School of Molecular and Life Sciences
Faculty of Science and Engineering

Environmental DNA (eDNA) metabarcoding for detecting arthropod pollinators, pests and parasites of a horticultural species

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This thesis is presented for the Degree of
Doctor of Philosophy
Of
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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for any other degree or diploma in any university.

SIGNED | Joshua Kestel

DATED | 14th November 2023

Abstract

Global food production is increasingly threatened as the ecological services that underpin crop yields are affected by an array of biotic and abiotic stressors. Beneficial (e.g. pollinators and predators) and antagonistic arthropods (e.g. pests and pathogens) are an important biotic component of agroecosystems, although monitoring these arthropod communities using conventional methods has often been difficult in large scale agriculture. This thesis explores environmental DNA (eDNA) metabarcoding, as a survey tool to detect ecologically significant arthropods and the resources upon which they rely in agroecosystems.

Environmental DNA metabarcoding is capable of amplifying trace deposits of DNA (e.g. hair, saliva, scales, pollen etc.) from a wide variety of substrates ranging from soil to air. The first stage of this thesis (Chapter 3) compares eDNA metabarcoding of *Persea americana* ('Hass' avocado) flowers with two conventional methods used in agriculture, digital video recording (DVR) devices and pan traps, to contrast the arthropod diversity measures generated. Here, eDNA metabarcoding produced similar diversity measures to conventional methods, although each method identified unique arthropod assemblages. To capture the widest breadth of arthropod diversity, eDNA-based surveys should be complemented with conventional monitoring methods. These results were then applied to a baseline study, where I combined eDNA metabarcoding of *P. americana* flowers with DVRs to assess the temporal and spatial shifts in beneficial and antagonistic arthropods in two orchards in south west Western Australia (Chapter 4). Here, arthropod pollinators, pests and predators were shown to increase in diversity and abundance with greater *P. americana* flowering, while between the study orchards, arthropod community similarity increased during peak *P. americana* flowering. At a fine spatial-scale, the relative abundance of some arthropod groups (e.g. Hymenoptera) were found to vary within individual trees, likely in response to the distribution of flowers within the canopies.

Diverse foraging resources (i.e. pollen, nectar and plant tissue) can increase the presence and persistence of arthropods within agroecosystems, making them important indicators of ecosystem health. To identify managed (e.g. European honey bee) and unmanaged (e.g. wild bees, flies and moths) arthropods, as well as the foraging resources upon which they rely, I used a conventional survey tool (pan traps) in a novel way, by metabarcoding pan-trap water (Chapter 5). In doing so, I aimed to minimise the contribution from gut contents of captured arthropods, while keeping whole specimens for abundance data. With this approach, plant taxa were detected in all of the pan trap samples, the most commonly detected plant families were all known to require arthropods to facilitate or enhance fruit set. Unfortunately, arthropod taxa were not consistently detected from

pan-trap water, likely due to the arthropod exoskeletons preventing the exchange of DNA with the water substrate. Given the results from Chapter 5, pan-trap water metabarcoding was complemented with morphological identifications of arthropods to evaluate if the presence of adjacent natural vegetation positively affects the diversity of orchard arthropod communities and the foraging resources upon which they rely.

Adjacent natural vegetation can provide habitat and foraging resources that may encourage the persistence of arthropods within agroecosystems during crop flowering and may ultimately contribute to greater crop yields. To explore this, in the final data chapter (Chapter 6), six orchards were sampled, adjacent to either to pasture or natural vegetation, at four *P. americana* flowering intensities. With this approach, I wanted to determine if the presence of adjacent natural vegetation affected arthropod community diversity and enhanced arthropod-plant foraging during the *P. americana* flowering season. Within orchards, the presence of adjacent natural vegetation only affected arthropod community composition and did not enhance species richness or abundance. For the plant detections however, I found that at low *P. americana* flowering, pan-trap water samples in orchards adjacent to natural vegetation, contained significantly greater arthropod-plant foraging diversity, compared to orchards adjacent to pasture. These findings indicate that, at certain periods, natural vegetation may increase the resilience of arthropod communities in agroecosystems by providing more consistent sources of nectar, pollen and nesting materials, compared to pasture habitats.

The studies presented herein illustrate how eDNA metabarcoding can be used to improve monitoring of arthropod-plant interactions in agroecosystems; by identifying entire arthropod communities, detecting beneficial and antagonistic arthropod species, as well as the foraging resources and habitat preferences they rely upon. With further methodological refinement, eDNA-based surveys will form a strong complement to current arthropod monitoring approaches in agriculture and may help guide management decisions that support biodiversity, whilst informing more effective control of pests and pathogens.

Acknowledgements

"*We can forgive a man for making a useful thing as long as he does not admire it. The only excuse for making a useless thing is that one admires it intensely. All art is quite useless*."

– Oscar Wilde

From the cataclysmic depths of the SARS-CoV-2 pandemic, it was beyond the realms of my imagination that three and a half years later I would be nearing the end of my PhD, let alone acquiring such a degree of knowledge about avocados than I ever knew was possible. This PhD has been an extraordinary leap into the world of agriculture, something which I knew very little about when I started, and it has required the support from a village of people to help me arrive at this point. These remarkable people are my collaborators, supervisors, colleagues, friends and loved ones.

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Statement of Contributions

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Chapter 3: Kestel, J.K., Bateman, P.W., Field, D.L., White, N.E., Lines, R., & Nevill, P. eDNA metabarcoding of avocado flowers: 'Hass' it got potential to survey arthropods in food production systems? *Molecular Ecology Resources*, 23(7), 1473-1755. doi:10.1111/1755-0998.13814

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Chapter 4: Kestel, J.K., Bateman, P.W., Field, D.L., White, N.E., & Nevill, P. Spatio-temporal variation in arthropod-plant interactions identified using eDNA and digital video recordings. *Agriculture, Ecosystems & Environment*, (In review).

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Chapter 5: Kestel, J.K., Field, D.L., Bateman, P.W., White, N.E., Bell, K.L., & Nevill, P. (2024). Environmental DNA metabarcoding of pan-trap water to monitor arthropod-plant interactions, *Environmental DNA.* 6, e527. doi:10.1002/edn3.527

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Chapter 6: Kestel, J.K., Bateman, P.W., Field, D.L., White, N.E., & Nevill, P. Come Together: Adjacent natural vegetation affects arthropod community composition and alters arthropod-plant foraging diversity in 'Hass' avocado orchards, *Agriculture, Ecosystems & Environment,* (In preparation).

