using DNA metabarcoding to investigate honey bee foraging reveals limited flower use despite high floral availability	UK (Wales), Europe	pollen from honey	*rbcL*	Specific ecological question	How does foraging change over time and space?	To investigate pollen foraging by honeybees
Dew et al.	2020	Diverse Diets with Consistent Core Microbiome in Wild Bee Pollen Provisions	USA, North America	pollen from nests	*rbcL*	Specific ecological question	What is the relationship between plant use and pollinator health? & How does foraging change throughout time and space?	To assess how pollen species used varies across eastern US and whether this is linked to bacterial species present in larval provisions of a solitary bee, *Ceratina calcarata*
Donkersley et al.	2017	Nutritional composition of honey bee food stores vary with floral composition	UK (England), Europe	pollen from bee bread	ITS2	Specific ecological question	What is the relationship between plant use and pollinator health?	To identify the plant species in bee bread and analyse its nutritional value
Eeraerts et al.	2021	Landscapes with high amounts of mass-flowering fruit crops reduce the reproduction of two solitary bees	Belgium, Europe	pollen from nests	ITS2	Specific ecological question	How does foraging change over time and space?	To identify the diet of Osmia bees
Elliott et al.	2021	Pollen diets and niche overlap of honey bees and native bees in protected areas	Australia, Australia	pollen from legs	*rbcL*	Specific ecological question	How are resources partitioned between species and individuals in a plant-pollinator network?	To identify and compare the pollen diets of honeybees and native bees (species and genus level analysis)
Fahimee et al.	2021	Metabarcoding in diet assessment of Heterotrigona itama based on trnl marker towards domestication program	Malaysia, Asia	DNA extracted from whole body?	trnL	Identifying floral resource use		To identify the plant species foraged and used by *Heterotrigona itama*

Galimberti et al.	2014	A DNA barcoding approach to characterize pollen collected by honeybees	Italy, Europe	pollen from legs	*rbcL* and trnH-psbA	Method development		To assess the effectiveness of DNA barcoding to identify species from pollen collected by honeybees
Galliot et al.	2017	Investigating a flower-insect forager network in a mountain grassland community using pollen DNA barcoding	France, Europe	pollen from whole body	ITS2	Method development		To develop a method to track pollen transfer in a plant-pollinator network
Gous et al.	2019	Plant–pollinator interactions over time: Pollen metabarcoding from bees in a historic collection	South Africa, Africa	pollen from scopa	ITS1, ITS2, *rbcL*	Method development		To develop methods for obtaining metabarcodes from historic specimens
Gous et al.	2021	Floral hosts of leaf-cutter bees (Megachilidae) in a biodiversity hotspot revealed by pollen DNA metabarcoding of historic specimens	South Africa, Africa	pollen from scopa	ITS2	Specific ecological question	How does foraging change over time and space?	To investigate whether the floral choice of Megachilidae differs between two regions in Suth Africa (one diverse, one less-diverse)
Gresty et al.	2018	Flower preferences and pollen transport networks for cavity-nesting solitary bees: Implications for the design of agri-environment schemes	UK (England), Europe	pollen from nests	ITS2	Identifying floral resource use		To identify the key plant species used by cavity-nesting solitary bees and evaluate the suitability of forage resource provision in UK agri-environment schemes
Hawkins et al.	2015	Using DNA metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences	UK (Wales and England), Europe	pollen from honey	*rbcL*	Specific methodological question	How do the results from metabarcoding and microscopy compare?	To assess the potential of using DNA metabarcoding to characterise the floral composition of honey in order to investigate honey bee foraging
Jones et al.	2021	Shifts in honeybee foraging reveal historical changes in floral resources	UK (England, Wales, Scotland, Ireland), Europe	pollen from honey	ITS2, *rbcL*	Specific ecological question	How does foraging change over time and space?	To compare honeybee foraging between 1952 and 2017
Kaluza et al.	2017	Generalist social bees maximize diversity intake in plant species-rich and resource-abundant environments	Australia, Australia	pollen from legs	ITS2	Method development		To validate colour assessment of pollen loads collected by *Tetragonula carbonaria*
Kamo et al.	2018	A DNA barcoding method for identifying and quantifying the composition of pollen species collected by european honeybees, apis mellifera (Hymenoptera: Apidae)	Japan, Asia	pollen from legs	trnL	Specific ecological question and method development	How does foraging change over time and space?	To develop a DNA barcoding method for identifying species in honeybee pollen and to demonstrate seasonal changes in species composition

Keller et al.	2015	Evaluating multiplexed next-generation sequencing as a method in palynology for mixed pollen samples	Germany, Europe	pollen from legs & pollen from nests	ITS2	Method development		To develop methods for next-gen sequencing of mixed pollen samples and bioinformatic workflow
Klecka et al.	unpublished	Individual-level specialisation and interspecific resource partitioning in bees revealed by pollen DNA metabarcoding	Czech Republic, Europe	pollen from nests	ITS2	Specific ecological question	How are resources partitioned between species and individuals in a plant-pollinator network?	To assess whether three species of *Ceratina* bee differ in their foraging preferences (species and individual level analysis)
Kratschmer et al.	2020	Pollen availability for the Horned mason bee (Osmia cornuta) in regions of different land use andlandscape structures	Austria, Europe	pollen from nests	trnL	Specific ecological question	How does foraging change over time and space?	To use metabarcoding as a supplement to microscopy to confirm taxa
Leidenfrost et al.	2020	Analyzing the Dietary Diary of Bumble Bee		pollen from legs	ITS2	Identifying floral resource use and method development		To ientify pollen source species of B. terrestris in agricultural landscapes using MinION
Lu et al.	2021	Metabarcoding Analysis of Pollen Species Foraged by Osmia excavata Alfken (Hymenoptera: Megachilidae) in China	China, Asia	pollen from nests	ITS2	Specific ecological question	How is foraging affected by resource availability?	To identify pollen plants of *Osmia excavata* over farmland, semi-natural habitats and orchards
Lucas et al.	2018	Floral resource partitioning by individuals within generalised hoverfly pollination networks revealed by DNA metabarcoding	UK (Wales), Europe	pollen from whole body	*rbcL*	Specific ecological question	How are resources partitioned between species and individuals in a plant-pollinator network?	To investigate pollen transport in hoverfly communities (species and genus level analysis)
Lucas et al.	2018	Generalisation and specialisation in hoverfly (Syrphidae) grassland pollen transport networks revealed by DNA metabarcoding	UK (Wales), Europe	pollen from whole body	*rbcL*	Specific ecological question	How are resources partitioned between species and individuals in a plant-pollinator network?	To investigate pollen transport network of Eristalis hoverflies (species and individual level analysis)
Lucek et al.	2019	Metabarcoding of honey to assess differences in plant-pollinator interactions between urban and non-urban sites	Switzerland, Europe	pollen from honey	ITS2	Specific ecological question	How is foraging affected by resource availability?	To investigate honeybee foraging in urban and non-urban sites
Macgregor et al.	2019	Construction, validation and application of nocturnal pollen transport networks in an agro-ecosystem: a comparison using microscopy and DNA metabarcoding	UK (England), Europe	pollen from proboscis	*rbcL*	Specific methodological question and identifying floral resource use	How do the results from metabarcoding and microscopy compare?	To construct nocturnal pollination networks and compare microscopy and metabarcoding

MacIvor	2016	DNA barcoding to identify leaf preference of leafcutting bees	Canada, North America	leaf tissue from nests	ITS2, *rbcL*	Identifying floral resource use		To identify the leaf preference of leafcutter bees (*Megachile* spp.) and assess whether they rely on related species
Mayr et al.	2021	Cryptic species and hidden ecological interactions of halictine bees along an elevational gradient	Tanzania, Africa	pollen from crop	ITS2	Specific ecological question	How does foraging change over time and space?	To assess whether pollen composition changes across an elevational gradient
McFrederick & Rehan	2016	Characterization of pollen and bacterial community composition in brood provisions of a small carpenter bee	USA, North America	pollen from nests	*rbcL*	Specific ecological question	What is the relationship between plant use and pollinator health?	To investigate the relationship between pollen and bacteria in pollen provisions of a solitary bee, *Ceratina calcarata*
McFrederick & Rehan	2019	Wild Bee Pollen Usage and Microbial Communities Co-vary Across Landscapes	Australia, Australia	pollen from nests	ITS, *rbcL*	Specific ecological question	What is the relationship between plant use and pollinator health? & How does foraging change throughout time and space?	To assess how pollen composition within larval provisions of the small carpenter bee *Ceratina australensis* varies across habitat and whether pollen composition correlates with microbial community composition?
McMinn-Sauder	2020	Flowers in Conservation Reserve Program (CRP) Pollinator Plantings and the Upper Midwest Agricultural Landscape Supporting Honey Bees	USA, North America	pollen from legs	ITS2, *rbcL*, trnL	Identifying floral resource use		To identify floral resources most valuable to honeybees and also to assess the extent of seed mix utilisation
Michelot-Antalik et al.	2021	Comparison of grassland plant-pollinator networks on dairy farms in three contrasting French landscapes	France, Europe	pollen from whole body	ITS2	Specific ecological question	How does resource availability affect foraging?	To supplement the use of pollen microscopy to identify how floral resource use is affected by availability
Muller & Richter	2018	Dual function of Potentilla (Rosaceae) in the life history of the rare boreoalpine osmiine bee Hoplitis (Formicapis) robusta (Hymenoptera, Megachilidae)	Switzerland, Europe	leaf tissue from nests	ITS2, trnL	Identifying floral resource use		To identify the larval diet and nest building material of *Hoplitis robusta*
Muller et al.	2019	Nesting in bark – the peculiar life history of the rare boreoalpine osmiine bee Osmia (Melanosmia) nigriventris (Hymenoptera, Megachilidae)	Switzerland, Europe	leaf tissue from nests	ITS2, trnL	Identifying floral resource use		To identify the source of nest building material used by *Osmia nigriventris*

Noël et al.	unpublished	Pollen meta-barcoding reveals foraging preferences of honeybees (Apis mellifera L.) along space-time gradient in Japan	Japan, Asia	pollen from legs	ITS1	Specific ecological question	How does foraging change over time and space? & How is foraging affected by resource availability?	To explore the temporal and spatial foraging habits of *Apis mellifera*
Nurnberger et al.	2019	Honey bee waggle dance communication increases diversity of pollen diets in intensively managed agricultural landscapes	Germany, Europe	pollen from legs	ITS2	Other		To study how dance communication affects the diversity of pollen diets
Park et al.	2017	Seasonal trends in honey bee pollen foraging revealed through DNA barcoding of bee-collected pollen	USA, North America	pollen from legs	matK, *rbcL*	Specific ecological question	How does foraging change over time and space?	To determine the seasonal patterns in colony recruitment dancing for pollen
Piko et al.	2021	Effects of three flower field types on bumblebees and their pollen diets	Germany, Europe	pollen from whole body	ITS2	Identifying floral resource use		To assess the use of flower fields (used in agri-environment schemes)
Piot et al.	2021	More is less: mass-flowering fruit tree crops dilute parasite transmission between bees	Belgium, Europe	pollen from nests	ITS2	Specific ecological question	What is the relationship between plant use and pollinator health?	Do investigate whether parasites are transmitted between *Osmia* nests through the visitation of mass-flowering crops?
Pornon et al.	2016	Using metabarcoding to reveal and quantify plant-pollinator interactions	France, Europe	mock pollen samples, & pollen from whole body	trnL, ITS1	Specific methodological question	Is DNA metabarcoding quantitative?	To investigate the relationship between trnL and ITS1 sequences yielded from mock samples and whether sequences are quantitative from insect loads
Pornon et al.	2017	DNA metabarcoding data unveils invisible pollination networks	France, Europe	pollen from whole body	trnL, ITS1	Specific methodological question	How do the results from metabarcoding and observation compare?	To compare metabarcoding to visit surveys to detect links
Pornon et al.	2019	Pollinator specialization increases with a decrease in a mass-flowering plant in networks inferred from DNA metabarcoding	France, Europe	pollen from whole body	ITS1, trnL	Specific ecological question	How are resources partitioned between species and individuals in a plant-pollinator network?	To investigate the effects of mass-flowering plants on pollination networks (species and individual level analysis)
Potter et al.	2019	Pollen metabarcoding reveals broad and species-specific resource use by urban bees	UK (England), Europe	pollen from whole body	*rbcL*	Identifying floral resource use		To investigate the foraging preferences of bees feeding in sown wildflower strips

Quinlan et al.	2021	Honey bee foraged pollen reveals temporal changes in pollen protein content and changes in forager choice for abundant versus high protein flowers	USA, North America	pollen from legs	ITS2	Specific ecological question	How does foraging change over time and space? & How is foraging affected by resource availability?	To investigate how pollen protein changes over space (colonies,apiaries and land-use categories) and time (annually and seasonally) by estimating protein content through identifying plant taxa. To assess the relationship between floral abundance and collected plants.
Richardson et al.	2015	Application of ITS2 Metabarcoding to Determine the Provenance of Pollen Collected by Honey Bees in an Agroecosystem	USA, North America	pollen from legs	ITS2	Method development		Method development for honey metabarcoding
Richardson et al.	2015	Rank-based characterization of pollen assemblages collected by honey bees using a multi-locus metabarcoding approach	USA, North America	pollen from legs	ITS2, matK, *rbcL*	Specific methodological question	Is DNA metabarcoding quantitative?	To improve understading of the quantitative capacity of barcode regions and primer sets
Richardson et al.	2019	Quantitative multi-locus metabarcoding and waggle dance interpretation reveal honey bee spring foraging patterns in Midwest agroecosystems	USA, North America	pollen from legs	ITS2, *rbcL*, trnL, trnH-psbA	Specific ecological question	How does foraging change over time and space?	To investigate pollen foraging by honeybees across space
Richardson et al.	2021	Application of plant metabarcoding to identify diverse honeybee pollen forage along an urban–agricultural gradient	USA, North America	pollen from legs	ITS2, *rbcL*, trnL	Specific ecological question	How is foraging affected by resource availability?	To investigate how foraging relates to landscapes of varying urbanisation?
Sickel et al.	2015	Increased efficiency in identifying mixed pollen samples by meta-barcoding with a dual-indexing approach		pollen from nests	ITS2	Method development		To develop protocol for highly multiplexed pollen sequencing utilising a dual-indexing strategy
Simanonok et al.	2021	A century of pollen foraging by the endangered rusty patched bumble bee (Bombus affinis): inferences from molecular sequencing of museum specimens	USA, North America	pollen from legs	ITS2	Specific ecological question	How does foraging change over time and space?	To construct a historical foraging profile of Bombus affinis from 1913-2013 using historical specimens
Smart et al.	2017	A comparison of honey bee-collected pollen from working agricultural lands using light microscopy and its metabarcoding	USA, North America	pollen from legs	ITS1, ITS2	Specific ecological question	How is foraging affected by resource availability?	To investigate pollen use over a land use gradient and to compare microscopy and metabarcoding
Sponsler et al.	2020	A screening-level assessment of the pollinator-attractiveness of ornamental nursery stock using a honey bee foraging assay	USA, North America	pollen from legs	ITS2	Identifying floral resource use		To assess whether ornamental plants are attractive to honeybees?

Sponsler et al.	2020	Characterizing the floral resources of a North American metropolis using a honey bee foraging assay	USA, North America	pollen from legs	ITS1, ITS2, trnL	Specific ecological question	How does foraging change over time and space?	To use pollen samples to monitor resource use and availability over time and space
Suchan et al.	2018	Pollen metabarcoding as a tool for tracking long-distance insect migrations	Spain, Europe	pollen from whole body	ITS2	Specific ecological question	How does foraging change over time and space?	To investigate migration patterns through the identification of pollen from *Vanessa cardui*
Swenson & Gerneinholzer et al.	2021	Testing the effect of pollen exine rupture on metabarcoding with Illumina sequencing	N/A	mock pollen samples	ITS1, ITS2, *rbcL*	Specific methodological question	How do varying DNA metabarcoding methods affect results?	To investigate the effect of exine rupture and lysis incubation time on extraction and sequencing
Tanaka et al.	2020	Using pollen DNA metabarcoding to profile nectar sources of urban beekeeping in Kōtō-ku, Tokyo	Japan, Asia	pollen from honeycomb	*rbcL*	Identifying floral resource use		To investigate pollen foraging by honeybees
Tommasi et al..	2021	Impact of land use intensification and local features on plants and pollinators in Sub-Saharan smallholder farms	Tanzania, Africa	pollen from whole body	ITS2	Specific ecological question	How is foraging affected by resource availability?	To investigate how plant-pollinator assemblages are shaped by land use intensification?
Tremblay et al.	2019	High-resolution biomonitoring of plant pathogens and plant species using metabarcoding of pollen pellet contents collected from a honey bee hive	Canada, North America	pollen from legs	ITS	Method development		To develop methods for using pollen pellet contents to monitor plant pests
Trinkl et al.	2020	Floral species richness correlates with changes in the nutritional quality of larval diets in a stingless bee	Australia, Australia	pollen from nests	ITS2	Specific ecological question	What is the relationship between plant use and pollinator health?	To investigate whether differences in plant species richness correlate with variation in floral diversity and nutritional quality of larval provisions in *Tetragonula carbonaria*
Valentini et al.	2010	DNA barcoding for honey biodiversity	N/A	pollen from honey	trnL	Method development		Method development for honey metabarcoding
Vaudo et al.	2020	Introduced bees (Osmia cornifrons) collect pollen from both coevolved and novel host-plant species within their family-level phylogenetic preferences	USA, North America	pollen from nests	ITS2	Identifying floral resource use		To assess whether Osmia cornifrons has a preference for plants which are found in native range, or a phylogenetic affinity which is independent of the geographic origin of host plant

Voulgari-Kokota et al.	2019	Linking pollen foraging of megachilid bees to their nest bacterial microbiota	Germany, Europe	pollen from nests	ITS2	Specific ecological question	What is the relationship between plant use and pollinator health?	To investigate the relationship between pollen and bacteria in pollen provisions of a range of solitary bee species
Wilson et al.	2010	Pollen foraging behaviour of solitary Hawaiian bees revealed through molecular pollen analysis	USA, North America	pollen from crop	ITS	Identifying floral resource use and method development		To identify floral resource use of *Hylaeus* bees
Wilson et al.	2021	Many small rather than few large sources identified in long-term bee pollen diets in agroecosystems	Australia, Australia	pollen from nests	ITS2, *rbcL*	Specific ecological question	How does foraging change over time and space? & How is foraging affected by resource availability?	To assess how foraging of *Tetragonula carbonaria* changes over time and land use and to improve the understanding of its diet

Chapter Three

Gardening for pollinators: DNA metabarcoding shows seasonal progression and differences in major floral resource use in bees and hoverflies

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Lowe, A.[1,2], Jones, L.[1], Brennan, G.[3], Creer, S.[2], & Vere, N.[4] (2022). Seasonal progression and differences in major floral resource use by bees and hoverflies in a diverse horticultural and agricultural landscape revealed by DNA metabarcoding. *Journal of Applied Ecology*. https://doi.org/10.1111/1365-2664.14144

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The study was conceived by A.L., N.dV., and S.C. Data collection and lab work was carried out by A.L. The data were compiled by A.L. and analysed by A.L. and L.J. with suggestions from N.dV., S.C. and G.B. The manuscript was written by A.L. with contributions from all the authors.

3.1. Abstract

Gardens are important habitats for pollinators, providing floral resources and nesting sites. There are high levels of public support for growing 'pollinator-friendly' plants but whilst plant recommendation lists are available, they are usually inconsistent, poorly supported by scientific research and target a narrow group of pollinators. In order to supply the most appropriate resources, there is a clear need to understand foraging preferences, for a range of pollinators, across the season within garden environments. Using an innovative DNA metabarcoding approach, we investigated foraging preferences of four groups of pollinators in a large and diverse, garden landscape, across the flowering season and over two years, significantly improving on the spatial and temporal scale that can be achieved using observational studies. Bumblebees, honeybees, non-corbiculate bees, and hoverflies visited 191 plant taxa. Overall floral resources were shared between the different types of pollinators, but significant differences were seen between the plants used most abundantly by bees (Hymenoptera) and hoverflies (Diptera). Floral resource use by pollinators is strongly associated with seasonal changes in flowering plants, with pollinators relying on dominant plants found within each season, with preferences consistent across both years. The plants identified were categorised according to their native status to investigate the value of native and non-native plants. The majority of floral resources used were of native and near-native origin, but the proportion of horticultural and naturalised plants increased during late summer and autumn. We recommend that plant lists should distinguish between bees and hoverflies as pollinators and provide evidence-based floral recommendations throughout the year that include native as well as non-native plants for use in the UK and Northern Europe. Specific management recommendations include reducing mowing to encourage plants such as dandelion *Taraxacum officinale,* buttercups *Ranunculus* spp., and reducing scrub management to encourage bramble *Rubus fruticosus*.

3.2. Introduction

The decline in pollinating insects is well documented globally, leading to potentially severe impacts on floristic biodiversity and human health due to the loss of pollination ecosystem services (Klein et al., 2007; Lundgren et al., 2016; Smith et al., 2015). Pollinator declines have occurred due to a combination of habitat loss, climate change, pests and diseases and the use of pesticides (Potts et al., 2010). As the availability of floral resources limits pollinators (Goulson et al., 2015), understanding foraging preferences is a key knowledge need for their effective conservation.

Gardens are important, heterogenous habitats, covering significant areas in urban landscapes (Loram et al., 2007). Gardens can provide pollinators with pollen, nectar, and nesting sites (Osborne et al., 2008), supporting pollinators in agricultural (Timberlake et al., 2020) and urban (Potter et al., 2019) settings whilst increasing habitat connectivity within the landscape (Goddard et al., 2009).

The limited number of studies in the UK (Wignall et al., 2019) and elsewhere in Northern Europe (Schonfelder & Bogner, 2017) on the public perception of pollinators suggests that attitudes towards their conservation is very positive. However, whilst there is a wealth of information available on the best plants for pollinators, only a small number of recommendation lists are based on empirical evidence (Garbuzov & Ratnieks, 2014), with most plants sold in UK garden centres relatively unattractive to flower-visiting insects (Garbuzov et al., 2017). Moreover, these lists broadly target pollinators, leading to generalisation across a wide range of functional groups and species.

Consequently, there is a clear need to provide scientific evidence for effective floral use in gardens to support pollinators. Although foraging can vary between

pollinator groups (Bänsch et al., 2020), most studies in gardens focus on a single group (de Vere et al., 2017). Honeybees and bumblebees are the most frequently studied, however, non-corbiculate bees and hoverflies have important roles in pollination and ecosystem function (Klein et al., 2007). Additionally, seasonality and annual variation can influence forage choice (Petanidou et al., 2014), highlighting the need to provide information on floral use throughout the year.

There are conflicting perspectives as to whether native or non-native plants are preferred by pollinators (Salisbury et al., 2015), but it is imperative to understand this for effective conservation. When surveying pollinator visits to a variety of plants, Salisbury et al. (2015) found a greater abundance of pollinators on native and near-native taxa than those defined as exotic. Additionally, introduced plant species have been shown to attract fewer species of flower visitors than natives and those closely related to natives (Memmott & Waser, 2002).

DNA metabarcoding has been used to identify pollen within honey (de Vere et al., 2017; Jones et al., 2021a), from the bodies of insects (Lucas et al., 2018b; Richardson et al., 2021), and from brood provision in nests (Vaudo et al., 2020). The advantages of pollen metabarcoding approaches include increased taxonomic resolution (Brennan et al., 2019) and the elimination of the taxonomic expertise required for pollen microscopy (Hawkins et al., 2015). DNA metabarcoding overcomes the limitations of observational methods by revealing interactions previously unseen due to spatial and temporal limitations (Arstingstall et al., 2021), however, it must be accompanied by a comprehensive reference library to ensure accurate identification. In the UK, the Barcode UK project provides 98% coverage of all native flowering plants and conifers using three plant DNA barcode markers, *rbcL*, matK and ITS2, allowing reliable identification at the species and genus level for the majority of plants (de Vere et al., 2012; Jones et al., 2021b).

3.2.1 Aims and Objectives

This study identifies plants used by pollinating insects in an extensive, well characterised, and complex garden landscape, using a multi-locus (*rbcL* and ITS2) pollen DNA metabarcoding approach. We specifically answer the following questions:

Do Diptera (hoverflies) and Hymenoptera (bees) have distinct foraging preferences?

a) How does foraging differ between broad pollinator groups (bumblebees, honeybees, non-corbiculate bees, and hoverflies)?
b) Do ecological functional categories within these groups (related to tongue length in bumblebees, body size in non-corbiculate bees and larval requirements in hoverflies) affect the plant taxa used?
c) How does foraging change over the flowering season and year?
d) Do pollinators prefer native or non-native plants?

The results are used to present novel recommendations for gardeners, landowners and conservation organisations based on time resolved, empirical data, to support pollinator populations and ensure effective conservation.

3.3. Materials and methods

3.3.1. Insect sampling

Bees and hoverflies were sampled monthly from March to October during 2018 and 2019 at the National Botanic Garden of Wales, UK (51°50'33.4"N 4°08'49.2"W). The site is a diverse landscape (230 ha) set within a predominately semi-improved (based on the extent of agricultural improvement) landscape and consists of formal garden and organic farmland, designated as a National Nature Reserve (Waun Las NNR) (Fig. 3.1). The Botanic Garden contains over 5000 plant taxa from throughout the world, including many horticultural plants grown throughout Western Europe. Eight areas were selected for pollinator sampling covering broadleaved woodland and hedgerows, horticultural, and grassland habitat. These areas were selected in order to capture pollinators in a diversity of habitats. Within each sampling area, a 210 m x 2 m transect was established and divided into 3 x 70 m sections, walked independently of each other. Transect walks were preferentially undertaken between 11:00 and 15:00 when the temperature was over 10 °C. When this was not possible, transects were walked on dry days with little wind. All bees and hoverflies seen on the transect were caught individually and stored at -20 °C prior to pollen removal. Further information on field sampling is provided in Supporting Information.

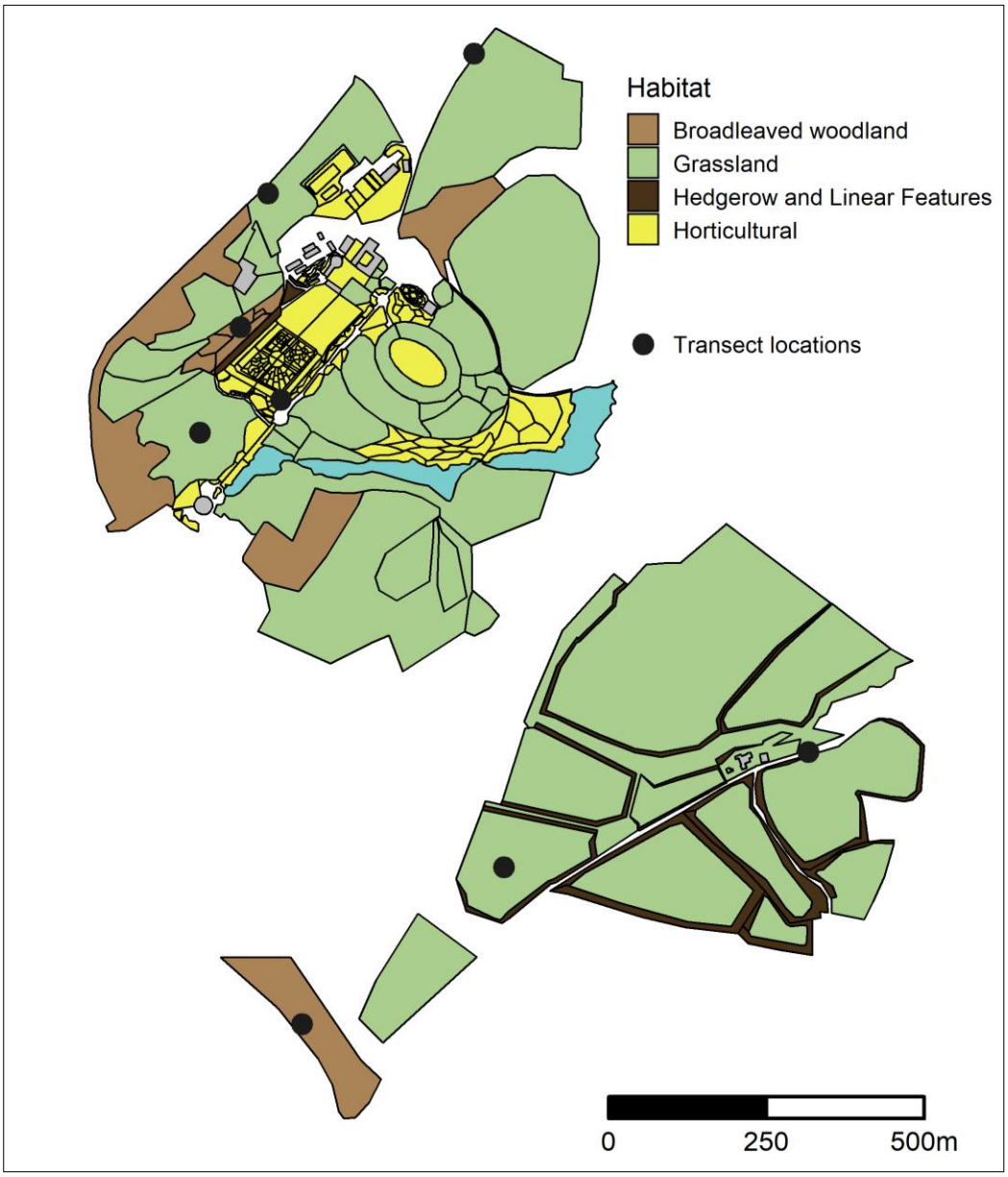

Figure 3.1: Habitat map of the National Botanic Garden of Wales and Waun Las National Nature Reserve showing location of transects where pollinators were collected. The grassland in the study site is mainly composed of semi-improved grassland and lowland hay meadows. Maps were created in QGIS v. 3.6.1 and R v. 4.0.2 from OS data © Crown Copyright (2021) licensed under the Open Government Licence.

3.3.2. Pollen removal

Pollen was washed from insects following a modified version of the protocol described by Lucas et al. (2018b). Insects were first transferred to a sterile 1.5 ml collection tube using sterile forceps and cleaned with 70% ethanol between each insect. The tube used to catch insects was washed with 1 ml of 1% sodium dodecyl sulphate (SDS) and 2% polyvinylpyrrolidone (PVP) solution, ensuring any pollen residue on the sides was collected and transferred to the tube containing the insect. Samples were shaken using a TissueLyser II (Qiagen) for 1 minute at 8.5 Hz, stood at room temperature for 5 minutes, then shaken again for 20 seconds at 8.5 Hz. Each insect was removed using sterile forceps and placed into a 1.5 ml microcentrifuge tube containing 70% ethanol, prior to species identification (see Taxonomic assignment of insects, Supporting Information). The tube containing the detergent and pollen pellet was centrifuged at 13000 rpm for 5 minutes and the supernatant removed. The pollen pellet was resuspended in 400 µl buffer, made up of 400 µl AP1 from the DNeasy 96 Plant Kit (Qiagen) and 80 µl (1 mg/ml) of Proteinase K (Qiagen).

3.3.3. DNA extraction

A modified version of the DNeasy 96 Plant Kit was used for DNA extraction. Samples were incubated in a water bath at 65 °C for 1 hour and 1 µl RNase (Qiagen) added before disruption using a TissueLyser II for 4 minutes at 30 Hz with 3 mm tungsten carbide beads. The remaining steps were carried out according to the manufacturer's protocol, excluding the use of the QIAshredder and the second wash stage. A negative control was included within each extraction.

3.3.4. Amplification and sequencing

Two barcode regions, *rbcL* and ITS2 were amplified via a two-step PCR protocol (de Vere et al., 2017) (Table S3.1, Supporting Information). The initial PCR used a final volume of 20 µl: 2 µl template DNA, 10 µl of 2x Phusion Hot Start II High-Fidelity Mastermix (New England Biolabs UK), 0.4 µl (2.5 µM) forward and reverse primers, and 7.2 µl of PCR grade water. Each PCR was repeated twice more and pooled before purification using the Illumina 16S metabarcoding protocol, with a 1:0.6 ratio of product to Agencourt AMPure XP beads (Beckman Coulter). The purified product was amplified further to anneal custom unique and matched i5 and i7 indices to each sample (Ultramer, Integrated DNA Technologies). This second stage PCR used a final volume of 25 µl: 5 µl of purified first-round PCR product, 12.5 µl of 2x Phusion Hot Start II High-Fidelity Mastermix (New England Biolabs UK), 1 µl of i5 and i7 Index Primer, and 6.5 µl of PCR grade water. All thermal cycling conditions are available in Supporting Information. Tag addition was confirmed with visualisation on a 1% agarose gel. A second Illumina clean-up stage was followed with a 1:0.8 ratio of product to beads. Products were quantified using a Qubit 4.0 (Thermo Fisher Scientific) and pooled at equal concentrations. The negative extraction and PCR controls from each plate were sequenced with the pollen samples on an Illumina MiSeq (2 x 300 bp). Laboratory contamination controls can be found in Supporting Information.

3.3.5. Sequence analysis

Sequence reads were processed following Ford & Jones (2020). Initially, raw sequences were trimmed to remove low quality regions, paired, and merged. Only sequences greater than 450 bp (*rbcL*) and 350 bp (ITS2) were used in downstream analysis. Identical reads were dereplicated within each sample and clustered at 100% identity across all samples with singletons (sequence reads occurring once across all samples) removed. Sequences were compared to a custom reference

library containing 5,887 plant species (Jones et al., 2021a), comprising native plants of the UK (Stace, 2019), naturalised and alien species (Preston et al., 2002) and horticultural species from the IRIS BG database at the National Botanic Garden of Wales.

3.3.6. Assigning taxonomic classifications

Sequences were compared against the reference library using blastn, summarising the top 20 BLAST hits and combining all sequences with identical BLAST results across all 20 hits. Sequences with bit scores below the 1st percentile were excluded. Sequences were assigned so that if the top bitscore matched a plant species, the sequence was assigned to that species. If the top bitscore matched different species within the same genus, the sequence was assigned to that genus. If the top bitscore belonged to multiple genera of the same family, then a family designation was made for that sequence. Sequences returning top bitscores of multiple families within different orders were removed, assuming that these were poor-quality sequences. The botanical veracity of the plants identified across all insect samples was assessed by considering whether those plants were present within the study site and wider landscape. Taxonomic assignment of sequences was compared between markers on a sample-by-sample basis for further verification.

Once the identifications were complete, a consensus identification was reached to combine the taxa identified by both markers at differing taxonomic resolution using a rule-based, objective, and conservative decision process (see Using *rbcL* and ITS2 markers, Supporting Information). The number of *rbcL* and ITS2 sequences for each consensus taxon within a sample were then summed to combine the results of each marker. Sequences assigned to taxa identified using one marker alone were retained. Plants identified to genus and species were assigned to a status category following Stace (2019). The category 'native and near native' comprised native species and also genera that include native species and

horticultural varieties which are functionally similar. Naturalised plants were those which have been introduced and become widespread and self-perpetuating in the wild. All remaining non-native plants were classified as horticultural.

3.3.7. Statistical analysis

The DNA metabarcoding data was treated as semi-quantitative with relative read abundance used for all analyses (Deagle et al., 2019), either using the proportion of taxa as a percentage or, for the models, the number of sequences, controlling for sequencing depth by setting the total number of sequences per sample as an offset (comparable to proportion) (Jones et al., 2021a) (Supporting Information).
Using the package 'mvabund' (Wang et al., 2012), a multivariate generalised linear model with a negative binomial distribution was used to understand how pollen load composition changed through time. The data best fit a negative binomial distribution due to the strong mean-variance relationship (Fig. S3.1, Supporting Information), likely from distributions of rare taxa where mean abundance is low, a common observation in multivariate abundance data.

To understand the effect of time and pollinator type on plant composition, the effect of season (coded as 1-3, starting with spring), year, and pollinator group/order were included as predictor variables, with the number of sequence reads for each plant taxon set as the multivariate response variable. The number of reads per sample was included as an offset to control for differences in sampling depth (Deagle et al., 2019; Jones et al., 2021a). Seasonal changes in the composition of pollen loads were visualised using non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarity indices (based on the proportion of reads returned for each plant taxa), using the vegan package (Dixon, 2003). A chi-squared contingency test was used to investigate differences in major taxa (constituting over 5% of sequences) between pollinator orders (based on the relative read abundance overall), with Holm correction for multiple testing. Each

pollinator group was split into categories based on a unique ecological functional trait (see Functional diversity analysis, Supporting Information) and chi-squared contingency tests were used to investigate differences in taxa constituting over 1% of sequences between functional categories within broader groups.

To investigate the change in use of native plants over time, the plant taxa were grouped by their status categories. A multivariate generalised linear model was run, with season and year included as predictor variables and the response variable being the number of reads, retaining the use of the offset. All statistical analyses were carried out in R v 4.0.2 using the consensus identification. Analysis of *rbcL* and ITS2 was also carried out separately to support the use of combining markers (Supporting Information).

3.4. Results

3.4.1. Overview

Throughout the study, 382 insects were caught with successful sequencing of pollen from 369 individuals (Table 3.1). No insects were caught in October despite surveys being carried out. Pollinators were grouped into hoverflies (Syrphidae, n =195), bumblebees (Bombus spp., n = 108), honeybees (*Apis mellifera*, n = 44) and all other non-corbiculate bees (n = 22) (Table S3.2, Supporting Information). A total of 40,800,709 reads were returned with 22,510,682 remaining after stringent quality control (11,305,697 *rbcL* and 11,204,985 ITS2). Using the *rbcL* and ITS2 regions combined, 191 plant taxa were identified with the majority of taxa identified at genus level. Six taxa were found on over 50% of insects sampled: bramble (*Rubus* spp.), thistles, knapweeds, and cat's ear (*Cirsium*/*Centaurea*/*Hypochaeris* spp.), buttercups and lesser celandine (*Ranunculus*/*Ficaria* spp.), angelica and hogweed (*Angelica*/*Heracleum* spp.), daisy family Asteraceae, and meadowsweet (*Filipendula ulmaria*) (Fig. 3.2). An average of 17 (SD = 9.76) plant taxa were found on each individual insect with an average of 4 (SD = 2.55) taxa contributing >1% of reads (Table 3.1).

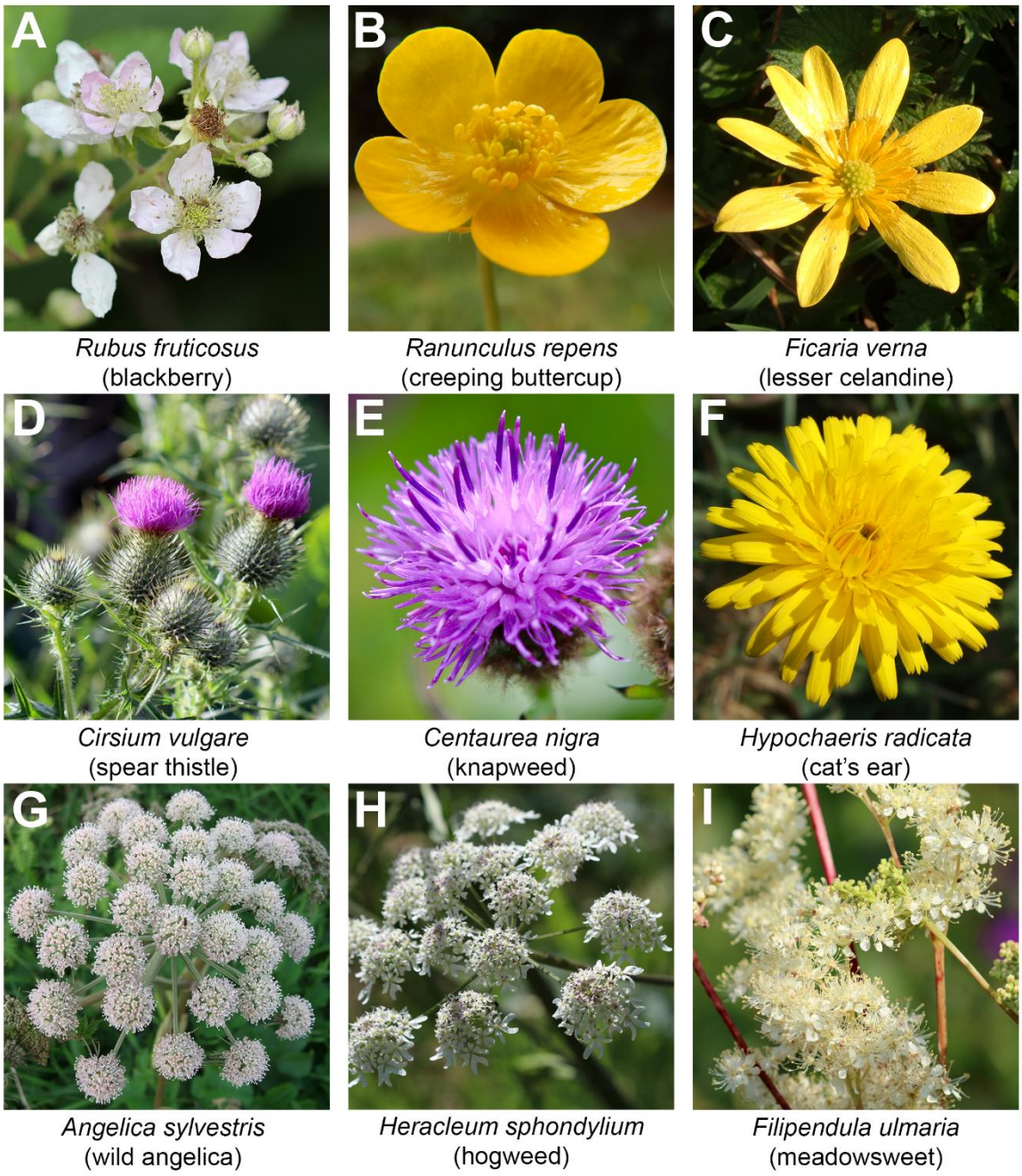

Figure 3.2: Plant taxa found in over 50% of pollen samples retrieved from pollinators. Those plants identified to species or genus level only are illustrated, with species given as an example of taxa represented. (A, D, E, H, I) by Natasha de Vere All rights reserved (B) by Matt Lavin CC BY-SA 2.0 (C, F, G) by Bruce Langridge All rights reserved. All images have been cropped and adjusted.

Table 3.1: Summary of the number of insects sampled along with the mean and standard deviation (SD) of plant taxa identified from the pollen on their bodies.

Order	Group	Successfully sequenced	Sequencing success rate (%)	Number of pollinator species (successful samples)	Mean number of plant taxa identified per individual	Mean number of plant taxa identified per individual (>1% sequence reads)	Plant taxa unique to group (%)
Diptera	Hoverfly (Syrphidae)	195	95.1	41	14 (SD = 7.76)	4 (SD = 2.45)	11.7
Hymenoptera	Bumblebee (*Bombus* spp.)	108	100	6	21(SD = 11.31)	4 (SD = 2.72)	5.8
	Honeybee (*Apis mellifera*)	44	97.8	1	20 (SD = 9.20)	3 (SD = 2.77)	4.3
	Non-corbiculate bee (*Andrena, Lasioglossum, Halictus, Nomada*)	22	91.7	9	13 (SD = 8.30)	4 (SD = 2.04)	0.0

3.4.2. Variation in foraging between pollinators

Overall, we found little variation in foraging habits between the four pollinator groups. Neither pollinator group nor pollinator order predicted pollen composition when all plant taxa were included in the model (pollinator group: $LR_{1,363} = 1753.8$, $P = 0.999$, order: $LR_{1,365} = 953.9$, $P = 1.000$). The ability of the model to predict pollen composition was greatest when characterising pollinators by their taxonomic order (Diptera, Hymenoptera) rather than group (bumblebees, honeybees, non-corbiculate bees, and hoverflies) (Table S3.3, Supporting Information). There was, however, a significant difference in the composition of plant taxa constituting over 5% of sequences carried by Diptera and Hymenoptera ($x^2 = 46.26$, d.f. = 5, $P < 0.001$) (Fig. 3.1). A large proportion of pollen sequences from hoverflies (Diptera) belonged to *Angelica*/*Heracleum* spp., but these were not found to be as valuable for bees (Hymenoptera). *Cirsium*/*Centaurea*/*Hypochaeris* spp. contributed a large proportion of sequences for bees but made up a lower proportion of sequences for hoverflies while hoverflies used *Ranunculus*/*Ficaria* spp. more abundantly than bees.