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Abbreviations

ANOVA	Analysis of variance
ASV	Amplicon sequence variant
ATL	Tissue lysis buffer
AUD	Australian Dollar
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Database
bp	Base pairs
BSA	Bovine Serum Albumin
COI	Mitochondrial cytochrome oxidase subunit I
Ct	Fluorescence threshold
DAWE	Department of Agriculture, Water and the Environment
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DPIRD	Department of Primary Industries and Regional Development
DVR	Digital Video Recording
eDNA	Environmental DNA
FAO	Food and Agriculture Organisation
HTS	High-throughput Sequencing
MAGs	Metagenome-Assembled Genomes
MEGAN	Metagenome Analyser
$MgCl_2$	Magnesium Chloride
MID-tag	Multiplex identifier tag
mtDNA	Mitochondrial DNA
NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencing
nMDS	Non-metric multidimensional scaling
OTU	Operational Taxonomic Unit
PCoA	Principal coordinates analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational analysis of variance
qPCR	Quantitative PCR

rbcL	Ribulose-1,5-bisphosphate carboxylase-oxygenase
RT-PCR	Real-time PCR
SE	Standard Error
SIMPER	Similarity percentage analysis
SWCC	South West Catchments Council
SWWA	South West Western Australia
UN	United Nations
TrEnD	Trace and Environmental DNA Laboratory
trnL	*trnL* (UAA) intron region
USD	United States Dollar
UV	Ultra violet
WA	Western Australia
ZOTU	Zero-radius operational taxonomic unit

Chapter 1. General Introduction

1.1 Introduction

Global arthropod biomass, abundance and diversity is under threat from a mixture of biotic and abiotic stressors (Wagner, 2020; Wagner, Grames, Forister, Berenbaum, & Stopak, 2021). These threats are varied, but some of the most significant include climate change (e.g. drought), habitat loss (e.g. agricultural intensification), pollution (e.g. pesticides), as well as the spread of invasive pest species (e.g. *Varroa* mite) (Potts, Imperatriz-Fonseca, & Ngo, 2016; Wagner, 2020; Wagner et al., 2021). Arthropods are intrinsic to the health of both natural (e.g. native habitats) and cultivated ecosystems (e.g. agricultural areas) through the delivery of beneficial ecosystem services (e.g. pollination and arthropod predation), as well as herbivory and pathogen transmission (Potts et al., 2016; Skendži´c et al., 2021; Tscharntke et al., 2012). In Australia, agriculture is the dominant land use for approximately half of the country's landmass (Australian Bureau of Statistics, 2021). Unfortunately, the practices associated with agriculture often harm arthropod biodiversity and reduce the delivery of beneficial ecosystem services where they are needed most (Benton, Vickery, & Wilson, 2003; Cresswell, Janke, & Johnston, 2021; Wagner, 2020). Despite these circumstances, efforts to incorporate regular arthropod monitoring into agriculture have remained the exception, rather than the rule.

Surveys and monitoring of arthropods have largely been omitted from agricultural practices and farm management decisions (Martínez-Sastre, García, Miñarro, & Martín-López, 2020; Rader et al., 2016). Arthropods are, however, critical for delivering pollination services that support or enhance fruit and seed set for approximately three quarters of all crop species (Klein et al., 2007; Kremen, 2018). Additionally, these taxa can improve crop production by removing crop pests and reducing pathogen transmission, services which have been valued at US$4.49 billion annually in the United States alone (Furlong, 2015; Losey & Vaughan, 2006). At the other extreme, one-tenth of all agricultural pests have spread to more than half of the countries that grow their host crops (Bebber, Holmes, & Gurr, 2014) and these antagonistic species continue to cause annual crop losses between 20 – 40% (Flood, 2010). Despite an increasing awareness in recent years about the need for greater monitoring efforts of these beneficial and antagonistic taxa, arthropods are often managed without *a posteriori* community data (Kestel et al., 2022; Potts et al., 2016). Pollination services are increased by hiring more honey bee hives (Garratt, Brown, Hartfield, Hart, & Potts, 2018; Klein et al., 2007), crop pests are reduced with generalised/prophylactic pesticide applications (Atwood & Paisley-Jones, 2017; Leskey, Lee, Short, & Wright, 2012) and arthropod predators remain largely unknown (Furlong, 2015; Martínez-Sastre et al., 2020). Furthermore, among the existing literature measuring arthropods in agroecosystems, there is bias towards managed honey bees (*Apis mellifera*) and significant knowledge gaps for unmanaged taxa (e.g.

native bees, moths, flies and wasps) (Kestel et al., 2022; Macgregor et al., 2019; Rader et al., 2016). Indeed, Rader et al. (2016) identified that the pollination services delivered by unmanaged non-bee taxa (e.g. flies, beetles, moths, and butterflies) may be like those delivered by managed honey bees and that these taxa may be more robust to changes in land use, compared to honey bees. Surveys for arthropods are therefore a key tool for helping identify and ultimately conserve the beneficial ecosystem services provided by these managed and unmanaged taxa, while also reducing the presence of antagonistic pests and the pathogens they transmit.

Arthropods in agroecosystems have traditionally been monitored using both active (e.g. sweep netting) and/or passive (e.g. pan traps) sampling methods, followed by morphological identification (Gervais, Chagnon, & Fournier, 2018; Kearns & Inouye, 1993; Shi et al., 2022). To date, these conventional approaches have proven useful over small-scales to detect pollinators, predators, crop pests and pathogens (Maistrello, Dioli, Bariselli, Mazzoli, & Giacalone-Forini, 2016; Shi et al., 2022; Vu et al., 2018). However, at the large scale of some intensive agricultural practices, which on average occupy an area of 51 ha (see Adamopoulos & Restuccia, 2014), conventional survey methods may be difficult to implement (though see; Biaggini et al., 2007) (Kestel et al., 2022). Specifically, such surveys may require extensive time commitments (e.g. Westphal et al., 2008), taxonomic expertise to morphologically identify arthropod specimens, which may not always be readily available (e.g. Biaggini et al., 2007), and potentially limited samples sizes for some key taxa (Prendergast, Menz, Dixon, & Bateman, 2020). Therefore, alternative high-throughput surveys have gained increasing attention as a complementary or standalone method to detect arthropods in agroecosystems.

Environmental DNA (eDNA) metabarcoding is a molecular method capable of characterising trace amounts of intracellular and extracellular environmental DNA (i.e. hair, saliva, faeces, pollen, etc.) from a broad array of terrestrial and aquatic substrates (Clare et al., 2021; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). Once collected, taxonomically informative 'barcode' regions of the preserved, but often degraded DNA can be targeted and amplified for specific-species, known as 'barcoding', or amplified for entire groups (i.e. arthropods), known as 'metabarcoding', using high-throughput sequencing (HTS) platforms (Saccò et al., 2022; Taberlet et al., 2012). Initial applications of eDNA metabarcoding helped to classify ancient DNA for plant and animal communities (Haile et al., 2009; Sonstebo et al., 2010; Willerslev et al., 2003). Since then, eDNA has been expanded to monitor a broad diversity of mammal (Abrams et al., 2019), plant (Johnson, Fokar, Cox, & Barnes, 2021), reptile (Ryan, Bateman, Fernandes, van der Heyde, & Nevill, 2022), fungal (Yan et al., 2018) and arthropod communities (Thomsen & Sigsgaard,

2019). Though applications of eDNA metabarcoding have expanded rapidly in natural systems, this molecular method remains novel in agriculture (Kestel et al., 2022). The ability to rapidly sample over large areas and generate identifications of species which may otherwise be difficult to observe (Macgregor et al., 2019; Valentin, Fonseca, Nielsen, Leskey, & Lockwood, 2018), does, however, make eDNA an appealing method for monitoring arthropods in agriculture, one that is particularly well-suited to agroecosystems in Australia.

Australia is home to approximately 320,000 arthropod species, of which an estimated 35% have been described (Cresswell et al., 2021). Unfortunately, almost 60% of Australia's biodiversity is affected by agricultural activity (e.g. habitat clearing, pesticide applications, etc.), with arthropod taxa being some of the most significantly affected (Cresswell et al., 2021; Kearney et al., 2019). In Western Australia, this issue is noteworthy as many agricultural crops (e.g. blueberries – *Vaccinium corymbosum*, apples - *Malus domestica* and avocado – *Persea americana*) are reliant on arthropods to facilitate cross pollination (DPIRD, 2016; Lacey & Sutton, 2017; Mccarthy & McCauley, 2020), while yields from these species are threatened by numerous emerging pest species (Herron & Rophail, 1998; Mccarthy & McCauley, 2020; Subhagan, Dhalin, & Humar, 2020). In particular, *P. americana* yields are highly variable in the south west of Western Australia (SWWA), where the crop is primarily grown, leading to large annual fluctuations in yield (Mccarthy & McCauley, 2020). With emerging research suggesting that unmanaged arthropods may play a role in successful pollination for this species (Cook et al., 2020; Sagwe, Peters, Dubois, Steffan-Dewenter, & Lattorff, 2022), *P. americana* presents an ideal species to apply the use of eDNA metabarcoding survey methods to measure the diversity of arthropod pollinations, predators, crop pests and pathogens, as well as the foraging resources upon which they rely.

1.2 Thesis overview

This thesis aims to explore the technical and methodological considerations when undertaking eDNA-based surveys in agroecosystems (Figure 1.1). I begin with a search of the current literature relating to eDNA-based surveys in agriculture to determine if there are any biases, knowledge gaps, or emerging areas of research (Chapter 2). The literature review included in Chapter 2 forms part of the introduction to this thesis. In Chapter 3, I use eDNA metabarcoding of *P. americana* flowers to detect flower-visiting arthropod taxa and compare these detections with two conventional methods used in agriculture, digital video recording (DVR) devices and pan traps, to see if the three methods identify similar arthropod taxa. With the emergence of eDNA-based surveys in agricultural (see Kestel et al., 2022), it is necessary to benchmark new substrates against conventional approaches (see Ryan et al., 2022), to detect any potential biases or limitations of this molecular method.

In Chapter 4, I extend the application of eDNA metabarcoding crop flowers, by combining these detections with DVRs to assess the community shifts of arthropod pollinators, pests and predator taxa. This chapter aims to assess whether beneficial and antagonistic arthropod species composition is similar between low and peak crop flowering, within trees, as well as between orchards. Finally, in Chapters 5 and 6, I use eDNA in a novel way, by metabarcoding pan-trap water to detect plant foraging resources upon which orchard arthropods rely. Chapter 5 evaluates the use of pan-trap water as a substrate to detect both arthropods and the plant material they forage upon (i.e. pollen, nectar and plant tissue), and Chapter 6 explores the use of pan-trap water to detect arthropod foraging preferences in response to the presence of natural vegetation adjacent to *P. americana* orchards. This chapter presents the first application of eDNA metabarcoding pan-trap water to assess community level foraging across an entire agroecosystem. Finally in Chapter 7, I discuss the lessons, key findings, conclusions and future directions which have evolved from this research.

This thesis explores five key research aims:

1) Determine how eDNA is being used for monitoring of agroecosystems, what current limitations exist, and how these could be managed to expand applications into the future;
2) Understand the extent to which the arthropod detections from eDNA metabarcoding, DVRs and pan traps complement one another in SWWA and how this molecular method may improve the monitoring of plant–animal interactions in agroecosystems;
3) Identify which arthropods providing beneficial ecosystem services (i.e. potential pollinators and predators), as well as those antagonistic species which may affect crop

yields (i.e. pests and pathogens), are present across two orchards in SWWA and determine how the abundance and diversity of these taxa vary over time and space;

4) Explore the use eDNA metabarcoding of pan-trap water, combined with marble trap detections of ambient plant DNA, to survey arthropod species and the plant resources they utilise in an agricultural landscape; and
5) Investigate how the presence of natural vegetation adjacent to orchards affects arthropod communities and foraging diversity across the *P. americana* flowering season.

Chapters 1 and 2 have been published within the peer-reviewed literature, Chapters 3 and 4 are currently under peer review and Chapter 5 is in preparation for submission to peer review.