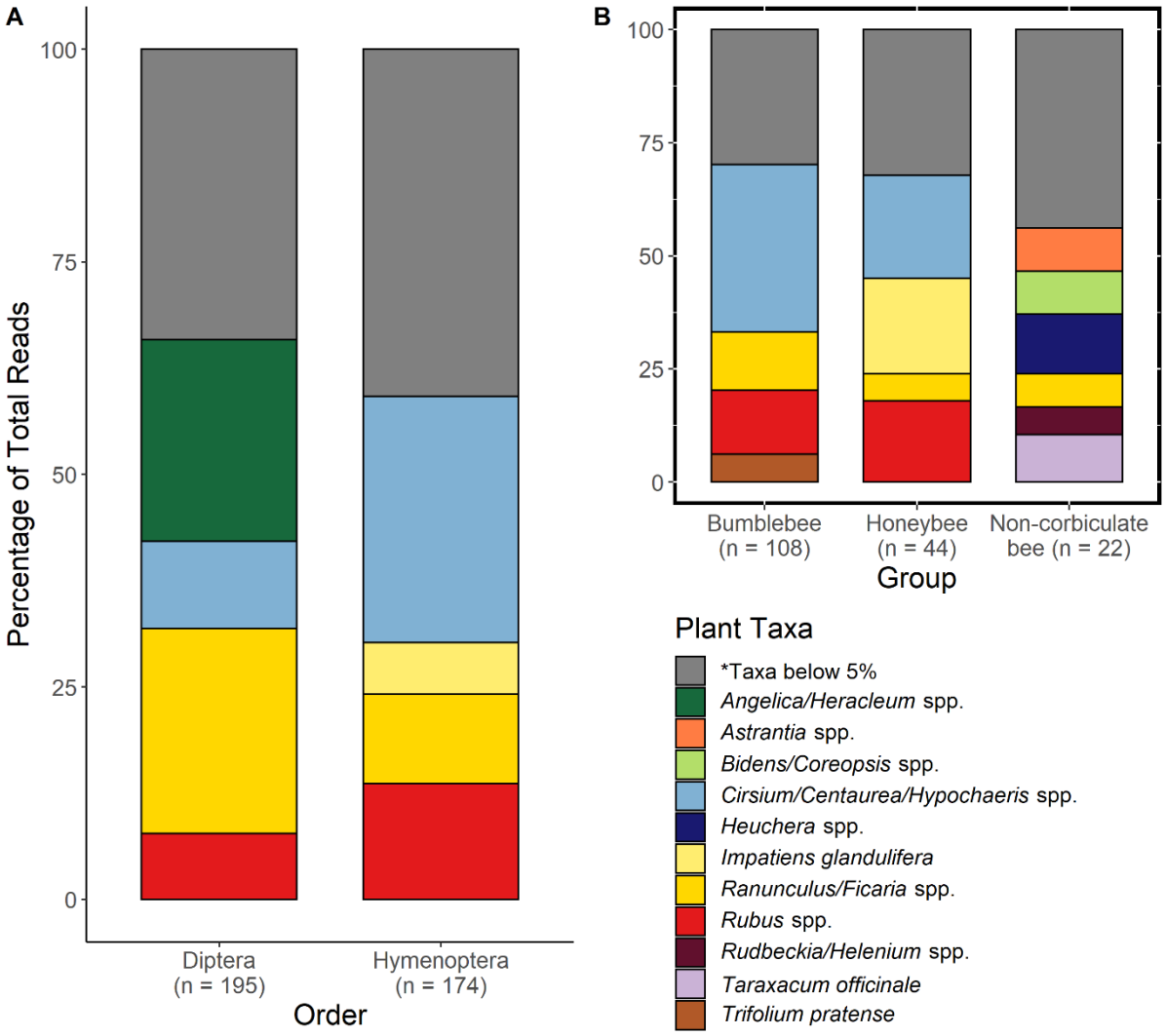

Figure 3.3: Plant taxa represented by >5% of total sequence reads for each pollinator order, where Diptera includes hoverflies only, and Hymenoptera comprises bumblebees, honeybees, and non-corbiculate bees. The proportion of taxa illustrated was significantly different between orders (x^2 = 46.26, d.f. = 5, P < 0.001). **B:** Plant taxa represented by >5% of total sequence reads for each pollinator group within Hymenoptera.

Within pollinator groups, differences in foraging were found between ecological functional categories (Fig. 3.4). A significant difference was found in the composition of plant taxa represented by over 1% of sequences from short- and long- tongued bumblebees (x2 = 50.179, d.f. = 20, P < 0.001). A large proportion of pollen was attributed to *Ranunculus/Ficaria* spp. across short-tongued species (*Bombus hypnorum, B. lapidarius, B. pratorum, B. terrestris/lucorum* agg.) whilst long-tongued species (*B. hortorum, B. pascuorum*), utilised more *Trifolium pratense* and *Rubus* spp. (Fig. 3.4). Honeybees' foraging habits were broadly similar to bumblebees but utilised a greater proportion of *Impatiens glandulifera* than any other group (Fig. 3.4). Within the non-corbiculate bees, the total proportion of pollen collected was significantly different between body size groups (x2 = 433.01, d.f. = 52, P < 0.001), with extra small bees carrying mostly Heuchera spp., small-sized carrying mostly *Rudbeckia/Helenium* spp., and medium-sized carrying mostly *Taraxacum officinale*. Pollen composition from hoverfly species differed between various larval requirements (x2 = 235.4, d.f. = 48, P < 0.001), with carnivorous and detritivorous species utilising a greater diversity of plant taxa than herbivorous species (Fig. 3.4).

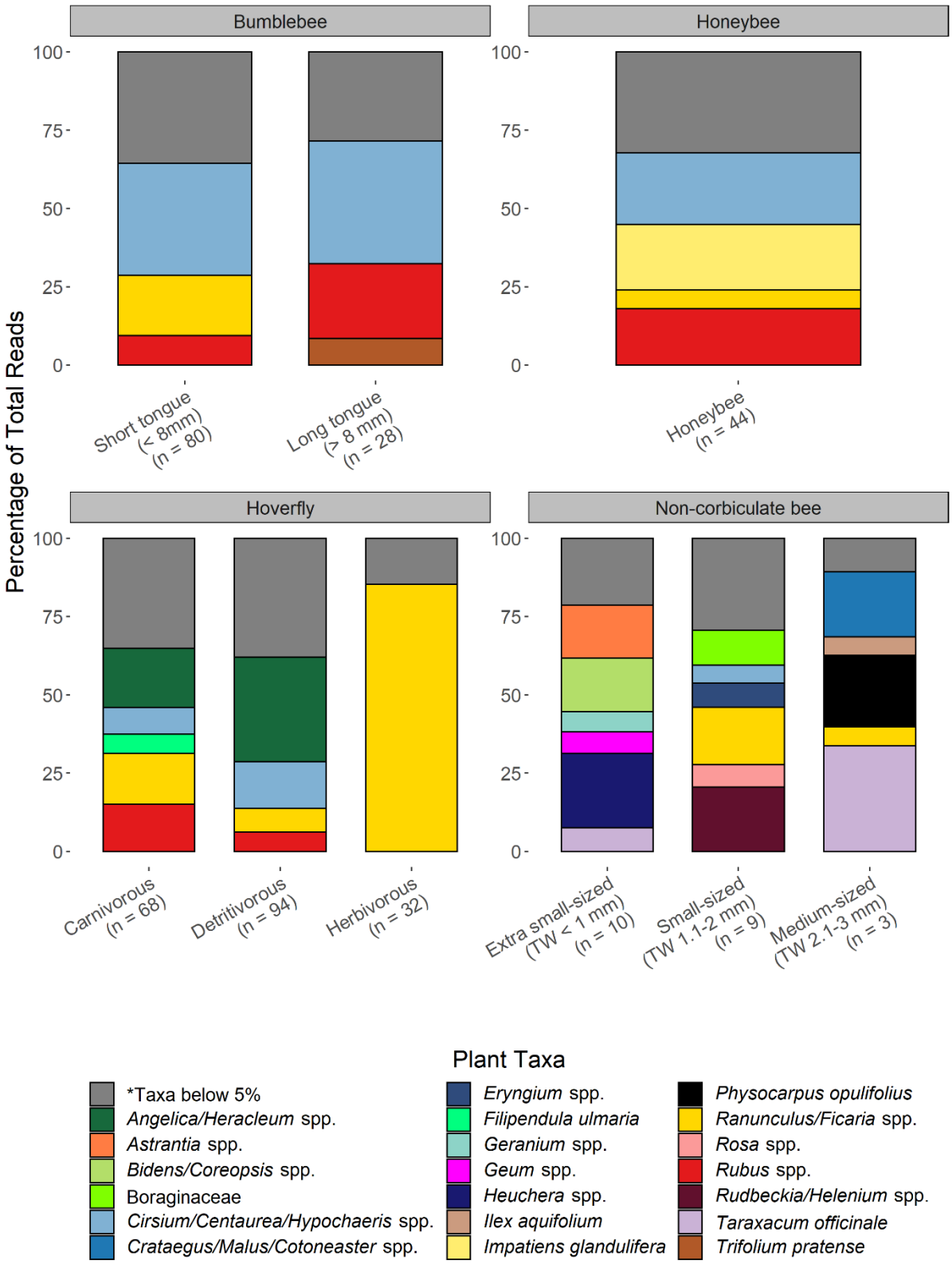

Figure 3.4: Plant taxa represented by over 5% of sequence reads for ecological functional categories within bumblebees, honeybees, hoverflies, and non-corbiculate bees (Table S3.2, Supporting Information).

3.4.3. Annual and seasonal variation in pollinator foraging

Season was a good predictor of pollen composition ($LR_{2,367}$ =2632.8, $P < 0.001$), regardless of year of sampling ($LR_{2,366}$ = 816.2, $P = 0.828$) (Figs S3.2-4). There were 147 taxa found in 2018 and 170 in 2019, and of these 71 were identified in both years. NMDS ordination scaling shows that pollen samples collected in the same season are most similar to each other (Fig. 3.5). Seasonal progression is visible for each pollinator group when assessing the most abundantly foraged plants throughout the year (Table 3.2) using the consensus data and *rbcL* and ITS2 separately (Figs S3.5-7, Supporting Information).

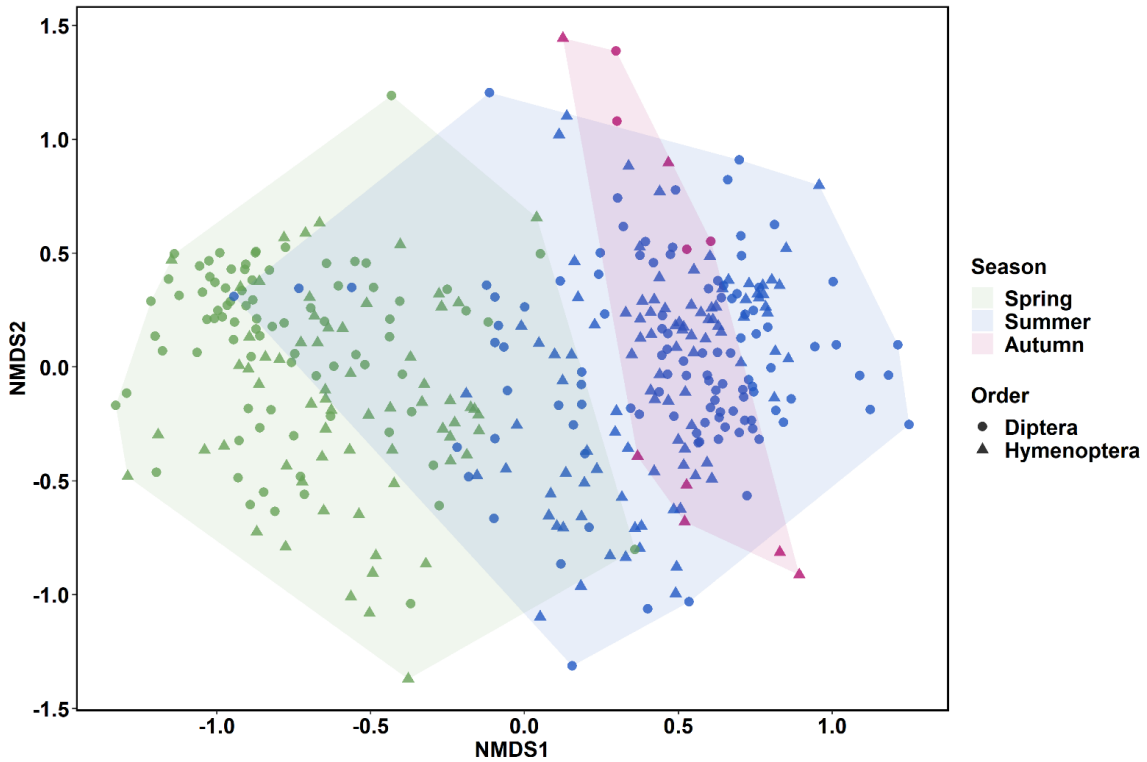

Figure 3.5: non-metric multidimensional scaling (NMDS) plot of pollen samples in relation to season of collection and insect order.

Table 3.2: Major plant taxa constituting over 10% of sequence reads in each season, using the consensus taxa which combines *rbcL* and ITS2. Reads for 2018 and 2019 were combined as year was not found to have a significant effect on pollen composition.

	Spring	Summer	Autumn
Bumblebee 	*Ranunculus/Ficaria* spp. *Rubus* spp.	*Cirsium/Centaurea/Hypochaeris* spp. *Rubus* spp.	*Aster* spp. *Clematis* spp. *Rubus* spp.
Honeybee 	*Ranunculus/Ficaria* spp. *Taraxacum officinale*	*Cirsium/Centaurea/Hypochaeris* spp. *Impatiens glandulifera* *Rubus* spp.	*Actaea* spp. *Heuchera* spp. *Impatiens glandulifera*
Non-corbiculate bee 	*Geum* spp. *Ranunculus/Ficaria* spp. *Taraxacum officinale*	*Bidens/Coreopsis* spp. *Heuchera* spp. *Rudbeckia/Helenium* spp.	*Astrantia* spp.
Hoverfly 	*Ranunculus/Ficaria* spp.	*Angelica/Heracleum* spp. *Cirsium/Centaurea/Hypochaeris* spp. *Rubus* spp.	*Angelica/Heracleum* spp. *Bidens/Coreopsis* spp. *Rudbeckia/Helenium* spp.

3.4.4. Relationship to native status

The largest proportion of DNA reads returned from pollinators were attributed to native and near-native plants (Fig. 3.6). Native and near-native plants were predominately used in the spring and the use of naturalised and horticultural plants increased during the summer and autumn ($LR_{1,367}$ = 58.10, P = 0.001) (Fig. 3.6), regardless of year of sampling ($LR_{1,366}$ = 3.14, P = 0.369). In transects with more horticultural plants, we see that pollinators use a diverse array of plants with no dominant taxa identified (Fig. S3.9, Supporting Information), compared to predominately native areas (Figs S3.8, 3.10-12, Supporting Information).

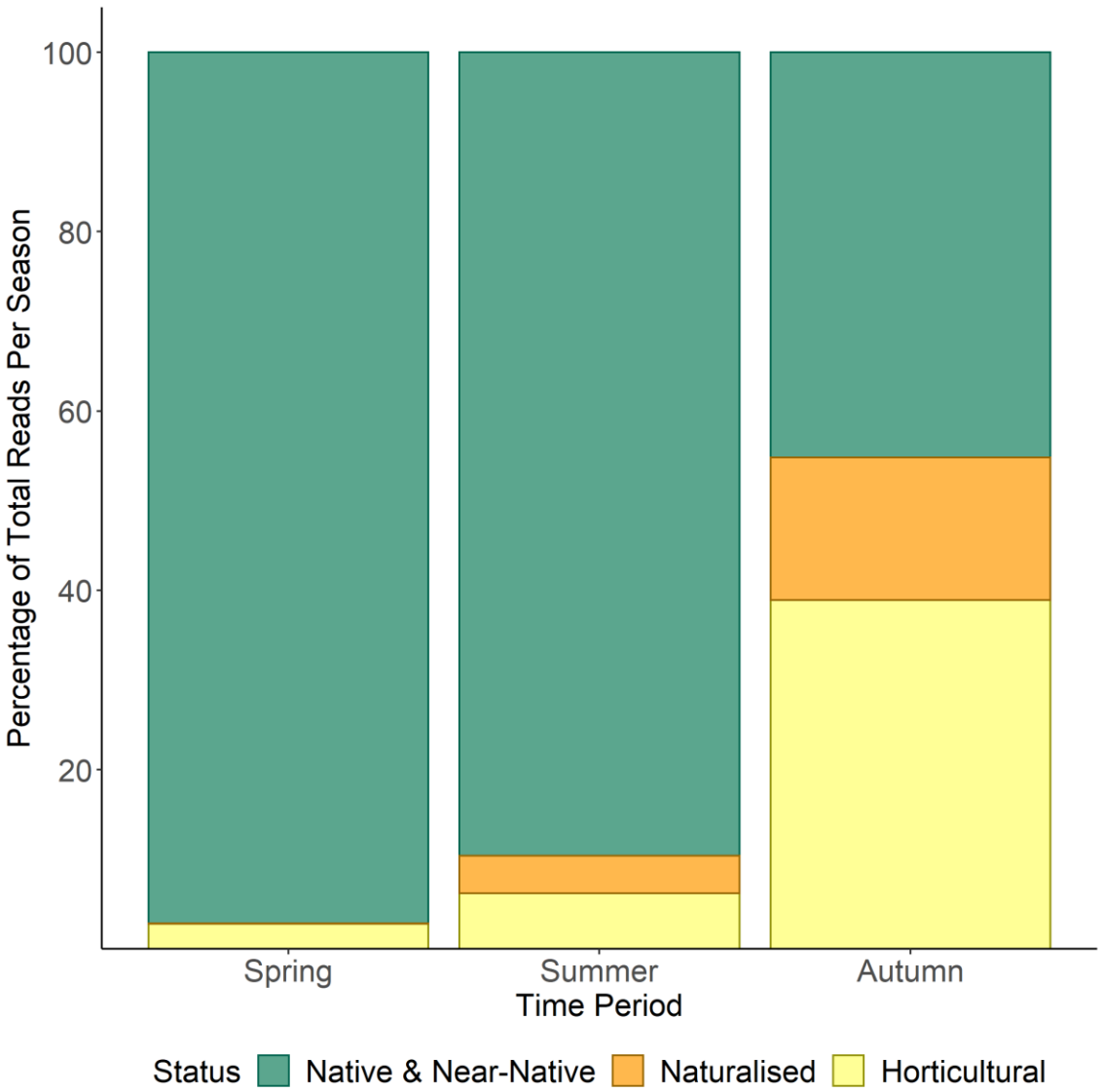

Figure 3.6: Proportion of sequence reads assigned to the native status of identified plants for all pollinators combined, by season. Plant taxa that were unable to be assigned a native status due to level of taxonomic rank were excluded. The use of plants within each category was affected by season of collection ($LR_{2,367}$ = 64.97, P < 0.001) but not by year of collection ($LR_{2,367}$ = 59.48, P < 0.001). Spring (n = 148), summer (n = 210), autumn (n = 11).

3.5. Discussion

Using DNA metabarcoding, we reveal the most frequently visited plants by key pollinator groups, across a broad taxonomic range covering bumblebees, honeybees, non-corbiculate bees, and hoverflies. We show that whilst common resources are shared across all groups, differences are seen in the major taxa visited by hoverflies (Diptera) and bees (Hymenoptera) and between ecological functional categories within. This choice in foraging is strongly influenced by season, with clear changes in floral use through the year. Pollinators were shown to predominately utilise native and near-native plants, with increased use of horticultural and naturalised plants towards the end of the season.

3.5.1. Pollinators share resources with differences between insect orders in major taxa

Floral resources were shared overall amongst pollinator groups (hoverflies, bumblebees, honeybees, and non-corbiculate bees), but clear differences were seen between the taxa used most abundantly by Diptera (hoverflies) and Hymenoptera (bees). In comparison to hoverflies, bees utilised thistles more and umbelliferous plants less. A possible explanation for the preference differences between the major plants of Diptera and Hymenoptera is that the accessibility of nectar may be limited by the morphology of the plants, influencing which plants are visited by pollinators. The hoverflies recorded here generally have shorter tongues than bees (King, 2012), and may have difficulty fully removing nectar from the long corollas found in the genera *Cirsium, Centaurea* and *Hypochaeris*. Whilst hoverflies are evidently able to utilise this resource, the issue of accessibility may be a reason for hoverflies prioritising the shorter, open flowers of *Angelica/Heracleum* spp.

We demonstrate that within broad pollinator groups, resources may be partitioned further based on ecological functional traits shared by species. When studying the

diversity and abundance of pollen collected by insects, Cullen et al. (2021) found that traits had a greater impact than local floral diversity itself, highlighting the importance of understanding this relationship. Tongue length is widely known to affect forage choice in bumblebees, and is thought to influence species' vulnerability to extinction as long-tongued species tend to specialise more on species with long corollae (Goulson et al., 2005). Whilst we did find differences in forage relating to bee size in non-corbiculate bees, the small sample size and long sampling period mean these results must be interpreted with caution and further work is required. Non-corbiculate bees comprise a cosmopolitan suite of ecologically distinct taxa in the UK. However, this study was limited to bees within Halictidae and *Andrena*, along with the kleptoparasitic *Nomada* which are all relatively small (thoracic width < 3 mm) making comparisons within this group difficult. As body size limits the foraging distance of bees (Greenleaf et al., 2007), the floral resources used by these species may have been predicted by the species immediately available to them in the area sampled. The relationship with floral resources is more complex in hoverflies, as larval requirements influence the habitats in which species occupy (Schirmel et al., 2018) although the link between these requirements and floral resources used is little studied. Whilst we identified differences in floral resource use between these functional guilds, we also highlight that hoverflies use plants for mate seeking, therefore additional work is required to fully understand which plants are being used for food, breeding sites or oviposition (in phytophagous species) (Rotheray & Gilbert, 2011a).

3.5.2. Plant use changes throughout the season

Season of collection was found to be the biggest predictor of plant use, with pollinators relying on key plants within each season (see Supporting Information for detailed discussion). The phenological patterns of plants result in shifting of floral availability, temporally limiting the foraging habits of insects. These shifts in

available resources require pollinators to alter their use of resources throughout the season to survive, with those with long flight periods utilising a greater diversity of plant taxa than those with short flight periods (Ogilvie & Forrest, 2017).

3.5.3. Dependence of pollinators on native and near-native plants

Pollinators use native and near native plants more often than non-native plants, however the non-native plants play a key role at the end of the flowering season. These findings are supported by Salisbury et al. (2015) who showed that native and near-native plants attracted a greater number of pollinators than non-native plants in a garden, however the non-native plants extended the flowering period. The greater use of naturalised plants in summer and autumn can be attributed to the high use of *Impatiens glandulifera* by honeybees, highlighting the importance of this species for nectar provision. However, such an observation comes with a broader conservation caveat since *I. glandulifera* is a highly invasive, non-native plant and so it must not be encouraged in gardens due to its ability to displace other plant species (Chittka & Schürkens, 2001). Whilst a lower proportion of non-native plants were used compared to native and near-native plants, they may contribute by increasing the diversity of pollinator diets. For example, *Taraxacum officinale* is used abundantly in the spring however it must be supplemented with additional resources as it lacks essential amino acids needed for pollinator health (Génissel et al., 2002).

3.5.4. Using DNA metabarcoding to study plant-pollinator interactions

The multi-locus metabarcoding approach used here allows the relationship between plants and pollinators to be studied on a fine scale, improving both the number of plant taxa that can be detected and the level of discrimination achievable with the use of one marker alone (Jones et al., 2021b) or alternative methods (Brennan et al., 2019). We highlight the ability of DNA metabarcoding to

not only provide a greater depth of information, but to confirm knowledge provided by traditional techniques, for example here the frequent use of taxa with large open inflorescences by hoverflies (Branquart & Hemptinne, 2000). Due to potential biases in sampling, along with extraction, amplification, and sequencing of DNA (Bell et al., 2016), the data should be treated as semi-quantitative, with the abundance of DNA reads treated as estimates of relative abundance (see Analysing DNA metabarcoding data using semi-quantitative approaches, Supporting Information). Frequent taxa may be overrepresented, and rare taxa more difficult to detect, however, this is also the case using pollen microscopy (Hawkins et al., 2015). Recent developments suggest that in some cases metabarcoding data may be quantitative (Baksay et al., 2020; Richardson et al., 2021), particularly regarding the most abundant taxa in a sample (Bänsch et al., 2020), however further work is needed to fully understand this relationship (Piñol et al., 2019). Further, species-level discrimination in plants using DNA metabarcoding is challenging due to no single marker meeting the requirements for an ideal barcode (CBOL Plant Working Group, 2009). While genus-level designations have limitations in understanding fine-scale plant-pollinator interactions, these provide a conservative approach to identification, using the most universal and discriminative plant DNA markers available, to provide accurate taxonomic information across a wide study scale (Jones et al., 2021b). Our conclusions therefore focus on the plants most abundantly used by pollinators, and how we can provide these in gardens and wider landscapes.

3.5.5. Synthesis and applications

As public awareness and enthusiasm for pollinator conservation increases, improving plant recommendation lists for gardeners and encouraging suitable management practices has the potential to support pollinator populations at risk. This study provides an evidence base for recommendations that will support pollinators.

We recommend that plants for pollinator lists should:

- distinguish between bees and hoverflies as a minimum
- provide recommendations throughout the seasons
- include native as well as non-native plants

Native and near-native plants can be provided in gardens by planting or through changing garden management regimes. For example, reducing mowing to encourage plants such as dandelion *Taraxacum officinale* and buttercups *Ranunculus* spp., and reducing scrub management to encourage bramble *Rubus fruticosus*. Whilst the availability of floral resources may limit pollinators, we also highlight the importance of providing suitable nesting habitat within gardens. In particular, providing pre-existing hollow cavities will support aerial nesters, whilst having a variety in sward length within grassland will benefit ground-nesting bees. Egg-laying in hoverflies can be encouraged by providing a diversity of floral resources, aquatic habitats, and decaying wood to support the diversity of larval requirements.

The results of this study allow us to provide an evidence-based plant recommendation list to support a range of pollinators throughout the season including native and horticultural plants across a range of growth forms (Table S3.4, Supporting Information). We improve on previous lists by providing foraging information from the perspective of the insect, increasing both the temporal and spatial scope possible compared to using observations of plants. This

recommendation list is based on taxa found within the UK, with relevance to Northern Europe and can be used by gardeners, land managers, plant producers and policy makers to inform decisions on planting within gardens and urban greenspace to ensure pollinators are appropriately supported.

3.6. Acknowledgements

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3.8. Supporting Information

3.8.1. Supporting Methods

Field sampling

Transects were set up so that the entire route was situated in the same habitat, either following linear features, footpaths, or a line in the shape of a 'Z' within open fields. Insects were caught individually within a sterile 1.5 ml or 15 ml tube by placing a net with the tube held inside above, allowing the insect to fly upwards into the tube (Lucas et al., 2018a). Bumblebee queens were excluded from sampling. To minimise cross-contamination, one sterile net was used per transect and nets were replaced if there was any suspected pollen transfer from insects.

Taxonomic assignment of insects

Due to difficulties separating workers of the *Bombus lucorum* complex (including *B. cryptarum* (Fabricius), *B. lucorum* (L.) and *B. magnus* (Vogt) and *B. terrestris* (L.), all males and females within this species complex were grouped together as *Bombus lucorum/terrestris* agg. Females of *Cheilosia albitarsis* (Meigen) and *C. ranunculi* (Doczkal) cannot currently be distinguished using morphology, therefore all specimens were grouped as *C. albitarsis sensu lato* (*s.l.)*. Bees were identified using Falk and Lewington (2015) and hoverflies using Stubbs and Falk (2002). Voucher specimens were retained in a reference collection at the National Botanic Garden of Wales.

Laboratory contamination controls

All laboratory procedures prior to the initial amplification were conducted in a dedicated pre-PCR lab, which was thoroughly cleaned with 10% bleach solution before and after in order to avoid contamination. The second PCR and library preparation was conducted in a dedicated post-PCR lab with the same stringent cleaning procedures. Filter tips were used for all molecular procedures.

Thermal cycling conditions

Thermal cycling conditions for the first *rbcL* PCR were: 98 °C for 30 s, 95 °C for 10 min; 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min (35 cycles); 72 °C for 10 min, 30 °C for 1 min. Thermal cycling conditions for the first ITS2 PCR were: 98 °C for 30 s, 95 °C for 10 min; 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min (40 cycles); 72 °C for 10 min, 30 °C for 1 min. Index PCR thermal cycling conditions were: 98 °C for 30 s; 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s (8 cycles); 72 °C for 5 min, 4 °C for 10 min.

Using *rbcL* and ITS2 markers

rbcL and ITS2 are both recommended as standard barcodes for plant DNA barcoding (CBOL Plant Working Group, 2009; Chen et al., 2010). The two markers may identify the same taxa at varying taxonomic levels, or additional taxa unique to one marker (Jones et al., 2021b). We analysed the results of each marker combined and separately. Using a combined approach increases the taxonomic scope of the study as the additional taxa which are detected using both markers are retained. In some cases, ITS2 can improve the taxonomic discrimination of taxa identified by *rbcL*, however, in this case we used an objective, conservative approach to combine the results for each marker. The following rules were applied:

- For taxa identified to genus level with *rbcL* and species using ITS2, taxa were moved up a taxonomic rank to genus level for the consensus.
- If one marker identified a single genus but the other identified multiple, closely related genera, then the consensus listed all of the genera. e.g., ITS2 identified *Sambucus nigra* and *Viburnum* spp. however *rbcL* only identified *Sambucus*/*Viburnum* spp. therefore the consensus was *Sambucus*/*Viburnum* spp.
- Taxa identified at genus or species level with ITS2 and only identified at tribe level with *rbcL* were moved to tribe level designation e.g., ITS2

identified multiple genera within Anthemideae however *rbcL* was unable to distinguish between these. The consensus was therefore Anthemideae.

- Taxa identified at genus or species level with ITS2 and only identified at family level with *rbcL* were moved to family level designation e.g., ITS2 identified multiple Asteraceae taxa which could not be grouped with any *rbcL* Asteraceae taxa identified.
-

The relationship between the relative read abundance of each consensus taxon identified using both markers within each sample was tested using Spearman's rank correlation with Holm correction for multiple testing to confirm the suitability of combining both markers.

Analysing DNA metabarcoding data using semi-quantitative approaches

Deagle et al. (2019) showed that using relative read abundance as a measure provided a more sensitive representation of diet compared to using presence/absence (frequency of occurrence). Furthermore, metabarcoding datasets often feature a long tail of increasingly rare taxa. Using a presence/absence method overstates rare taxa and devalues the most abundant taxa in a sample.

Using the proportion of plant taxa in a sample is an effective way of controlling for differences in sequencing depth between samples (Brennan et al., 2019; Deagle et al., 2019; McMurdie & Holmes, 2014). We therefore analyse our data using relative read abundance – either the proportion of taxa as a percentage (in the graphs and NMDS plots) or as the number of sequences with an offset of the total number of sequences per sample, comparable to proportion (in the statistical models).

As each sample has a different total number of sequences (variable sequencing depth), this must be accounted for in the model. To allow for this, the standard approach is to include an offset variable in the linear predictor during fitting to calculate the rate of presence (comparable to proportion) (Hardin & Hilbe, 2007; Pendegraft et al., 2019). Here, we use the number reads for each taxon within each

sample as the multivariate response variable and the total number of sequences obtained from each sample set as an offset (Jones et al., 2021a).

Functional diversity analysis

In order to investigate pollen use within pollinator groups, insects were categorised by unique ecological functional traits. Bumblebees were defined as short-tongued species if proboscis length was less than 8 mm, and long-tongued if over 8 mm, with values taken from Goulson et al. (2005) and Persson et al. (2015). The thoracic widths of non-corbiculate bee specimens were measured, and species were grouped as extra-small if the average thoracic width was less than 1 mm, small if between 1.1- and 2-mm, and medium if between 2.1- and 3-mm. Hoverflies were grouped by larval habits: herbivorous, fungivorous, carnivorous or detritivorous following Stubbs & Falk (2002). As only one species of hoverfly was classified as fungivorous, this category was omitted from functional diversity analysis.

3.8.2. Supporting Results

Comparison of statistical analysis using both markers

180 plant taxa were identified using the *rbcL* marker and 186 using the ITS2 marker. Of the taxa identified using *rbcL*, 12% were identified to family, 3% to tribe, 67% to genus and 18% to species. Using ITS2, 1% taxa were identified to family, 65% to genus and 34% to species. Following the creation of a consensus identification, there was a strong correlation between the proportion of sequences of each matched taxon (n = 105) found within each sample using both *rbcL* and ITS2 (Spearman correlation coefficient $r_s = 0.601$, $P < 0.001$).

Modelling changes in pollen composition using the *rbcL* and ITS2 marker separately revealed no relationship between pollinator group and plant taxa (*rbcL*: $LR_{3,363} = 1606.6$, $P = 1.000$, ITS2: $LR_{3,363} = 1441.0$, $P = 1.000$). No relationship was found

between pollinator order and pollen composition using the *rbcL* marker ($LR_{1,365}$ =828.2, P = 1.000) but the ITS2 marker revealed a significant effect of pollinator order ($LR_{1,365}$ =806.9, P = 0.015). Season was found to be a significant factor affecting pollen composition using both markers (*rbcL*: $LR_{1,367}$ =2339.4, P < 0.001, ITS2: $LR_{1,367}$ =2237.8, P < 0.001) however year was not found to significantly affect pollen composition using either marker (*rbcL*: $LR_{2,366}$ =905.2, P = 0.998, ITS2: $LR_{1,366}$ =875.3, P = 1.000). Modelling the proportion of plant use relating to their native status revealed a significant effect of season using both markers separately (*rbcL*: $LR_{2,367}$ = 28.80, P < 0.001, ITS2: $LR_{2,367}$ = 35.46, P < 0.001) but no annual effect (*rbcL*: $LR_{2,366}$ = 3.04, P = 0.386, ITS2: $LR_{2,366}$ = 1.53, P < 0.679).

3.8.3. Supporting Discussion

Pollinators rely on key plants within each season

A small number of plant taxa were found to dominate pollen loads in the spring. The high use of *Ranunculus*/*Ficaria* spp. and *Taraxacum officinale* is likely due to a combination of their high nectar production (Hicks et al., 2016) and mass-flowering events, emerging when little other rewarding forage is available (Timberlake et al., 2019).

Species within the *Cirsium, Centaurea* and *Hypochaeris* grouping have been found to be extremely valuable forage during the summer due to the high volumes of nectar and pollen they produce (Baude et al., 2016; Hicks et al., 2016),. with species such as *Centaurea nigra* and *Hypochaeris radicata* recommended for use in wildlife seed mixes and agri-environment schemes (Pywell et al., 2011; Wood et al., 2017). The semi-improved and species-rich grasslands within the study site contain an abundance of *Cirsium arvense, C. vulgare*, *Centaurea nigra*, and *Hypochaeris radicata,* whilst horticultural varieties of *Cirsium* and *Centaurea* are planted in the formal garden areas. The large proportion of *Rubus* spp. reads during the summer are likely to be identified as the apomictic group *R. fruticosus* agg., due to its

abundance within the study site where occurrence of cultivated species e.g. *R. idaeus* are much less prevalent (Preston et al., 2002). *R. fruticosus* produces rewarding nectar, both in large volumes and of high sugar content (Baude et al., 2016; Fowler et al., 2016) and its ecological value to a range of pollinators is recognised in the literature (Jones et al., 2021a; Lucas et al., 2018b, 2018a; Wignall et al., 2020a).

Towards the end of the season, the naturalised, invasive species, *Impatiens glandulifera* can be an important nectar source for honeybees (Jones et al., 2021a). From hoverflies, we identify a large proportion of reads from horticultural species including *Bidens*, *Coreopsis*, *Rudbeckia* and *Helenium* species, providing an additional floral resource when other flowers are becoming limited. There was however a considerable drop in the number of insects collected during autumn compared to spring and summer, due to pollinator activity diminishing by this period (Balfour et al., 2018). Caution should therefore be taken in interpretating the results obtained later in the season due to these smaller sample sizes. These seasonal changes emphasise the need to supply a diversity of plants in gardens to ensure that optimal floral resources are available throughout the year.

In addition to seasonal changes in resources, variable weather can also affect foraging. Changes in temperature, precipitation and sunlight, can affect the availability of resources (Takkis et al., 2018) and foraging behaviour (Inouye et al., 2015). Whilst no annual variation in the floral composition of pollen collected from insects was found in this study, Jones et al. (2021a) monitored honeybee foraging over two years and revealed fluctuations in forage use between years. Longer time-series of foraging behaviour, collected over multiple years, may reveal patterns corresponding to weather variation which would allow recommendations to be made to reduce the effect of stochastic weather events.

3.8.4. Supporting Tables and Figures

Table S3.1: Primer sequences used to amplify the *rbcL* and ITS2 barcode regions. A 6N sequence was added between the forward template specific primer and the universal tail to improve clustering on the Illumina MiSeq.

Primer name	Universal Tail	6N Sequence	Primer sequence
*rbcL*af (Kress & Erickson, 2007)	**ACACTCTTTCCCTACACGACGCTCTTCCGATCT**	NNNNNN	ATGTCACCACAAACAGAGACTAAAGC
*rbcL*r506 (de Vere et al., 2012)	**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**		AGGGGACGACCATACTTGTTCA
ITS2F (Chiou et al., 2007)	**ACACTCTTTCCCTACACGACGCTCTTCCGATCT**	NNNNNN	ATGCGATACTTGGTGTGAAT
UniPlantR (Moorhouse-Gann et al., 2018)	**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**		CCCGHYTGAYYTGRGGTCDC

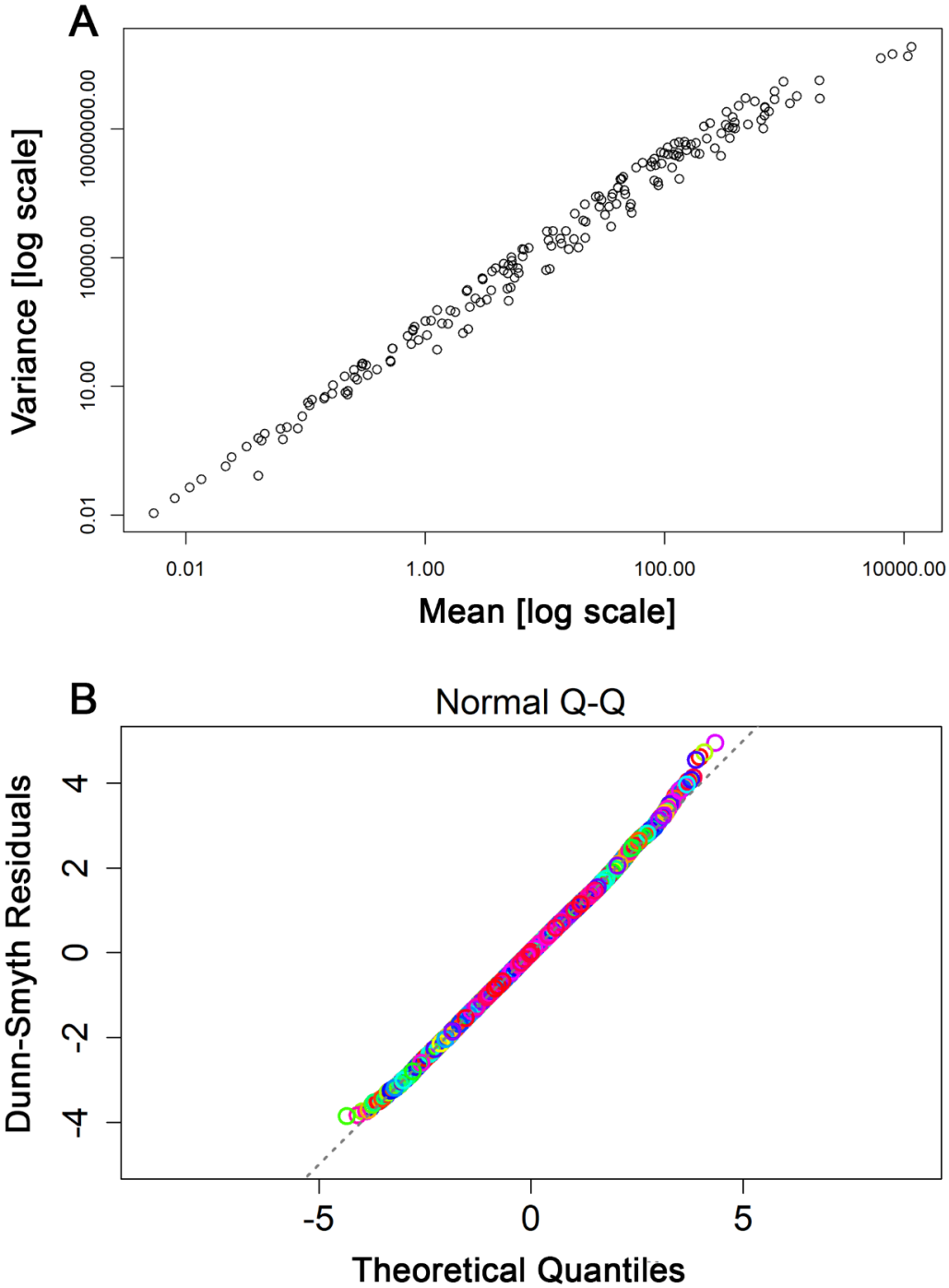


Figure S3.1: A: There was a strong relationship between the mean proportion of sequences and the variance of the proportion of sequences using the consensus dataset. **B:** Plot of theoretical quantile values against the residuals output from the optimal model used to fit the sequence data produced by the metabarcoding data (*rbcL* and ITS2 combined). Minimal deviations from the straight line suggest the model is plausible and the mean-variance assumption of the negative binomial regression is correct.

Table S3.2: List of pollinator species sampled from March to September across 2018 and 2019. Bumblebee tongue lengths were taken from Goulson et al. (2005) and Persson et al. (2015) and hoverfly larval descriptions from Stubbs & Falk (2002).

Group	Ecological functional category	Species	Total
Bumblebee	Long tongue (> 8 mm)	*Bombus hortorum*	6
		Bombus pascuorum	22
	Short tongue (< 8 mm)	*Bombus hypnorum*	4
		Bombus lapidarius	29
		Bombus pratorum	12
		Bombus terrestris/lucorum agg.	35
Honeybee	Honeybee	*Apis mellifera*	44
Hoverfly	Carnivorous	*Dasysyrphus venustus*	1
		Episyrphus balteatus	7
		Eupeodes corollae	1
		Eupeodes latifasciatus	4
		Leucozona lucorum	1
		Melangyna umbellatarum	1
		Melanostoma mellinum	9
		Melanostoma scalare	7
		Meliscaeva auricollis	1
		Meliscaeva cinctella	1
		Platycheirus albimanus	14
		Platycheirus clypeatus	4
		Platycheirus granditarsus	1
		Platycheirus tarsalis	2
		Sphaerophoria scripta	2
		Syrphus ribesii	6
		Syrphus vitripennis	5
		Trichopsomyia flavitarsis	1
	Detritivorous	*Eristalis arbustorum*	7
		Eristalis horticola	9
		Eristalis intricaria	2
		Eristalis nemorum	7
		Eristalis pertinax	28
		Eristalis tenax	12
		Ferdinandea cuprea	1
		Helophilus hybridus	1
		Helophilus pendulus	2
		Helophilus trivittatus	1
		Melanogaster hirtella	6
		Rhingia campestris	7
		Sericomyia superbiens	2
		Sphegina elegans	1
		Syritta pipiens	8
	Fungivorous	*Cheilosia longula*	1
	Herbivorous	*Cheilosia albitarsis s.l.*	21
		Cheilosia griseiventris/latifrons	1
		Cheilosia illustrata	1
		Cheilosia pagana	2
		Cheilosia proxima	1
		Cheilosia vernalis	1
		Merodon equestris	5
Non-corbiculate bee	Extra small-sized (TW <1 mm)	*Lasioglossum smeathmanellum*	8
		Nomada fabriciana	2
	Medium-sized (TW 2.1-3 mm)	*Andrena haemorrhoa*	2
		Andrena scotica	1
	Small-sized (TW 1.1-2 mm)	*Andrena bicolor*	3
		Andrena congruens	1
		Halictus tumulorum	1
		Lasioglossum calceatum	3
		Lasioglossum zonulum	1

Table S3.3: Model selection showing the most appropriate model contained pollinator order rather than pollinator group. Model 2 was used in analysis.