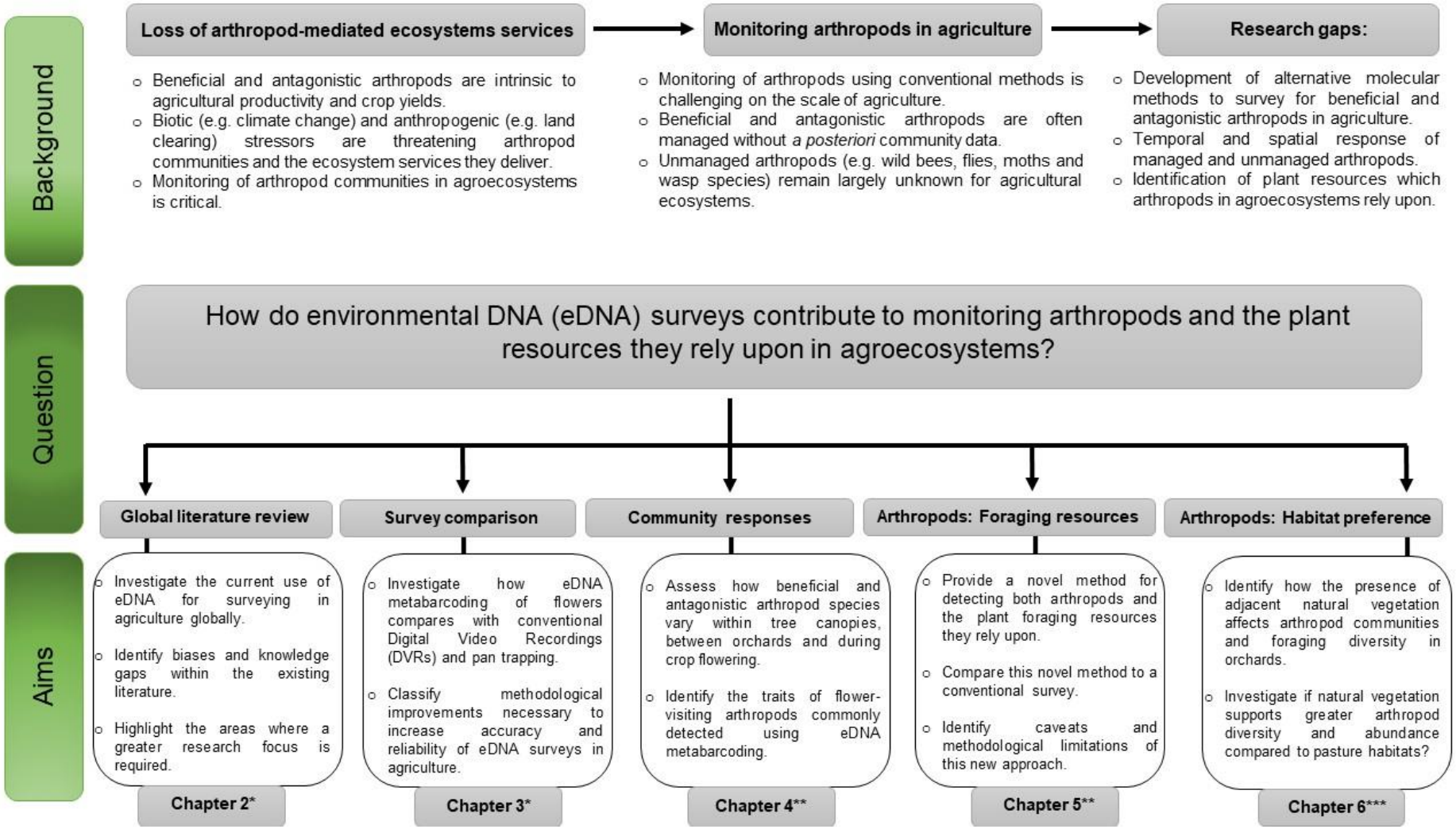

Figure 1.1 Conceptual framework of the research questions and aims of the present thesis. *Published, ** Under peer-review and *** In preparation..

1.3 Study area and focal species

The research presented herein was undertaken in the Manjimup-Pemberton region in SWWA, approximately 325 km south of Perth (34°26'50"S, 116°01'47"E). The climate of this study region is temperate, with an average yearly rainfall of ca. 1185 mm (Bureau of Meteorology, http://www.bom.gov.au/climate/data/). I selected six orchards separated by an average distance of 15.55 km, the largest distance between orchards was 28.24 km and the smallest distance was 7.19 km (Orchard MB - 34°18′52 S, 116°08′36 E; Orchard BA - 34°25'30 S, 116°01'23 E; and Orchard BD - 34°26'28 S, 115°54'02 E, Orchard SD - 34°22′55 S, 115°57′47 E, Orchard PB 34°22′29 S, 116°12′00 E, Orchard DC 34°18′19 S, 116°03′10 E) (Figure 1.2). *Persea americana* trees in each study orchard were similar in age (2 – 7 years). Three of the sample orchards were situated within 50 m of pasture habitat used for grazing cattle or sheep (Figure 1.3). Pasture habitats were primarily dominated by *Arctotheca calendula*, *Trifolium subterraneum* and various exotic flowering herbs and grass species. While, the other three orchards were situated within 50 m of secondary growth tall forest (Figure 1.3), dominated by *Eucalyptus diversicolor* and various flowering native shrubs.

Persea americana is a major agricultural crop in Western Australia and the second largest growing region for this species in Australia (Mccarthy & McCauley, 2020). Between October and December each year, mature *P. americana* trees produce ca. one million flowers over a month long period (Bender, 2013). During flowering, *P. americana* exhibits a synchronous protogynous dichogamous breeding system, whereby each flower opens twice, once as functionally female and once again as functionally male, generally for four to six hours per phase (Alcaraz & Hormaza, 2009; Stout, 1922). The flowers of *P. americana* are 1 mm in diameter and produce small quantities of pollen and nectar (Ish-Am, 2005). In their native habitat in Central America, *P. americana* is pollinated by native stingless bees (e.g. Apidae and Meliponinae), and wasps (e.g. *Brachygastra mellifica*) (Ish-Am, Barrientos-Priego, Castaneda-Vildozola, & Gazit, 1999). In Australia, *A. mellifera* hives are hired to facilitate cross-pollination, with variable levels of success (Mccarthy & McCauley, 2020; Pattemore et al., 2018).

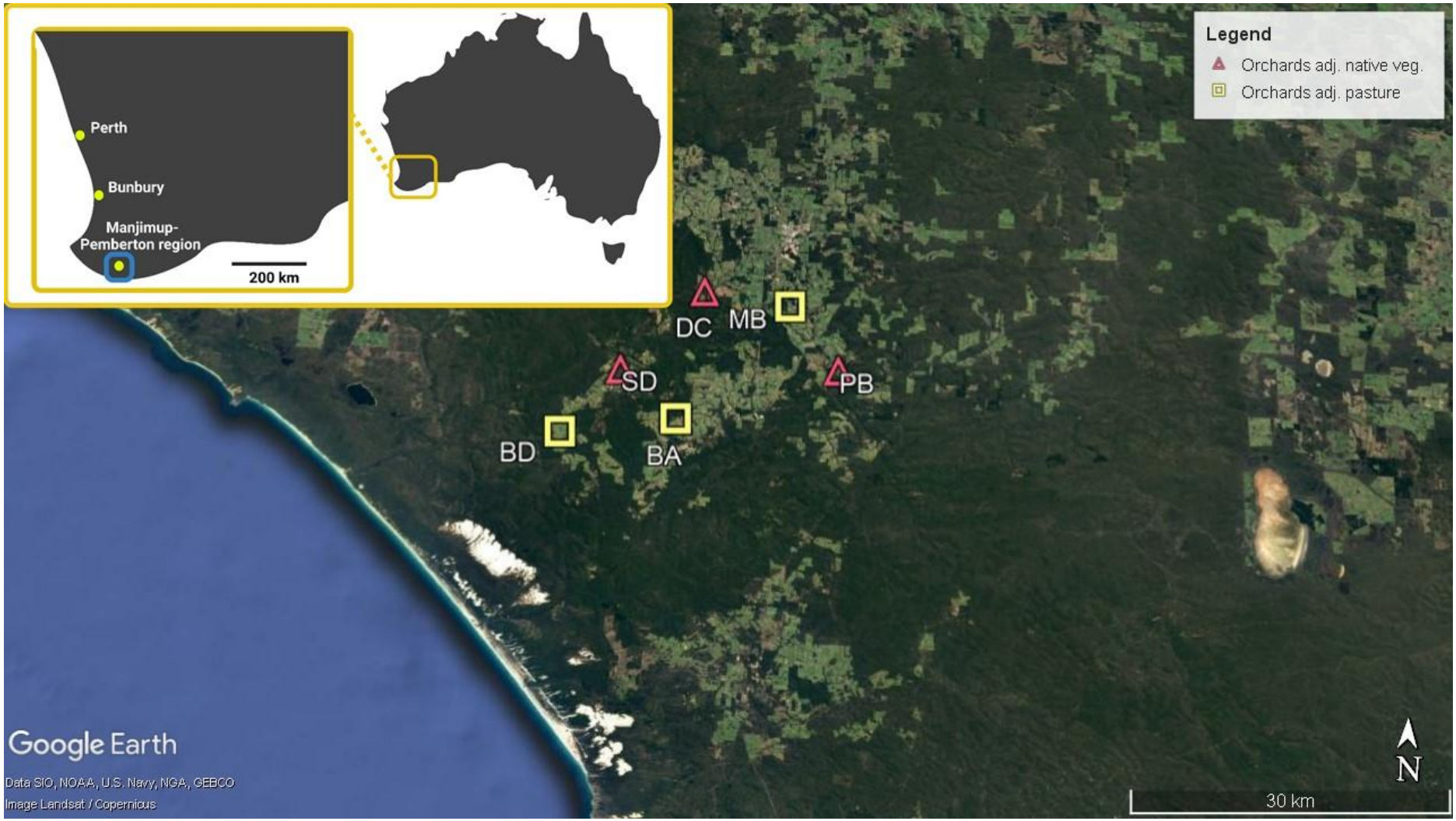

Figure 1.2. Location of the six study orchards, with the dominant adjacent habitat denoted by symbol. All orchards were located in the Manjimup-Pemberton region of south west Western Australia. Figure created in Biorender.