Model	Variables	AIC
1	Season, year, group	105465.8
2	**Season, year, order**	**105233.3**

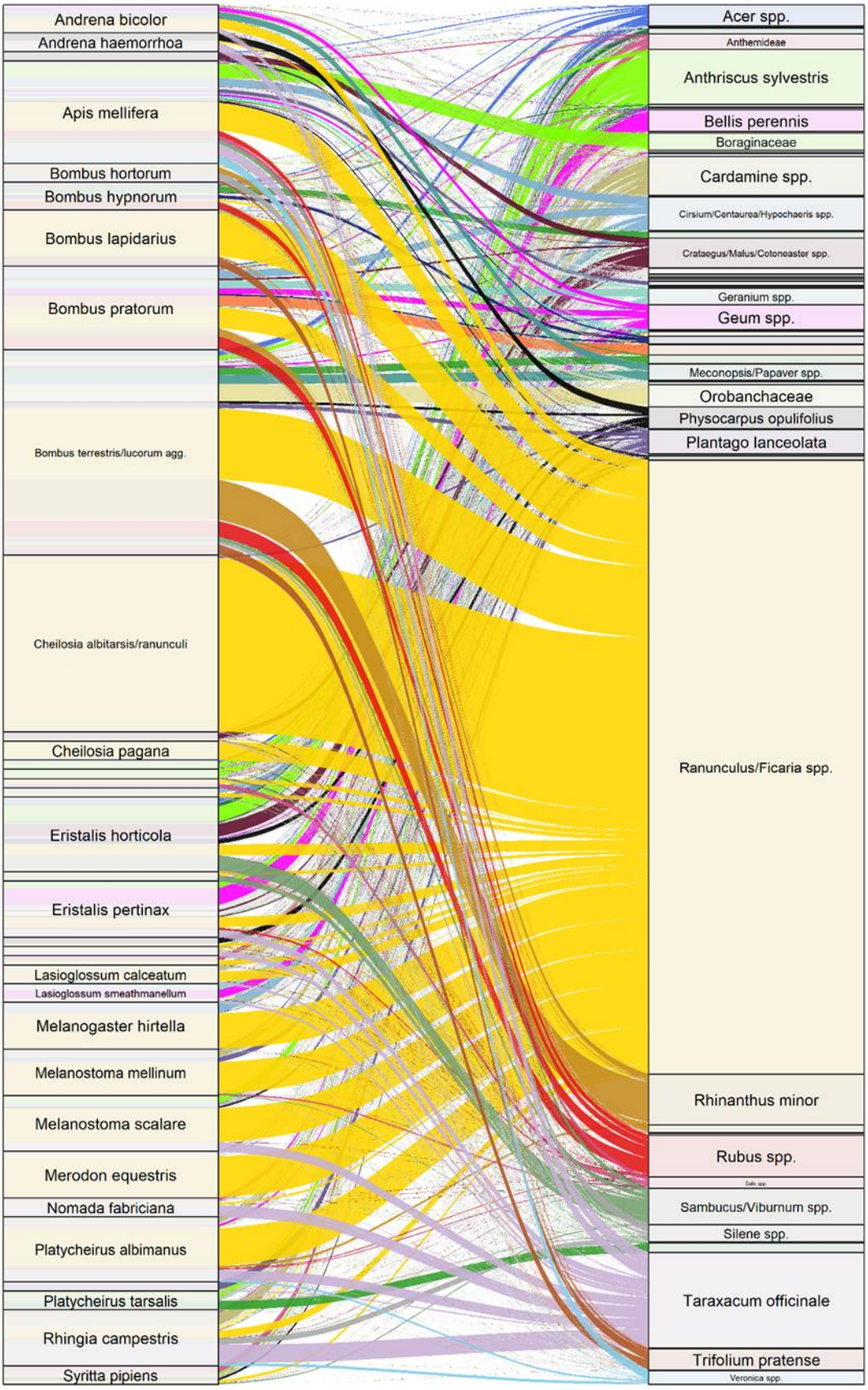


Figure S3.2: Plant-pollinator network during spring 2018 and 2019 (March, April and May, n = 148) based on pollen recovered from the bodies of insects, identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction.

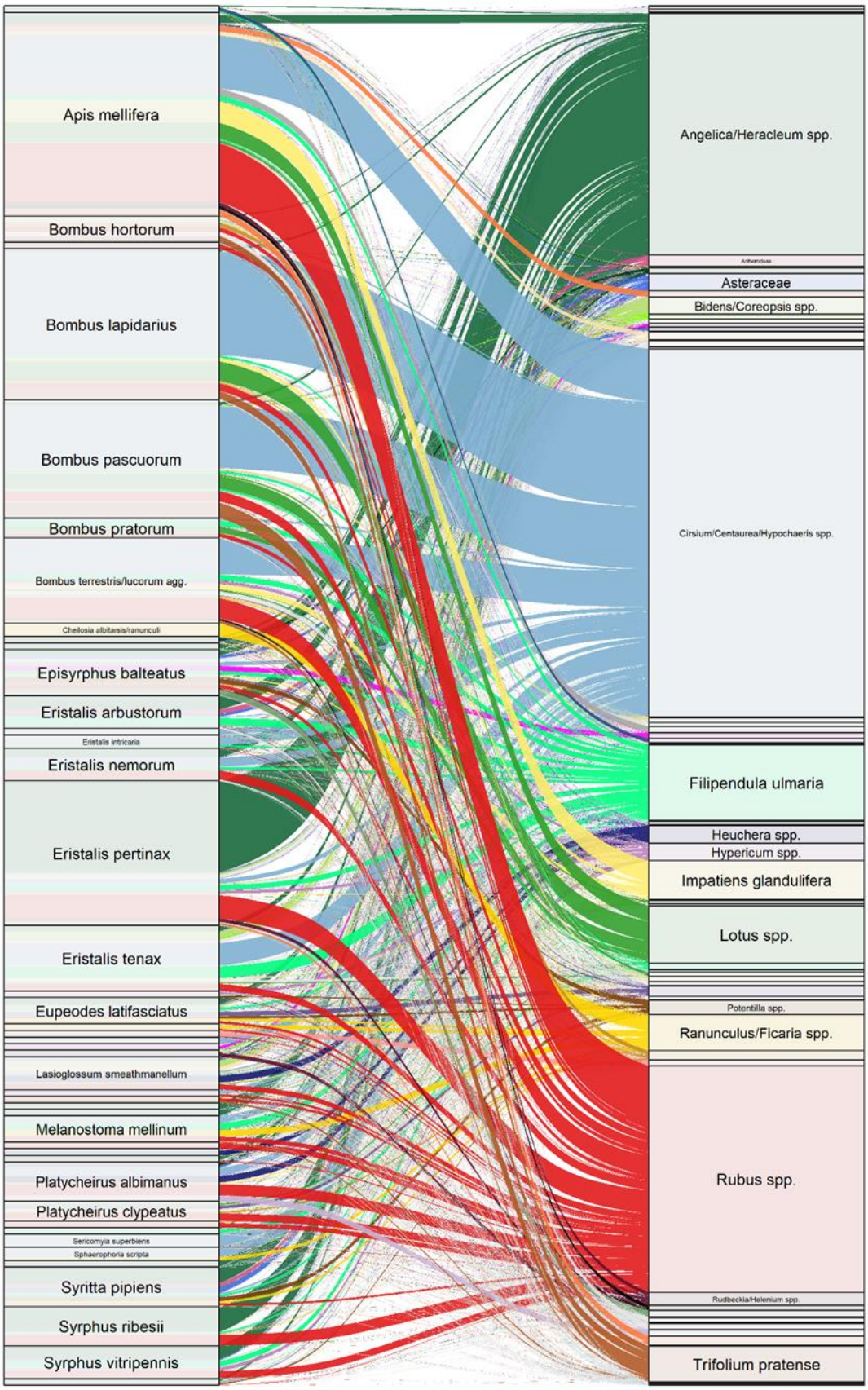


Figure S3.3: Plant-pollinator network during summer 2018 and 2019 (June, July and August, n = 210) based on pollen recovered from the bodies of insects, identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction.

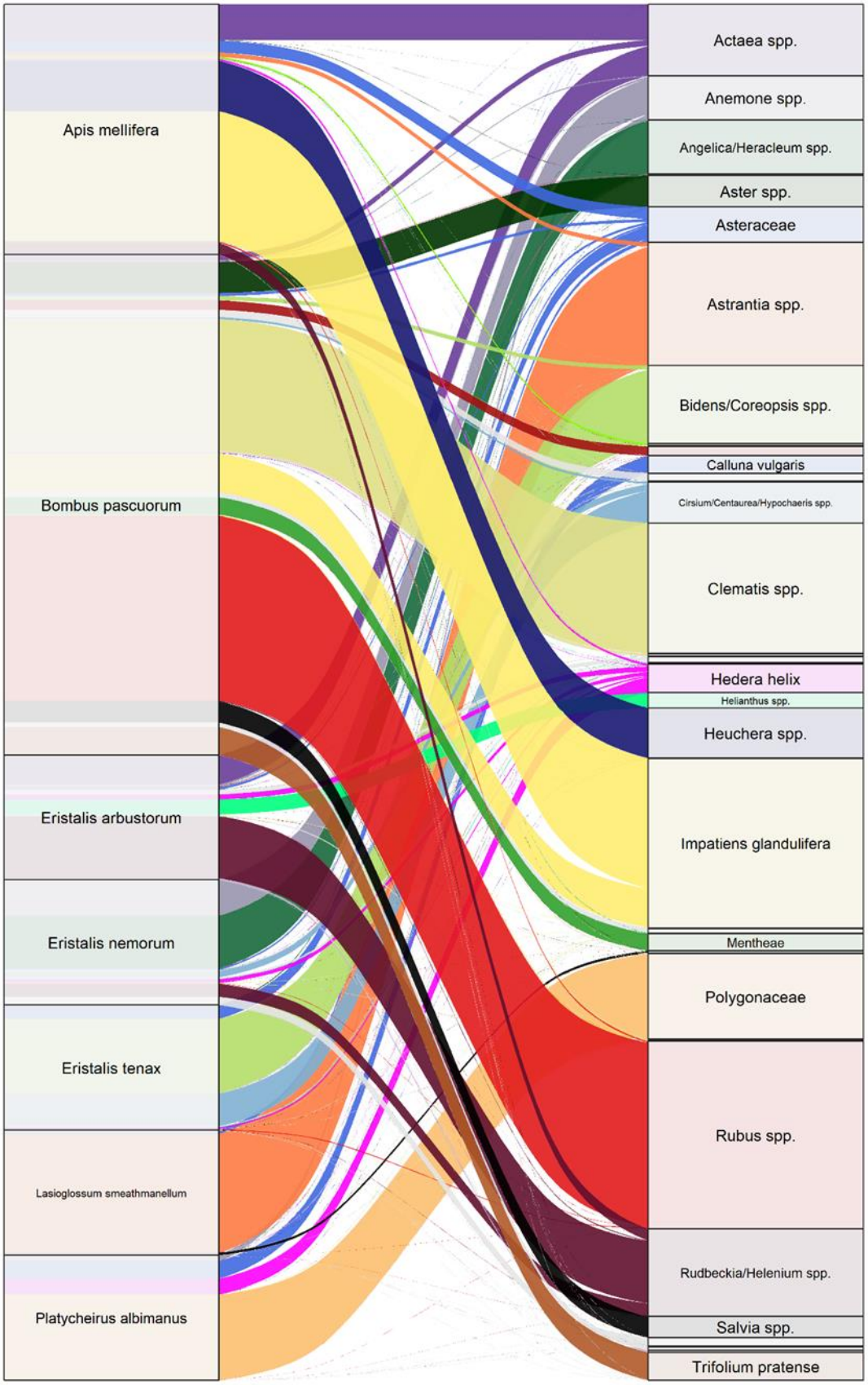


Figure S3.4: Plant-pollinator network during autumn 2018 and 2019 (September, n = 11) based on pollen recovered from the bodies of insects, identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction.

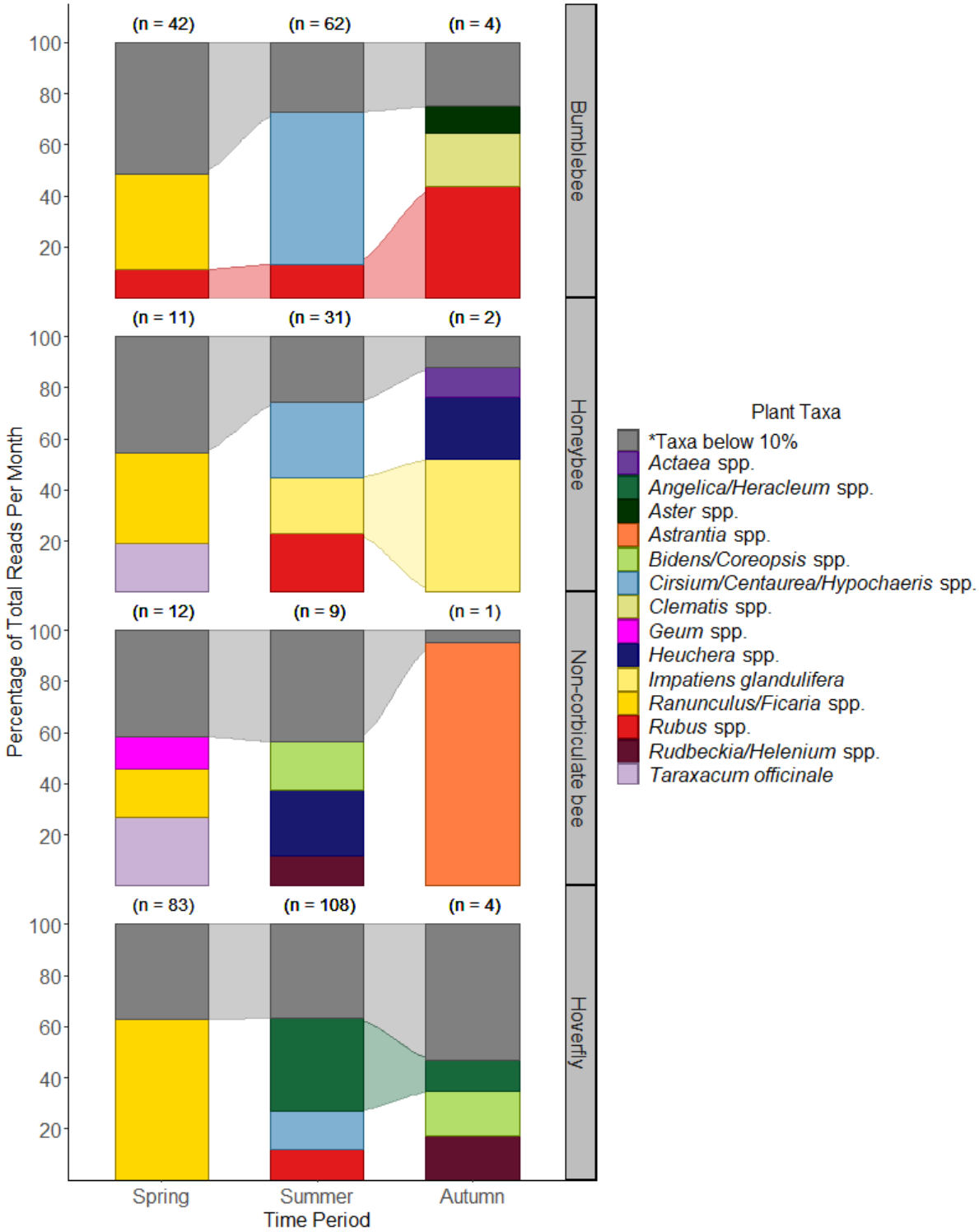


Figure S3.5: Major plant taxa constituting over 10% of sequence reads in at least one season, using the consensus taxa which combines *rbcL* and ITS2. Reads for 2018 and 2019 were combined as year was not found to significantly affect pollen composition.

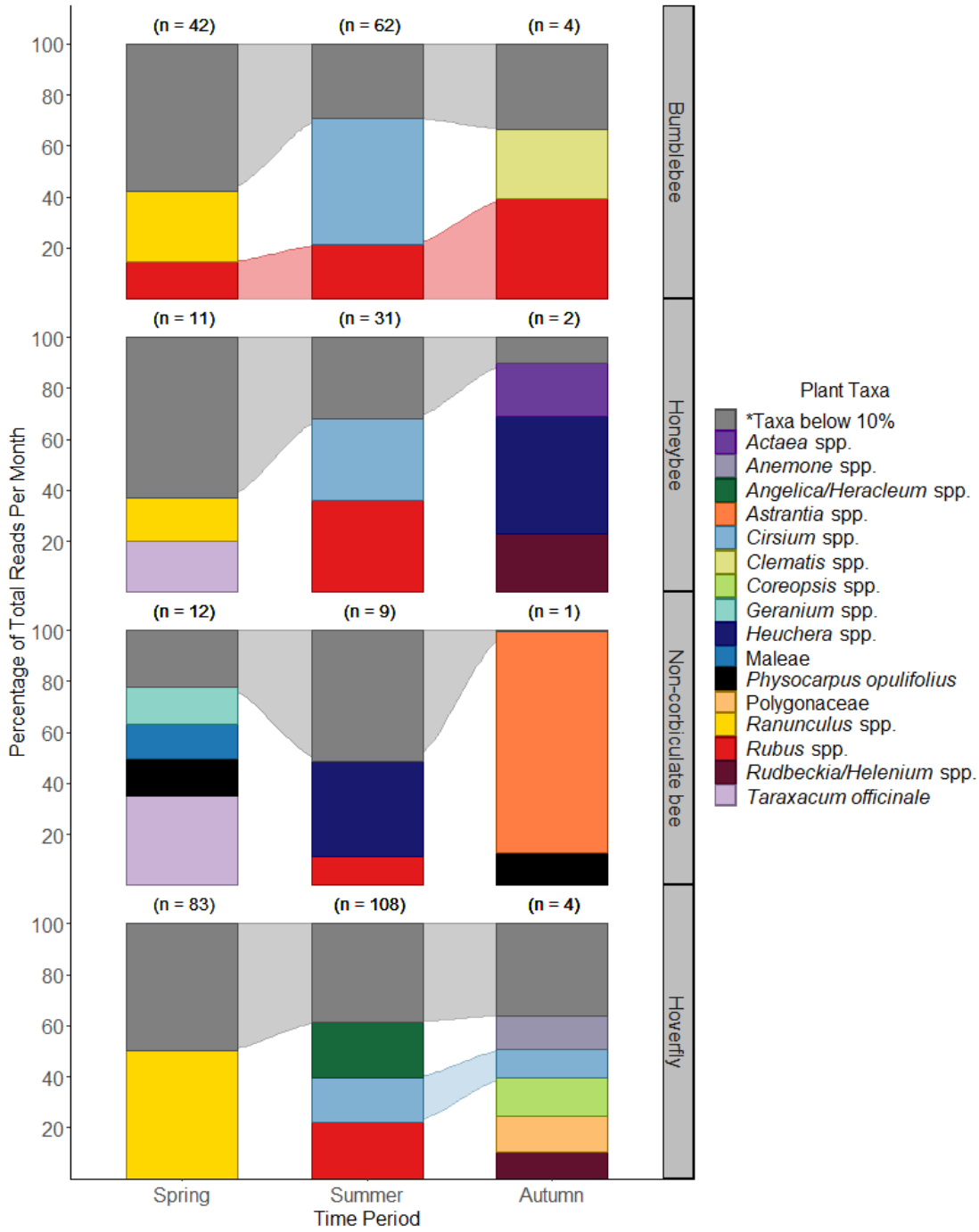


Figure S3.6: Major plant taxa constituting over 10% of sequence reads in at least one season, using the *rbcL* marker. Reads for 2018 and 2019 were combined as year was not found to significantly affect pollen composition.

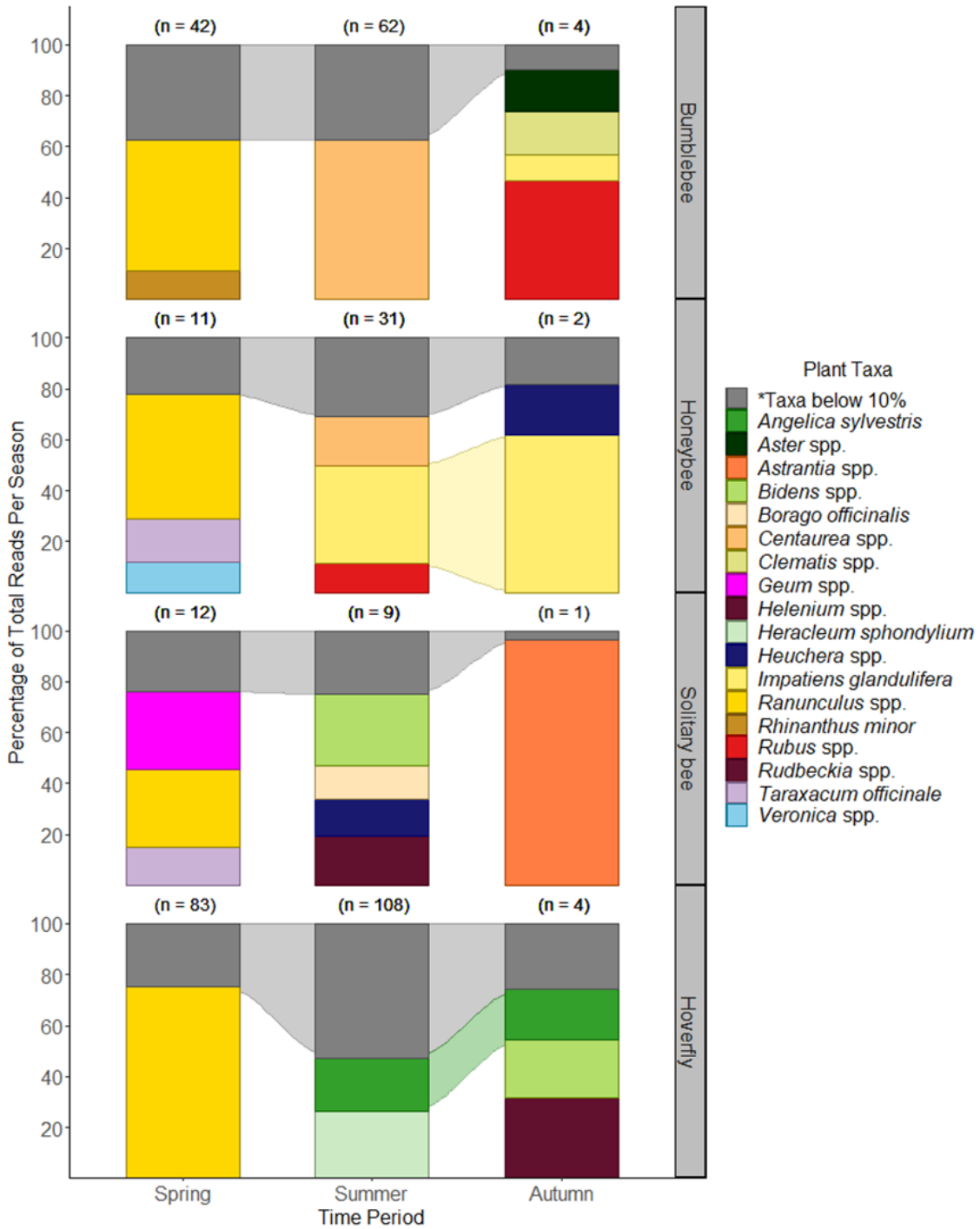
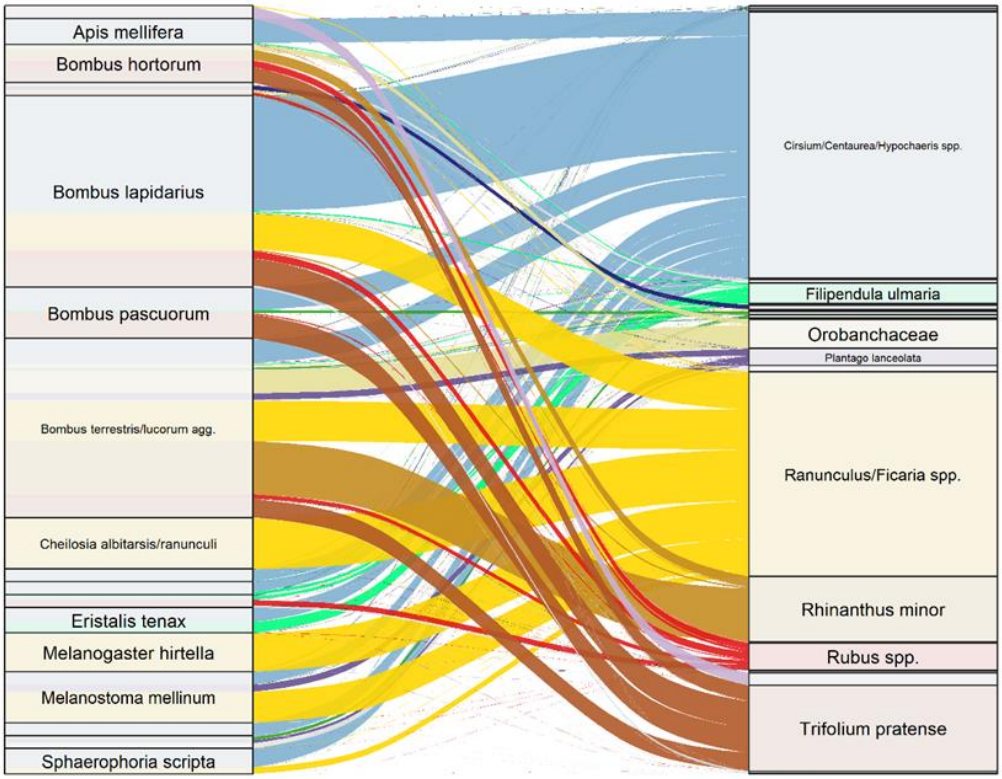


Figure S3.7: Major plant taxa constituting over 10% of sequence reads in at least one season, using the ITS2 marker. Reads for 2018 and 2019 were combined as year was not found to significantly affect pollen composition.

Transect 1 (Grassland, Nature Reserve)



Transect 2 (Grassland, Botanic Garden)

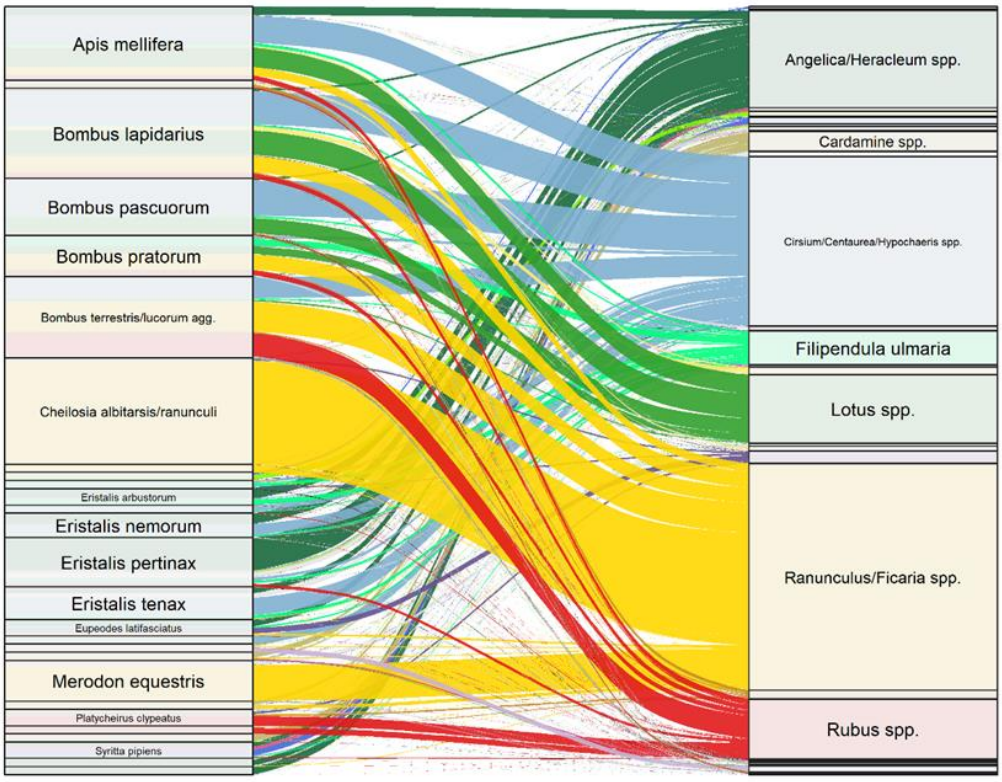


Figure S38: Plant-pollinator networks within Transect 1 (n = 60) and 2 (n = 94) from March to September 2018 and 2019 based on pollen recovered from the bodies of insects, identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction.

Transect 3 (Horticultural, Botanic Garden)

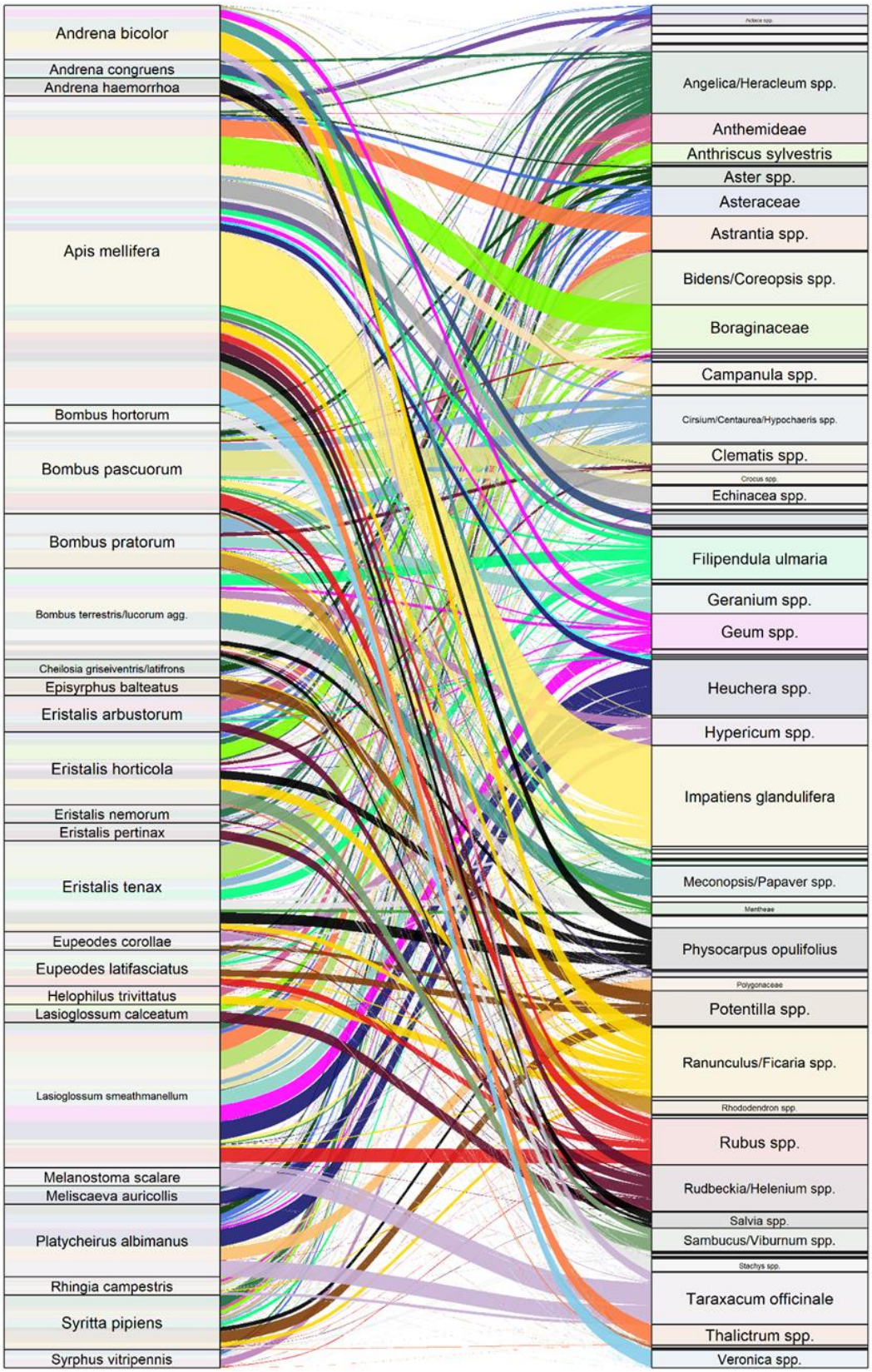
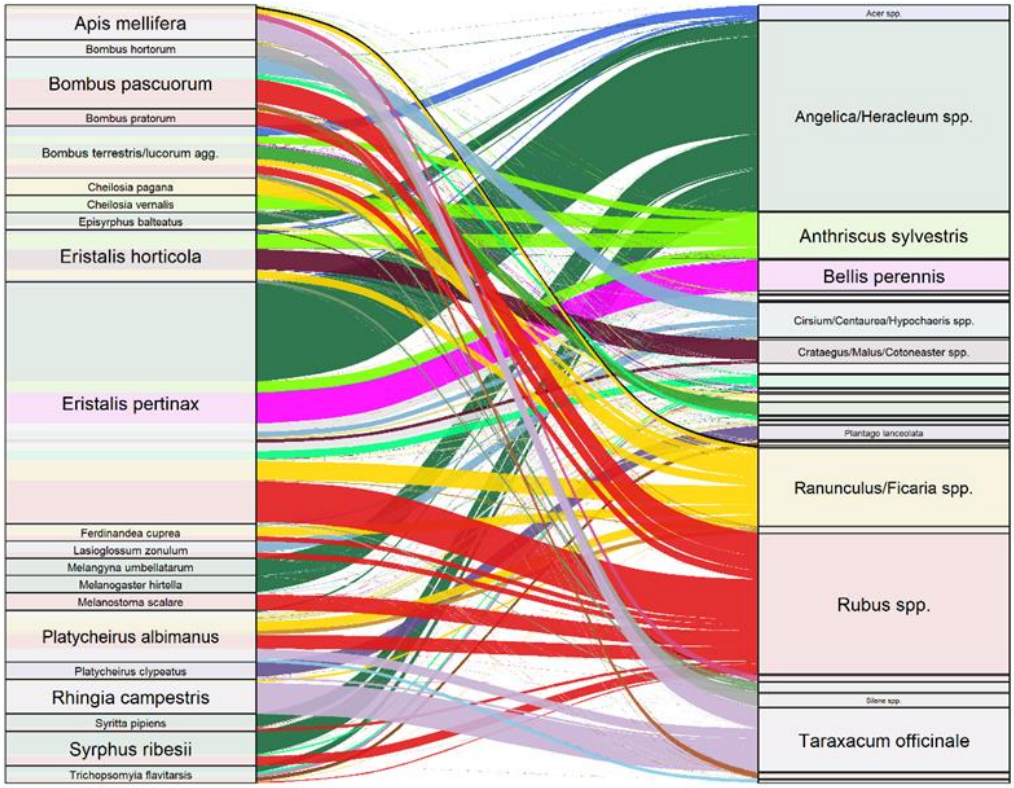


Figure S3.9: Plant-pollinator network within Transect 3 (n = 75) from March to September 2018 and 2019 based on pollen recovered from the bodies of insects, identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction.

Transect 4 (Broadleaved woodland and linear features, Nature Reserve)



Transect 5 (Broadleaved woodland and linear features, Botanic Garden)

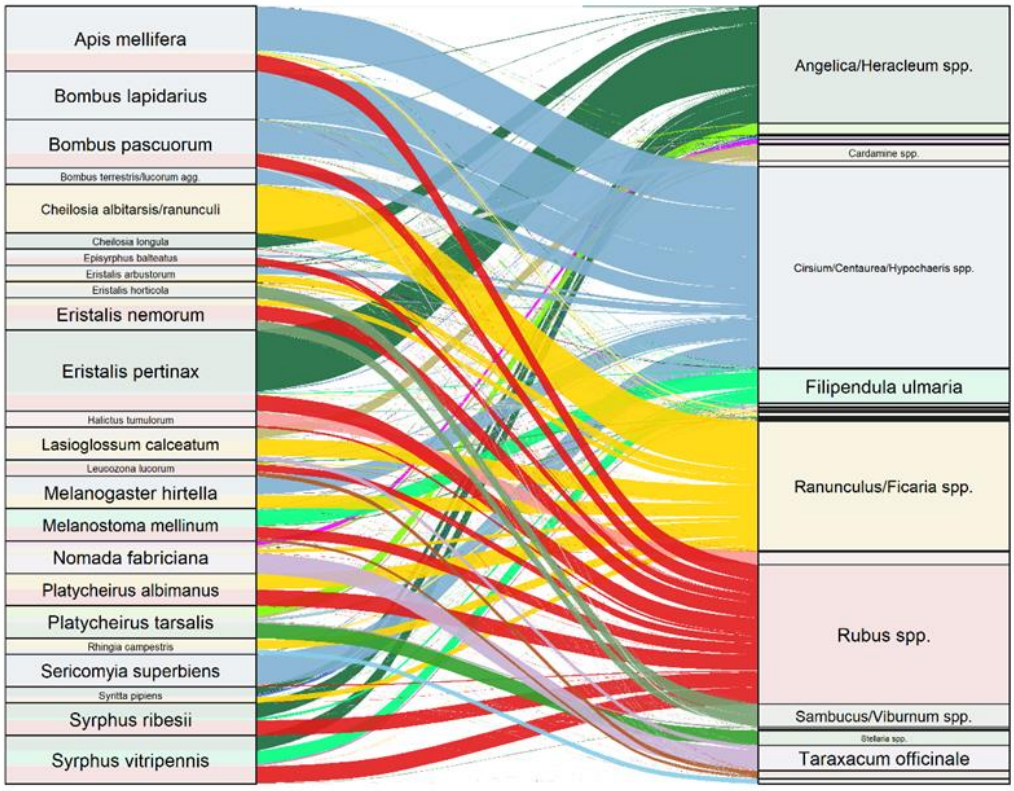
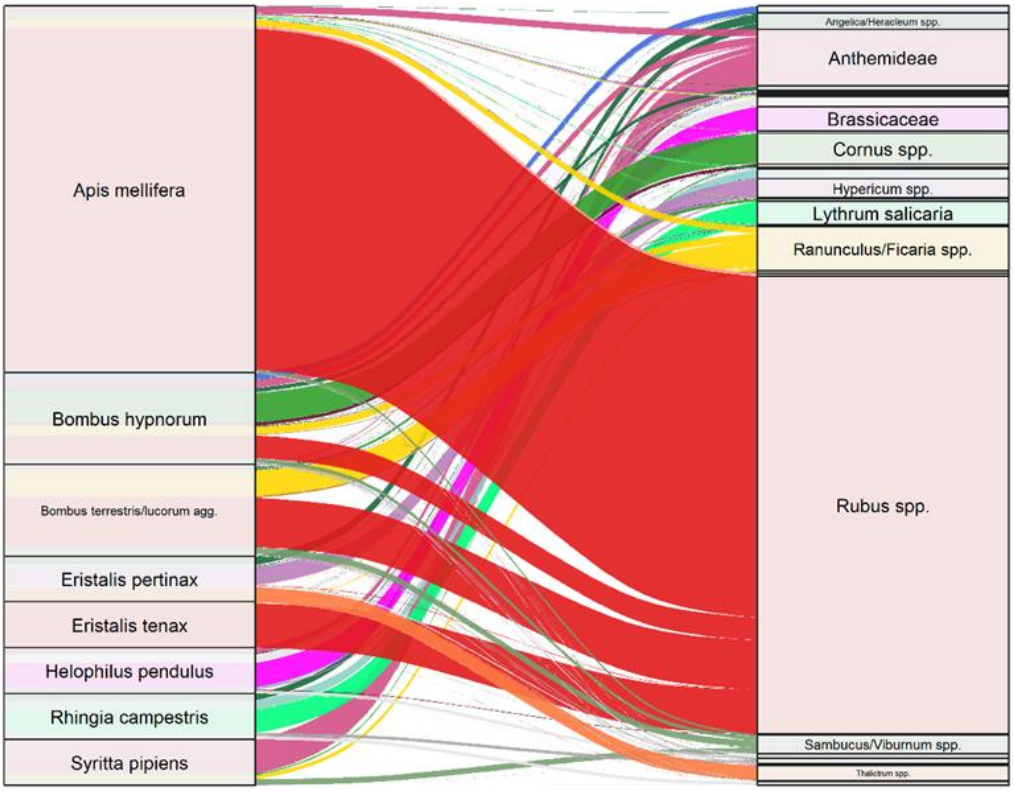


Figure S3.10: Plant-pollinator network within Transect 4 (n = 45) and 5 (n = 48) from March to September 2018 and 2019 based on pollen recovered from the bodies of insects, identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction.

Transect 6 (Broadleaved woodland and linear features, Botanic Garden)



Transect 7 (Broadleaved woodland and linear features, Botanic Garden)

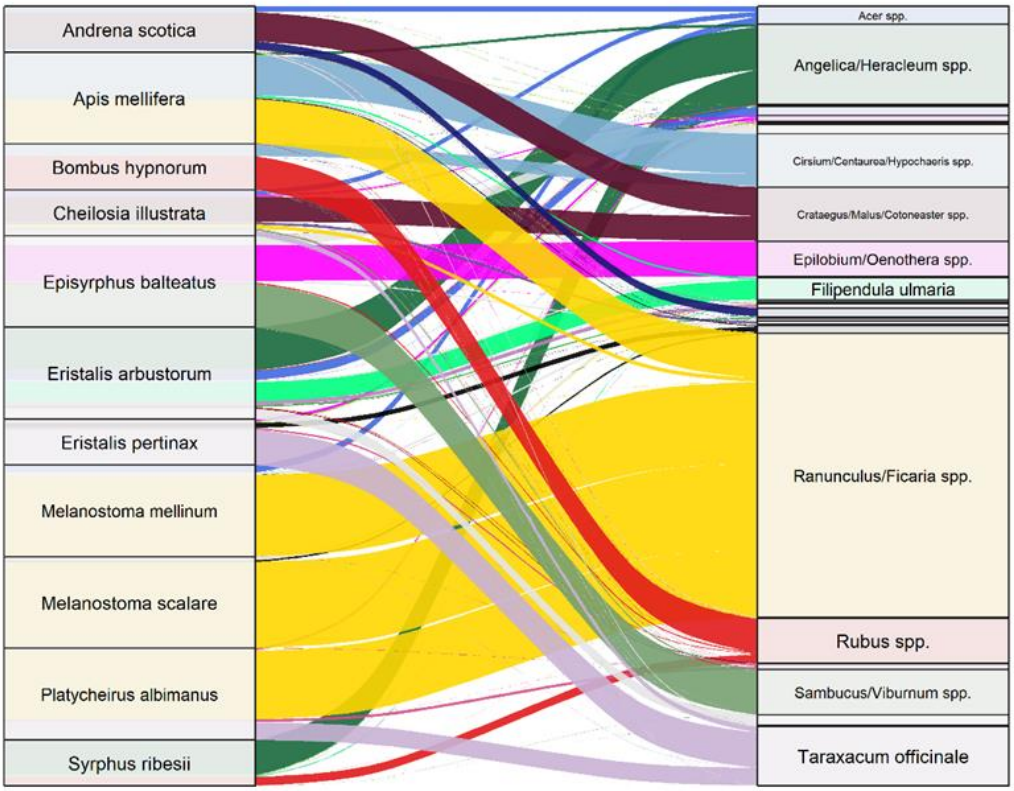


Figure S3.11: Plant-pollinator network within Transect 6 (n = 17) and 7 (n = 17) from March to September 2018 and 2019 based on pollen recovered from the bodies of insects, identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction.