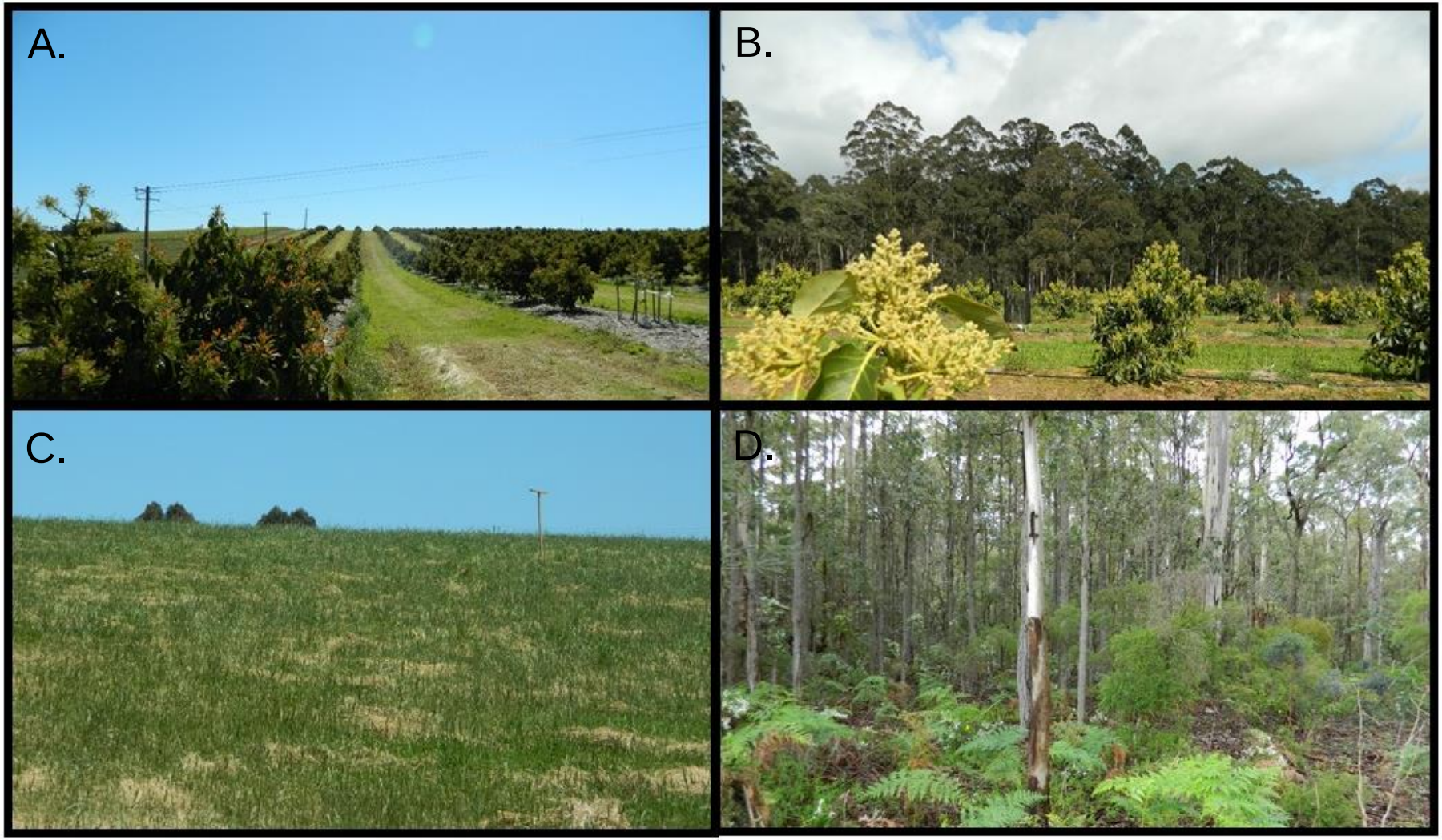

Figure 1.3: Sample orchards in the Manjimup-Pemberton region were located adjacent to either pasture habitat (A & C) or natural vegetation (B & D).

1.4 References

A thorough effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Chapter 2. Applications of environmental DNA (eDNA) in Agricultural Systems: current uses, limitations and future prospects

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2.1 Abstract

Global food production, food supply chains and food security are increasingly stressed by human population growth and loss of arable land, becoming more vulnerable to anthropogenic and environmental perturbations. Numerous mutualistic and antagonistic species are interconnected with the cultivation of crops and livestock and these can be challenging to identify on the large scales of food production systems. Accurate identifications to capture this diversity and rapid scalable monitoring are necessary to identify emerging threats (i.e. pests and pathogens), inform on ecosystem health (i.e. soil and pollinator diversity), and provide evidence for new management practices (i.e. fertiliser and pesticide applications). Increasingly, environmental DNA (eDNA) is providing rapid and accurate classifications for specific organisms and entire species assemblages in substrates ranging from soil to air. Here, we aim to discuss how eDNA is being used for monitoring of agricultural ecosystems, what current limitations exist, and how these could be managed to expand applications into the future. In a systematic review we identify that eDNA-based monitoring in food production systems accounts for only 4 % of all eDNA studies. We found that the majority of these eDNA studies target soil and plant substrates (60 %), predominantly to identify microbes and insects (60 %) and are biased towards Europe (42 %). While eDNA-based monitoring studies are uncommon in many of the world's food production systems, the trend is most pronounced in emerging economies often where food security is most at risk. We suggest that the biggest limitations to eDNA for agriculture are false negatives resulting from DNA degradation and assay biases, as well as incomplete databases and the interpretation of abundance data. These require in silico, in vitro, and in vivo approaches to carefully design, test and apply eDNA monitoring for reliable and accurate taxonomic identifications. We explore future opportunities for eDNA research which could further develop this useful tool for food production system monitoring in both emerging and developed economies, hopefully improving monitoring, and ultimately food security.

2.2 Introduction

Global food production faces increasing threats from both environmental and human-induced stressors (Cole, Augustin, Robertson, & Manners, 2018; Grafton, Daugbjerg, & Qureshi, 2015; Grubisic, van Grunsven, Kyba, Manfrin, & Hölker, 2018; Yue et al., 2020). These stressors have curtailed efforts to meet the United Nations Sustainable Development Goal and reduce the 8.9 percent of the global population that are currently malnourished (FAO, 2020; United Nations, 2015). The failure to reduce this malnutrition rate has emphasised the challenge of achieving widespread access (physical, social and economic) to safe nutritious food, known as food security (Isvilanonda & Bunyasiri, 2009; Sarkar et al., 2020; Torquebiau, 2016). Improving global food security is a substantial challenge that will become more difficult to achieve as food production systems (used interchangeably with agricultural systems) around the world are threatened by climate change (Lesk & Anderson, 2021), loss of arable land (Hossain et al., 2020), increases in water scarcity (Wada et al., 2016), greater threats from pests and pathogens (Savary et al., 2019), and the loss of pollinating species (Lippert, Feuerbacher, & Narjes, 2021). These threats will likely inflate global food commodity prices, thereby further restricting food security to only those who can afford it (Beydoun, Powell, Chen, & Wang, 2011; Green et al., 2013; Pollard & Booth, 2019). Responding and adapting to these emerging threats will require a whole systems approach that strengthens current measures by accounting for the inherent biological complexity within food production systems.

Escalating global food demands will need to be met with further intensification of production systems across agricultural and horticultural sectors (FAO, 2020), production which relies upon a combination of soil health/plant nutrition, suppression of disease pressure, and promotion of the presence of beneficial organisms (e.g. nodulating bacteria, pollinators, etc.) (Amari, Huang, & Heinlein, 2021; Mbow & Rosenzweig, 2019; Potts, Imperatriz-Fonseca, & Ngo, 2016). Detection and identification of these mutualistic and antagonistic species is largely reliant on labour intensive processes (Kudoh, Minamoto, & Yamamoto., 2020; George et al., 2017; Ashfaq et al.. 2016). Indeed, manual identifications have historically been the standard procedure for identifying meso- and macrofauna within soil (Gerlach, Samways, & Pryke, 2013; Menta & Remelli, 2020), crop and animal pests/pathogens (Tsoi, Šlapeta, & Reynolds, 2020; Vu et al., 2018), as well as pollinating species (Macgregor et al., 2019; Pardo & Borges, 2020). Nevertheless, taxonomic and specialist expertise are becoming increasingly rare, and the effort required to identify organisms to species-level based on morphological characteristics is often time-consuming. Upscaling such detections to large agricultural and horticultural practices is often not economically viable, and some traditional monitoring methods are only effective for a small fraction of the total diversity

present (i.e. cultivating bacteria) (Bell et al., 2016; Kudoh et al., 2020; Rappé & Giovannoni, 2003). Consequently, a significant barrier currently exists for efficiently detecting and classifying soil, pest, and beneficial species within food production systems. Increasingly, environmental DNA (eDNA) is being used as a tool to detect taxa from trace DNA deposits, potentially offering a strong complement for monitoring in agricultural ecosystems.