Transect 8 (Broadleaved woodland and linear features, Nature Reserve)

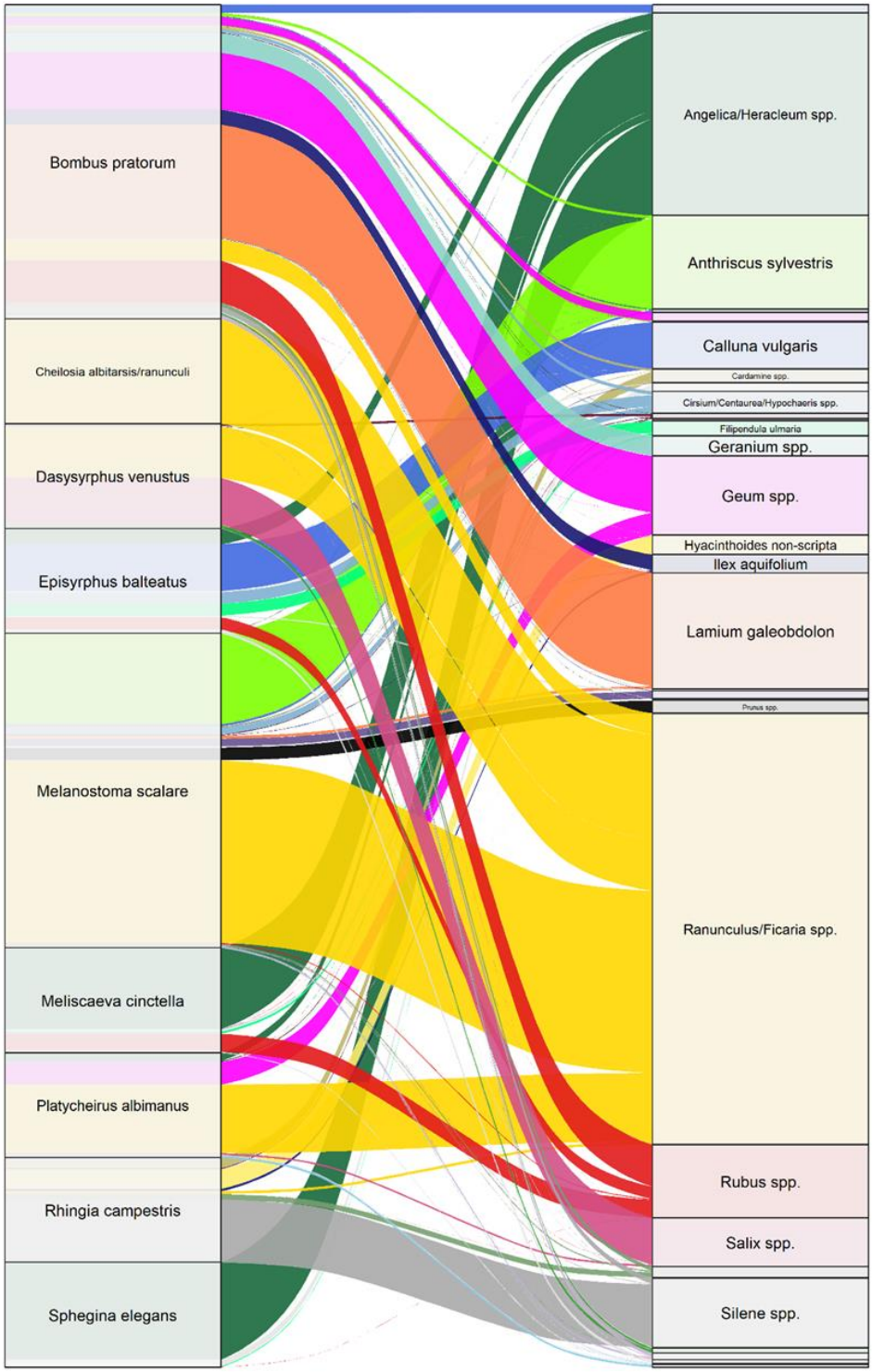


Figure S3.12: Plant-pollinator network within Transect 8 (n = 13) from March to September 2018 and 2019 based on pollen recovered from the bodies of insects, identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction.

Table S3.4: Plants for Pollinators Recommendation List grouped by season and pollinator group based on plants grown in the UK, with relevance to Northern Europe. Note that Impatiens glandulifera is used by all pollinators in the summer but cannot be recommended due to its invasive status in the UK. The suitability of each plant must be checked prior to use.

Plants for Pollinators Recommendation List

Plant taxa	Common name	Spring				Summer				Autumn				Notes
		Bumblebees	Honeybees	Non-corbiculate bees	Hoverflies	Bumblebees	Honeybees	Non-corbiculate bees	Hoverflies	Bumblebees	Honeybees	Non-corbiculate bees	Hoverflies	
Acer spp.	Maple	1	1											Native & Near-Native
Actaea spp.	Baneberry									1	3			Horticultural
Anemone spp.	Anemone												1	Native & Near-Native
Angelica sylvestris	Wild angelica					1	1	1	3	1			3	Native & Near-Native
Anthemideae	Mayweed tribe											1	1	Native & Near-Native
Anthriscus sylvestris	Cow parsley	1												Native & Near-Native
Aster spp.	Aster									2			1	Horticultural
Astrantia spp.	Masterwort											1		Horticultural
Bidens spp.	Bidens							3		1			2	Horticultural
Brassica spp.	Brassica												1	Native & Near-Native
Cardamine spp.	Bittercress		1	1	1									Native & Near-Native
Centaurea spp.	Knapweeds	1				3	3	1	3	1			1	Native & Near-Native
Cichorieae	Chicory tribe												1	Native & Near-Native
Cirsium spp.	Thistles	1				3	3	1	3	1			1	Native & Near-Native
Clematis spp.	Clematis									2		1		Native & Near-Native
Coreopsis spp.	Tickseed							3		1			2	Horticultural
Cotoneaster spp.	Cotoneaster		1											Native & Near-Native
Crataegus spp.	Hawthorn		1											Native & Near-Native
Eupatorium spp.	Eupatorium												1	Native & Near-Native
Ficaria verna	Lesser celandine	3	3	3	3					1				Native & Near-Native
Filipendula ulmaria	Meadowsweet					1	1		1	1			1	Native & Near-Native
Geum spp.	Geum			2							1			Native & Near-Native
Hedera helix	Ivy									1	1		1	Native & Near-Native
Helenium spp.	Sneezeweed							2		1			3	Horticultural
Heracleum sphondylium	Hogweed					1	1	1	3	1			3	Native & Near-Native
Heuchera spp.	Coral bells							2			2			Horticultural
Hypochaeris spp.	Cat's-ear	1				3	3	1	3	1			1	Native & Near-Native
Lotus spp.	Bird's-foot trefoil					1								Native & Near-Native
Malus spp.	Apple		1											Native & Near-Native
Meconopsis spp.	Poppy									1				Native & Near-Native
Mentheae	Mint tribe									1				Native & Near-Native
Papaver spp.	Poppy									1				Native & Near-Native
Physocarpus opulifolius	Common ninebark											1		Horticultural
Plantago lanceolata	Ribwort plantain	1	1		1									Native & Near-Native
Ranunculus spp.	Buttercups	3	3	3	3					1				Native & Near-Native
Rhinanthus minor	Yellow rattle	1												Native & Near-Native
Rubus spp.	Bramble	3	1			3	3	1	3	3	1		1	Native & Near-Native
Rudbeckia spp.	Rudbeckia							2		1			3	Horticultural
Salix spp.	Willow		1											Native & Near-Native
Salvia spp.	Salvia									1				Native & Near-Native
Sambucus nigra	Elder	1	1											Native & Near-Native
Senecio spp.	Ragwort/Groundsel									1			1	Native & Near-Native
Silene spp.	Campion	1												Native & Near-Native
Stachys spp.	Hedgenettle									1				Native & Near-Native
Taraxacum officinale	Dandelion		3	3						1				Native & Near-Native
Trifolium pratense	Red clover	1				1								Native & Near-Native
Trifolium repens	White clover					1								Native & Near-Native
Urtica dioica	Common nettle	1				1			1					Native & Near-Native
Viburnum spp.	Viburnum	1	1											Native & Near-Native

1 **Frequent** (Plants present on over 50% of individuals)

2 **Abundant** (Plants represented by over 10% of sequencing reads)

3 **Frequent and Abundant**

Bumblebees Honeybees Non-corbiculate bees Hoverflies

3.8.5. Supporting Information References

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Chapter Four

Short-term specialisation of individuals within a plant-pollinator network revealed by DNA metabarcoding

This manuscript is in preparation

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The study was conceived and designed by A.L., N.dV., and S.C; A.L. collected the data, undertook the lab work, and carried out statistical analysis with L.J., with suggestions from N.dV., S.C. and G.B. Writing of the manuscript was led by A.L. with all authors advising on drafting of the manuscript.

4.1. Abstract

The relationships between pollinators and plants underpin global biodiversity and key ecosystem services. Consequently, understanding the fine-scale details of interactions is vital for effective conservation of biodiversity, ecosystem services and human health. The degree of resource specialisation by pollinators can affect resilience against environmental change and can be measured at varying hierarchical levels from the community to the individual. At each level, pollinators may be specialised in two ways: either through floral fidelity or by occupying distinct dietary niches. However, plant-pollinator interactions are typically assessed at the level of species, potentially concealing intraspecific differences in forage. We employed a multi-locus (*rbcL* and ITS2) metabarcoding method to characterise the pollen assemblage on 369 individuals across two taxonomic orders (Diptera and Hymenoptera) including four taxonomic groups (hoverflies, bumblebees, honeybees and non-corbiculate bees) throughout the flowering season and over two years. We found that Diptera and Hymenoptera shared 143 plant taxa at the order level and niche overlap was high at the taxonomic group level. Nevertheless, unique interactions were identified within each group. Generalism in floral resource use was consistent at the species level, with few interactions unique to any species, yet the overall composition of diets was distinct. At the individual level, pollinators exhibited specialisation both in terms of floral fidelity and the dietary niche they occupy in comparison to other individuals within the same species. Our study therefore demonstrates that plant-pollinator networks are composed of individuals which exhibit short-term specialisation, a pattern which is undetected at the species level. As the degree of specialisation and generalisation within a network can affect its vulnerability to environmental change, we highlight the value of DNA metabarcoding for providing detailed individual-level information, significantly advancing our knowledge of how plant-pollinator networks are structured.

4.2. Introduction

Plant-pollinator interactions form complex ecological networks which underpin global biodiversity whilst providing key ecosystem services (Potts et al., 2016). The stability of these networks is threatened by species extinctions and phenological mismatches, which can be caused by environmental stressors such as climate change and a reduction in floral availability (Mathiasson & Rehan, 2020). Ensuring that these important interactions are maintained requires a deeper understanding of how plant-pollinator relationships are organised within a network, which can be used to predict the resilience of both species and networks to environmental change (Memmott et al., 2004).

The interactions between pollinators and plants can be studied at different levels of biological hierarchy, from the whole community to the individual (Brosi, 2016). Diet specialisation and generalisation can vary at each level and can be characterised either considering niche breadth or the degree of niche partitioning (Blüthgen et al., 2006). For example, each hierarchical pollinator group (order, family, genus, species) may be classed as specialised if utilising one or few plant hosts (exhibiting floral fidelity) or generalised if using many resources. In addition, if the group in question uses a small subset of the total resources used by others at the same hierarchical level, it is considered to have a specialised dietary niche within the network even if utilising a large number of resources (Bolnick et al., 2002). High degrees of specialisation are thought to have evolved to minimise competition between species, increasing the efficiency of foraging whilst also improving plant reproductive success by transferring more conspecific pollen between flowers (Brosi & Briggs, 2013). Specialisation can occur as a result of plants possessing a particular morphology which limits species interactions based on body size (Peat et al., 2005) or mouthpart shape (Gilbert, 1981), having restricted blooming periods (McGinley, 2003), and adopting specific floral rewards such as

oil to attract certain visitors (Minckley & Roulston, 2006). The degree of specialisation of an individual, species and/or network can have consequences for its robustness against environmental change, with specialised networks and species being more susceptible (Goulson et al., 2005; Memmott et al., 2004) whilst generalists may be more resilient (Biesmeijer et al., 2006).

4.2.1. Specialisation within a community (between broad taxonomic groups)

Within plant-pollinator networks, insects may be grouped by taxonomy; for example, order, family, or genus, depending on the complexity of the network. At the community level, networks tend to be generalised, as multiple pollinator species forage on a range of plants, and plants in turn receive visits from many insect visitors (Waser et al., 1996). Network patterns such as connectance, nestedness, and modularity are often used to assess network structure, and are influenced by the number of plant and pollinator species present (Olesen et al., 2007; Bascompte et al., 2003). Plant-pollinator networks are typically highly nested, with specialists interacting with only a subset of species which interact with more generalist species (Bascompte et al., 2003). These interactions are organised into subsets of species which are interlinked (modules), and the extent of organisation of modules is known as the modularity of the network (Olesen et al., 2007). The relationship between nestedness and modularity is complex, however, in pollination networks, modularity is complementary to nestedness (Olesen et al., 2007).

Partitioning of floral resources between insects of different taxonomic groups can be shaped by inherent biological attributes that may lead to specialisation. For example, bees (Hymenoptera) and hoverflies (Diptera) differ markedly in their life history; with bees being central-place foragers, collecting pollen and nectar to

return to the nest to rear young and as such are restricted in their forage preferences by the distance from the nest. In contrast, hoverflies visit plants for mating and egg-laying as well as for pollen and nectar feeding and may travel across larger distances than bees as they do not operate from a central location (Meyer et al., 2009). Moreover, morphological features such as body size and pilosity (hairiness) (Cullen et al., 2021), or tongue length (Gilbert, 1981; Goulson et al., 2008) can drive differences in pollen collection between bees and hoverflies by affecting the abundance of pollen sampled and access to certain plants.

4.2.2. Specialisation within a taxonomic group (between species)

Pollinators can also display preferences for particular plants at a species level, with this preference either being learned (Dobson et al., 2012) or innate (Praz et al., 2008). A measure of floral fidelity which is often used to describe species-level specialisation is pollen diet breadth, defined by a set of terms relating to the number of and relationship between plant hosts. Broadly speaking, foragers which collect pollen from many different pollen sources are 'polylectic', those which utilise a few species of the same genus or family are described as 'oligolectic' and those which completely specialise on a single plant species are 'monolectic' (Cane & Sipes, 2006). This specialisation may be influenced by spatiotemporal variation in resources, for example monolecty can just be the result of isolation from other closely related pollen sources, as opposed to a preference by the species itself (Cane & Sipes, 2006). In addition, plants visited for nectar consumption may be overlooked, making species seem more specialised than they are (Arstingstall et al., 2021). Diet breadth has been widely studied in bees, with most species classed as polylectic, although high incidences of specialism have been found in some bee communities (Moldenke, 1976). In contrast, few authors have attempted to classify hoverfly species by this terminology, yet it is thought that they are less likely to display strong preferences for a particular plant species (Amy et al., 2018; Branquart & Hemptinne, 2000).

4.2.3. Specialisation within a species (individual specialisation)

Individual specialisation in pollinators has been typically measured by assessing the diversity and abundance of plant sources visited by an individual within a foraging bout, with individuals classed as specialised when using one or few sources in a single trip. Short-term specialisation of individuals may arise as a result of learned and innate preferences, spatiotemporal restrictions to alternative resources or memory constraints (Amaya-Márquez, 2009; Brosi, 2016; Waser, 1986). The term floral constancy is often used to describe a behaviour in which individuals visit solely one plant species in a foraging trip (Lucas et al., 2018b), however, many authors explicitly distinguish this term from learned and innate preferences and reserve its use for a behaviour driven by cognitive constraints (Amaya-Márquez, 2009; Brosi, 2016; Waser, 1986). We therefore use the term floral fidelity at both the species and individual level to describe the tendency to focus on one plant species, without explicitly considering the underlying mechanisms driving this behaviour.

Another important aspect of specialisation is the study of whether all individuals within a species have specialised dietary niches. Few studies have focussed on plant-pollinator networks at the individual level, however, those that have found that generalised networks can be composed of specialised individuals, each with variable diets (Lucas et al., 2018b; Tur et al., 2014). The analysis of plant-pollinator interactions at a species or network level may therefore be concealing individual patterns of specialisation (Brosi, 2016).

Individual specialisation in pollinators may be studied by observing pollinator behaviour during flight (Goulson et al., 1997) or by studying the composition of pollen carried (Smith et al., 2019). Most studies concern honeybees and bumblebees, with both groups found to exhibit high degrees of floral fidelity at the

individual level (Heinrich, 1976; Leonhardt & Blüthgen, 2012), although this behaviour has also been found in solitary or non-corbiculate bees (Bennet et al., 2018) and hoverflies (Goulson & Wright, 1998; Lucas et al., 2018b).

4.2.4. Network analysis using DNA metabarcoding

DNA metabarcoding, the use of high-throughput sequencing to identify species in mixed samples by comparing sequences with known reference libraries (Ji et al., 2013) has recently been shown to be a useful tool for characterising the structure of ecological networks (Clare et al., 2019; Evans et al., 2016). In the UK, the Barcode UK project provides 98% coverage of all native flowering plants and conifers with three plant DNA markers, *rbcL*, *matK* and ITS2, ensuring accurate species identification of plants at species and genus level (Jones et al., 2021b). DNA metabarcoding overcomes the limitations of using traditional methods such as observational field studies and pollen microscopy to construct ecological networks by allowing a large number of samples to be analysed (Sickel et al., 2015), reducing sampling biases (Jordano, 2016) and minimising the level of expertise required to identify pollen grains (Hawkins et al., 2015). DNA metabarcoding has successfully been used to identify the foraging preferences of pollinators through analysis of pollen from legs (Bänsch et al., 2020; Richardson et al., 2021), nests (Gresty et al., 2018; Sickel et al., 2015), honey (Hawkins et al., 2015; Jones et al., 2021a), gut contents (Wilson et al., 2010) and the bodies of insects (Galliot et al., 2017; Lucas et al., 2018a).

The analysis of pollen using DNA metabarcoding facilitates a comprehensive, integrated spatiotemporal analysis of foraging on a fine scale, presenting an opportunity to transform our understanding of individual-level plant-pollinator interactions within a complex network. Enhancing our knowledge of floral resource partitioning and specialisation within networks gives an insight into which plants are most utilised by individuals and species, allowing us to

effectively conserve flora that are demonstrably important for forage for a diverse suite of pollinators, increasing the resilience of the network to environmental change.

4.2.5. Aims and Objectives

We aim to use DNA metabarcoding to understand how plant use is structured within an insect community at different hierarchical levels. We specifically address the following questions:

5. Within a plant-pollinator network, do taxonomic orders (Diptera and Hymenoptera) and groups (bumblebees, hoverflies, honeybees, and non-corbiculate bees) show floral specialisation?
6. What are the patterns of specialisation and generalisation between species?
7. Do individuals exhibit floral fidelity?
8. How do the foraging choices of individuals reflect the overall species patterns?

4.3. Materials and Methods

4.3.1. Pollinator sampling

The study took place during 2018 and 2019 at the National Botanic Garden of Wales, UK (51°50'33.4"N 4°08'49.2"W). The study area (230 ha) consists of formal garden including over 5000 global plant taxa, and organic farmland, designated as a National Nature Reserve (Waun Las NNR). The major habitat is semi-improved grassland, and pollinators were sampled in eight areas covering broadleaved woodland and linear features (hedgerows and walls), horticultural and grassland habitat. These areas were selected in order to capture pollinators in a diversity of habitats. Transect walks were established within each area, consisting of a 210 m x 2 m transect, divided into 3 x 70 m sections which were walked independently of each other (Chapter Three; Lowe et al., 2022). Bees and hoverflies were caught individually within a sterile 1.5 ml or 15 ml tube by placing a net with the tube held inside above the insect and stored at -20 °C prior to pollen removal.

4.3.2. Pollen removal

Pollen was removed from each insect following a modified protocol described by Lucas et al. (2018b). Insects were moved into sterile 1.5 ml collection tubes using sterile forceps and cleaned with 70% ethanol between each insect. A 1 ml solution of 1% sodium dodecyl sulphate (SDS) and 2% polyvinylpyrrolidone (PVP) was used to wash the tube the insects were caught in, collecting any pollen residue on the sides, and transferring into the tube now containing the insect. Samples were shaken using a TissueLyser II (Qiagen) at 8.5 Hz for 1 minute, stood at room temperature for 5 minutes, then shaken again at 8.5 Hz for 20 seconds. Each insect was removed using sterile forceps and placed in a 1.5 ml microcentrifuge tube containing 70% ethanol, prior to species identification. Bees were identified using Falk and Lewington (2015) and hoverflies using Stubbs and Falk (2003). Voucher specimens were retained in a reference collection at the National Botanic Garden

of Wales. The tube containing the detergent and pollen pellet was centrifuged at 13000 rpm for 5 minutes and the supernatant removed. The pollen pellet was resuspended in 400 µl buffer, made up of 400 µl AP1 from the DNeasy 96 Plant Kit (Qiagen) and 80 µl (1 mg/ml) of Proteinase K (Qiagen).

4.3.3. Molecular analysis

DNA was extracted using a modified version of the DNeasy 96 Plant Kit. Pollen samples were incubated in a water bath for 1 hour at 65 °C and 1 µl RNase (Qiagen) added before being shaken with 3 mm tungsten carbide beads for 4 minutes at 30 Hz using a TissueLyser II. The remaining steps of the protocol were followed, excluding the use of the QIAshredder and the second wash stage. A negative control containing 400 µl of the AP1 and Proteinase K buffer and 1 µl RNase only was included within each extraction. A two-step PCR protocol (Brennan et al., 2019) was used to amplify two barcode regions, *rbcL* and ITS2 using the primers *rbcL*af (Kress & Erickson, 2007) and *rbcL*r506 (de Vere et al., 2012); and ITS2F (Chiou et al., 2007) and UniPlantR (Moorhouse-Gann et al., 2018). A 6N sequence was added between the forward template specific primer and the universal tail to improve clustering on the Illumina MiSeq (Table S4.1, Supporting Information)

A final volume of 20 µl was used for the initial PCR: 2 µl template DNA, 10 µl of 2x Phusion Hot Start II High-Fidelity Mastermix (New England Biolabs UK), 0.4 µl (2.5 µM) forward and reverse primers, and 7.2 µl of PCR grade water. The thermal cycling conditions for the first *rbcL* PCR were: 98 °C for 30 s, 95 °C for 10 min; 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min (35 cycles); 72 °C for 10 min, 30 °C for 1 min. Thermal cycling conditions for the first ITS2 PCR were: 98 °C for 30 s, 95 °C for 10 min; 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min (40 cycles); 72 °C for 10 min, 30 °C for 1 min. The initial PCR was carried out three times and pooled prior to purification using Illumina's 16S Metagenomic Sequencing Library preparation protocol using a 1:0.6 ratio of product to Agencourt AMPure XP beads

(Beckman Coulter). This purified product was amplified further with a second PCR to add sample-specific i5 and i7 indices (Ultramer, Integrated DNA Technologies), using a final volume of 25 µl: 5 µl of purified first-round PCR product, 12.5 µl of 2x Phusion Hot Start II High-Fidelity Mastermix (New England Biolabs UK), 1 µl of i5 and i7 Index Primer, and 6.5 µl of PCR grade water. Thermal cycling conditions for the index PCR were as follows: 98 °C for 30 s; 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s (8 cycles); 72 °C for 5 min, 4 °C for 10 min. The Illumina protocol for the second PCR clean-up stage was followed using a 1:0.8 ratio of product to beads. Products were quantified using a Qubit 4.0 (Thermo Fisher Scientific) and pooled at equalimolar concentrations. The negative extraction and PCR controls from each plate were sequenced with the pollen samples on an Illumina MiSeq (2 x 300 bp).

4.3.4. Bioinformatic analysis

Sequence reads were processed following Ford & Jones (2020). Raw sequences were trimmed to remove low quality regions, paired, and merged, retaining only sequences greater than 450 bp (*rbcL*) and 350 bp (ITS2) for downstream analysis. Within each sample, identical reads were dereplicated and clustered at 100% identity across all samples with singletons (sequence reads occurring once across all samples) removed. Sequences were compared to a custom reference library of 5,887 plant species for identification (Jones et al., 2021a), comprising native plants of the UK (Stace, 2019), naturalised and alien species (Preston et al., 2002) and horticultural species from the IRIS BG database at the National Botanic Garden of Wales.

Sequences were compared to the reference library using blastn and a summary of the top 20 BLAST hits was created, with all sequences obtaining identical BLAST results across all 20 hits combined. Sequences with bit scores below the 1st percentile were excluded. Assignment of plant taxa followed so that if the top

bitscore matched a plant species, the sequence was assigned to that species. If the top bitscore matched different species within the same genus, the sequence was assigned to that genus. If the top bitscore belonged to multiple genera of the same family, then a family designation was made for that sequence. Sequences returning top bitscores of multiple families within different orders were removed, assuming that these were poor-quality sequences. Each identification was checked for botanical veracity, considering the species presence within the study site and wider landscape. Taxonomic assignment of sequences was compared between markers on a sample-by-sample basis for further verification.

Following assignment of species, taxa identified by both markers at differing taxonomic resolution were compared and a consensus identification was reached using a rule-based, objective, and conservative decision process (Chapter Three; Lowe et al., 2022). Spearman's rank correlation with Holm correction for multiple testing was used to test the relationship between the proportion of read abundance of matched plant taxa detected by both *rbcL* and ITS2 within each sample. Following this, the number of *rbcL* and ITS2 sequences for each consensus taxon within a sample were summed to combine the results of each marker.

Plants identified to genus and species were assigned to a status category following Stace (2019). The category 'native and near native' comprised native species and also genera that include native species and horticultural varieties which are functionally similar. Naturalised plants were those which have been introduced and become widespread and self-perpetuating in the wild. All remaining non-native plants were classified as horticultural.

4.3.5. Statistical analysis

The data were treated as semi-quantitative with the proportion of taxa in a sample used as a measure of relative read abundance (Deagle et al., 2019; Jones et al.,

2021a). The appropriateness of analysing data from females and males together was assessed by visual inspection of differences in pollen composition between sexes using non-metric multidimensional scaling plots. We compiled three bipartite networks: one for all pollinator species within the network, one for Dipteran (hoverfly) species only and one for Hymenopteran (bee) species only. Sampling completeness was estimated using the Chao2 incidence-based estimator (Macgregor et al., 2017). Network metrics H_2' and d' (Blüthgen et al., 2006) were calculated using the bipartite package (Dormann et al., 2008). H_2' measures specialisation within a network and varies from 0 (complete generalisation – all plant interactions are shared equally across all pollinator species) to 1 (complete specialisation – no plant interactions are shared across pollinator species). d' is a measure of the degree of exclusivity of interactions by each species in comparison to others in the same network ranging from 0 (complete generalisation – the plant interactions of each pollinator species are identical) to 1 (the plant interactions of each pollinator species are unique).

We focused our analysis on the most species-rich and abundant taxonomic groups, here hoverflies and bumblebees, with additional analysis on honeybees and non-corbiculate bees where possible. H_2' was calculated for all three networks whilst d' values were calculated for hoverflies and bumblebees separately within their respective networks to account for phylogenetic relationships within the same taxonomic group (Blüthgen et al., 2006). d' values were also calculated to measure the exclusivity of interactions between genera within Hymenoptera. The statistical significance of H_2' was tested using permutation tests with 2000 iterations. The statistical significance of intraspecific differences in diet composition within bumblebee and hoverfly groups were tested using permutational analysis of variance (PERMANOVA) with 999 permutations using the 'adonis' function in the package vegan (Oksanen et al., 2019). The niche overlap between Diptera and Hymenoptera and each combination of subgroup within (hoverflies, bumblebees,

honeybees and non-corbiculate bees) was calculated using the bipartite package (Dormann et al., 2008), based on Horn's index (Horn, 1966), where values range from 0 (no overlap in dietary niche) to 1 (total dietary niche overlap).

We measured the degree of individual specialisation (IS) within a species using the 'PSicalc' function within the RInSP package (Zaccarelli et al., 2013) which calculates the proportional similarity (PSi), adapted from Schoener (1968) (Bolnick et al., 2002):

$$PS_i = 1 - 0.5 \sum_j |p_{ij} - q_j|$$

in which p_{ij} is the frequency of floral resource j in the individual i's diet, and q_j is the frequency of floral resource j in the population as a whole. If individuals are specialised on one plant taxa j, then $PS_i = q_j$, and if the diet of an individual is directly proportional to the diet of the species as a whole, then $PS_i = 1$ (generalised). The individual specialisation index (IS) was calculated for each species by averaging the proportional similarity (PS_i) across each individual within a species. To calculate the significance of IS we used a nonparametric Monte Carlo procedure using 2000 simulations. All statistical analyses were carried out in R v 4.0.2 (R Core Team, 2020) using the consensus identification. Analysis of *rbcL* and ITS2 was also carried out separately to support the use of combining markers (Chapter Three; Lowe et al., 2022).

4.4. Results

4.4.1. Overview

Pollen loads from 369 insects were sequenced. Insects were grouped according to five hierarchical levels: taxonomic order (Diptera, Hymenoptera), taxonomic group (hoverflies, bumblebees, honeybees, and all other non-corbiculate bees), genus, species, and individual (Fig. 4.1). Diptera were represented by 195 hoverflies of 41 species and Hymenoptera consisted of 108 bumblebees (6 species), 44 honeybees (1 species) and 22 non-corbiculate bees (9 species). The most abundant species collected were *Apis mellifera* (n = 44), *Bombus terrestris/lucorum* agg. (n = 35), *Bombus lapidarius* (n = 29), *Eristalis pertinax* (n = 28), *Bombus pascuorum* (n = 22) and *Cheilosia albitarsis s.l.* (n = 21). A total of 148 insects were collected in spring (March – May), 210 in summer (June – August) and 11 in autumn (September) (Fig. S4.1, Supporting Information).

22,510,682 sequences were returned (11,305,697 *rbcL* and 11,204,985 ITS2) and 191 plant taxa were identified using both markers combined. There was a strong correlation between the proportion of sequences of each matched taxa (n = 105) found within each sample using both *rbcL* and ITS2 (Spearman correlation coefficient $r_s = 0.601$, $P < 0.001$), supporting the use of combining markers. Overall, the most frequently found taxa across all samples were *Rubus* spp., *Cirsium/Centaurea/Hypochaeris* spp., *Ranunculus/Ficaria* spp., *Angelica/Heracleum* spp., Asteraceae and *Filipendula ulmaria*. Network completeness with respect to Chao2 richness estimations was 67.8% (Macgregor et al., 2017) for the entire plant-pollinator network. No distinct separation was found between pollen composition of males and females across all taxonomic groups therefore both sexes were analysed together (Fig. S4.2, Supporting Information).

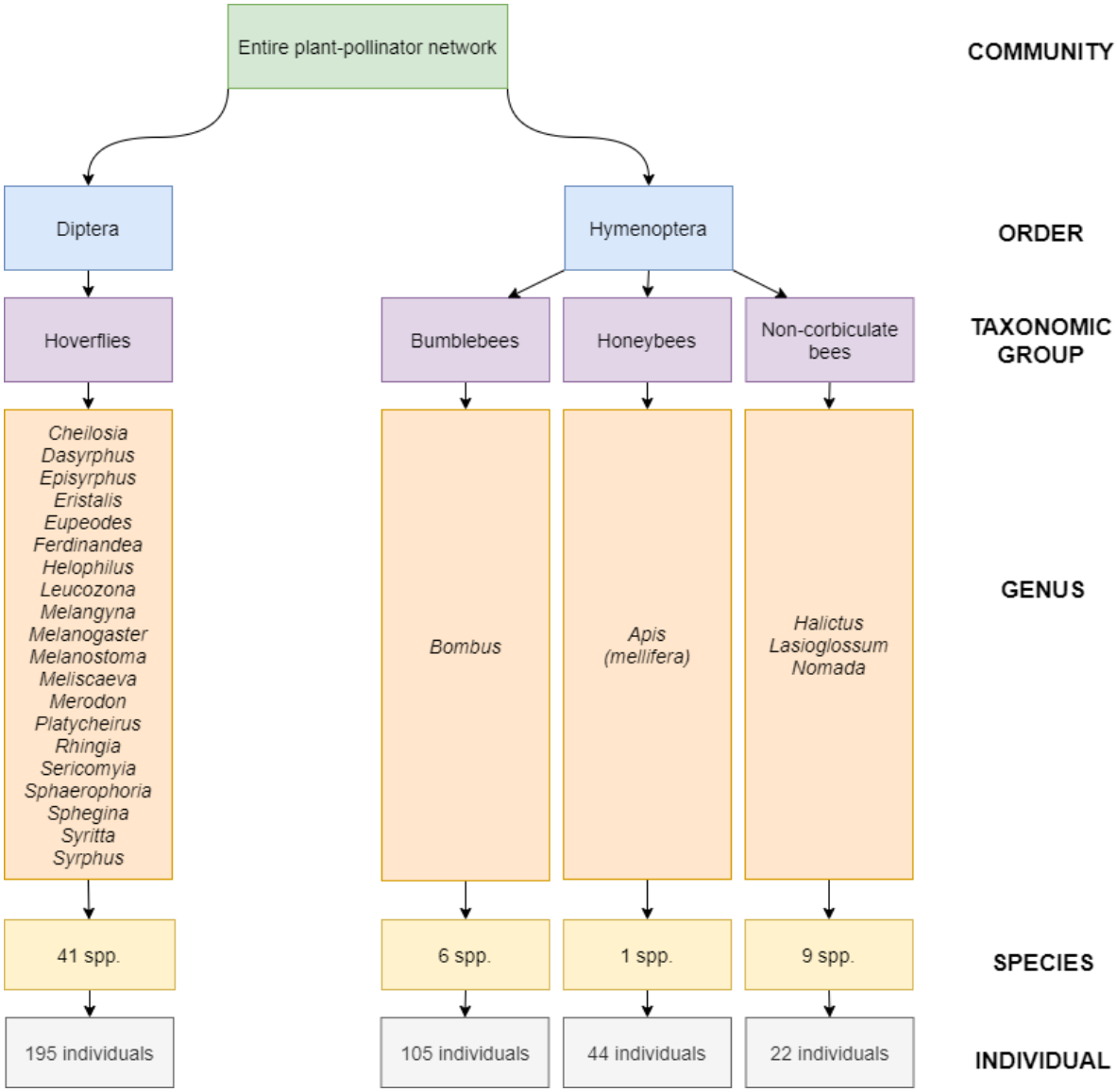

Figure 4.1: The hierarchical levels of insects used to analyse interactions between plants and pollinators within a network, from the community to individual level.

4.4.2. Within a plant-pollinator network, do bumblebees, hoverflies, honeybees, and non-corbiculate bees show specialisation?

The network specialisation metric H_2' revealed that overall, at the community level, the plant-pollinator network showed more of a tendency toward specialisation than generalisation (H_2' = 0.64, P < 0.001) (Fig. 4.2). A total of 162 taxa were identified from Diptera (hoverflies), and 172 from Hymenoptera (bumblebees, honeybees, and non-corbiculate bees), with 143 taxa shared between both. When analysing Diptera and Hymenoptera networks separately, both networks showed the same degree of specialisation as the community level (H_2' = 0.65 for both) (Fig. S4.3, Supporting Information). For all three networks, we found that H_2' differed significantly from null models (P < 0.001), demonstrating that the values did not arise by chance. Diptera and Hymenoptera had a niche overlap of 0.62 and similar values were found between each taxonomic group within (Table S4.2, Supporting Information).

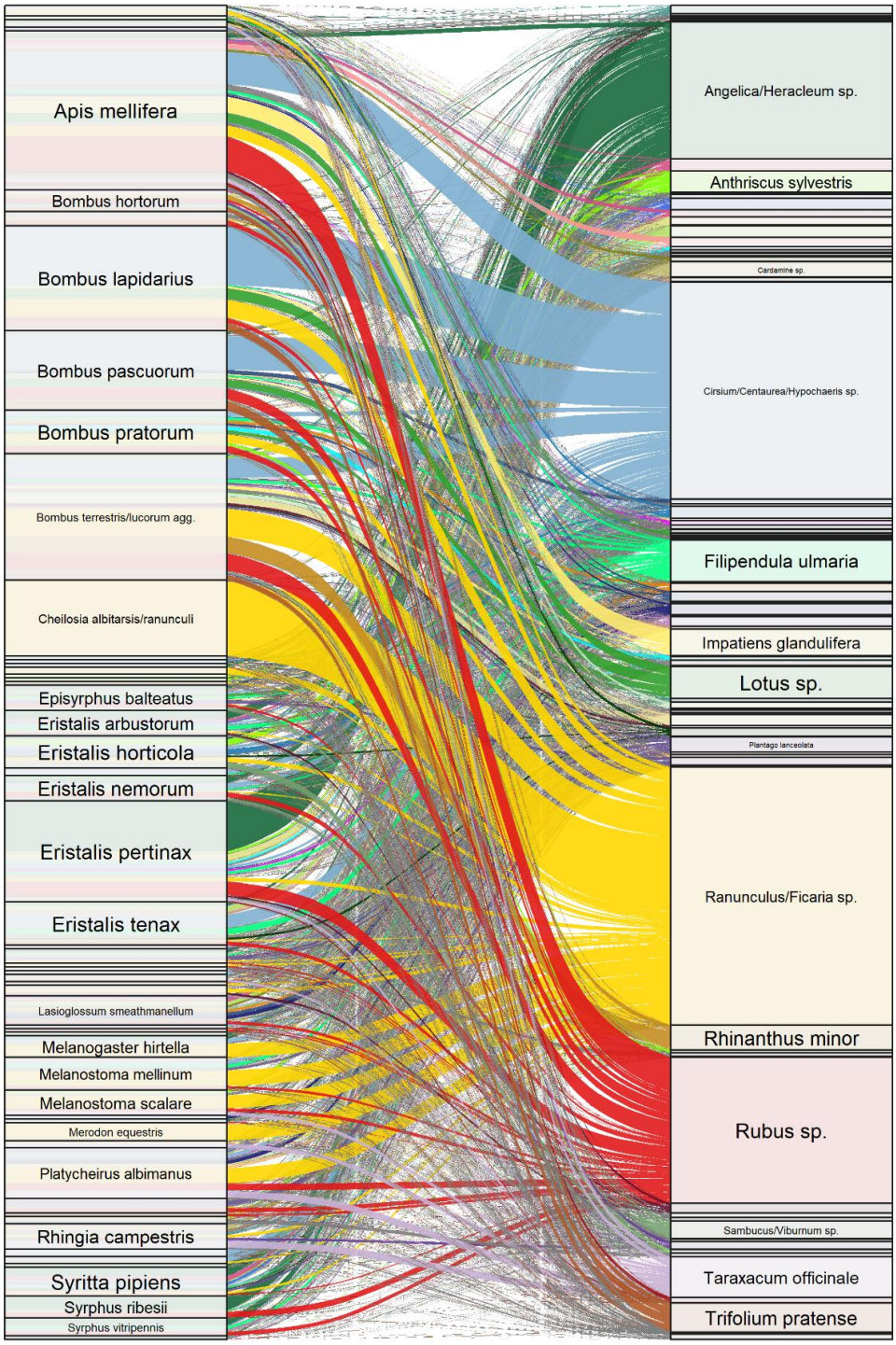

Figure 4.2: Plant-pollinator network based on pollen recovered from bodies of insects (n = 369) and identified by DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and pollinators is based on the strength of the interaction. Pollinators were collected across seven sampling periods from March to September across 2018 and 2019.

4.4.3. What are the patterns of specialisation and generalisation between species?

Only species represented by over five individuals were included in further analysis of species-level specialisation. This comprised sixteen species of hoverfly and five species of bumblebee. The number of taxa found from each species ranged from 26 to 141 and was reduced to one to 18 when assessing those that contributed over 1% of reads per species (Table 4.1). The hoverfly *Cheilosia albitarsis s.l.* appears to be oligolectic on *Ranunculus/Ficaria* spp. with 98% of all sequence reads returned from this taxon (Fig. S4.4, Supporting Information). All other bee and hoverfly species were found to be polylectic, utilising taxa from a range of plant families (Figs S4.4-4.5, Supporting Information).

For bumblebees, the number of taxa identified from each species ranged from 56 to 114 (Table 4.1). Values of *d′* ranged from 0.19 (*Bombus terrestris/lucorum* agg.) to 0.56 (*Bombus hortorum*), illustrating that few bumblebee-plant interactions were exclusive to any particular species (Table 4.1). PERMANOVA analysis detected that diet composition significantly differed between bumblebee species which were represented by over five individuals ($R_2 = 0.123$, d.f. = 4, $P < 0.001$). Taxa such as *Cirsium/Centaurea/Hypochaeris* spp. and *Rubus* spp. were most shared between species, with *Bombus hortorum* visiting a visibly different suite of taxa often in comparison to other species (Fig. S5.5, Supporting Information).

Within the hoverfly group, the number of taxa identified from each species ranged from 26 to 92 for hoverflies (Table 4.1). *d′* values ranged from 0.18 (*Melanogaster hirtella*) to 0.66 (*Rhingia campestris*), demonstrating a greater range in species-level specialisation than seen in bumblebees (Table 4.1). Diet composition significantly differed between hoverfly species represented by over five individuals ($R_2 = 0.34$, d.f. = 15, $P < 0.001$). Taxa such as *Ranunculus/Ficaria* spp. and *Angelica/Heracleum*

spp. were shared across multiple species, however, *R. campestris* was the only species to use *Lythrum salicaria, Cardamine* spp., *Silene* spp., and *Geranium* spp. abundantly (Fig. S4.4, Supporting Information).

As only one species of non-corbiculate bee met this criterion, and honeybees constitute one species alone in this instance, resource partitioning was assessed between genera within Hymenoptera represented by over five individuals (*Apis, Bombus, Lasioglossum,* and *Andrena*) (Fig. S4.1, Supporting Information). Some genus-level specialisation was identified within Hymenoptera, with *d'* values ranging from 0.42 to 0.6 (Table S4.3, Supporting Information). *Apis* and *Bombus* shared a large proportion of major taxa however *Andrena* and *Lasioglossum* were more variable in their diet, utilising taxa such as *Physocarpus opulifolius* and *Heuchera* spp. respectively, which were not shared by others (Fig. S4.6, Supporting Information).

Table 4.1: For each species represented by over five individuals, the network metrics *d'* and IS were calculated. *d'* is a measure of the degree of unique interactions within a species in comparison to others (within the same taxonomic group) and ranges from 0 (complete generalisation) to 1 (complete specialisation). IS measures of individual specialisation, with IS = 1 when the average diet of an individual is directly proportional to the diet of the species as a whole (generalisation) and nears towards 0 when each individual has its own unique diet. *d'* values were not calculated for *Apis mellifera* and *Lasioglossum smeathmanellum* due to low or missing samples of other species within the same group.

Taxonomic group	Species	F	M	n	Number of plant taxa identified	Number of plant taxa contributing >1% total reads	*d'*	IS
Honeybee	*Apis mellifera*	44	0	44	141	16	NA	0.18 (P < 0.001)
Bumblebee	*Bombus hortorum*	6	0	6	56	12	0.56	0.38 (P < 0.001)
	Bombus lapidarius	21	8	29	75	7	0.34	0.41 (P < 0.001)
	Bombus pascuorum	20	2	22	90	13	0.28	0.37 (P < 0.001)
	Bombus pratorum	8	4	12	106	13	0.34	0.26 (P < 0.001)
	Bombus terrestris/lucorum agg.	31	4	35	114	16	0.19	0.26 (P < 0.001)
Hoverfly	*Cheilosia albitarsis s.l.*	8	13	21	56	1	0.44	0.98 (P < 0.001)
	Episyrphus balteatus	4	3	7	39	12	0.32	0.23 (P < 0.001)
	Eristalis arbustorum	2	5	7	61	10	0.25	0.38 (P < 0.001)
	Eristalis horticola	6	3	9	66	10	0.31	0.43 (P < 0.001)
	Eristalis nemorum	4	3	7	61	11	0.19	0.42 (P < 0.001)
	Eristalis pertinax	8	20	28	92	9	0.25	0.45 (P < 0.001)
	Eristalis tenax	3	9	12	82	10	0.33	0.30 (P < 0.001)
	Melanogaster hirtella	5	1	6	26	3	0.18	0.56 (P < 0.001)
	Melanostoma mellinum	8	1	9	45	8	0.24	0.36 (P < 0.001)
	Melanostoma scalare	3	4	7	41	7	0.36	0.37 (P < 0.001)
	Merodon equestris	1	4	5	40	3	0.44	0.85 (P < 0.001)
	Platycheirus albimanus	9	5	14	67	15	0.22	0.25 (P < 0.001)
	Rhingia campestris	4	3	7	61	10	0.66	0.32 (P < 0.001)
	Syritta pipiens	5	3	8	55	9	0.26	0.38 (P < 0.001)
	Syrphus ribesii	3	3	6	38	5	0.27	0.81 (P < 0.001)
	Syrphus vitripennis	5	0	5	35	7	0.27	0.40 (P < 0.001)
Non-corbiculate bee	*Lasioglossum smeathmanellum*	6	2	8	48	12	NA	0.17 (P < 0.001)

4.4.4. Do individuals exhibit floral fidelity?

Bumblebees, honeybees, and hoverflies all had high occurrences of floral fidelity, with the largest proportion of individuals having 90-100% of their pollen load dominated by a single plant taxon (Fig. 4.3). Non-corbiculate bees, however, tended to have a lower proportion of their pollen load dominated by a single taxon, with 60-69% being the most abundant occurrence.