DNA-based approaches offer an efficient means to characterise biodiversity, establish diversity thresholds, and to monitor community changes as a result of activities or management decisions. Trace amounts of intracellular and extracellular DNA can be isolated and characterised from biological substrates including; soil, scats (faeces), plant material, water, or air are collectively referred to as environmental DNA (eDNA) (Levy-Booth et al., 2007; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). This also extends to DNA obtained from bulk samples (e.g. a collection of whole insects from pitfall traps; see Rasmussen et al., 2021; Young et al., 2021) (Taberlet et al., 2012a; Taberlet et al., 2018). Once captured, the preserved, but often degraded DNA provides a means to rapidly and accurately identify taxa and survey biological communities (Ficetola, Miaud, Pompanon, & Taberlet, 2008; Taberlet et al., 2007). When combined with High-Throughput Sequencing (HTS) technologies, the large eDNA data volumes can provide a wealth of information on, for example, community composition, food web dynamics, animal diet, the recovery (or otherwise) of ecosystems following restoration, and invasive or pest species presence/absence (Ruppert, Kline, & Rahman, 2019; Taberlet et al., 2012a). A significant strength of eDNA-based monitoring is the ability to tailor surveys to detect either single species or whole biological communities.

Environmental samples may be targeted and amplified with either a barcoding or metabarcoding approach depending on how many taxa are of interest. DNA barcoding, otherwise known as "targeted-PCR", provides a single taxon identification and is often used in combination with Sanger sequencing, while eDNA metabarcoding targets many DNA fragments, and therefore many taxa, from mixed biological samples, the amplified fragments are then sequenced on an HTS platform, a process which is sometimes described as "metabarcoding" (Saccò et al., 2022). DNA barcoding is frequently applied to eDNA samples with single-species probe assays (i.e. Valentin et al., 2016), to determine the presence or absence of species via quantitative PCR. In contrast, eDNA metabarcoding uses "universal" primer sets (i.e. assays) to bind to a conserved homologous region of a gene shared by numerous species or groups of taxa using PCR (i.e. Miya et al., 2020) (Saccò et al., 2022). Subsequently, the variable region is amplified (known as an "amplicon"), arranged into libraries and sequenced on an HTS platform (i.e. Illumina MiSeq, Oxford MinION,

etc.), the millions of short DNA sequences generated are filtered using a bioinformatics pipeline that can then be used to assess diversity by assigning taxonomic identifications, Operational Taxonomic Units (OTUs) (i.e. Jiang et al., 2014), or Amplicon Sequence Variants (ASVs) (Callahan, McMurdie, & Holmes, 2017; Callahan et al., 2016) (Figure 2.1). The choice of assay for both barcoding and metabarcoding depends on the availability of reference sequences (i.e. Cytochrome c Oxidase subunit I (COI) generally favoured for insects) as well as the presence and suitability of a locus to target (Saccò et al., 2022). Unfortunately, because the genes containing the homologous regions targeted using universal assays evolve and mutate at different rates (see Kocher et al., 1989), no single universal assay exists that can capture all prokaryotic or eukaryotic diversity within a mixed biological environmental sample (Alexander et al., 2020). Instead, multiple assays are often used to accurately capture the taxonomic diversity and monitor community composition with eDNA samples (e.g. Makiola et al., 2019). eDNA can provide rapid, scalable monitoring which can support current techniques used for food production systems with bulked samples and accurate molecular identifications.

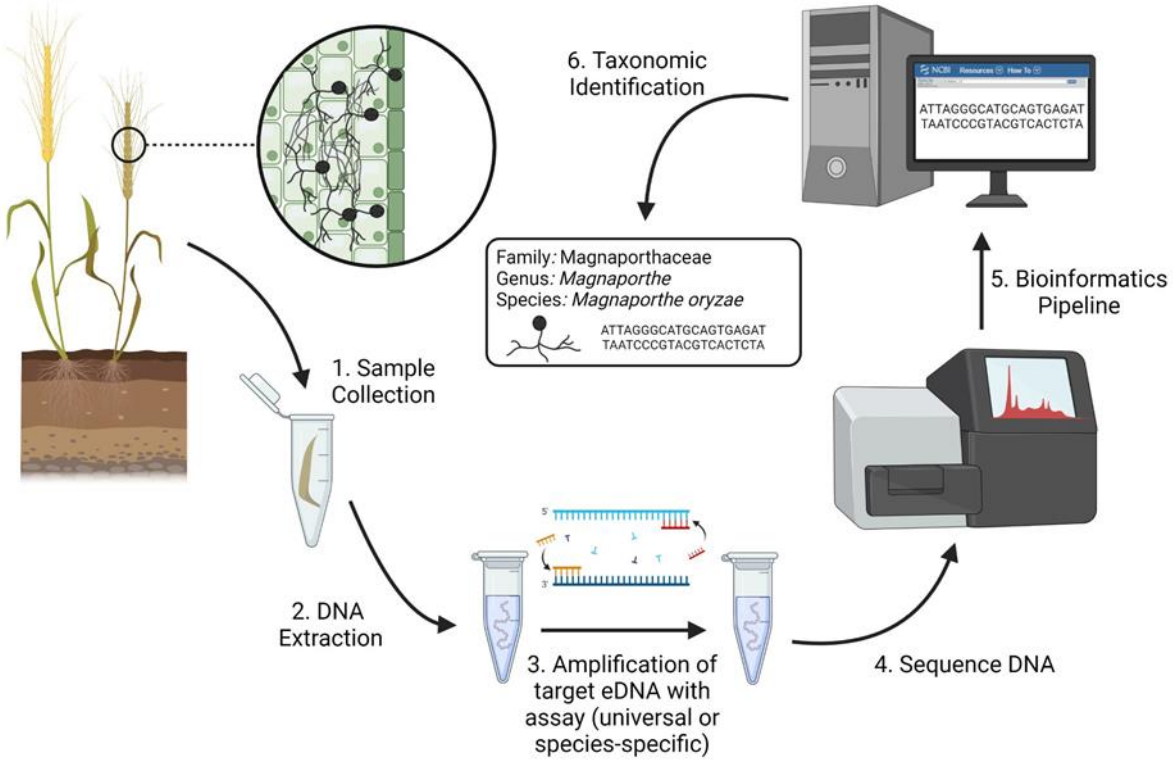

Figure. 2.1. An example workflow for eDNA-based monitoring to measure the species identity of a fungal pathogen infecting wheat (*Triticum aestivum*). Leaf samples are collected from the infected plants (1), these samples may be placed on ice, or immediately processed for DNA extraction (2). Following extraction, the target DNA of interest, in this case fungal DNA, is amplified with Polymerase Chain Reaction (PCR) using either species-specific or universal

primers (3). The amplified products are then cleaned, purified and arranged into libraries prior to sequencing on a Next Generation Sequencing (NGS) platform (4). The reads generated are then filtered using a bioinformatics pipeline (5) and compared to reads from either online databases, or a custom Barcode Reference Library (BRL) to provide taxonomic identifications (6). Graphic created using BioRender.