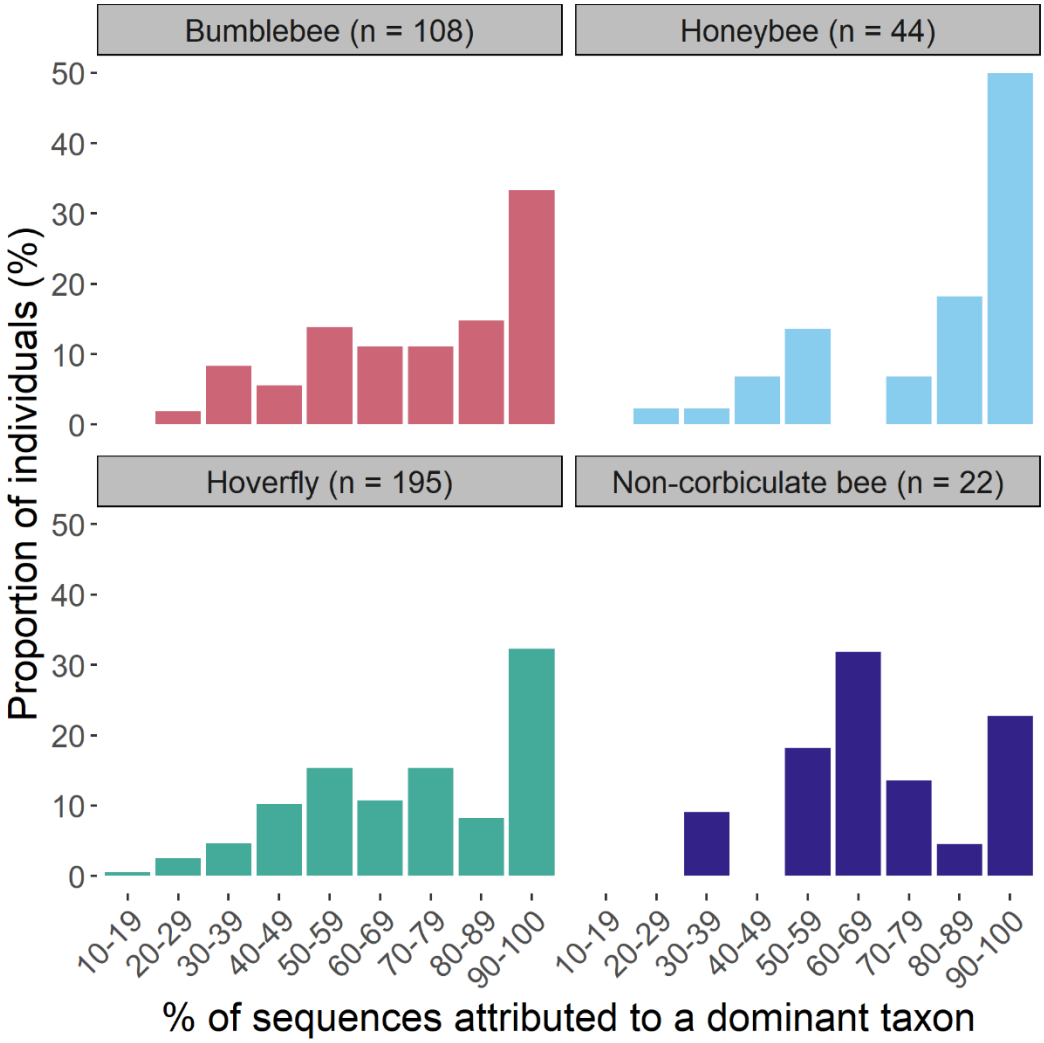

Figure 4.3: Proportion of pollinator individuals (n = 369) displaying floral fidelity at a range of scales.

4.4.5. How do the foraging choices of individuals reflect the overall species patterns?

Individuals within each bumblebee species were found to have a specialised dietary niche (IS < 0.5) (Table 4.1). The species found to be most specialised at the individual level was *Bombus pratorum* (IS = 0.26), with each individual using a subset of the total resources used by the entire species (Fig. 4.4). The species with the most generalised individuals was *Bombus lapidarius* (IS = 0.41), as a result of a large proportion of individuals showing floral fidelity to *Cirsium/Centaurea/Hypochaeris* spp. and a small number of other taxa.

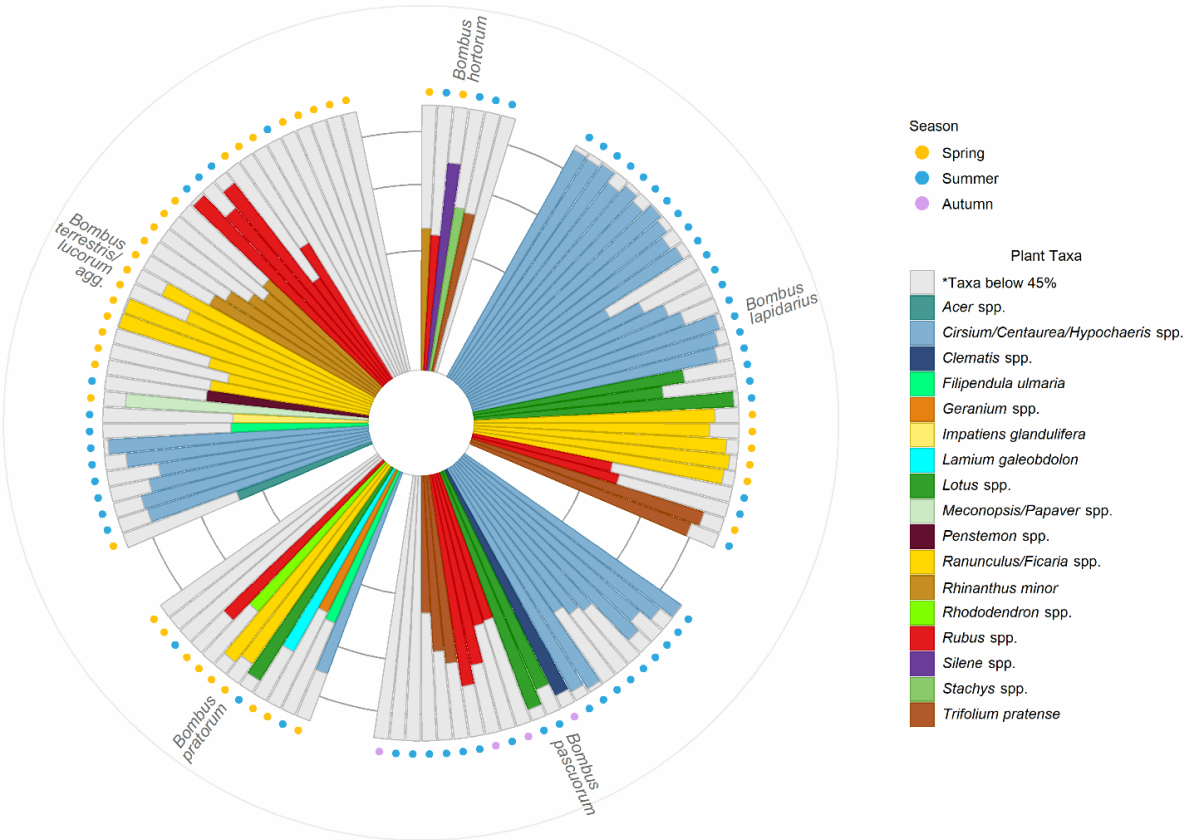

Figure 4.4: Proportion of taxa contributing over 45% of reads for each individual bumblebee within a species. Only species which were represented by >5 individuals were included. The inner circular line reflects the 45% threshold, middle line 70% and top illustrates 90%.

The level of dietary niche specialisation in individual hoverflies was dependent on the species, ranging from relatively specialised when all individuals forage differently (IS = 0.23 in *Episyrphus balteatus*) to extremely generalised when all individuals have almost the exact same diet (IS = 0.93 in *Cheilosia albitarsis s.l.*) (Table 4.1). *E. balteatus* foraged on a diverse suite of plant species, with no dominant taxa shared between individuals (Fig. 4.5). Conversely, *Cheilosia albitarsis s.l.* carried an average of 98% *Ranunculus/Ficaria* spp. pollen per individual, with each individual possessing a pollen load consisting of over 85% of this species alone.

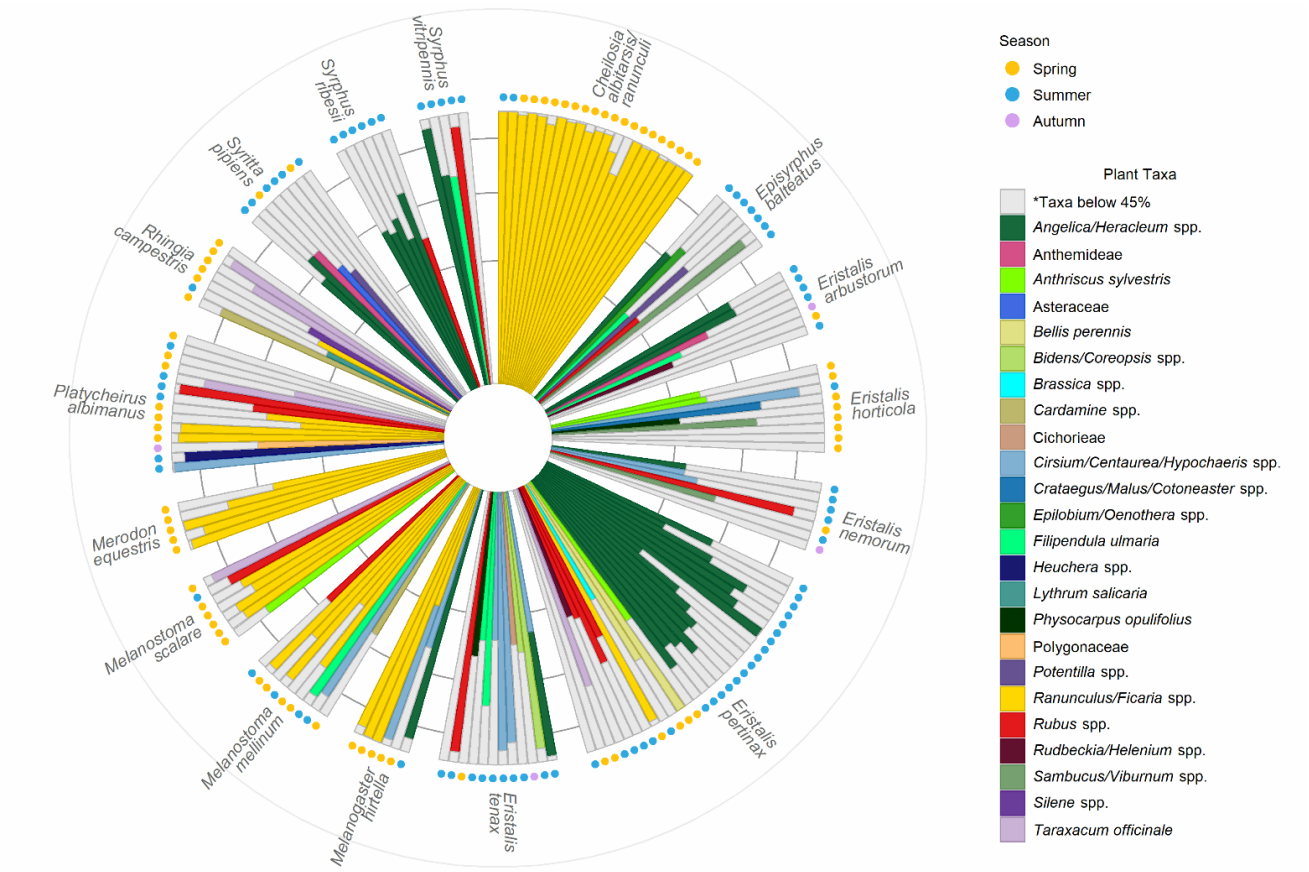

Figure 4.5: Proportion of taxa contributing over 45% of reads for each individual hoverfly within a species. Only species which were represented by >5 individuals were included.

Honeybees showed high levels of individual specialisation (IS = 0.18), with a broad range of taxa used across all individuals, although many displayed floral fidelity particularly to *Rubus* spp., and *Cirsium*/*Centaurea*/*Hypochaeris* spp. (Fig. S4.7, Supporting Information). Likewise, *L. smeathmanellum* was relatively specialised (IS = 0.17), with the dominant taxon of each individual being different (Fig. S4.8, Supporting Information).

4.5. Discussion

We use DNA metabarcoding to unravel the mechanisms underpinning plant-pollinator networks to assist our understanding of how robust species may be under environmental change. We reveal links which may have previously been undetectable using traditional methods, whilst vastly reducing the effort previously required to build complex networks. We find that specialisation can occur at each hierarchical level and can either depend on the specificity of pollen types used, or the dietary niche in which the hierarchical group occupies in comparison to others in the same network. We present a detailed discussion on the level of specialisation and generalisation found at three hierarchical levels: order, genera and species, and individuals.

4.5.1. Dietary overlap between Dipteran and Hymenopteran pollinators

We found that overall, the plant-pollinator network was slightly more specialised than generalised, and this was consistent when investigating the two taxonomic orders (Diptera and Hymenoptera) as separate networks. The slight specialisation indicates that some pollinator species in the network visit plants not often visited by other species. Plant-pollinator networks typically have a generalised structure (Waser et al., 1996), most likely due to the fact that even when specialised interactions occur, these are usually between a specialist pollinator and a generalist plant (Guimarães et al., 2006). The degree of specialisation seen here may be

influenced by the spatiotemporal arrangement of pollinators and floral resources (Waser et al., 1996) for example if rare pollinators were sampled in an area containing rare plants, then the degree of specialised interactions may be overestimated.

Although both Hymenoptera and Diptera were found to utilise a broad range of resources, we found moderate overlap of floral resource use between orders, as expected from incidences of generalism identified in previous studies of plant-pollinator networks (Lucas et al., 2018a; Pornon et al., 2019). We found that niche overlap was found to be moderate between each combination of taxonomic group (hoverflies, bumblebees, honeybees and non-corbiculate bees), with the exception of honeybees and non-corbiculate bees, where overlap was reduced. Whilst high niche overlap may result in competition for resources, any negative effect on fitness is dependent on the availability of resources (Mallinger et al., 2017; Wignall et al., 2020b). Indeed, the abundance of resources has been found to influence niche overlap, with high floral abundance resulting in lower niche overlap between pollinators (Tommasi et al., 2021).

4.5.2. Species within a taxonomic group tend to be generalised

When considering host specificity, only one species (*Cheilosia albitarsis s.l.)* was found to be specialised and therefore oligolectic. Utilising a wide range of resources allows species to adapt in response to spatiotemporal changes in resource availability, a trait which is particularly beneficial for insects with long flight periods (Ogilvie & Forrest, 2017). Conversely, specialised relationships between a pollinator specFies and its host plant can aid pollination by facilitating transfer of conspecific pollen, however, can narrow the spatiotemporal range of an insect visitor (Minckley & Roulston, 2006). The classification of *Cheilosia albitarsis s.l.* as broadly oligolectic is consistent with the literature, as the larvae of *C. albitarsis/ranunculi* develop in roots of *Ranunculus* spp. and adults are thought to

forage solely on plants within this genus (Haslett, 1983). However, whilst most of the pollen identified from *Cheilosia albitarsis s.l.* was from *Ranunculus*/*Ficaria* spp., additional taxa were detected at low levels which suggests that this species might visit other plants for pollen or nectar.

Within the two taxonomic groups for which species-level analysis was possible (hoverflies and bumblebees), species had distinct diets, however, few interactions were exclusive to any species, illustrating generalisation at the species level. The partitioning of resources by hoverfly species may be explained by differences in life history. The larval feeding requirements of hoverflies are diverse (Rotheray & Gilbert, 2011b), which may drive plant choice, for example, phytophagous species will seek out specific host plants to lay their eggs (e.g., *Cheilosia albitarsis s.l.*) whilst = hoverflies with different larval habits may be more generalised in their preferences. Tongue length may also play a role in differences in diet between both bumblebee and hoverfly species respectively. We found that the species with the most unique interactions within each taxonomic group were long-tongued species *Rhingia campestris* and *Bombus hortorum,* which may be due to their ability to access taxa with long corollae e.g., *Silene* spp. that are less accessible to species with shorter tongues.

Whilst morphological or life history differences may play an important role in driving differences in diet between species, interspecific competition may also contribute (Inouye, 1978). There is evidence that bees switch their foraging efforts to replace favoured resources when subject to competition from other species, which may increase diet breadth of the species (Frund et al., 2013), however this phenomenon is not well studied in hoverflies. The degree of interspecific competition will be influenced by the availability of target resources, with greater competition when resources are limited.

4.5.3. Generalised species can be composed of highly specialised individuals

At the individual level, we found high occurrence of specialisation in relation to floral fidelity, which tended to be hidden at the species level. For both bumblebees and hoverflies, over a third of individuals sampled had over 90% of their pollen dominated by one species. Inferring floral fidelity from pollen loads is frequent in studies of bumblebees (Carvell et al., 2006; Heinrich, 1976), but much less so in hoverflies and rarely are the two compared within the same network (Cullen et al., 2021). In a study across four bumblebee species in Belgium, Somme et al. (2014) found that 60.2% of pollen loads collected were composed of over 95% of a single-plant taxa, demonstrating high levels of floral fidelity. In comparison, Lucas et al. (2018b) found that 24-42% of hoverflies caught in Welsh grasslands had 90% or more of their pollen originating from a single plant host.

Individuals within bumblebee and hoverfly species were also found to exhibit dietary specialisation in comparison to other individuals of the same species. This demonstrates that whilst most pollinators were found to be generalised at the species level, specialisation occurs in the short term (e.g., an individual's life) and generalised in the long term (e.g., across a species' flight period). Increased diet breadth between individuals within a species increases the range of plants that a species relies on, which may provide an advantage in the case of any loss of plant species (Goulson et al., 2005). *Cheilosia albitarsis s.l.* showed the lowest degree of individual specialisation (all individuals had almost identical diet), even after sampling a substantial number of individuals. However, both individuals and the species itself are classed as specialised when considering host specificity and floral fidelity to *Ranunculus/Ficaria* spp., demonstrating that the classifications of specialisation and generalisation are interchangeable depending on the context and hierarchical level studied.

Similar to species-level specialisation, individual specialisation may also be influenced by intraspecific competition (Tur et al., 2014) or morphological traits (Cullen et al., 2021). Another factor which may increase individual specialisation is the flight period of the species itself. Insects with long flight periods that can forage great distances are usually generalists, adapting to a range of plant phenologies throughout the year (Ogilvie & Forrest, 2017). Szigeti et al (2019) found that individual specialisation could be explained in a butterfly species when considering the temporal variation in both the occurrence of individuals and their food resources. For example, individuals collected within the same time period had more closely related diets than those collected at other time periods, demonstrating that had this temporal occurrence not been considered, individuals would have seemed to occupy more specialised niches than in reality (Szigeti et al., 2019). The sample size of individuals and the period in which they are caught may therefore overestimate the degree of specialisation in terms of the number of plant sources used, with rare species caught within a short period seeming more specialised than in reality (Vázquez & Aizen, 2004). In contrast, abundant individuals allow a greater sampling depth, therefore more links are uncovered and generalism increases. Our study aimed to achieve a global picture of individual specialisation within a species therefore sampling was undertaken across the entire flowering season, over two years. Indeed, the species which showed the highest degree of individual specialisation, *Bombus pratorum, B. lapidarius, Episyrphus balteatus* and *Platycheirus albimanus* all have long flight periods, however, only *P. albimanus* was represented by individuals across a large proportion of its flight season. It should be noted that *E. balteatus* has an exceptionally long flight period, occurring throughout the year in the UK, yet this species was only sampled between June and August, allowing only a snapshot into its lifelong diet breadth. Moreover, further work could be done to increase sample size to investigate how temporal occurrence affects individual specialisation in the species studied.

4.5.4. Conclusion

The levels of specialisation and generalisation within a plant-pollinator network can vary from the level of community to the individual. We found that although the structure of the overall network tended more toward specialisation, most bumblebee and hoverfly species were generalised in comparison to other species within the same group. Concurrent with previous studies of plant-pollinator networks, we find that most individual pollinators are specialised when considering both the prevalence of floral fidelity and the dietary niche they occupy within the species, widening the diet breath of the species as a whole and therefore driving generalisation at the species level. As the removal of specialists affects the behaviour of generalists in a network (Brosi & Briggs, 2013), our work highlights the need to study individual interactions within a network in order to detect specialism at high resolution.

4.6. References

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4.7. Supporting Tables and Figures

Table S4.1: Primer sequences used to amplify the *rbcL* and ITS2 barcode regions. A 6N sequence was added between the forward template specific primer and the universal tail to improve clustering on the Illumina MiSeq

Primer name	Universal Tail	6N Sequence	Primer sequence
*rbcL*af (Kress & Erickson, 2007)	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	NNNNNN	ATGTCACCACAAACAGAGACTAAAGC
*rbcL*r506 (de Vere et al., 2012)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		AGGGGACGACCATACTTGTTCA
ITS2F (Chiou et al., 2007)	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	NNNNNN	ATGCGATACTTGGTGTGAAT
UniPlantR (Moorhouse-Gann et al., 2018).	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		CCCGHYTGAYYTGRGGTCDC

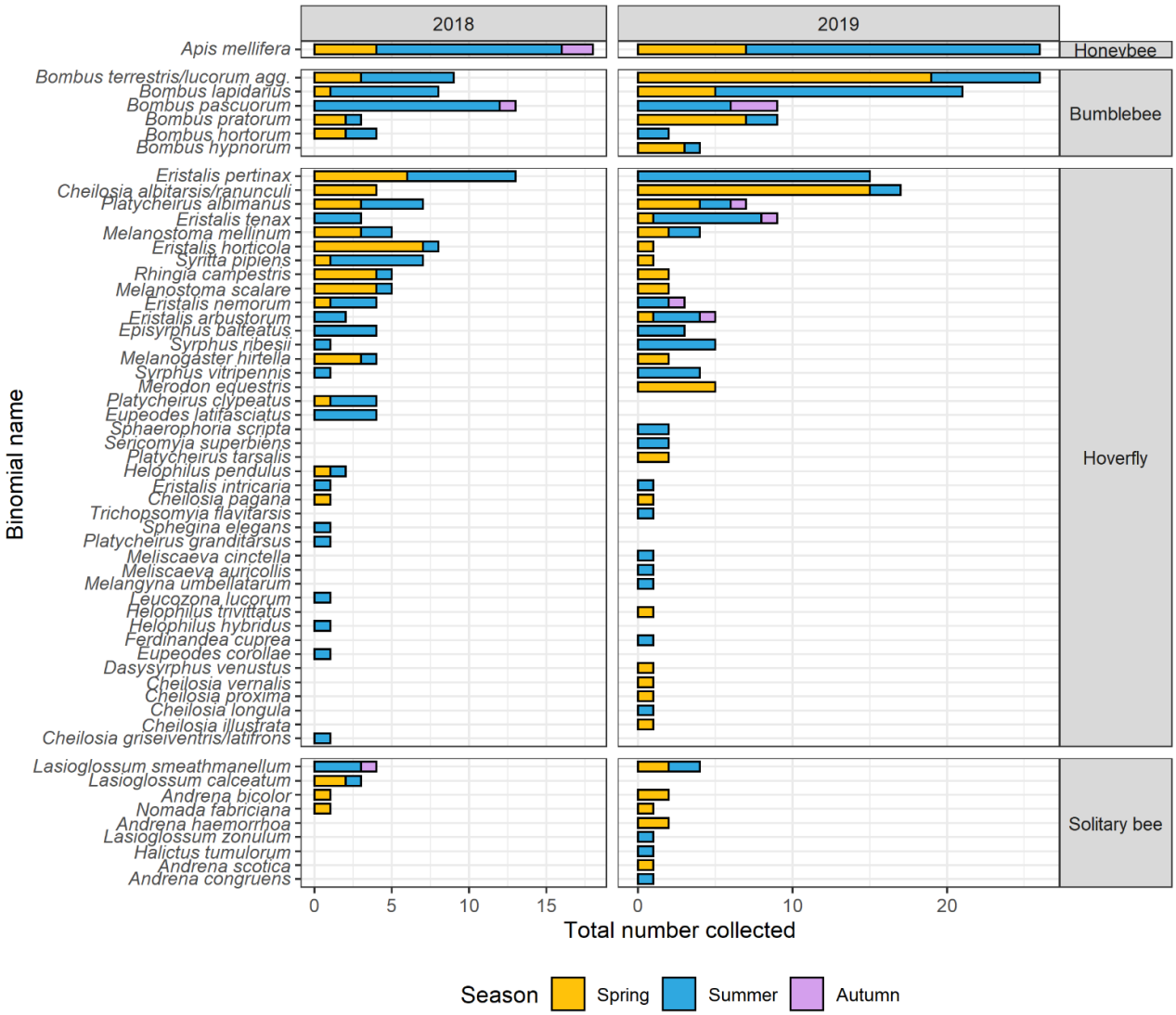

Figure S4.1: Number of each pollinator species collected within each season across 2018 and 2019. Spring consisted of months March to May, summer encompassed June to August and autumn represented September.

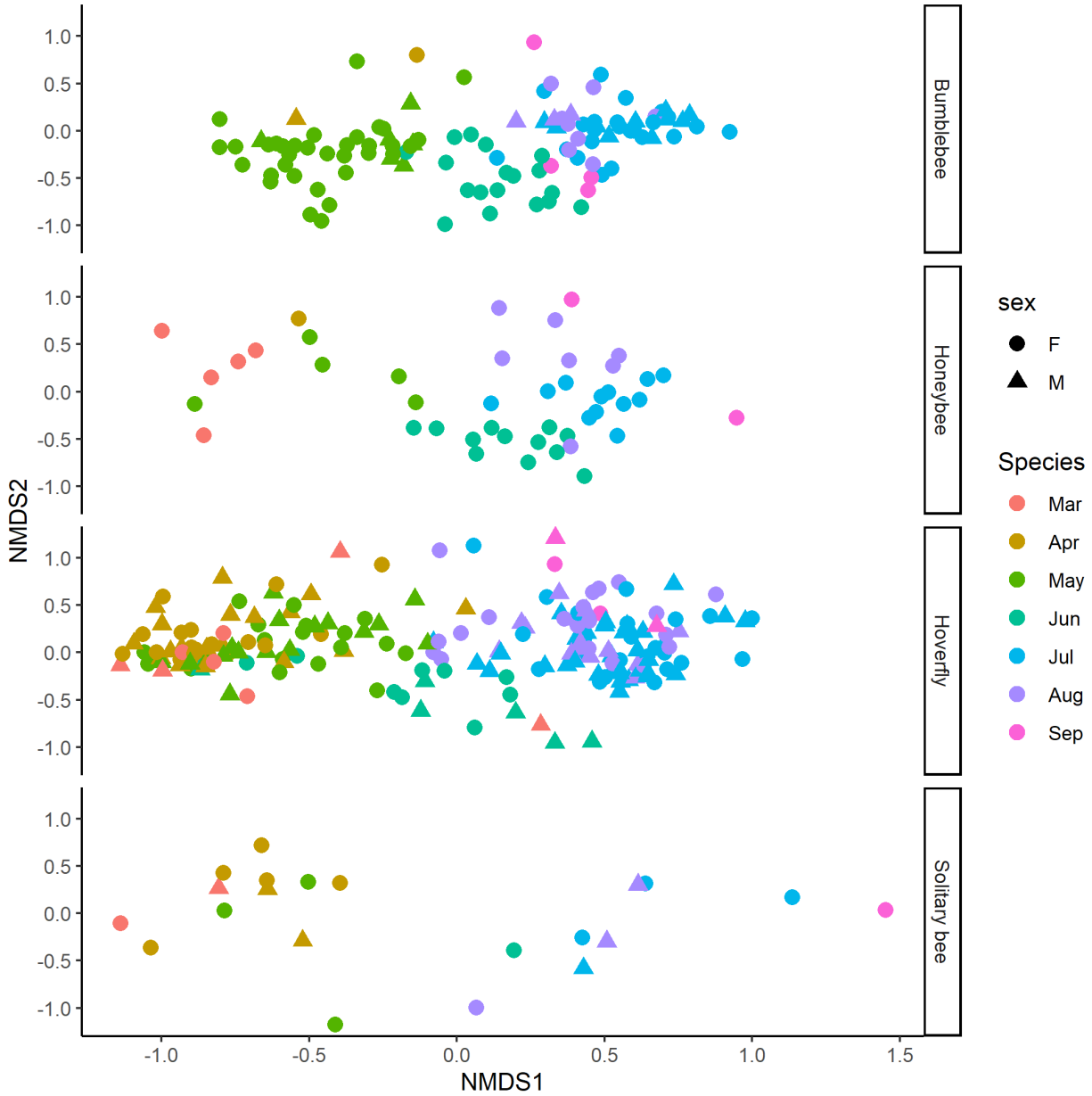

Figure S4.2: Non-metric multidimensional scaling (NMDS) of pollen samples, with colour indicating the season of collection and shape indicating sex of the insect.

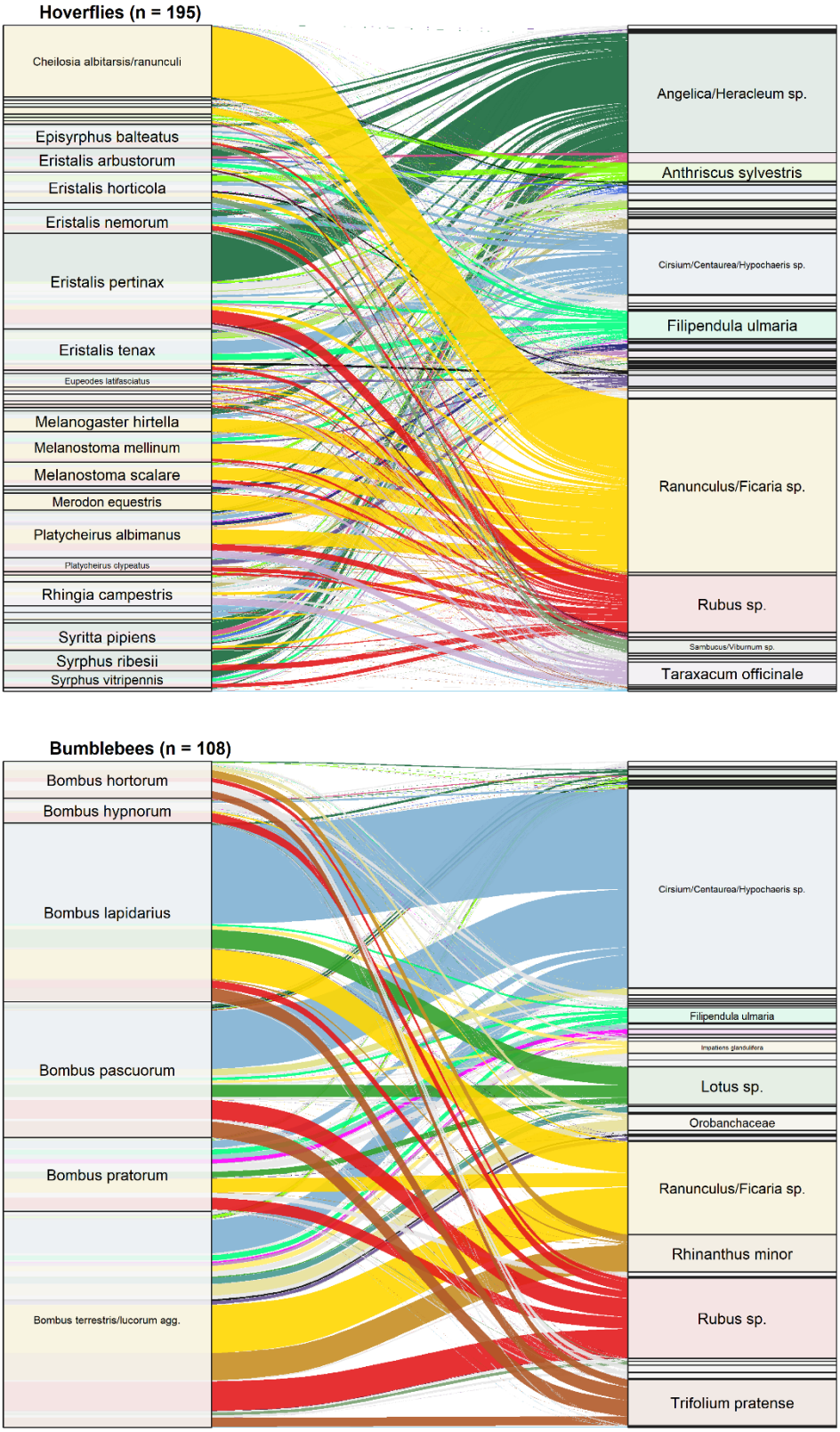

Figure S4.3: Plant-hoverfly and Plant-bumblebee networks, constructed from pollen recovered from insects collected from March to September 2018 and 2019, identified using DNA metabarcoding. The size of each insect bar relates to the number caught, whilst the size of each plant bar relates to the proportion of sequences recovered. The connecting alluvial between plants and insects is based on the strength of the interaction.

Table S4.2: Niche overlap values represent diet comparison between two taxonomic orders (Diptera and Hymenoptera) and four taxonomic groups within (hoverflies, bumblebees, honeybees and non-corbiculate bees), calculated from Horn's index (Horn, 1966). Values range from 0 (no common use of niches) to 1 (perfect niche overlap).

Comparison		**Niche overlap**
Diptera (n = 195)	Hymenoptera (n = 174)	0.62
Hoverflies (n = 195)	Bumblebees (n = 195)	0.59
Hoverflies (n = 195)	Honeybees (n = 44)	0.63
Hoverflies (n = 195)	Non-corbiculate bees (n = 22)	0.68
Bumblebees (n = 108)	Honeybees (n = 44)	0.62
Bumblebees (n = 108)	Non-corbiculate bees (n = 22)	0.66
Honeybees (n = 44)	Non-corbiculate bees (n = 22)	0.60

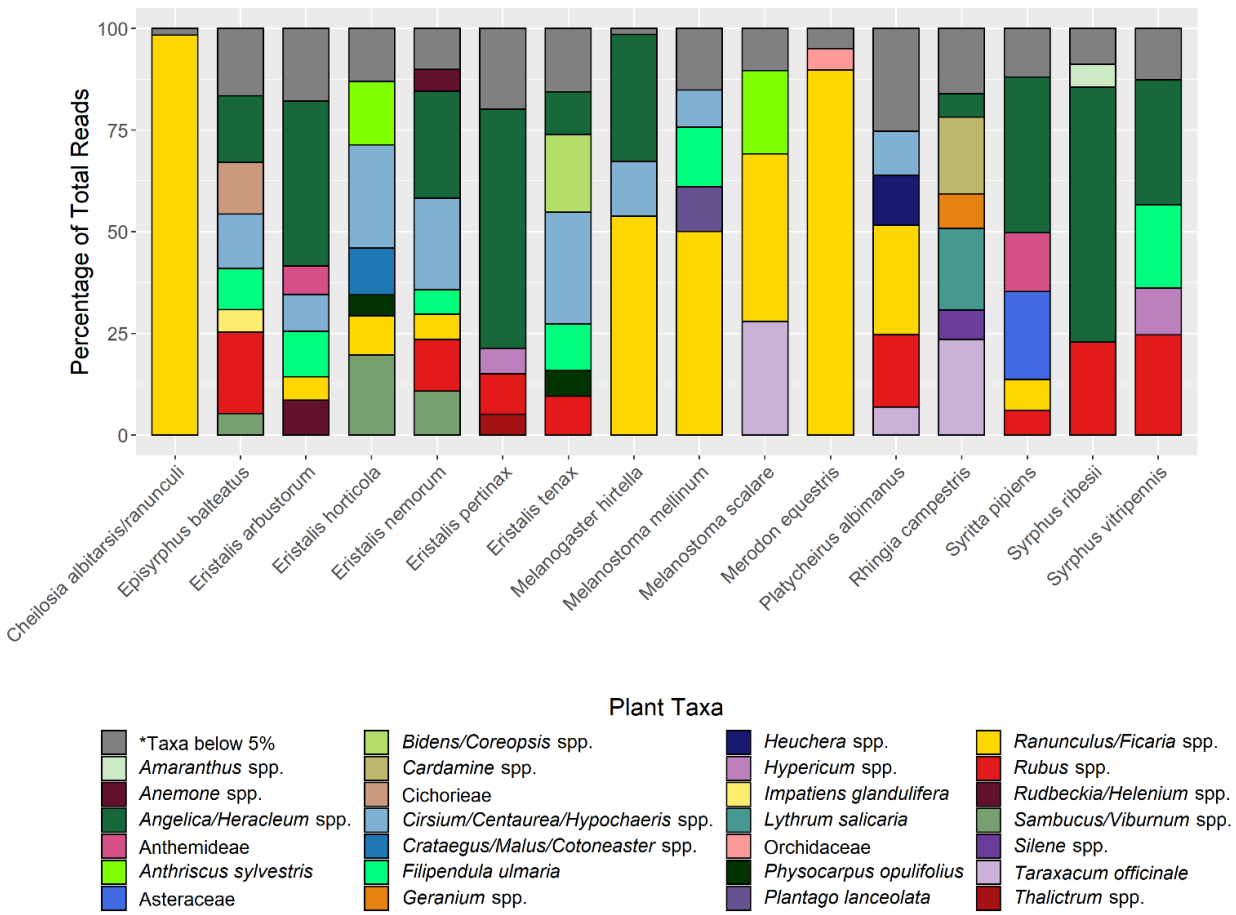

Figure S4.4: Plant taxa contributing over 5% of total reads for each hoverfly species represented by over five individuals. Diet composition differed between hoverfly species (R_2 = 0.345, d.f. = 15, P < 0.001).

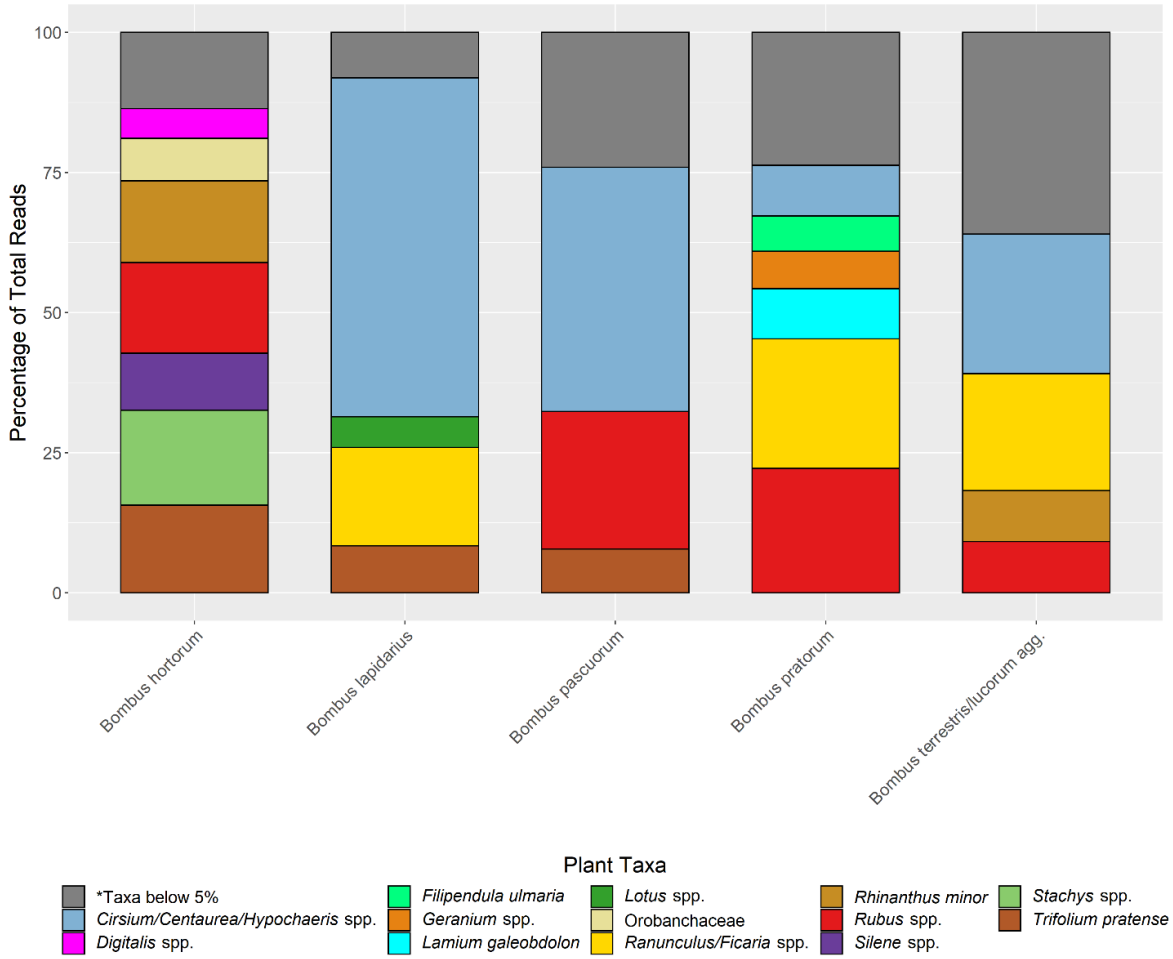

Figure S4.5: Plant taxa contributing over 5% of total reads for each bumblebee species represented by over five individuals. Diet composition differed between bumblebee species (R_2 = 0.123, d.f. = 4, P < 0.001).

Table S4.3: Values of *d'* for each Hymenoptera genera represented by over five individuals. *d'* measures the degree of unique interactions within a genus in comparison to all interactions within the same taxonomic order, and ranges from 0 (complete generalisation) to 1 (complete specialisation).

Genus	n	*d'*
Andrena	7	0.56
Apis	44	0.42
Bombus	108	0.48
Lasioglossum	12	0.59

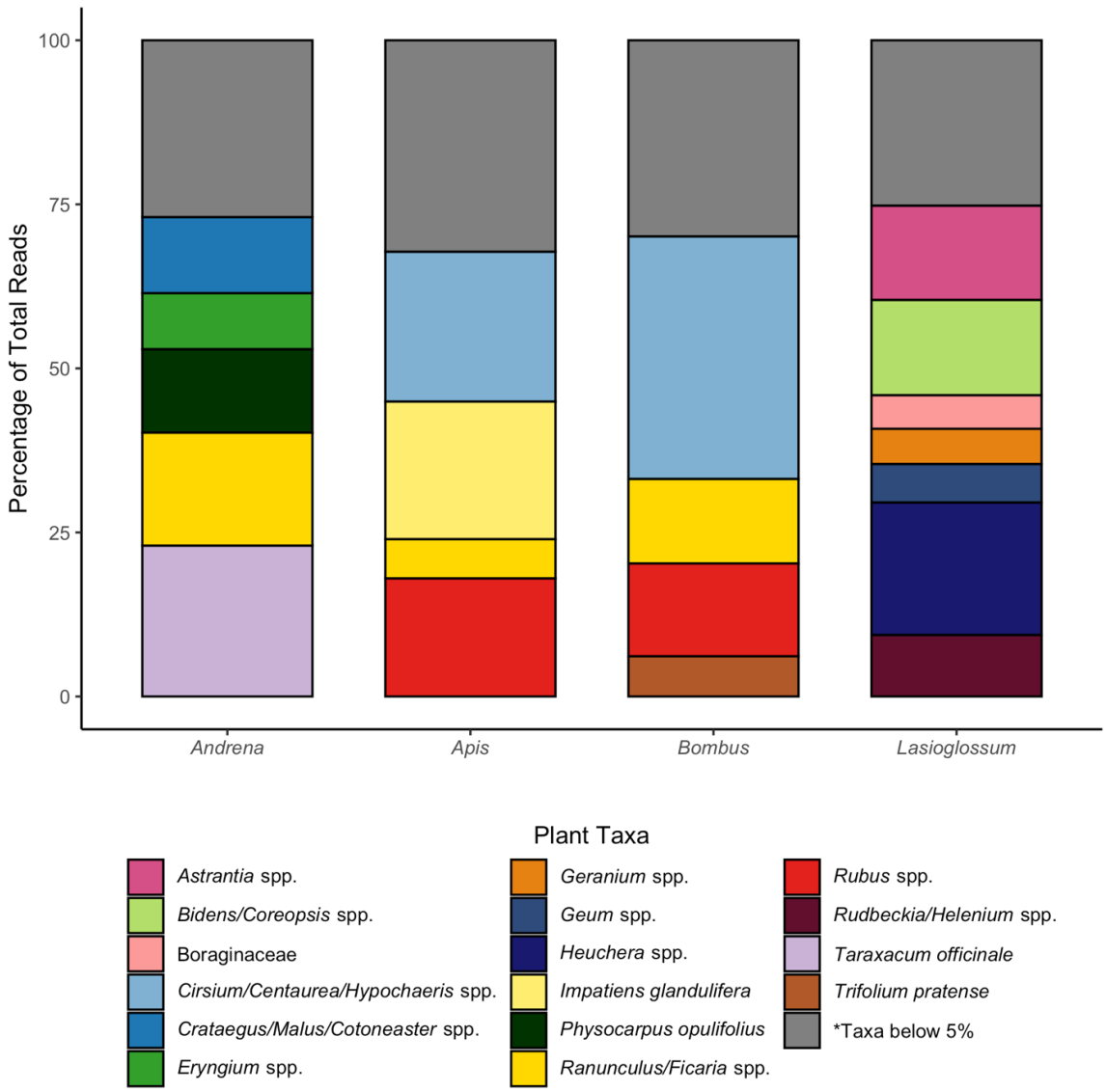

Figure S4.6: Plant taxa contributing over 5% of total reads for each genus represented by over five individuals within Hymenoptera (bees).

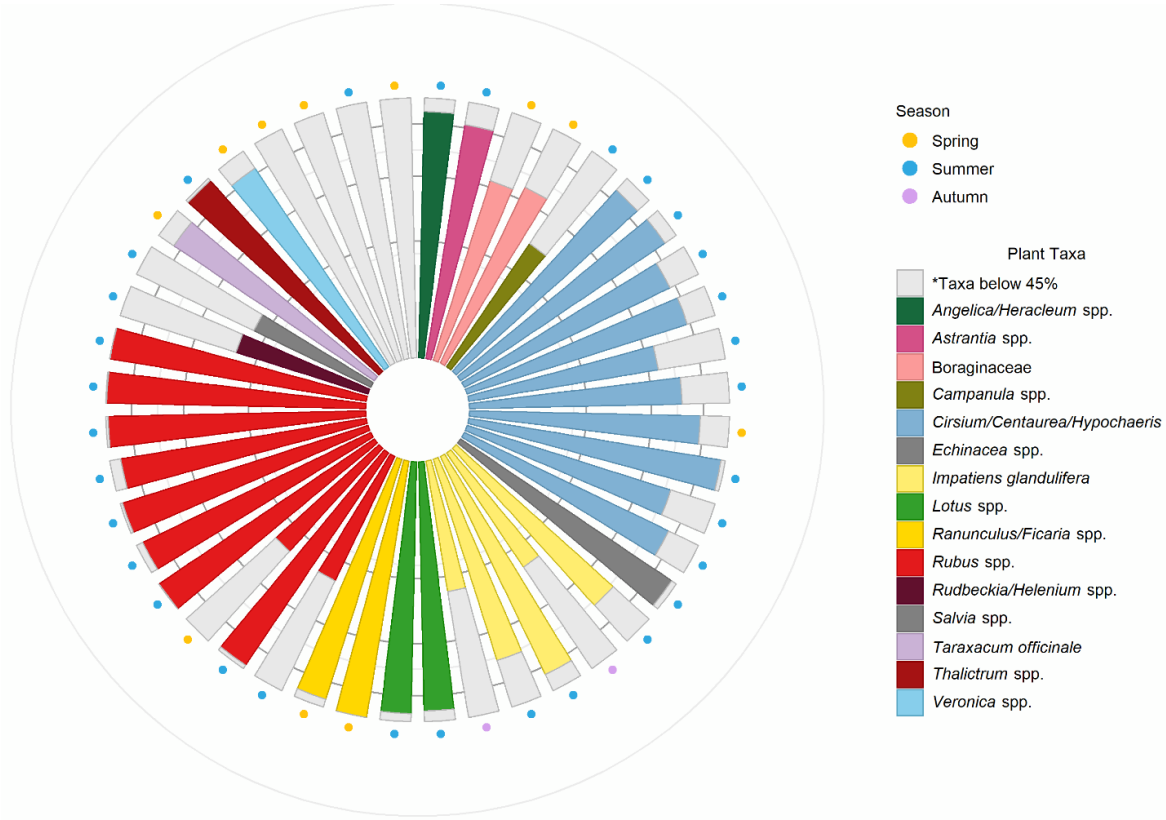

Figure S4.7: Proportion of taxa contributing over 45% of reads for each individual honeybee (*Apis mellifera*). The inner circular line reflects the 45% threshold, middle line 70% and top illustrates 90%.

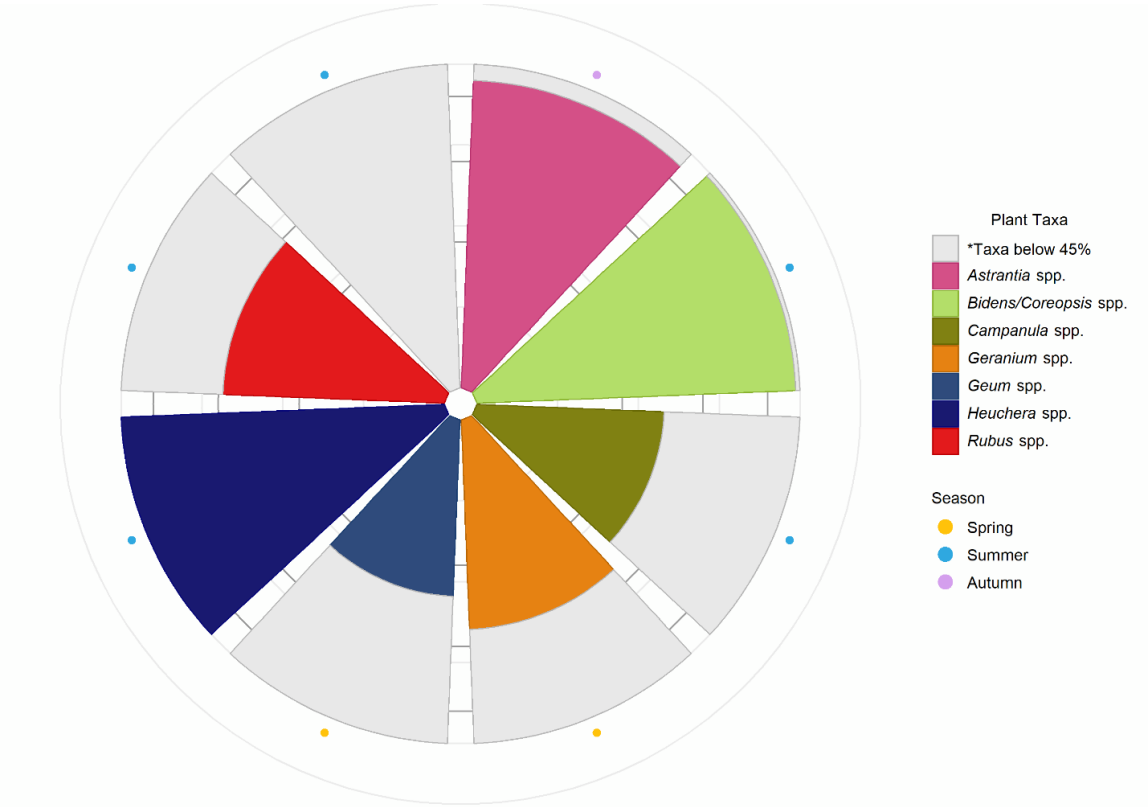

Figure S4.8: Proportion of taxa contributing over 45% of reads for each individual *Lasioglossum smeathmanellum,* the only non-corbiculate bee of which over five individuals were samples. The inner circular line reflects the 45% threshold, middle line 70% and top illustrates 90%.

Chapter Five

Seasonal specialisation in floral resource use by honeybee colonies reveals periods of food shortage in a diverse agricultural and horticultural habitat

This manuscript has been submitted to *Molecular Ecology*

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The study was designed by N.dV., L.J. and A.L. Hive management and sample collection was undertaken by beekeeper Lynda Christie and a team of volunteers. Lab work was carried out by A.L. The data were compiled by A.L. and analysed by A.L. and L.J. with suggestions from N.dV., S.C. and G.B. The manuscript was written by A.L. with contributions from all the authors.

5.1. Abstract

Availability of suitable floral resources for nectar and pollen foraging is a limiting factor for pollinator survival, with both overall resource levels and provision of sufficient resources throughout the season being critical. In order to ensure floral resource continuity, more information is needed on how the selection of floral resources change over time, and how this relates to floral availability within the landscape. Multi-locus DNA metabarcoding was used to characterise the pollen present in honey samples from April to September, over two years. We compared the plants found to their availability within the surrounding landscape using floral surveys. Honeybees used a total of 143 plant taxa, but only 10 of these were major sources over the entire season, and total plant use represented a very small proportion of the available floral resources within the landscape (23% of available genera). Distinct differences in diet occurred between honeybee colonies during June and August representing periods of resource limitation. Honeybees showed a preference for flowering trees in the spring, followed by shrubs and herbs in summer and used native and near-native plants, more than horticultural plants, as major food sources. Our results highlight the importance of providing sufficient floral resources throughout the season.

5.2. Introduction

Insect pollinators are crucial components of our ecosystems and contribute demonstrably to human health and well-being due to their role in pollinating important food crops (Klein et al., 2007; Smith et al., 2015). Lack of suitable foraging resources is a key driver of pollinator decline and ill health (Goulson et al., 2008; Potts et al., 2010). Most pollinators require pollen and nectar for survival, with nutritional requirements fluctuating throughout their life cycle (Russell et al., 2013), creating a demand for sufficient resources throughout the year. Availability of floral resources can vary (Ogilvie & Forrest, 2017), with interruptions to resource continuity having negative effects on reproduction and colony resilience in honeybees (Horn et al., 2016; Requier et al., 2017) and bumblebees (Requier et al., 2020; Rotheray et al., 2017). Understanding how foraging preferences change throughout the year, and how flower visitation relates to floral availability in the landscape allows critical periods of resource limitation to be identified and potentially mitigated against using supplemental planting in agricultural and amenity landscapes.

Periods of resource limitations for managed honeybee colonies have long been described by beekeepers, where colonies require supplemental feeding as a result of low food stores. A prominent food shortage is thought to occur between periods of strong spring and summer nectar flow, anecdotally known as the "June Gap" within the UK (Crane, 1976; Suryanarayana & Singh, 1989). Like most bees, honeybees are central-place foragers, gathering nectar and pollen from the landscape surrounding their nest and returning to provide for their brood (Michener, 2000). Honeybees are complex, social insects, able to communicate the location and quality of resources to other members of the colony through the "waggle-dance", with recruitment determined by a combination of the quality and quantity of the resource, and the distance from the colony (Seeley, 1995).

Honeybees often forage within a kilometre of the colony (Couvillon et al., 2014; Garbuzov et al., 2015), however, they will travel further to reach large quantities of rewarding resource or when local availability is low (Beekman & Ratnieks, 2000).

Optimal foraging theory (OFT) states that the selection of resources by foragers is determined by the overall net energetic gain, which itself is calculated from the value of the reward against the energy required to locate and extract it (Araújo et al., 2011; Schoener, 1971). OFT predicts that when preferred resources are abundant, foragers utilise few resources, and when resources become limited, dietary breadth is increased as the diet is supplemented with lower value resources (MacArthur & Pianka, 1966; Svanbäck & Bolnick, 2007). Whilst optimal foraging theory is mostly used to understand dietary breadth, it also can affect how resources are partitioned between individuals and species (Stephens et al., 2019). In periods of low resource availability, foragers must become opportunistic as the search time for rewarding resources increases, resulting in decreased niche overlap (Stephens et al., 2019). Consequently, identifying how the partitioning of resources between honeybee colonies fluctuates throughout the year could be used to identify periods of food shortage, if it is assumed that diet variation between colonies will be higher in periods of resource limitation.

The wide geographical distribution (Hung et al., 2018) and large foraging range of the honeybee, *Apis mellifera,* make it an excellent study species for investigating floral resource use (de Vere et al., 2017) and relating this to changes in floral abundance (Jones et al., 2021a). Despite management by humans, honeybees face many threats including pesticide use (Lu et al., 2020), pests and disease (in particular the mite *Varroa destructor*) (Rosenkranz et al., 2010), stress from apicultural mis-management e.g., regular transportation to provide pollinator services (Simone-Finstrom et al., 2016) and poor diet through lack of suitable

forage (Scofield & Mattila, 2015). These stressors are known to interact, increasing pressure on colonies, resulting in ill health and colony loss (Potts et al., 2010). Furthermore, a reduction in suitable forage for honeybees can lead to increased competition with wild pollinators for floral resources (Herbertsson et al., 2016), whilst also increasing susceptibility to disease (Dolezal et al., 2019), which may spill over into wild populations (Graystock et al., 2014). The wide diet breadth and high abundance of honeybees in a landscape are often used to argue for competitive exclusion of wild pollinators (Mallinger et al., 2017). However, exploitative competition between bumblebees and honeybees has been shown to vary, driven by limitations in floral resources (Wignall et al., 2020c) and landscape context (Herbertsson et al., 2016).

A. mellifera is widely considered to be a super-generalist (Corbet, 2006; Hung et al., 2018; Memmott & Waser, 2002; Potts et al., 2010), although, there is increasing evidence to suggest that a small number of plants are used most frequently (de Vere et al., 2017; Jones et al., 2021a). Whilst knowledge of honeybee forage plants is increasing, little is known about the drivers behind floral selection. Particularly, if visitation is directly linked to the abundance of plants in the landscape, or whether native or horticultural plants are preferred.

Traditionally, floral use by pollinators has been investigated using observations of plants (Carvell et al., 2006; Rollings & Goulson, 2019), or microscopy of pollen obtained from the bodies of insects (Hennessy et al., 2020; Köppler et al., 2007) or honey (Coffey & Breen, 1997; Ponnuchamy et al., 2014). DNA metabarcoding provides an alternative to these methods and has been successfully used to identify pollen within nests (Gresty et al., 2018; McFrederick & Rehan, 2016) and from the bodies of insects (Danner et al., 2017; Lucas et al., 2018a; Potter et al., 2019). In addition, DNA metabarcoding is an established tool for the identification of pollen within honey (Valentini et al., 2010; Hawkins et al., 2015; Prosser & Hebert, 2017),

and has been used to identify temporal (de Vere et al., 2017; Jones et al., 2021a) and spatial (Lucek et al., 2019) patterns in honeybee foraging (explored in further detail in Chapter Two). The metabarcoding method allows a large number of samples to be processed (Sickel et al., 2015), increases plant species discrimination (Brennan et al., 2019) and reduces the need for the taxonomic expertise required for pollen microscopy (Hawkins et al., 2015).

A key advantage of using DNA metabarcoding over observational techniques for investigating diet preferences is the increased spatial and temporal resolution available as a result of revealing inaccessible interactions (Arstingstall et al., 2021; Olesen et al., 2011). Whilst metabarcoding can overcome the limitations of alternative methods, it must be accompanied by a comprehensive reference library to ensure accurate identification (Jones et al., 2021b). In the UK, the Barcode Wales and Barcode UK projects provide 98% coverage of all native flowering plants and conifers using three plant DNA markers, *rbcL*, *matK* and ITS2, allowing reliable identification at the species and genus level (de Vere et al., 2012; Jones et al., 2021b).

5.2.1. Aims and objectives

Here, we aim to investigate floral resource use in a social central-placed forager, the honeybee, *Apis mellifera*. Specifically, we ask the following questions:

- What are the seasonal changes in honeybee foraging in a diverse area of agricultural habitat and horticultural planting?
- Can periods of resource limitation be identified by assessing the level of diet specialisation between honeybee colonies throughout the year?
- Can preference analysis identify plants which are used more or less than expected by chance given their relative abundance in the landscape?
- Is the use of plants by honeybees proportional to their relative abundance in the landscape with regard to their growth form (tree/shrub/herb) or native status?

5.3. Materials and Methods

5.3.1. Study site

The study was conducted at the National Botanic Garden of Wales, UK (51°50'33.4"N 4°08'49.2"W), a diverse landscape (230 ha) consisting of formal garden and organic farmland, designated as a National Nature Reserve (Waun Las NNR) (Fig. S5.1, Supporting Information). The botanic garden contains over 5000 plant taxa from throughout the world, including many horticultural plants grown throughout Western Europe. It is set within an agricultural area in South West Wales, UK, with semi-improved grassland being the major habitat. Two apiaries are located within the site, 1 km apart, one within the Botanic Garden with close access to horticultural plants and the other at the edge of the Nature Reserve (Fig. S5.1, Supporting Information). All colonies sampled had identical management practices, housed within British Standard National hives.

5.3.2. Floral surveys

To estimate the availability of floral resources, the outside areas of the Botanic Garden and Nature Reserve were surveyed monthly from April to September throughout 2018 and 2019. The site was split into 287 survey zones each of 18 ha and classified according to habitat. The range of habitats surveyed consisted of broadleaved woodland and linear features (hedgerows and walls), horticultural, and grassland (mostly semi-improved). Each of the zones was mapped using QGIS v. 3.6.1 and R v. 4.0.2. For each zone, all plant species in flower were noted, and for each, an estimate of the percentage cover of available flowers within the zone was recorded. This percentage cover value was multiplied by the area of each zone to measure the approximate total area covered by each plant taxa.

5.3.3. Honey sampling and DNA extraction

Throughout the same time period, honey was sampled from six colonies (three from each apiary) during the last week of each month. 30 ml of honey was sampled from a comb in each colony using a sterile 50 ml centrifuge tube. Wax was removed using sterile forceps and 10 g of each honey sample was weighed into a new sterile 50 ml centrifuge tube. A modified version of the Qiagen DNeasy 96 Plant Kit was used for DNA extraction. The 10 g of honey was made up to 30 ml with molecular grade water (Sigma) and placed in a water bath at 65 °C for 30 min, shaking each sample at 10-minute intervals. Samples were transferred to 50 ml Nalgene round-bottomed tubes and placed in a high-speed centrifuge (Sorvall RC-5B) at 15,000 rpm for 30 min. The supernatant was discarded, and the pellet resuspended in 400 µl of buffer, made up of 400 µl AP1 from the DNeasy Plant Mini Kit (Qiagen), 80 µl proteinase K (1 mg/ml) (Qiagen) and 1 µl RNase A (Qiagen). Samples were incubated for 60 min at 65 °C and then disrupted in the TissueLyser II (Qiagen) at 30 Hz for 4 min. The remaining steps were carried out according to the manufacturer's protocol, excluding the use of the QIAshredder and the second wash stage. The OneStep PCR Inhibitor Removal Kit (Zymo Research) was used to purify the DNA extract prior to diluting 1 in 10.

5.3.4. Amplification and Sequencing

The plastid gene *rbcL* and the nuclear-transcribed region ITS2 were amplified across all samples following a two-step PCR protocol (Brennan et al., 2019). The primers *rbcL*aF (Kress and Erickson 2007) and *rbcL*r506 (de Vere et al. 2012), and ITS2F (Chiou et al. 2007) and UniPlantR (Moorhouse-Gann et al. 2018) were used to amplify the barcode regions (Table S5.1, Supporting Information). The first PCR used a final volume of 20 µl: 2 µl template DNA, 10 µl of 2x Phusion Hot Start II High-Fidelity Mastermix (New England Biolabs UK), 0.4 µl (2.5 µM) forward and reverse primers, and 7.2 µl of PCR grade water. A negative control was included

within each PCR to test for cross-contamination and reagent contamination. Thermal cycling conditions for the first *rbcL* PCR were: 98 °C for 30 s, 95 °C for 2 min; 95 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s (40 cycles); 72 °C for 5 min, 30 °C for 10 s. Thermal cycling conditions for the first ITS2 PCR were as follows: 98 °C for 30 s, 95 °C for 10 min; 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min (40 cycles); 72 °C for 10 min.

Agarose gel electrophoresis was used to visualise PCR products and to assess whether amplification was successful. The PCR was carried out three times, and the products of each PCR were pooled. The pooled products were purified using Illumina's 16S Metagenomic Sequencing Library preparation protocol using a 1:0.6 ratio of product to Agencourt AMPure XP beads (Beckman Coulter). A second PCR was carried out on the purified product to anneal custom unique and matched i5 and i7 indices to each sample (Ultramer, Integrated DNA Technologies). The second PCR used a final volume of 25 µl consisting of: 5 µl of pooled purified first-round PCR product, 12.5 µl of 2x Phusion Hot Start II High-Fidelity Mastermix (New England Biolabs UK), 1 µl of i5 and i7 Index Primer, and 6.5 µl of PCR grade water. Thermal cycling conditions for the index PCR were as follows: 98 °C for 30 s; 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s (8 cycles); 72 °C for 5 min, 4 °C for 10 min.

The index PCR product was compared to the cleaned-up product from the first PCR on a 1% agarose gel to confirm amplification of tags. A second clean-up stage was followed according to the Illumina protocol with a 1:0.8 ratio of product to beads. Products were quantified using a Qubit 4.0 fluorescence spectrophotometer (Thermo Fisher Scientific) and pooled at equal concentrations to create the final library for sequencing. The negative PCR controls from each plate were sequenced with the pollen samples on an Illumina MiSeq (2 x 300 bp).

5.3.5. Bioinformatic analysis

The Illumina sequence reads were processed following Ford & Jones (2020). Initially, raw sequences were trimmed to remove low quality regions, paired, and merged. Only sequences greater than 450 bp (*rbcL*) and 350 bp (ITS2) were used in downstream analysis. Identical reads were dereplicated within each sample and clustered at 100% identity across all samples with singletons (sequence reads occurring once across all samples) removed. Sequences were compared to a custom reference library for identification containing 5,887 plant species (Jones et al., 2021a), comprising native plants of the UK (Stace, 2019), naturalised and alien species (Preston et al., 2002) and horticultural species from the IRIS BG database at the National Botanic Garden of Wales.

5.3.6. Assigning plant taxa

Sequences were compared against the reference library using blastn, recording the top 20 BLAST hits and grouping together sequences with identical BLAST results across all 20 hits. The taxonomic identifications of these grouped sequences were then automatically assigned based on the highest bit score. If the top bitscore belonged to a species, the sequence was assigned to that species. If the top bitscore belonged to different species within the same genus, a genus designation was made for that sequence. If the top bitscore matched multiple genera of the same family, then a family designation was made for that sequence. Sequences returning top bit scores of multiple families within different orders were removed, assuming that these were poor-quality sequences. The identification of the sequences was then manually checked to ensure botanical veracity, relating to the plant's presence within the garden and wider landscape. A consensus identification for each taxon identified by *rbcL* and ITS2 was assigned based on a rule-based, objective, and conservative approach (Chapter Three; Lowe et al., 2022). The number of reads for each consensus taxon within a sample were then summed to combine the results

of each marker. The relationship between the proportion of read abundance of each matched taxa identified using both markers within a sample was tested using Spearman's rank correlation with Holm correction for multiple testing.

Plant taxa were assigned a native status and form, with those identified to family not categorised. Native status was assigned according to Stace (2019). The category 'native and near native' comprised native species and also genera that include native species and horticultural varieties which are functionally similar. Naturalised plants were those which have been introduced and become widespread and self-perpetuating in the wild. All remaining non-native plants were classified as horticultural. Taxa were grouped into three form categories: tree, shrub, or herb. Herbs were defined as non-woody species, shrubs were defined as woody species < 5 m tall, and trees were defined as woody species > 5 m, following the Royal Horticultural Society classification (Bricknell, 2010). The plant taxa found were labelled with four categories of abundance according to the total proportion of sequences contributed each month: major (≥10% sequences), secondary (≥1% and <10%), minor (≥0.01% and <1%) and occasional (>0% and < 0.01%).

5.3.7. Statistical analyses

The DNA metabarcoding data was treated as semi-quantitative with relative read abundance used for all analyses (Deagle et al., 2019), either using the proportion of taxa as a percentage or, for the models, the number of sequences, controlling for sequencing depth by setting the total number of sequences per sample as an offset (comparable to proportion) (Jones et al., 2021a; Lowe et al., 2022). To analyse how the floral composition of honey changes throughout time and space, the function manyglm within the R package 'mvabund' was used to run a generalised linear model, using all of the plant taxa present in each sample (Wang et al., 2012). The data best fit a negative binomial distribution due to the strong mean-variance

relationship within the data and the high number of zeros in the abundance data occurring from plant taxa absent in a sample. The number of sequence reads for each plant taxon was set as the multivariate response, with the effect of month (measured as the number in the calendar), year, and location of hives included as predictor variables. The number of reads per sample was included as an "offset" in the model to control for differences in the number of sequences between samples (Deagle et al., 2019; Jones et al., 2021a). To visualise seasonal changes in the composition of the honey over time, non-metric multidimensional scaling (NMDS) ordination was used based on the proportion of reads returned for each plant taxa. Ordinations were carried out using the metaMDS function in the vegan package in R (Dixon 2003), using Bray-Curtis dissimilarity indices. *Post hoc* pairwise comparisons were run using the anova.manyglm function to identify which seasons significantly differed from each other.

To visualise connections between each colony and plant taxa, a bipartite network was constructed using the proportion of sequences in a sample as a measure of relative abundance (Deagle et al., 2019). The metric *G*, a quantitative measure of the average number of effective plant taxa per colony per month, was calculated using the 'bipartite' package (Dormann & Strauss, 2014). The degree of diet variation was measured with the IS index, the mean proportional similarity (PS_i) between an individual's diet and the rest of the population (in this case, one colony's diet compared to the combined diet of all colonies during the same sampling period), using the 'RinSp' package (Zaccarelli et al., 2013) with a Monte Carlo resampling simulation using 2,000 iterations to test the significance of IS (Chapter Four). The value of IS = 1 when the average diet of each colony is directly proportional to the diet of all colonies, (meaning the diets are more similar to each other) and decreases towards 0 when each colony has a distinct diet (Bolnick et al., 2002). All statistical analyses were carried out in R (version 4.0.2).

5.3.8. Comparison with floral surveys

All plant taxa identified using DNA metabarcoding were matched to those recorded in floral surveys, for comparison of relative abundance in the honey and landscape. Plants which were identified to species using DNA were matched to the same species in the landscape. To account for the differing taxonomic levels at which plants were identified, those identified at genus level in the honey were matched to all species belonging to that genus in the floral surveys. Those identified as one or more genera were also matched by grouping each matched genus in the floral survey. Plants which were identified to tribe or family level were omitted.

The relationship between the proportion of sequence reads in each honey sample and the proportion of area flowering throughout the season for plant taxa contributing over 1% of sequence reads per month was assessed using Spearman's rank correlation with Holm correction for multiple testing. To test whether plant taxa were used more or less than expected by chance given their relative abundance in the landscape, null models were created using the package 'econullnetr' for preference analysis (Vaughan et al., 2018). Included in these models were both plants which either contributed more than 1% of sequences or over 1% of total flowering area in any given month. Fisher's exact test was used to test whether the abundance and richness of plants in the honey (characterised by native status and form) differed from the floral survey data, using 1) the relative read abundance and the proportion flowering in the landscape each month and 2) the generic richness of honey samples and the generic richness of the landscape each month.

5.4. Results

5.4.1. Overview

A total of 54 honey samples were collected throughout the survey period, returning 6,984,378 sequences after stringent quality control (3,996,872 *rbcL* and 2,987,506 ITS2). Due to a lack of stores, honey could not be sampled from every colony each month (12 occasions across the 2-year survey period). The mean number of sequences in a sample was 129,341 (SD = 24497), ranging from 57,495 to 167,861. The *rbcL* marker detected 99 taxa at the following taxonomic ranks: 3 family, 2 tribe, 75 genus and 19 species. ITS2 identified 120 taxa consisting of 94 genera and 26 species. Following the matching of taxa identified by both markers at varying discrimination, there was a strong correlation between the proportion of sequences for each matched taxon (n = 59) found within each sample using both *rbcL* and ITS2 (Spearman correlation coefficient r_s = 0.604, P < 0.001) (Fig. S5.2, Supporting Information).

5.4.2. Question 1: What are the seasonal changes in honeybee foraging in a diverse area of agricultural habitat and horticultural planting?

143 plant taxa were identified using the *rbcL* and ITS2 regions combined with 79% of taxa identified to genus. Of these 143, only 15 were found to contribute more than 1% of total sequence reads across all samples (Table 5.1). Included in this list are plants used abundantly by honeybees and those with long flowering periods. Over both years, 10 plant taxa were classified as major (≥10% of reads in at least one month), 30 as secondary taxa (≥1% and <10% of reads in at least one month), 66 minor taxa (≥0.01% and <1% of reads in at least one month) and 37 as only ever occurring occasionally (>0% and <0.01% of reads in at least one month) (Table S5.2, Supporting Information). The most abundantly used plants across the season were bramble *Rubus* spp., Himalayan balsam *Impatiens glandulifera*, sycamore and maples *Acer* spp., cherries and plums *Prunus* spp., and white clover *Trifolium repens*.

Table 5.1: List of plant taxa found in the honey using the *rbcL* and ITS2 DNA barcode markers. Per month, taxa contributing to 10% of sequences or over are categorised as major plant taxa, below 10% or equal to 1% are secondary taxa, below 1% or equal to 0.01% are minor taxa, and below 0.01% are occasional. Those noted as 0.00 were present but at very low values.

Major (≥10%)	Secondary (≥1% and <10%)	Minor (≥0.01 and <1%)	Occasional (>0 and <0.01%)

		2018						2019					
Family	Taxa	Apr	May	Jun	Jul	Aug	Sep	Apr	May	Jun	Jul	Aug	Proportion of total reads (%)
Rosaceae	*Rubus* spp.		0.00	33.83	52.22	34.99	25.08	0.04	0.02	14.12	72.80	65.73	**33.15**
Balsaminaceae	*Impatiens glandulifera*				0.01	28.46	52.33	0.93	0.01	0.01	0.42	4.84	**9.83**
Sapindaceae	*Acer* spp.		13.58	7.40	1.03			15.07	41.05	17.42	0.06	0.08	**8.48**
Rosaceae	*Prunus* spp.	44.15	5.67	3.99	0.00			30.85	11.24	12.56	0.02	0.35	**6.66**
Fabaceae	*Trifolium repens*			0.45	8.92	5.12	0.62			0.06	15.14	9.78	**4.81**
Salicaceae	*Salix* spp.	7.43	2.68	1.91				19.77	7.87	7.96	0.02	0.15	**3.73**
Asteraceae	*Taraxacum officinale*	6.09	22.64	13.99		0.00		6.05	4.04	6.09	0.03	0.00	**3.61**
Rosaceae	*Filipendula ulmaria*				7.02	6.08	3.88			0.01	4.29	3.72	**2.88**
Ranunculaceae	*Ranunculus/Ficaria* spp.	5.21	6.66	5.28	0.27			4.23	4.99	7.10	0.59	0.48	**2.44**
Asteraceae	*Cirsium/Centaurea/Hypochaeris* spp.			0.93	12.15	4.72	0.33	0.00	0.00	1.36	1.17	1.45	**2.40**
Plantaginaceae	*Plantago lanceolata*		0.63	0.53	0.34	7.61	7.69	0.10	3.05	0.70	0.07	2.00	**2.39**
Boraginaceae	*Pulmonaria* spp.	13.63	12.83	4.38	1.33			3.32	4.41	0.23	0.00	0.10	**2.22**
Rosaceae	Maleae (*Crataegus/Malus/Cotoneaster* spp.)	0.41	4.37	1.40	1.45			0.55	10.11	4.44	0.05	0.54	**2.07**
Brassicaceae	*Cardamine* spp.	4.81	6.55	2.66	0.02			4.93	3.82	6.08		0.18	**1.99**
Hyacinthaceae	*Hyacinthoides non-scripta*		1.12					2.29	1.22	9.51			**1.17**

Both month ($LR_{1,52}$ = 800.8, P < 0.001) and year ($LR_{1,51}$ = 408.9, P < 0.001) were found to strongly predict the pollen composition of honey, irrespective of apiary location ($LR_{1,50}$ = 412.4, P = 0.149). The most abundantly used plants were the same in 2018 and 2019 but 26 taxa were unique to 2018 and 36 to 2019 (Table S5.2, Supporting Information). Non-metric multidimensional ordination scaling (NMDS) shows that samples collected in the same month are most similar to each other, with samples collected later in the season becoming increasingly divergent (Fig. 5.1). *Post hoc* pairwise comparison revealed that pollen composition differed between all pairs of seasons (spring vs summer: sum-of-LR = 571.9, P < 0.001; summer vs autumn: sum-of-LR = 231.3, P = 0.005; autumn vs spring: sum-of-LR = 729.2, P < 0.001), illustrating a transition in honeybee forage choice between these periods. The plants used by each colony varied throughout the year, resulting in phenological shifts discernible at the colony and network level (Figs 5.2-5.3).

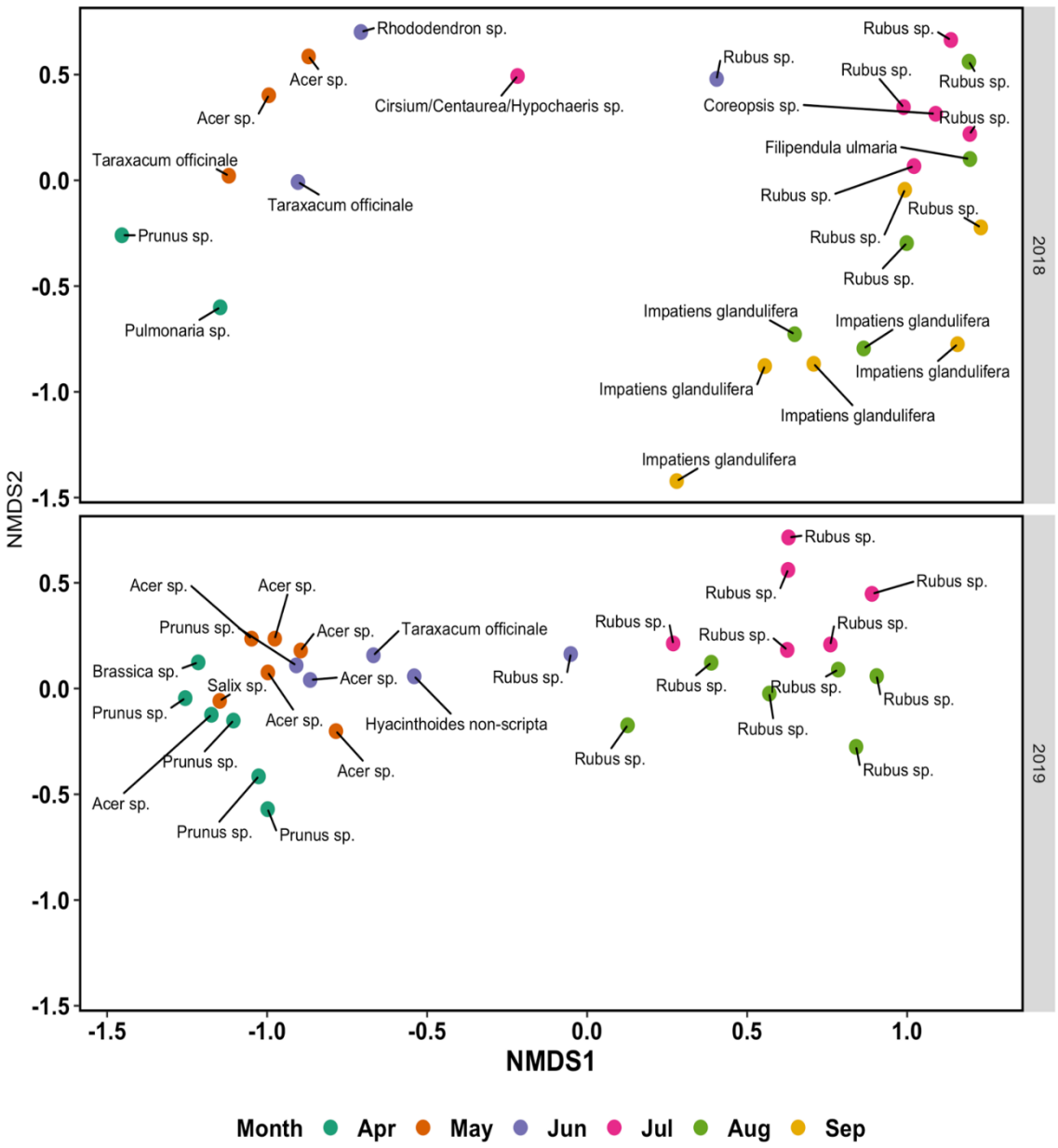

Figure 5.1: Non-metric multidimensional scaling (NMDS) plot of honey samples in relation to month of collection, with each sample labelled with the most abundant taxa in terms of proportion of sequence reads.

5.4.3. Question 2: Can periods of resource limitation be identified by assessing the level of diet specialisation between honeybee colonies?

Inter-individual, or inter-colony specialisation (*IS*) measures the degree of similarity between the diet of a colony and the total diet of all colonies during the same sampling period, and = 1 when all colonies have the same diet, decreasing toward 0 when each colony has its own unique diet. During spring (April and May), each colony's diet was strongly similar to the total dietary niche of all colonies sampled during the same time period (*IS* > 0.5) (Table 5.2). This is a result of all colonies utilising woody tree species such as cherries and plums *Prunus* spp. along with sycamore and maples *Acer* spp. and herbs dandelion *Taraxacum officinale* and *Pulmonaria* spp. abundantly. During this same time period, the average number of effective taxa per colony (*G*) was between 5 and 6 as colonies except for in May 2018, where each colony used 9.85 effective taxa (Table 5.2).

Table 5.2: Network metrics quantifying phenological changes in foraging behaviour. Inter-colony specialisation (IS) = 1 when the average diet of a colony is directly proportional to the diet of all colonies during the same sampling period and nears towards 0 when each colony has its own unique diet. The metric *G*, generality, is a quantitative measure of the average number of effective plant taxa per colony per month. Honey was not sampled in September 2019 due to lack of stores.

Year	Month	Inter-colony specialisation (IS)	Generality (*G*)
2018	Apr	0.66 (< 0.001)	5.47
	May	0.65 (< 0.001)	9.85
	Jun	0.42 (< 0.001)	6.06
	Jul	0.58 (< 0.001)	4.22
	Aug	0.52 (< 0.001)	4.21
	Sep	0.59 (< 0.001)	2.75
2019	Apr	0.62 (< 0.001)	5.75
	May	0.72 (< 0.001)	6.18
	Jun	0.56 (< 0.001)	7.6
	Jul	0.84 (< 0.001)	2.45
	Aug	0.76 (< 0.001)	3.59
	Sep	NA	NA

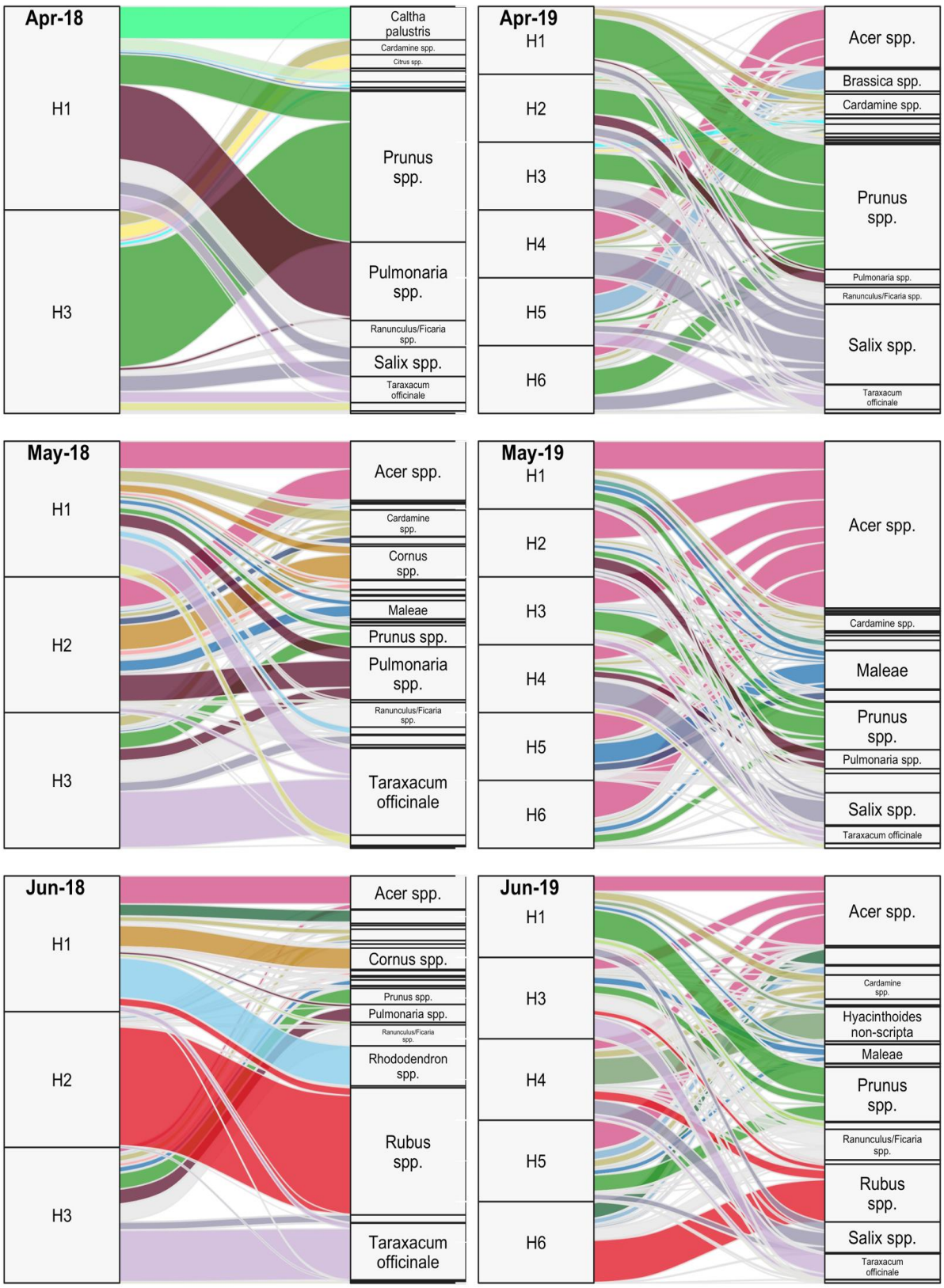

Figure 5.2: Apis *mellifera* pollen transport networks from up to six colonies, collected from April to June in 2018 and 2019. Missing samples were not collected due to limited stores. The width of the connecting ribbon is based on the total proportion of sequences from each honey sample and coloured depending on the plant taxa found.

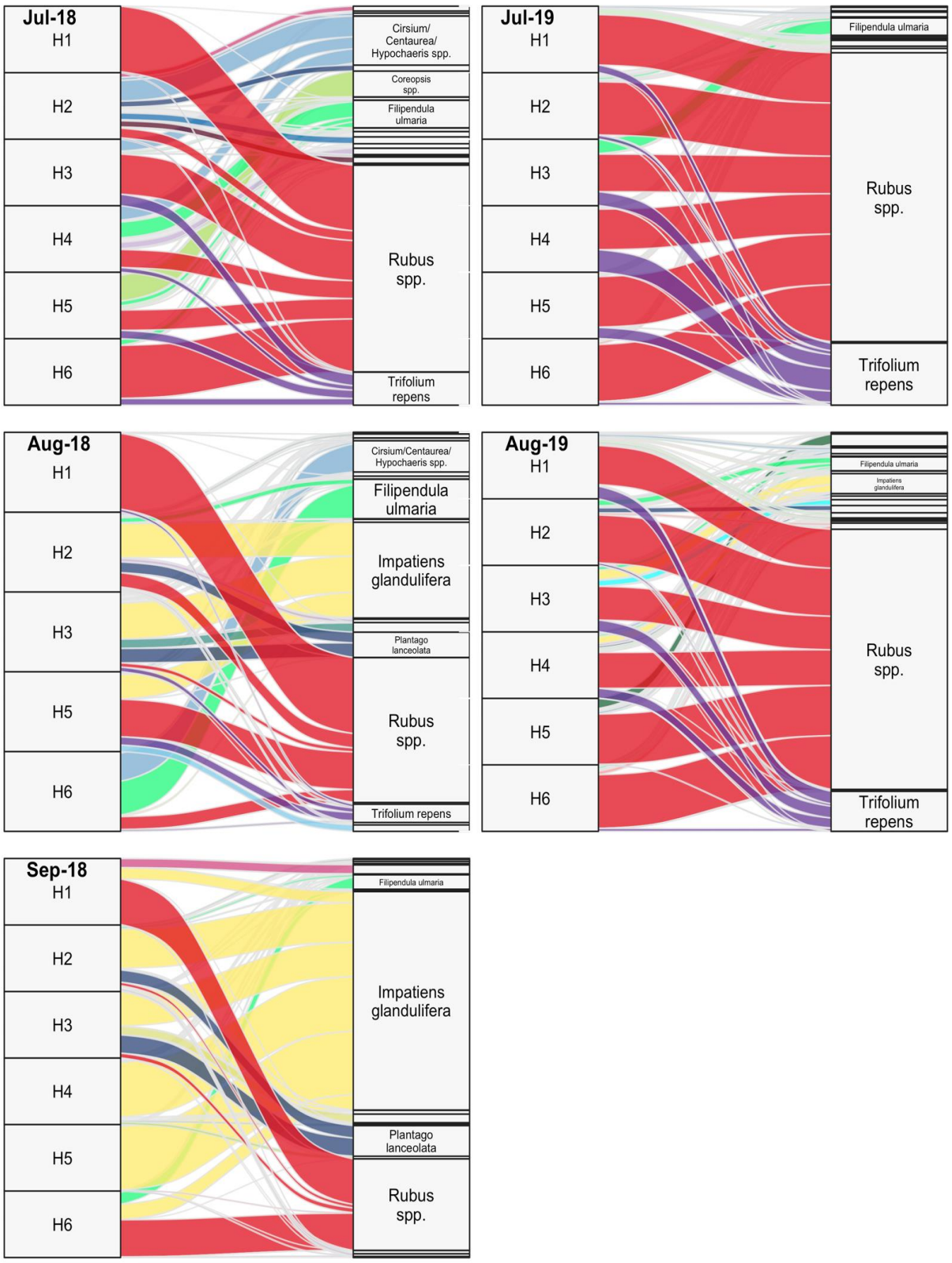

Figure 5.3: *Apis mellifera* pollen transport networks from up to six colonies, collected from July to August in 2018 and 2019, and September 2018. Samples were not collected in September 2019 due to bad weather and limited stores. The width of the connecting ribbon is based on the total proportion of sequences from each honey sample and coloured depending on the plant taxa found.

In June, the values of IS decrease to 0.420 ($P < 0.001$) in 2018 and to 0.562 ($P < 0.001$) in 2019, demonstrating that the diets of colonies diverge and on average, the diet of each colony in comparison to the diet of all colonies together becomes more distinct than in previous months (Table 5.2). Whilst spring taxa such as *Acer* spp. and *Taraxacum officinale* are retained in the diet of all colonies, the introduction of *Rubus* spp. into the diet of some colonies marks a shift in resource use (Fig. 5.2). The number of effective taxa per colony remains comparable to previous months, with values of *G* being 6.0 and 7.8 for 2018 and 2019 respectively.

By July, *Rubus* spp. becomes the dominant taxa used by most colonies (Fig. 5.3), quantified by the low number of effective taxa per colony, in comparison to previous months (2018: $G = 4.2$, 2019: $G = 2.5$). Colonies used other summer resources such as *Cirsium/Centaurea/Hypochaeris* spp., and *Coreopsis* spp., abundantly in 2018 in addition to *Rubus* spp., *Trifolium repens* and *Filipendula ulmaria* which were used abundantly over both years, leading to higher inter-colony variation (lower IS) in 2018 compared to 2019 (2018: IS = 0.579, $P < 0.001$, 2019 (IS = 0.836, $P < 0.001$) (Fig. 5.3).

Inter-colony foraging differences increased again in August, illustrated by lower IS values in comparison to July (Table 5.2), as colonies retain the use of resources such as *Cirsium/Centaurea/Hypochaeris* spp., *F. ulmaria*, *Rubus* spp., and *T. repens,* whilst a subset of colonies begin using *Impatiens glandulifera* (Fig. 5.3). The average number of effective taxa per colony (*G*) remained the same between July and August in 2018 ($G = 4.2$) however increased slightly in 2019 ($G = 3.6$).

Honey was not collected in September 2019 due to lack of stores. However, during September 2018, inter-colony variation remained similar to August (IS = 0.764, $P < 0.001$), but the average number of effective taxa per colony reduced to 2.7, the lowest value seen throughout the season, with the majority of colonies using *I. glandulifera* most abundantly.

5.4.4. Question 3: Do honeybees visit plants more or less than expected by chance given their relative abundance in the landscape?

Over the study period, honeybees had access to a total of 1,498 unique plant taxa covering 613 genera, distributed across native habitats consisting of semi-improved grassland, woodland, and hedgerows, along with planted horticultural areas and amenity grassland. There was an average of 261 unique genera in flower each month (SD = 55.62), however, honeybees used a minority of available genera (Fig. 5.4), with each colony choosing an average of 17% of genera per month (SD = 6.75) (Table 5.3).

Table 5.3: Genera use compared to availability within the study site.

		2018						2019				
		Apr	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Apr**	**May**	**Jun**	**Jul**	**Aug**
Number of genera in flower		123	235	279	299	303	257	200	242	296	326	291
Number of genera in honey	Hive 1	9 (7%)	35 (15%)	36 (13%)	5 (2%)	7 (2%)	8 (3%)	36 (18%)	40 (17%)	38 (13%)	16 (5%)	27 (9%)
	Hive 2		38 (16%)	24 (9%)	26 (9%)	31 (10%)	23 (9%)	35 (18%)	47 (19%)		28 (9%)	15 (5%)
	Hive 3	13 (11%)	26 (11%)	25 (9%)	7 (2%)	22 (7%)	19 (7%)	19 (10%)	37 (15%)	39 (13%)	20 (6%)	28 (10%)
	Hive 4				14 (5%)		12 (5%)	23 (12%)	22 (9%)	29 (10%)	15 (5%)	22 (8%)
	Hive 5				10 (3%)	22 (7%)	12 (5%)	26 (13%)	20 (8%)	32 (11%)	20 (6%)	11 (4%)
	Hive 6				11 (4%)	8 (3%)	12 (5%)	21 (11%)	26 (11%)	28 (10%)	30 (9%)	22 (8%)
Number of unique genera in honey		14 (11%)	51 (22%)	51 (18%)	40 (13%)	46 (15%)	39 (15%)	53 (27%)	57 (24%)	58 (20%)	58 (18%)	54 (19%)
Mean number of genera per colony		11 (SD = 2.83)	33 (SD = 6.24)	28 SD = 6.66	12 (SD = 7.47)	18 (SD = 10.2)	14 (SD = 5.54)	27 (SD = 7.23)	32 (SD = 10.9)	33 (SD = 5.07)	22 (SD = 6.19)	21 (SD = 6.68)

When considering taxa that contributed over 1% of sequence reads in at least one month (major and secondary taxa, n = 36), no significant relationship was found between the proportion of DNA sequences in the honey and abundance in the study site in any month, with the exception of July 2019 (Spearman correlation coefficient r_s = 0.68, P = 0.002) (Fig. S5.3, Supporting Information). Preference analysis showed that most plants were used more than expected given their abundance in the landscape, including the two key plants *Rubus* spp. and *Impatiens glandulifera* (Fig. S5.4, Supporting Information). The exceptions were *Ranunculus*/*Ficaria* spp. and *Cirsium*/*Centaurea*/*Hypochaeris* spp., which were used significantly less than expected given their abundance.

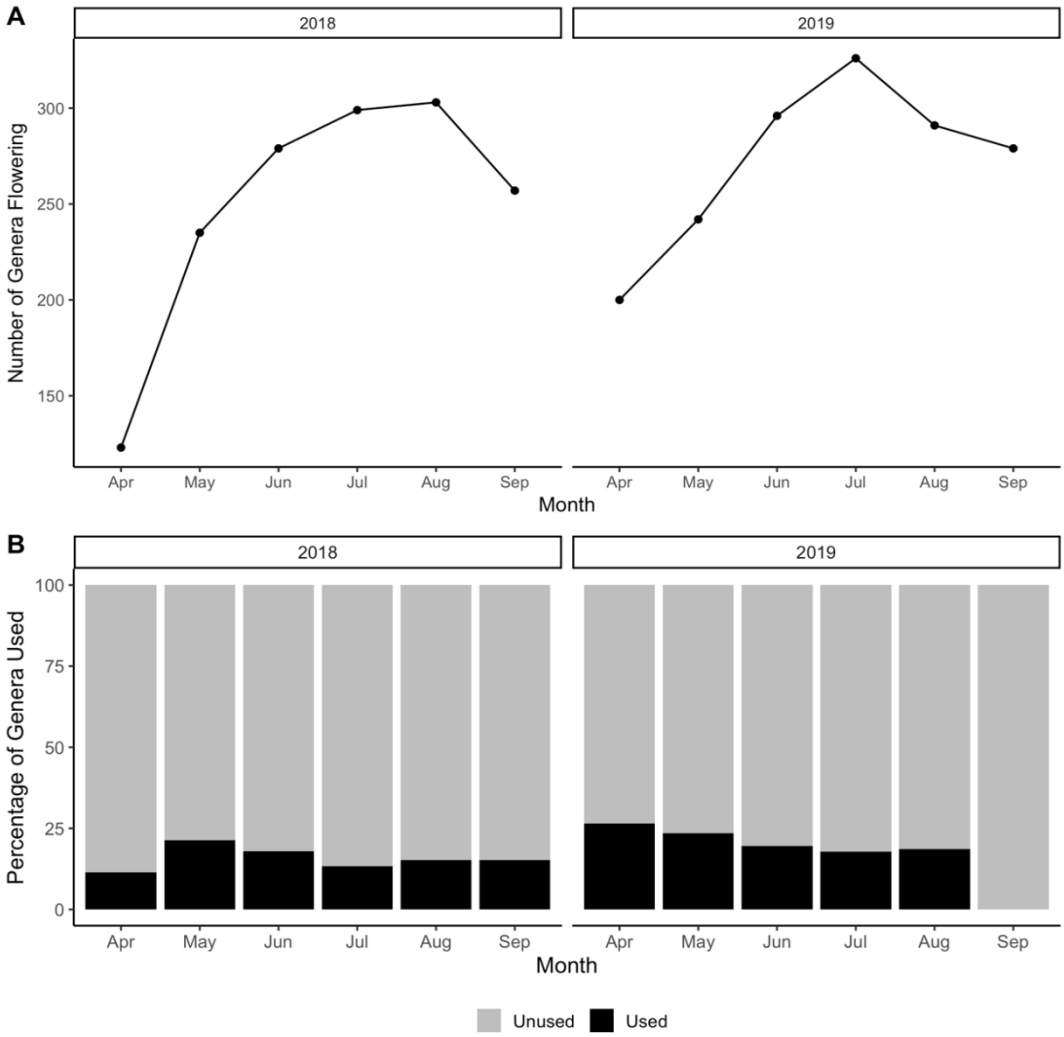

Figure 5.3: A) Total number of genera in flower in floral surveys from April to September. **B)** Proportion of genera found in DNA compared to those in flower. No honey samples were collected in September 2019 due to bad weather and limited stores.

5.4.5. Question 4: Is the use of plants by honeybees proportional to their relative abundance in the landscape, with regard to their growth form (tree/shrub/herb) or native status?

Forage preference of trees, shrubs, and herbs was driven by month ($LR_{1,48} = 74.79$, $P < 0.001$) and year ($LR_{1,47} = 11.33$, $P = 0.008$) (Fig. 5.5). Each month, the proportion of reads for each plant form identified in honey samples was significantly different to the proportion flowering within the landscape, with honeybees showing a preference for trees in April and May and switching to shrubs in June and herbs by September (Fig. 5.5). A similar trend was found when comparing the generic richness of each plant form category found in honey compared to its availability in the landscape each month (Fig. S5.5, Supporting Information).

Throughout the season, the most abundantly used plant taxa were native and near-native (Figs S5.6-5.7, Supporting Information). The use of plants in each status category varied by month ($LR_{1,48} = 70.98$, $P < 0.001$) but similar patterns were observed in 2018 and 2019 ($LR_{1,47} = 7.01$, $P = 0.062$) when modelling the proportion of reads, with a higher proportion of naturalised sequences found at the end of the season in both years. There was no significant difference found between the proportion of sequences attributed to native and near-native plants in the honey and the proportion flowering within the landscape in any month, except during August and September 2018 due to the increased use of the naturalised *Impatiens glandulifera* (Fig. S5.6, Supporting Information). However, when comparing generic richness of each status category in honey samples to generic richness availability in the landscape each month, honeybees used more native and near-native genera than expected by chance in all months except April 2019 (Fig. S5.7, Supporting Information).

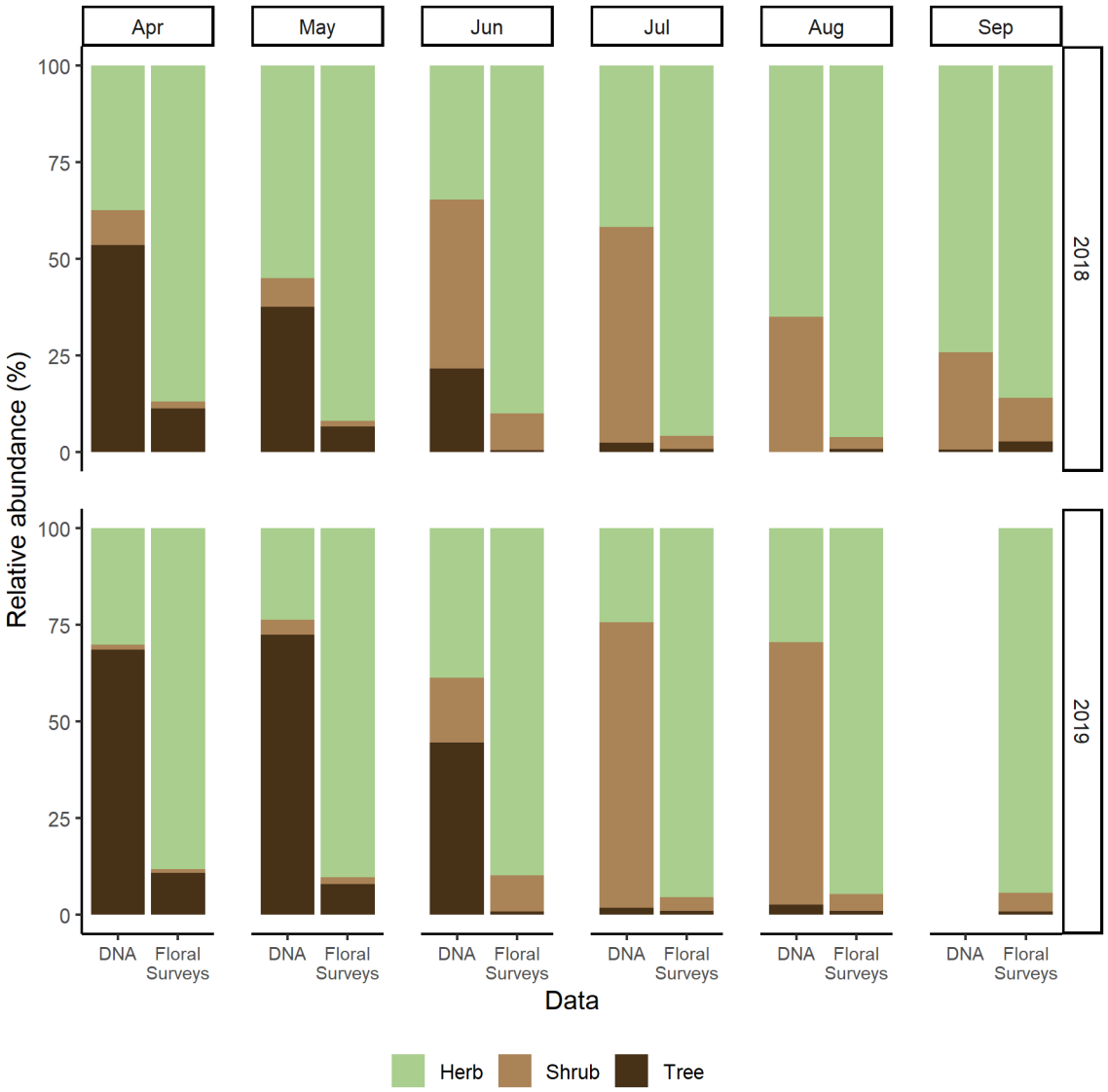

Figure 5.4: Relative abundance of each plant form in honey (DNA) compared to the relative abundance in flower (Floral Surveys) each month for 2018 and 2019. Using Fisher's exact test, a significant difference was found each month across both years. 2018: Apr ($P < 0.001$), May ($P < 0.001$), June ($P < 0.001$), July ($P < 0.001$), August ($P < 0.001$), September ($P = 0.018$). 2019: Apr ($P < 0.001$), May ($P < 0.001$), June ($P < 0.001$), July ($P < 0.001$), August ($P < 0.001$).

5.5. Discussion

In this study we use DNA metabarcoding to reveal patterns in floral resource use by a central-place forager, the honeybee (*Apis mellifera*). We identify that whilst honeybees forage on a large number of plant taxa, few plants make major contributions to their overall diet. The selection of resources is strongly influenced by month and year, as a result of the phenological progression of plants through the seasons and differences in minor taxa between years. Two key transition periods were identified in June and August as colonies diverge in their selection of resources before an increase in diet similarity and decrease in diet breadth in July and September. These seasonal patterns of specialisation infer that there may be underlying periods of resource limitation which drive opportunistic patterns in foraging. Honeybees predominately used trees in April and May, switching to shrubs in June, consistently selecting these plant forms more than expected given their abundance in the landscape. This study highlights the importance of semi-natural habitats such as woodland, hedgerows and scrub which provide the major plants used to support honeybee colonies.

5.5.1. Honeybees use a large number of plants throughout the season, but very few are major resources

The plants identified from honey samples include those used for both nectar and pollen (Ponnuchamy et al., 2014). The floral resources selected by honeybees cover a diverse taxonomic breadth, making them generalist in their preferences. This is expected in social bees with long foraging periods, allowing species to adapt to available resources throughout the season to survive (Ogilvie & Forrest, 2017). Many authors regard honeybees as a "super-generalist" due to their large diet breadth (Corbet, 2006; Hung et al., 2018; Memmott & Waser, 2002; Potts et al., 2010), however, our results are consistent with those that have assessed frequency of use, suggesting that a core number of species are crucial to their diet (de Vere et

al., 2017; Hawkins et al., 2015; Jones et al., 2021a; Richardson et al., 2021). Although only a small number of plants are used often, it is thought that the high diversity of taxa utilised enables honeybees to meet the complex nutritional needs throughout the lifecycle and provide protection from stress and disease (Vaudo et al., 2015).

Eight out of the 10 major taxa identified across the year are identified as important contributors to the total nectar availability within the UK (Baude et al., 2016) and most are also valuable pollen sources (Hicks et al., 2016; Percival, 1947). There is strong phenological progression throughout the season as honeybees change their foraging preferences to adapt to seasonal changes in resources. As the selection of resources can be influenced by the quality of nectar or pollen (Vaudo et al., 2015), this suggests that honeybees alter their foraging behaviour to select the most rewarding plants to maintain nutritional requirements throughout the year (Danner et al., 2017). The most frequently used plants in this study corresponded closely with Jones et al. (2021a), who sampled honey from beekeepers across the UK in the first national assessment of honeybee foraging since 1952, with the top five plants identified across 441 samples found to be *Rubus* spp., *Trifolium repens, Brassica* spp., Maleae group (*Crataegus*/*Malus*/*Cotoneaster*) spp. and *Acer* spp.

5.5.2. Seasonal variation in diet specialisation by honeybees suggests potential periods of food shortages

Amongst the overall seasonal variations in resource use, the way resources were partitioned between honeybee colonies also varied throughout the year. Two key transition periods were revealed, where colonies diverged in their use of resources and then converged again, exploiting fewer major resources. The most notable of these periods was during June, where the greatest variation in foraging between individual colonies was found, across both years. Requier et al (2015) also found

that the foraging preferences of honeybee colonies were most dissimilar in June, between peak flowering of two crop blooms. Under optimal foraging theory, individuals increase dietary breadth when abundant resources are low, which causes a reduction in niche overlap (Stephens et al., 2019). Our results suggest a period of food shortage in June as diet similarity between colonies becomes more varied, with honeybees beginning to use bramble *Rubus* spp. at low levels as it begins to flower and narrowing their food niche as it becomes abundant, resulting in a period of high diet similarity between colonies in July. This change in foraging behaviour in response to varying plant abundance has also been recorded in bumblebees, hoverflies, and butterflies, widening their forage choice as the density of their preferred resource decreases (Goulson, 1999). Whilst less pronounced than the transition period in June, a similar pattern was found in August as honeybees switch to using Himalayan balsam *Impatiens glandulifera.* The low average number of effective plant taxa per colony found in the months of July and September suggest that honeybees make a distinct choice to forage on *Rubus* spp. and *I. glandulifera* during these periods. Both *Rubus fruticosus* and *I. glandulifera* produce rewarding nectar, both in large volumes and of high sugar content (Baude et al., 2016; Fowler et al., 2016) and their ecological value to a range of pollinators is recognised in the literature (Baldock et al., 2019; Jones et al., 2021a; Lucas et al., 2018b, 2018a; Wignall et al., 2020a).

The nectar collected by honeybees is processed and stored as honey to be used when foraging is not possible, for example through winter. As a result, honey stores increase during periods of high productivity. Although there was a high diversity of plants available throughout the season, failure to sample honey from multiple colonies in spring and summer across both years due to low stores suggests that there are indeed periods of low forage availability for honeybees in this landscape, supporting the anecdotal "June Gap" of floral resources (Crane, 1976; Suryanarayana & Singh, 1989). Requier et al. (2015) demonstrated that the

effects of food stress can occur seven months later, with food shortages in spring resulting in colony losses in winter. Seasonal food shortages are well documented in agricultural habitats across Europe and North America, following mass flowering of insect-pollinated crops (Couvillon et al., 2014; Jachuła et al., 2021; Timberlake et al., 2019). Increased diet specialisation occurs in June as a result of honeybees switching between trees and shrubs, supporting the idea that this "gap" in floral resources is a result of reduced floral availability between spring flowering trees and summer flowering shrubs and herbs (Balfour et al., 2018). This period of resource limitation may impact wild pollinators if preferred resources are shared with honeybees, by increasing exploitative competition between species (Wignall et al., 2020b). However, generalist pollinators can switch resources when required (Spiesman & Gratton, 2016), which may buffer populations against negative effects (Mallinger et al., 2017). It is likely that any gaps in floral resource availability will have a greater impact on species that lack the ability to store resources, in particular, those with short flight seasons such as solitary bees (Ogilvie & Forrest, 2017; Timberlake et al., 2019). A key area for further work would be to quantify the phenology of nectar and pollen sources to assess if there are periods of resource limitation in this complex garden landscape as our results may suggest (Timberlake et al., 2019). Our results suggest that the prevalence of intraspecific specialisation is a sign of food shortages, however, foraging in social insects is a complex behaviour and will also be influenced by the communication between individuals, environmental factors, and phenotypic or genotypic differences in individuals (Frank & Linsenmair, 2017).

5.5.3. Most plants are visited more often than expected given their relative abundance in the landscape

Within the diverse landscape studied, honeybees used a low proportion of available taxa each month. This is consistent with de Vere et al. (2017) who found

that in spring, only 11% of available genera were found in honey samples, demonstrating that foraging is limited to a few, select resources from the landscape. Whilst we found that the relative abundance of plants in honey samples was not related to their relative abundance in the landscape, the abundance of floral resources has been shown to influence foraging patterns (Nürnberger et al., 2019). Foragers tend to select larger patches of resources (Goulson et al., 1999), likely due to the increased likelihood of encountering the resource within a landscape. Although there is a relationship between patch size and recruitment in social species, it is not found to be proportional, with other factors such as density of flowers and resource quality also influencing selection (Fowler et al., 2016; Goulson, 1999). The major plants identified in this study tend to flower in dense patches, with flowering trees in particular being a large three-dimensional flowering patch in the landscape. Therefore, it would be beneficial for further work to investigate the relationship between patch size in the landscape and their use by honeybees. Alternatively, there is research to suggest that the selection of abundant resources fluctuates throughout the year, interacting with the nutritional needs of the colony (Quinlan et al., 2021). Percival (1947) found a strong relationship between the abundance of plants and their use by honeybees; however, a disproportionately smaller amount of pollen was collected from *Ranunculus bulbosus, Plantago lanceolata, Hyacinthoides non-scripta and Cirsium arvense* given their abundance. These findings are similar to ours given the low abundance of *Centaurea/Cirsium/Hypochaeris* spp. and *Ranunculus/Ficaria* spp. identified in honey samples.

5.5.4. Honeybees are selective in the type of plant they use (tree/shrub/herb) and native or horticultural, but this is strongly influenced by season and not related to their abundance in the landscape

Honeybees showed clear preferences for trees in early spring, although their use decreased considerably as the season progressed so that by early summer, shrubs made up the majority of honeybees' diet, supplemented with herbs which became dominant in the diet by September. Whilst this pattern is consistent with the peak flowering times of insect-pollinated trees, shrubs, and herbs in the UK (Balfour et al., 2018), we found that the preferences differed from expected given their abundance in the landscape. The importance of trees for pollinators is often overlooked, however, spring flowering trees provide a vital early season resource for a diversity of pollinators (de Vere et al., 2017; Urban-Mead et al., 2021). It is thought that trees and mass-flowering crops play a role in buffering spring-flying pollinators from the extinctions faced by summer-flying species by providing a high abundance of resources during the beginning of the pollinator flight season (Balfour et al., 2018).

The high use of native and near-native plants by the honeybees corresponded with the relative abundance of this plant type in the landscape. The pattern was different when assessing generic richness, as there were a high number of horticultural taxa available within the study site that occurred at low abundance. Landscapes with semi-natural habitats have been shown to support pollinators throughout the season (Mallinger et al., 2016), with horticultural plants playing a key role in supporting pollinators both as minor plants (de Vere et al., 2017) and by extending the season (Lowe et al., 2022; Salisbury et al., 2015). Honeybees rely on the non-native, *Impatiens glandulifera* at the end of the season, however this species is highly invasive in the UK and can displace other plant species (Chittka & Schürkens, 2001).

5.5.5. Conclusion

This study demonstrates that DNA metabarcoding is a useful tool that can be used to investigate plant-pollinator interactions across time and space. By using this technique, we have been able to gain an increased ecological insight into the foraging preferences of the honeybee, *Apis mellifera*, improving our understanding of floral resource selection by pollinators. Although *A. mellifera* is considered a super-generalist, we provide evidence of strong seasonal selection of resources and demonstrate that a low number of resources are used abundantly across all colonies. These minor resources likely reflect opportunistic choices made by each colony rather than preferred resources. The high level of inter-colony diet specialisation seen in June and August infers that there may be periods of resource limitation however, resource availability was found to be high throughout the year. This, along with the fact that the use of resources and their relative abundance in the landscape is not directly proportional suggests other drivers of floral selection. Our results show that the perceived resource limitation may be an effect of the progression from trees to shrubs and herbs from spring and summer. This study has implications for honeybee foraging but also for wild pollinators, as honeybees can travel further, communicate resources and store food they are more likely to be more resilient than solitary bees and other pollinators. We therefore highlight the need to ensure specific major resources are abundant throughout the year which can be achieved through supplemental planting.

5.6. Acknowledgements

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5.8. Supporting Tables and Figures

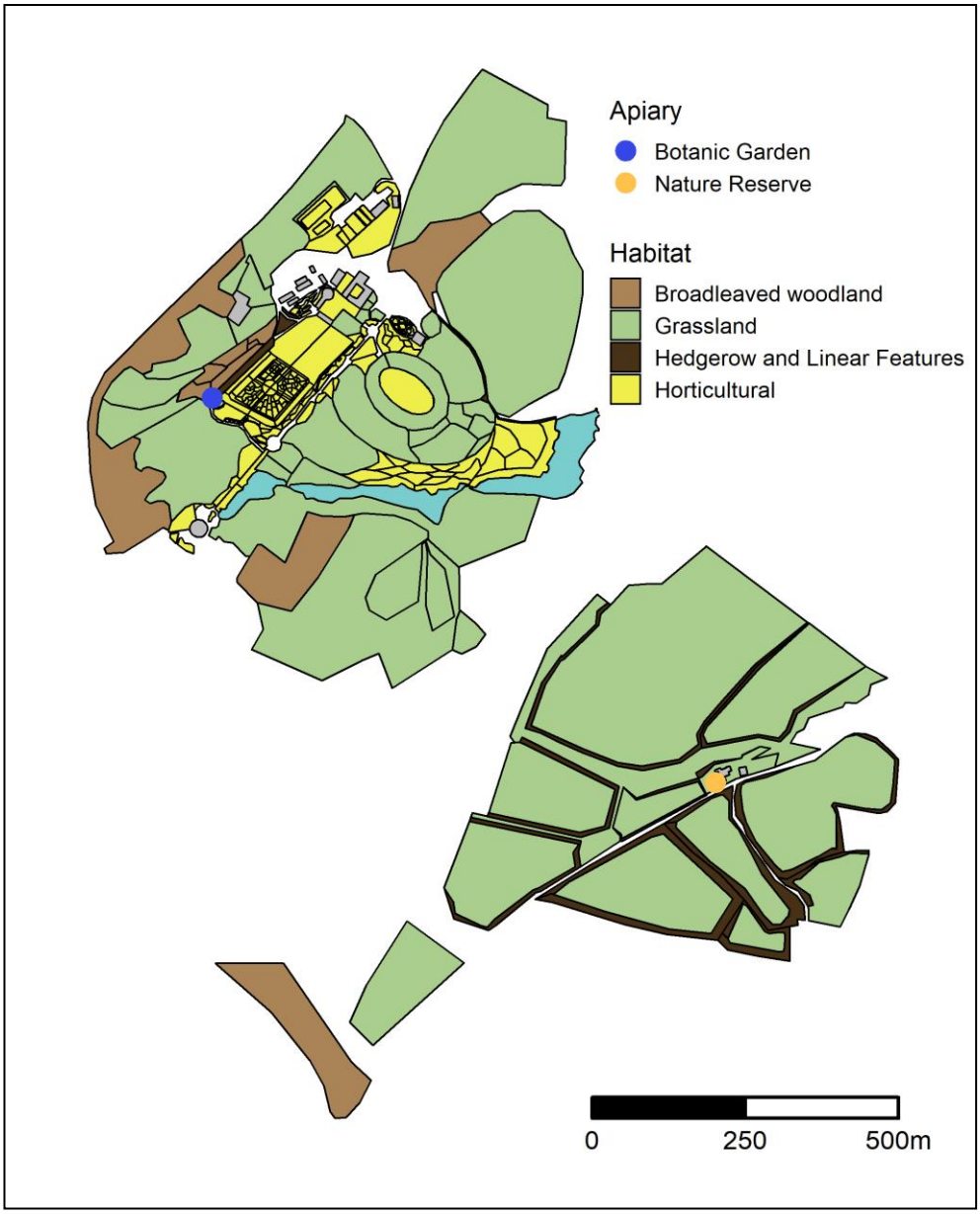

Figure S5.1: Habitat map of the National Botanic Garden of Wales and Waun Las National Nature Reserve showing the survey zones around the two apiaries. The grassland in the study site is mainly composed of semi-improved grassland and lowland hay meadows. Maps were created in QGIS v. 3.6.1 and R v. 4.0.2. from OS data © Crown Copyright (2021) licensed under the Open Government Licence.

Table S5.1: Primer sequences used to amplify th*e rbcL* and ITS2 barcode regions. A 6N sequence was added between the forward template specific primer and the universal tail to improve clustering on the Illumina MiSeq

Primer name	Universal Tail	6N Sequence	Primer sequence
*rbcL*af (Kress & Erickson, 2007)	**ACACTCTTTCCCTACACGACGCTCTTCCGATCT**	NNNNNN	ATGTCACCACAAACAGAGACTAAAGC
*rbcL*r506 (de Vere et al., 2012)	**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**		AGGGGACGACCATACTTGTTCA
ITS2F (Chiou et al., 2007)	**ACACTCTTTCCCTACACGACGCTCTTCCGATCT**	NNNNNN	ATGCGATACTTGGTGTGAAT
UniPlantR (Moorhouse-Gann et al., 2018)	**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**		CCCGHYTGAYYTGRGGTCDC

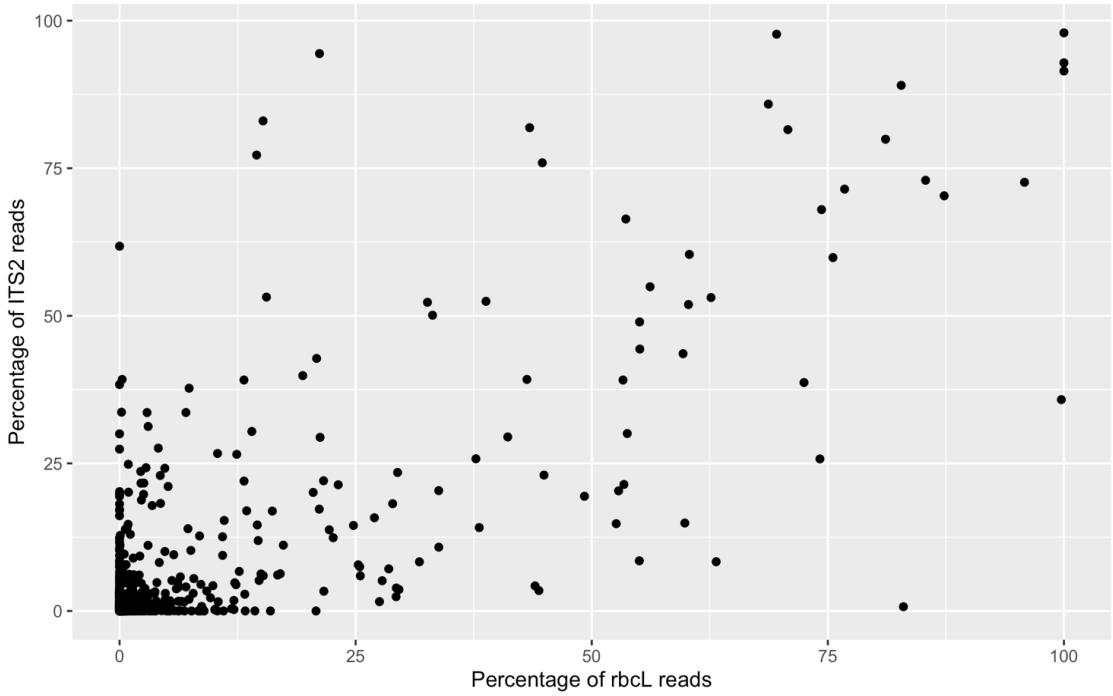

Figure S5.2: Comparison of the proportion of *rbcL* and ITS2 reads in each sample for taxa found using both markers. A strong correlation was found using Spearman's rank correlation (r_s = 0.663, P < 0.001, n = 59).

Table S5.2: List of plant taxa found in the honey using the *rbcL* and ITS2 DNA barcode markers. Per month, taxa contributing to 10% of sequences or over are categorised as major plant taxa, below 10% or equal to 1% are secondary taxa, below 1% or equal to 0.01% are minor taxa, and below 0.01% are occasional. Those noted as 0.00 were present but at very low values.

Major (≥10%)	Secondary (≥1% and <10%)	Minor (≥0.01 and <1%)	Occasional (>0 and <0.01%)

				2018						2019					
Family	Taxa	Status	Form	Apr	May	Jun	Jul	Aug	Sep	Apr	May	Jun	Jul	Aug	Proportion of total reads (%)
Rosaceae	*Rubus* spp.	Native and near-native	Shrub		0.00	33.83	52.22	34.99	25.08	0.04	0.02	14.12	72.80	65.73	**33.15**
Balsaminaceae	*Impatiens glandulifera*	Naturalised	Herb				0.01	28.46	52.33	0.93	0.01	0.01	0.42	4.84	**9.83**
Sapindaceae	*Acer* spp.	Native and near-native	Tree		13.58	7.40	1.03			15.07	41.05	17.42	0.06	0.08	**8.48**
Rosaceae	*Prunus* spp.	Native and near-native	Tree	44.15	5.67	3.99	0.00			30.85	11.24	12.56	0.02	0.35	**6.66**
Fabaceae	*Trifolium repens*	Native and near-native	Herb			0.45	8.92	5.12	0.62			0.06	15.14	9.78	**4.81**
Salicaceae	*Salix* spp.	Native and near-native	Tree	7.43	2.68	1.91				19.77	7.87	7.96	0.02	0.15	**3.73**
Asteraceae	*Taraxacum officinale*	Native and near-native	Herb	6.09	22.64	13.99		0.00		6.05	4.04	6.09	0.03	0.00	**3.61**
Rosaceae	*Filipendula ulmaria*	Native and near-native	Herb				7.02	6.08	3.88			0.01	4.29	3.72	**2.88**
Ranunculaceae	*Ranunculus/Ficaria* spp.	Native and near-native	Herb	5.21	6.66	5.28	0.27			4.23	4.99	7.10	0.59	0.48	**2.44**
Asteraceae	*Cirsium/Centaurea/ Hypochaeris* spp.	Native and near-native	Herb			0.93	12.15	4.72	0.33	0.00	0.00	1.36	1.17	1.45	**2.40**

Plantaginaceae	*Plantago lanceolata*	Native and near-native	Herb		0.63	0.53	0.34	7.61	7.69	0.10	3.05	0.70	0.07	2.00	**2.39**
Boraginaceae	*Pulmonaria* spp.	Native and near-native	Herb	13.63	12.83	4.38	1.33			3.32	4.41	0.23	0.00	0.10	**2.22**
Rosaceae	Maleae (*Crataegus/Malus/ Cotoneaster* spp.)	Native and near-native	Tree	0.41	4.37	1.40	1.45			0.55	10.11	4.44	0.05	0.54	**2.07**
Brassicaceae	*Cardamine* spp.	Native and near-native	Herb	4.81	6.55	2.66	0.02			4.93	3.82	6.08		0.18	**1.99**
Hyacinthaceae	*Hyacinthoides non-scripta*	Native and near-native	Herb		1.12					2.29	1.22	9.51			**1.17**
Apiaceae	*Angelica/Heracleum/ Oenanthe* spp.	Native and near-native	Herb			2.96	0.01	0.84	0.43			4.10	0.22	2.67	**0.99**
Brassicaceae	*Brassica* spp.	Native and near-native	Herb		1.46	0.49	0.60			5.52	0.45	2.40	0.26	0.02	**0.96**
Asteraceae	*Coreopsis* spp.	Horticultural	Herb				6.62			0.00					**0.79**
Cornaceae	*Cornus* spp.	Native and near-native	Tree		7.70	4.65	0.00				0.10				**0.68**
Ericaceae	*Rhododendron* spp.	Horticultural	Shrub		1.63	8.72				0.00	0.01	0.01			**0.57**
Rosaceae	*Rosa* spp.	Native and near-native	Shrub		0.21	0.50	0.18	0.01	0.01			1.08	1.12	1.74	**0.51**
Fagaceae	*Quercus* spp.	Native and near-native	Tree		0.65	0.56			0.63	0.73	1.08	1.47	0.00	0.30	**0.48**
Fabaceae	*Ulex* spp.	Native and near-native	Shrub	2.71	2.27	0.34				0.82	1.16	0.80			**0.47**
Onagraceae	*Oenothera* spp.	Native and near-native	Herb			0.01	1.59	0.89	0.12				0.19	0.89	**0.42**
Papaveraceae	*Papaver* spp.	Native and near-native	Herb		0.19		0.25	3.12	0.36				0.07	0.00	**0.37**
Salicaceae	*Populus* spp.	Native and near-native	Tree		0.00					0.09	0.03	0.09	1.45	1.23	**0.36**
Aquifoliaceae	*Ilex* spp.	Native and near-native	Shrub		0.17	0.09				0.00	2.34	0.67			**0.32**
Cucurbitaceae	*Cucumis/Cucurbita* spp.	Horticultural	Herb						2.51						**0.32**

Malvaceae	*Malva* spp.	Native and near-native	Herb						2.44						**0.31**
Ranunculaceae	*Caltha palustris*	Native and near-native	Herb	5.68	0.02					0.62	0.43	0.06			**0.25**
Ranunculaceae	*Clematis* spp.	Native and near-native	Shrub		1.69	0.00	1.28		0.00						**0.24**
Limnanthaceae	*Limnanthes douglasii*	Horticultural	Herb		1.05	0.66	1.13	0.00							**0.23**
Onagraceae	*Epilobium* spp.	Native and near-native	Herb			0.06	0.81	0.82	0.16				0.08	0.18	**0.22**
Boraginaceae	*Borago officinalis*	Horticultural	Herb		0.14			0.87	0.00	0.02	0.03		0.86	0.18	**0.22**
Oleaceae	*Fraxinus* spp.	Native and near-native	Tree	0.86	2.13	1.22	0.03			0.04	0.02	0.06	0.01		**0.22**
Fagaceae	*Fagus* spp.	Native and near-native	Tree		0.22	0.18			0.00	0.95	0.62	0.31	0.00	0.00	**0.20**
Asphodelaceae	*Phormium tenax*	Horticultural	Herb										0.19	1.41	**0.20**
Hypericaceae	*Hypericum* spp.	Native and near-native	Herb					0.99					0.14	0.68	**0.19**
Ranunculaceae	*Helleborus* spp.	Native and near-native	Herb	1.55	0.16	0.01				1.45	0.03	0.01			**0.18**
Plantaginaceae	*Veronica* spp.	Native and near-native	Herb			0.00		1.98	0.00				0.01	0.02	**0.18**
Araliaceae	*Hedera helix*	Native and near-native	Shrub	1.81		0.03	0.92		0.10	0.03	0.00			0.01	**0.17**
Euphorbiaceae	*Mallotus* spp.	Horticultural	Shrub				1.11								**0.13**
Lythraceae	*Lythrum salicaria*	Native and near-native	Herb					0.11	0.94					0.01	**0.13**
Ranunculaceae	*Anemone* spp.	Native and near-native	Herb		0.02		0.00	0.43	0.19	0.26	0.03	0.00	0.00	0.31	**0.13**
Iridaceae	*Crocus* spp.	Horticultural	Herb					1.36					0.02		**0.12**
Adoxaceae	*Sambucus/Viburnum* spp.	Native and near-native	Shrub		0.58	0.06	0.01		0.01	0.11	0.22	0.12	0.01	0.24	**0.11**
Rutaceae	*Citrus* spp.	Horticultural	Shrub	4.39											**0.11**

Amaryllidaceae	*Allium* spp.	Native and near-native	Herb							0.00	0.53	0.42		0.00	**0.09**
Asteraceae	*Sonchus* spp.	Native and near-native	Herb					0.00	0.68			0.01	0.00		**0.09**
Tropaeolaceae	*Tropaeolum* spp.	Horticultural	Herb					0.65	0.21	0.00	0.00				**0.08**
Paeoniaceae	*Paeonia* spp.	Horticultural	Herb		0.54	0.09	0.01			0.29	0.07	0.08			**0.07**
Apiaceae	*Conopodium majus*	Native and near-native	Herb		0.47	0.88					0.00				**0.07**
Ranunculaceae	*Thalictrum* spp.	Native and near-native	Herb		0.01		0.01	0.44			0.06	0.02	0.15		**0.07**
Asteraceae	*Calendula* spp.	Horticultural	Herb						0.53						**0.07**
Convolvulaceae	*Convolvulus/ Calystegia* spp.	Native and near-native	Herb											0.52	**0.06**
Asteraceae	*Achillea* spp.	Native and near-native	Herb				0.52	0.00							**0.06**
Campanulaceae	*Campanula* spp.	Native and near-native	Herb			1.00							0.04	0.00	**0.06**
Sapindaceae	*Aesculus hippocastanum*	Naturalised	Tree		0.40	0.24				0.07	0.13	0.06	0.00	0.00	**0.06**
Nothofagaceae	*Nothofagus* spp.	Horticultural	Tree	0.36	0.00	0.08				0.43	0.01	0.02			**0.05**
Lamiaceae	Mentheae	Native and near-native	Herb		0.06				0.18	0.08	0.08	0.01	0.02	0.00	**0.05**
Asteraceae	*Helenium* spp.	Horticultural	Herb					0.10	0.22						**0.04**
Rutaceae	*Zanthoxylum* spp.	Horticultural	Shrub		0.37					0.13	0.02	0.01			**0.03**
Asteraceae	*Bellis perennis*	Native and near-native	Herb			0.00				0.02	0.05	0.27	0.02	0.01	**0.03**
Cyperaceae	*Carex* spp.	Native and near-native	Herb								0.30				**0.03**
Asteraceae	*Helminthotheca/ Picris* spp.	Native and near-native	Herb					0.00	0.22						**0.03**
Poaceae	Poaceae	Not categorised	Not categorised	0.02	0.00	0.00	0.00			0.00	0.14	0.00	0.08	0.01	**0.03**

Rosaceae	*Sorbus* spp.	Native and near-native	Tree		0.20	0.08				0.00	0.05	0.05			**0.03**
Boraginaceae	*Echium* spp.	Native and near-native	Herb					0.00					0.15		**0.02**
Zingiberaceae	Zingiberaceae	Not categorised	Not categorised	0.80											**0.02**
Plantaginaceae	*Antirrhinum* spp.	Horticultural	Herb		0.27		0.02	0.01	0.01					0.00	**0.02**
Magnoliaceae	*Magnolia* spp.	Horticultural	Tree							0.06	0.04	0.07			**0.02**
Oleaceae	*Ligustrum* spp.	Native and near-native	Shrub		0.01		0.00	0.02	0.07		0.02	0.00	0.01	0.01	**0.02**
Polygonaceae	*Fagopyrum/Rheum/ Rumex* spp.	Native and near-native	Herb		0.00	0.05	0.05	0.07				0.00	0.00	0.00	**0.02**
Fabaceae	*Wisteria* spp.	Horticultural	Shrub		0.29	0.00									**0.02**
Malvaceae	*Tilia* spp.	Native and near-native	Tree										0.11	0.01	**0.02**
Nymphaeaceae	*Nymphaea* spp.	Native and near-native	Herb					0.17							**0.01**
Pinaceae	*Pinus* spp.	Native and near-native	Tree		0.09						0.06	0.00			**0.01**
Asteraceae	*Leucanthemum vulgare*	Native and near-native	Herb			0.19	0.00					0.00	0.00	0.00	**0.01**
Hydrangeaceae	*Hydrangea* spp.	Horticultural	Shrub			0.00		0.00						0.08	**0.01**
Commelinaceae	*Tradescantia* spp.	Horticultural	Herb										0.07		**0.01**
Cistaceae	*Cistus/ Helianthemum* spp.	Horticultural	Shrub		0.12										**0.01**
Rosaceae	*Sanguisorba* spp.	Native and near-native	Herb				0.04	0.01	0.01						**0.01**
Poaceae	*Dactylis glomerata*	Native and near-native	Herb			0.00	0.03					0.03	0.00		**0.01**
Crassulaceae	*Sedum* spp.	Native and near-native	Herb						0.00			0.07			**0.01**

Brassicaceae	*Isatis tinctoria*	Native and near-native	Herb		0.09	0.01						0.00			**0.01**
Asteraceae	*Tanacetum* spp.	Native and near-native	Herb											0.04	**0.00**
Asteraceae	*Artemisia* spp.	Native and near-native	Herb					0.02	0.00					0.02	**0.00**
Apiaceae	*Anthriscus sylvestris*	Native and near-native	Herb		0.01	0.02				0.00	0.03	0.00			**0.00**
Betulaceae	*Betula* spp.	Native and near-native	Tree						0.00	0.01	0.01	0.01		0.01	**0.00**
Boraginaceae	*Brunnera* spp.	Horticultural	Herb	0.08	0.00		0.00			0.01	0.00	0.01			**0.00**
Rosaceae	*Potentilla* spp.	Native and near-native	Shrub					0.04		0.00					**0.00**
Fabaceae	*Lotus* spp.	Native and near-native	Herb				0.02	0.01	0.00				0.00		**0.00**
Berberidaceae	*Berberis* spp.	Native and near-native	Shrub							0.03	0.00				**0.00**
Myrtaceae	*Eucalyptus/ Myrtus* spp.	Horticultural	Tree					0.00	0.02			0.00	0.00		**0.00**
Geraniaceae	*Geranium* spp.	Native and near-native	Herb										0.02		**0.00**
Plantaginaceae	*Cymbalaria muralis*	Naturalised	Herb		0.02	0.00					0.01	0.00	0.00		**0.00**
Brassicaceae	*Lunaria* spp.	Horticultural	Herb							0.02	0.00				**0.00**
Rosaceae	*Fragaria* spp.	Native and near-native	Herb					0.03							**0.00**
Euphorbiaceae	*Mercurialis perennis*	Native and near-native	Herb							0.02	0.00				**0.00**
Caryophyllaceae	*Silene* spp.	Native and near-native	Herb									0.02	0.00		**0.00**
Urticaceae	*Urtica* spp.	Native and near-native	Herb										0.01	0.00	**0.00**
Caryophyllaceae	*Cerastium/Stellaria* spp.	Native and near-native	Herb							0.00	0.02	0.00			**0.00**

Chenopodiaceae	*Atriplex* spp.	Native and near-native	Herb					0.02	0.00						**0.00**
Rosaceae	*Kerria japonica*	Horticultural	Shrub							0.00	0.00	0.01			**0.00**
Boraginaceae	*Trachystemon* spp.	Horticultural	Herb							0.01					**0.00**
Winteraceae	*Drimys* spp.	Horticultural	Tree							0.01					**0.00**
Plantaginaceae	*Digitalis* spp.	Native and near-native	Herb										0.01	0.00	**0.00**
Rosaceae	*Geum* spp.	Native and near-native	Herb		0.01	0.02	0.00				0.00				**0.00**
Asteraceae	*Echinacea* spp.	Horticultural	Herb					0.01	0.00				0.00		**0.00**
Asphodelaceae	*Hemerocallis* spp.	Horticultural	Herb										0.00		**0.00**
Theaceae	*Camellia* spp.	Horticultural	Shrub		0.00	0.01									**0.00**
Asteraceae	*Solidago* spp.	Native and near-native	Herb					0.01				0.00	0.00		**0.00**
Asteraceae	*Senecio* spp.	Native and near-native	Herb					0.00						0.00	**0.00**
Platanaceae	*Platanus* spp.	Horticultural	Tree							0.00		0.00			**0.00**
Brassicaceae	*Erysimum* spp.	Native and near-native	Herb			0.01									**0.00**
Ranunculaceae	*Aquilegia* spp.	Native and near-native	Herb					0.00				0.00	0.00		**0.00**
Brassicaceae	*Aubrieta* spp.	Horticultural	Herb		0.00	0.00					0.00				**0.00**
Asteraceae	*Dahlia* spp.	Horticultural	Herb					0.00						0.00	**0.00**
Brassicaceae	*Lobularia* spp.	Horticultural	Herb			0.00									**0.00**
Asteraceae	*Rudbeckia* spp.	Horticultural	Herb				0.00								**0.00**
Lardizabalaceae	Lardizabalaceae	Not categorised	Not categorised							0.00					**0.00**
Asparagaceae	*Cordyline* spp.	Horticultural	Herb								0.00				**0.00**
Lamiaceae	*Glechoma hederacea*	Native and near-native	Herb							0.00					**0.00**

Brassicaceae	*Arabis* spp.	Native and near-native	Herb							0.00	0.00				**0.00**
Ericaceae	*Enkianthus* spp.	Horticultural	Shrub								0.00				**0.00**
Boraginaceae	*Myosotis* spp.	Native and near-native	Herb		0.00					0.00	0.00	0.00	0.00		**0.00**
Phrymaceae	*Mimulus* spp.	Horticultural	Herb			0.00	0.00						0.00	0.00	**0.00**
Ranunculaceae	*Actaea* spp.	Horticultural	Herb						0.00						**0.00**
Apiaceae	*Astrantia* spp.	Horticultural	Herb										0.00		**0.00**
Asteraceae	*Eupatorium* spp.	Horticultural	Herb											0.00	**0.00**
Asteraceae	*Ligularia* spp.	Horticultural	Herb											0.00	**0.00**
Caprifoliaceae	*Lonicera* spp.	Native and near-native	Herb					0.00							**0.00**
Fabaceae	*Cytisus* spp.	Native and near-native	Shrub		0.00						0.00				**0.00**
Fagaceae	*Castanea sativa*	Native and near-native	Tree											0.00	**0.00**
Plantaginaceae	*Penstemon* spp.	Horticultural	Herb					0.00					0.00	0.00	**0.00**
Polemoniaceae	*Phlox* spp.	Horticultural	Herb							0.00					**0.00**
Rhamnaceae	*Frangula alnus/ Rhamnus cathartica*	Native and near-native	Shrub										0.00		**0.00**
Apiaceae	*Eryngium* spp.	Native and near-native	Shrub										0.00		**0.00**
Asteraceae	*Arctium* spp.	Native and near-native	Herb											0.00	**0.00**
Asteraceae	*Cosmos* spp.	Horticultural	Herb						0.00						**0.00**
Papaveraceae	*Eschscholzia californica*	Horticultural	Herb							0.00					**0.00**
Rubiaceae	*Galium* spp.	Native and near-native	Herb									0.00			**0.00**
Scrophulariaceae	*Verbascum* spp.	Native and near-native	Herb					0.00							**0.00**

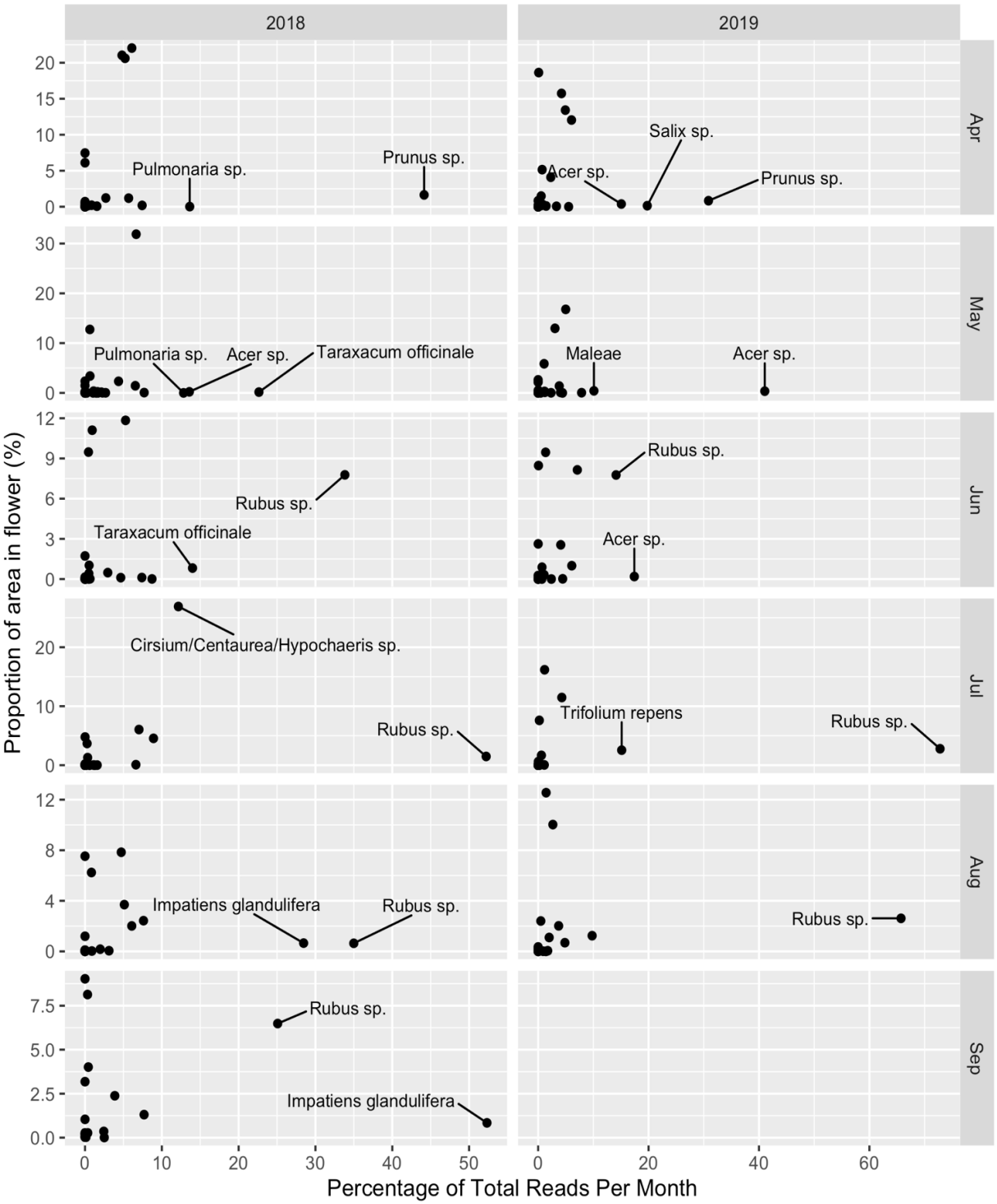

Figure S5.3: Relationship between the proportion of total sequence reads per month and the proportion of area in flower for each plant taxa contributing to over 1% of sequence reads in any one month. Four plant taxa were removed from this analysis (*Citrus* spp., *Crocus* spp., *Hedera helix, Populus* spp.) due to contributing >1% of sequence reads only when outside their flowering period. A strong relationship was found in July 2019 (Rs = 0.68, P = 0.002). No significant relationship was found in remaining months. 2018: April (Rs = 0.46, P = 0.033), May (Rs = 0.29, P = 0.342), Jun (Rs = 0.46, P = 0.127), July (Rs = 0.52, P = 0.074), August (Rs = 0.56, P = 0.058), September (Rs = 0.25, P = 0.574). 2019: April (Rs = 0.51, P = 0.058), May (Rs = 0.21, P = 0.574), Jun (Rs = 0.45, P = 0.127), August (Rs = 0.56, P = 0.054).

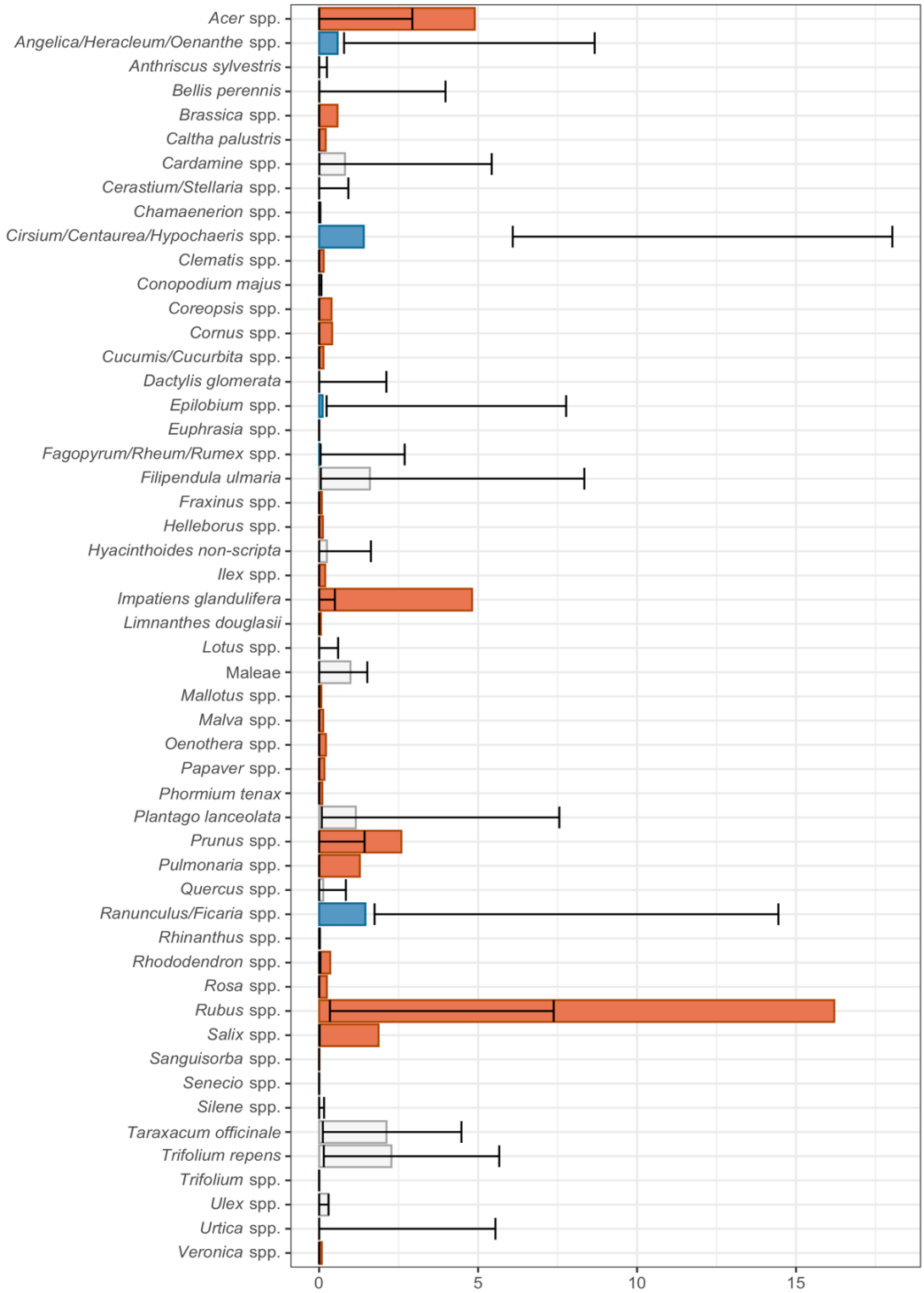

Figure S5.4: Dietary preference plot for the honeybee, *Apis mellifera*, identified by comparing observed usage (bars) with expected usage (horizontal lines of 95% confidence interval), calculated by a null model based on relative abundance of plants within the landscape. Plant taxa which were found at over 1% of sequence reads within honey samples or over 1% of flowering area in any month were included in the null model. Blue bars denote plant taxa which were used less than predicted given the null model, white bars represent taxa which were used as predicted and orange circles are those which were used more frequently than predicted.

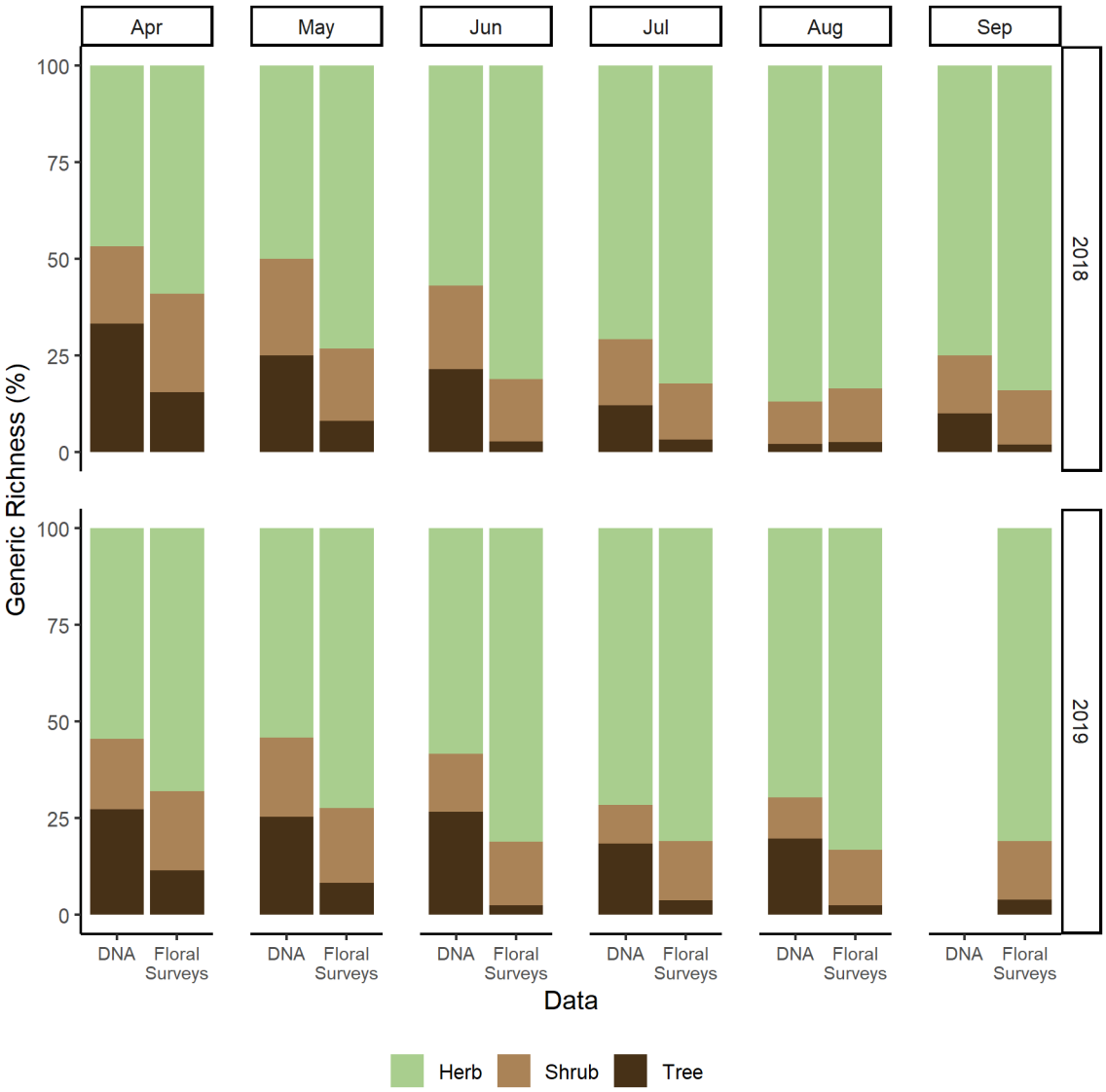

Figure S5.5: Generic richness of each plant form in honey (DNA) compared to the generic richness in flower (Floral Surveys) each month for 2018 and 2019, both calculated as a proportion. Using Fisher's exact test, a significant difference was found in May (2018: P = 0.009, 2019: 0.021), June (2018: P < 0.001, 2019: P < 0.001), July 2019 (P = 0.031), August 2019 (P = 0.001). No significant difference was found in April (2018: P = 0.111, 2019: P = 0.111), July 2018 (P = 0.112), August 2018 (P = 0.702), September 2018 (P = 0.112).

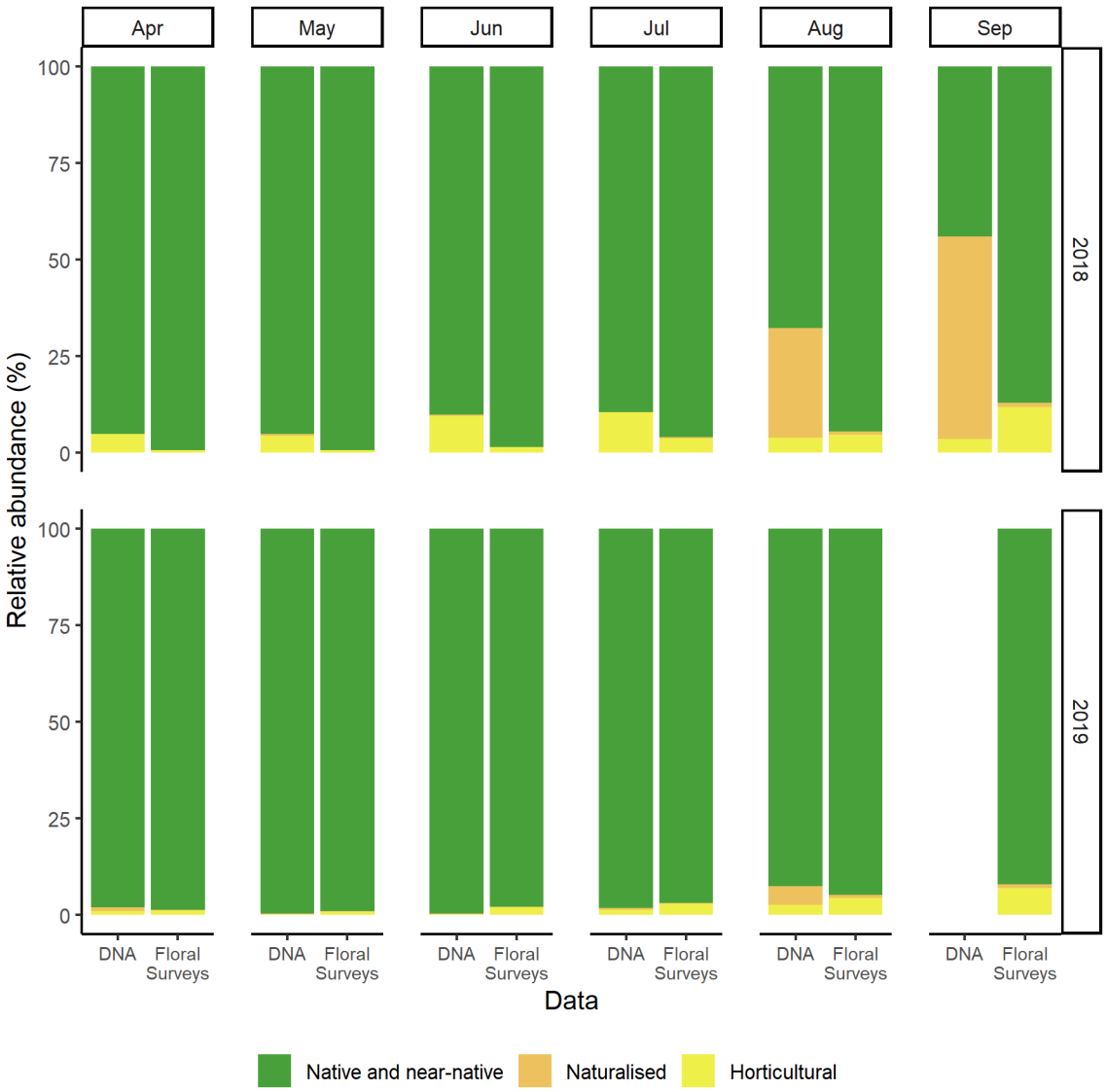

Figure S5.6: Relative abundance of each plant status in honey (DNA) compared to the relative abundance in flower (Floral Surveys) each month for 2018 and 2019. Using Fisher's exact test, a significant difference was found in August 2018 ($P < 0.001$) and September 2018 ($P < 0.001$). No significant difference was found in remaining months. 2018: April ($P = 1.00$), May ($P = 1.00$), June ($P = 0.297$), July ($P = 1.00$). 2019: April ($P = 1.00$), May ($P = 1.00$), June ($P = 1.00$), July ($P = 1.00$), August ($P = 1.00$).

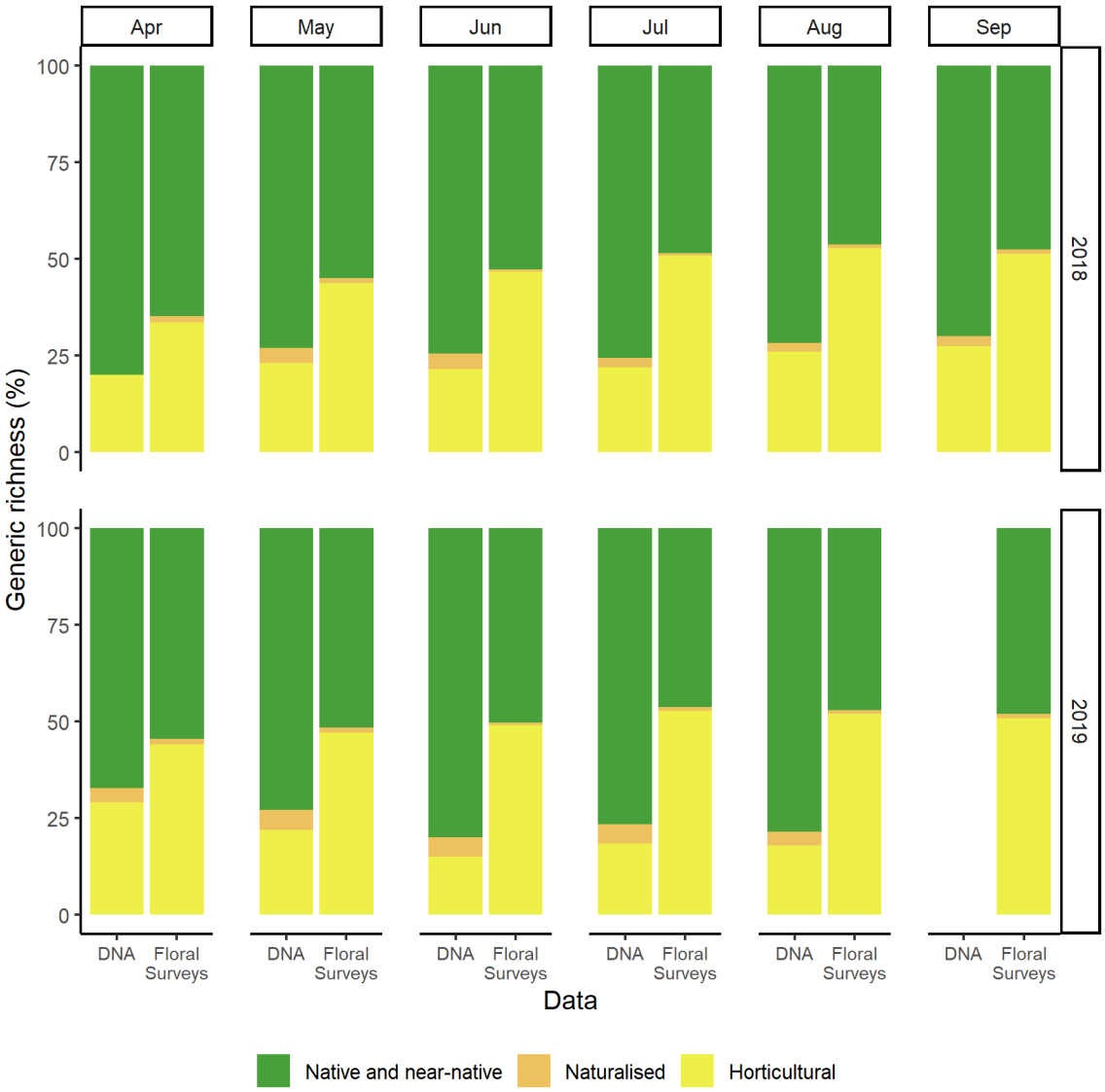

Figure S5.7: Generic richness of each plant status in honey (DNA) compared to the generic richness in flower (Floral Surveys) each month for 2018 and 2019, both calculated as a proportion. Using Fisher's exact test, a significant difference was found in April 2018 (P = 0.036), May 2018 (P = 0.010), June 2018 (P = 0.003), July 2018 (P < 0.001), August 2018 (P = 0.002), September 2018 (P = 0.008), May 2019 (P = 0.002), June 2019 (P < 0.001), July 2019 (P < 0.001), August 2019 (P < 0.001). No significant difference was found in April 2019 (P = 0.081).

Chapter Six

Synthesis

6.1. Introduction

The overall aim of this thesis was to extend our understanding of the relationships between pollinators and plants by using a DNA metabarcoding approach to increase the spatiotemporal scope of current knowledge. The thesis began with a review of the use of DNA metabarcoding for identifying floral visitation, thoroughly outlining the methodological steps required, and describing the range of ecological questions which may be answered. The following three data chapters used DNA metabarcoding to explore which plants are used by pollinators, how they are partitioned between species and individuals, and whether periods of resource shortage may be identified in a diverse landscape.

6.2. Summary of main findings

6.2.1. Chapter Two: Using DNA metabarcoding to identify floral visitation by pollinators

Chapter Two encompasses a review of the use of pollen DNA metabarcoding to identify floral visitation by pollinators. A description of the major ways in which floral resource use may be identified is provided, along with a comparison of molecular and non-molecular techniques. The current literature is found to be separated into four major ecological questions surrounding floral visitation, and the research belonging to each of these questions are described according to their methodological approaches and overarching results in an effort to build an understanding of the current knowledge base. A detailed methodological workflow is provided for researchers hoping to use pollen DNA metabarcoding techniques, with key considerations and guidance at each step. Finally, the future of DNA metabarcoding and related techniques for identifying floral visitation are discussed whilst emphasising the need for standardised approaches.

6.2.2. Chapter Three: Gardening for pollinators: DNA metabarcoding shows seasonal progression and differences in major floral resource use in bees and hoverflies

The aim of Chapter Three was to use pollen DNA metabarcoding to identify the floral resources visited by bumblebees, honeybees, non-corbiculate bees, and hoverflies throughout the season, and to use these data to investigate both dietary differences between pollinator groups and phenological changes in forage. It was revealed that although many resources were utilised by both bees and hoverflies, there were key differences in the major resources used, likely driven by morphological traits separating taxonomic orders. Within pollinator groups, ecological functional traits such as tongue length and larval habitat were found to influence the foraging preferences of species, highlighting the need to ensure resources for a range of species with fundamental ecological differences. Native and near-native plants were found to be essential for supporting pollinators throughout the year, but horticultural plants played a key role in extending the flowering season and improving resource availability. The strong seasonal changes in resource use emphasises the need for "plants for pollinators" lists to provide recommendations throughout the year and affirms the need to include native and horticultural species to support pollinators appropriately. The results of this chapter are synthesised to create an evidence-based recommendation list for gardeners, landowners, and conservation organisations which specify which plants can be provided during spring, summer, and autumn to suitably support pollinator populations and ensure their effective conservation.

6.2.3. Chapter Four: Short-term specialisation of individuals within a plant-pollinator network revealed by DNA metabarcoding

Chapter Four investigates how resources are partitioned within an insect community at varying hierarchical levels, from the community to the individual.

Two types of specialisation were investigated at each level: niche breadth specialisation (i.e. how many resources are utilised) and niche partitioning specialisation (i.e. how resource use compares to that of others at the same hierarchical level). At the order and group level, pollinators utilised numerous plant taxa, most of which were shared. Most species were polylectic and although some differences were found in the diets of species within the same group, few interactions were unique, revealing that species were generalised in their resource use. High incidences of specialisation were found within individuals, with many exhibiting floral fidelity and distinct diets in comparison to those within the same species. This work provides further evidence that generalised pollinator species are themselves composed of individuals displaying short-term specialisation. As incidences of specialisation and generalisation are likely to affect individuals and species' resilience towards environmental change, we highlight the ability of DNA metabarcoding to provide fine-scale information on an entire foraging trip to better understand ecological networks.

6.2.4. Chapter Five: Seasonal specialisation in floral resource use by honeybee colonies reveal periods of food shortage in a diverse agricultural and horticultural habitat

In Chapter Five, honey DNA metabarcoding was used to identify seasonal floral resource use by the European honeybee, *Apis mellifera,* utilising network metrics to understand how resources are partitioned between colonies throughout the year. Honey sampling and floral surveys were carried out monthly to quantify the availability of floral resources in the landscape and used to identify which plants were used more or less than expected given their abundance. Plant taxa were categorised according to their growth form and native status to further understand the drivers behind the seasonal selection of resources. Honeybees were found to be generalised in their selection of resources throughout the season, however diet

specialisation increased when assessing the plants used each month, with few taxa contributing a notable proportion of the diet. Whilst floral resource availability was plentiful throughout the year, the patterns of resource partitioning revealed between honeybee colonies suggest that there are periods of resource shortage in June and August causing opportunistic foraging. Plant use by honeybees was not found to be correlated with their relative abundance in the landscape, with most plants used more than expected. The patterns of selection of trees, shrubs and herbs suggest that seasonal shortages in food are driven by a resource gap between spring flowering trees and summer shrubs and herbs. Identifying periods of resource limitation for honeybees has implications for wild pollinators, particularly those which are not able to store resources as they are expected to be more vulnerable to food shortages. Plants for pollinators lists should prioritise resources which can be used to supplement these periods to ensure pollinator populations are supported throughout the season and to minimise competition between species.

6.3. How has the use of DNA metabarcoding advanced the field of pollinator ecology?

This work aimed to extend the understanding of the relationship between pollinators and plants by using a DNA metabarcoding approach to increase the spatiotemporal scope of current knowledge. We illustrate that DNA metabarcoding is a powerful tool that can be used to confirm information derived from observational methods, as well as increase the depth of existing information. In this thesis, plant use is explored within four broad pollinator groups (honeybees, bumblebees, non-corbiculate bees and hoverflies) throughout the year to provide empirical data for the creation of recommendation lists, and to further

understand resource partitioning between individuals and species in multifaceted plant-pollinator networks.

Prior to this work, there has been a substantial interest in identifying the floral resources used by honeybees using DNA metabarcoding due to their cultural and economic importance (Danner et al., 2017; de Vere et al., 2017; Richardson et al., 2015). The results from chapters three and five complement previous studies on honeybee forage plants, with strong similarities found in the most frequently used resources in the UK and beyond (Jones et al., 2021; Richardson et al., 2019). Although floral resource use by bumblebees is well-studied, the knowledge gained by identifying the entire foraging trip of bumblebee individuals in chapters three and four improves the accuracies of future recommendations. The substantial lack of research into floral use by hoverflies results in this work also provides a significant contribution to the understanding of foraging behaviour across this diverse family of pollinators. Although regrettably, sample sizes of non-corbiculate bees were low throughout this study due to the difficulties in sampling, we provide promising insights into the foraging behaviour of some lesser studied species and encourage further work to target the diversity of species outside honeybees and bumblebees to make valuable recommendations for conservation (Gresty et al., 2018). Further, DNA metabarcoding can be used to assess the effectiveness of seed mixes or wildflower strips aimed at supporting pollinator populations in a bid to improve their effectiveness (McMinn-Sauder et al., 2020; Witter, 2021).

The relatively recent advances in knowledge that pollen metabarcoding has provided the field of pollinator ecology has many applications for future work. It is imperative that we use the information gained not only to understand ecological interactions now, but how these may be shaped in the future, by using data to predict the consequences of future global change (Bell et al., in prep). For example,

recent advances in sequencing historic pollen specimens have highlighted an exciting opportunity to explore resource use outside the realms of the present day (Gous et al., 2019). By exploring past and present resource use in declining species, the mechanisms of decline may be further explored (Simanonok et al., 2021).

In addition, revealing specialisation across pollinator groups, species and individuals is beginning to transform our understanding of the structure of these complex networks and their vulnerability to ecological change (Evans et al., 2016). Incidences of short-term specialisation were identified in chapter four, supporting the growing understanding that generalised networks are themselves composed of specialised individuals (Klečka et al., 2021; Lucas et al., 2018a, 2018b; Pornon et al., 2019). As the removal of generalists affects the structure of a network (Brosi & Briggs, 2013), it is vital that we understand resource use by individuals to predict outcomes of species loss (Memmott et al., 2004).

Although pollen metabarcoding has advanced the field of pollinator ecology in recent years, there are still many research gaps which can be addressed using DNA metabarcoding and related techniques. At present, most studies of floral visitation by pollinators using DNA metabarcoding have been situated in Europe and North America (Chapter Two; Lowe et al., 2022). Whilst similarities in foraging can be found in species with large geographical ranges (Jones et al., 2021; Richardson et al., 2019), it would be interesting to investigate whether similar patterns of behaviour, particularly specialisation, are comparable across wide geographical scales and different ecological environments.

Much of the discussion within this thesis relates to the ability of DNA metabarcoding to improve the spatiotemporal scale of ecological questions related to pollinator foraging. However, whilst a large proportion of studies explore seasonal differences in forage, less attention is given to changes over short

temporal scales such as throughout a day. Foragers may exhibit short temporal preferences in nectar or pollen foraging (Fahimee et al., 2021); therefore, by further investigating resource use in comparison to time collected, a greater depth of information surrounding pollinator behaviour could be achieved.

In chapter five, honey metabarcoding revealed that each colony of honeybees makes distinct foraging decisions, however, key resources are shared across colonies. As bumblebees are also social foragers and are known to adapt their foraging based on the needs of the colony (Goulson et al., 2002), an interesting question is how colony membership is related to floral use (Saifuddin & Jha, 2014). Difficulties in locating nests (Kells & Goulson, 2003) or using pollen traps (Judd et al., 2020) mean that these colony-level differences are less explored in bumblebees than honeybees. However, if family relationships and colony membership are identified through molecular methods (Saifuddin & Jha, 2014), there is great potential for this to be combined with DNA metabarcoding to better understand how colonies co-exist in a landscape.

DNA metabarcoding provides great potential for further large-scale analyses of floral resource use across a wide range of taxonomic groups. At present, research is limited to a very small subset of all flower-visitors and has been particularly focussed on bees (Chapter Two; Lowe et al., 2022). As there are thought to be approximately 6000 species of floral visitors in the UK alone (Steven Falk, pers. communication), there remains a large research gap regarding unexplored interactions between insects and plants. Whilst the pollen metabarcoding workflow is not yet standardised, we encourage that the review provided in chapter two and recent reviews in various steps of the process (Swenson & Gemeinholzer, 2021; Tommasi et al., 2021) provides a beginning point to standardisation so that researchers are able to compare results between studies.

This will allow easy adoption of techniques to a range of insect species and groups across a diversity of habitats.

Lastly, we have outlined that the future of exploring pollinator foraging through molecular ecology will ultimately reside in the study of entire genomes which hold a greater source of information. However, routine large-scale use of entire genomic material is still not widely accessible and so DNA metabarcoding currently poses the most appropriate, accessible, and cost-effective tool to use to identify floral visitation by pollinators to yield valuable information on these important species interactions.

6.4. How can metabarcoding advance the broad field of ecology?

As well as transforming the field of pollinator ecology, DNA metabarcoding has a range of applications to advance the wider field of ecology (Deiner et al., 2017; Ruppert et al., 2019). As with the study of plant-pollinator interactions, the ability to greatly expand the spatiotemporal scope of biodiversity knowledge is a key benefit of the utility of DNA metabarcoding. Thus, these tools provide a huge potential for ecosystem monitoring for ecologists. Biodiversity within air, soil, and water can be monitored, where morphological approaches are limited (Bohmann et al., 2014; Deiner et al., 2017). In addition, the ability to identify taxa with limited morphological differences allows an acceleration of the description of global biodiversity (Hebert et al., 2004; Sheffield et al., 2019). Whilst the information gained from metabarcoding can be comparable to that achieved by traditional techniques, it is the scale, speed and resolution of the resultant information which makes these techniques particularly valuable (Deiner et al., 2017).

Moving forward, DNA metabarcoding can be scaled up further to assess biodiversity across entire ecosystems by using bulk community samples across huge spatiotemporal scales (Chain et al., 2016). One way this is currently being

achieved is through the BIOSCAN initiative and its regional extensions, which aim to use DNA metabarcoding of bulk invertebrate samples across the world to not only capture biodiversity but to describe entire symbiomes by using multiple DNA markers, revealing species interactions on a scale incomparable to any other (Arribas et al., 2021; Hobern & Hebert, 2019).

Lastly, comparing robust species assessments with past communities and using these to predict the impact of future changes in ecosystems due to climate change is fundamental for biodiversity conservation (Anderson-Carpenter et al., 2011). The identification of ancient DNA can be used as a valuable measure of previous ecological communities, providing a historical baseline which can be used to understand how communities have changed through time (Wilmshurst et al., 2014). In addition, metabarcoding can be used to identify diet preferences of extinct species which provides information about their role in past ecosystems (Polling et al., 2021) and has further application for monitoring and detection of future invasive species (Westfall et al., 2020).

Whilst there are still unresolved issues surrounding how quantitative DNA metabarcoding is (Chapter Two; Lowe et al., 2022), this technique remains an exciting tool to explore biodiversity with limitless opportunities surrounding terrestrial, marine, freshwater, and estuarine monitoring (Ruppert et al., 2019). Advances in molecular methods may identify more suitable techniques for particular studies, however, due to limitations in cost and the length of time required for techniques to become routine, it is expected that DNA metabarcoding will remain a standard technique for biodiversity monitoring and species assessment for the immediate future.

6.5. Conclusions

This thesis aimed to extend the understanding of the relationship between pollinators and plants by using a DNA metabarcoding approach to increase the spatiotemporal scope of current knowledge. We provide clear evidence for the utility of DNA metabarcoding to advance our knowledge of the intricate relationships between plants and pollinators, by reviewing the information which has been gained so far and further extending our knowledge by contributing to key research gaps. The plants used most frequently by honeybees, bumblebees, non-corbiculate bees, and hoverflies throughout the year are identified and this information is used to create the first evidence-based recommendation list to support a diversity of pollinator species throughout the year. This list may be used by gardeners, landowners, and conservation organisations. The identification of short-term specialisation of floral resources within a complex plant-pollinator network highlights the need to study individual interactions in order to detect specialism at higher resolution. In addition, the identification of periods of resource shortage in a diverse habitat demonstrates the need to further study whether other pollinators are also limited by resource availability, and the need to ensure there is an abundance of the most favoured resources throughout the year, which can be achieved through supplemental planting.

6.6. References

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Appendix 1: Shifts in honeybee foraging reveal historical changes in floral resources

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