A wide range of studies, mainly in natural ecosystems, have shown eDNA barcoding and metabarcoding to be an effective taxonomic identification tool for both micro- and macroorganisms (Buée et al., 2009; Clare et al., 2021; Miya et al., 2020; Ruppert et al., 2019; Taberlet, Coissac, Hajibabaei, et al., 2012). Microbiologists were the first to use DNA barcoding to target uncultivable microorganisms (Hugenholtz & Pace, 1996). By the early 2000's, barcoding of bacterial, fungal and eukaryotic DNA based on cloning technology had become common practice within microbiology, although the term 'eDNA' was not used in the discipline until 2009 (Buée et al., 2009; Rolf, 2005; Rondon et al., 2000). While for macroorganisms, the first applications of eDNA helped to reconstruct ancient plant and animal communities from permafrost, ice cores and cave sediment (Haile et al., 2009; Sonstebo et al., 2010; Willerslev et al., 2003). DNA–based assessment of palaeoecological communities (both barcoding and metabarcoding) provided higher taxonomic resolution compared to traditional identification and survey techniques (Haile et al., 2009; Sonstebo et al., 2010; Willerslev et al., 2003). These initial studies established eDNA tools as a fast and efficient means of classifying species assemblages directly from environmental samples (Taberlet et al., 2012b). Such promise made the application of eDNA for detecting extant biodiversity appealing, and it was first used to barcode tadpole DNA from aquarium water samples (Ficetola et al., 2008). Since these initial studies, eDNA-based surveys have been expanded to monitoring a wide range of animal (Lesk & Anderson, 2021), plant (Yoccoz et al., 2012), fungal (Yan et al., 2018), prokaryotic (Caldwell, Silva, Da Silva, & Ouverney, 2015) and viral communities (Miaud, Arnal, Poulain, Valentini, & Dejean, 2019). With this expansion however, an increasing awareness of the limitations of the technology has emerged. For instance, the basic biological processes that "feeds" DNA into the environment and the physical and chemical processes that determine its persistence in terrestrial, aquatic and aerial environments remain largely unexplored (Deiner et al., 2017). Further, not all taxonomic groups can be differentiated with commonly amplified barcoding regions such as COI, and false negatives (taxa present but genetically misclassified as absent), as well as false positives (taxa absent but genetically misclassified as present) are persistent issues in this research field (Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014; Deiner et al., 2017; Ficetola et al., 2015). These caveats highlight that although eDNA surveys are a powerful molecular tool, they will not apply equally

well to all ecosystems (Deiner et al., 2017; Taberlet et al., 2012a). In spite of these limitations, it remains necessary to continually test where eDNA is applicable and what targeted approaches for sampling and species detections are possible to further increase its utility for food production systems.

Traditional monitoring for agriculture has proved challenging to scale and is sometimes impossible because the majority of organisms cannot be cultivated or are difficult to rear (Kudoh et al., 2020; Rappé & Giovannoni, 2003). Detecting species from trace amounts of DNA or from a single bulked environmental sample offers an efficient, reproducible and cost-effective alternative (Kudoh et al., 2020; Valentin et al., 2018; Littlefair & Clare, 2016). For instance, manually testing individual plants or animals in large consignments for pests and diseases is often logistically impossible given time constraints and associated costs (Brunner, 2020; Ceresini et al., 2019). While for eDNA, one bulked sample (made up of many sub samples) provides a presence/absence measure for the entire consignment, allowing for a rapid general assessment (e.g. targeting Khapra beetle (*Trogoderma granarium*) in shipping containers using a species-specific assay; see DAWE, 2021) (Brunner, 2020; Valentin et al., 2018). eDNA-based detections can also be tailored for economically-important species or entire communities where morphology-based identification has proved problematic (see Aloo et al., 2020; Macgregor et al., 2019), and where microorganisms cannot be cultured easily using selective media (i.e. ≥ 99 % of bacteria are estimated to be unculturable; Rappé & Giovannoni, 2003) (see Sternhagen et al., 2020). Further, because of the high levels of mechanisation in modern agriculture, there are opportunities to integrate these eDNA-based sampling methods with existing machinery and infrastructure to detect these species of interest. Information on these generally 'invisible' organisms would enable better monitoring, and potentially better informed management for these species depending on their relationship to the cultivated animal or plant of interest (i.e. controlled pesticide application, reduced fertiliser input, etc.) (Menta & Remelli, 2020; Willcox et al., 2019). The ability to tailor eDNA sampling and specificity according to the species, community or system of interest has enabled non-invasive surveys in an array of different ecosystems and contexts, although despite this promise, eDNA surveys have remained novel for the field of agriculture.

Applications of eDNA surveys have almost exclusively occurred within natural ecosystems (Bohmann et al., 2014; Evans & Kitson, 2020; Ruppert et al., 2019; Taberlet et al., 2012a). Few studies have used eDNA in agricultural systems, although this is beginning to change (Figure 2.2). As far as we are aware, no systematic reviews of the applications of eDNA barcoding and metabarcoding for food production systems have been conducted (Figures 2.2 and 2.3); a

significant omission given that taxonomic identifications are necessary for monitoring in both natural and human-modified ecosystems (Memmott, Waser, & Price, 2004; Van Elsas, Garbeva, & Salles, 2002; Yue et al., 2020). Here, we conducted a systematic review to identify the current uses of eDNA-based monitoring in agriculture, the substrates and organisms routinely being targeted, and the geographical distribution of these studies. We also stress the most relevant challenges for implementing eDNA methods into food production systems and highlight the current and emerging solutions available. The complexities present within the eDNA workflow have merited numerous reviews over the last decade (i.e. Ruppert et al., 2019; Taberlet et al., 2018, 2012b). Within the constraints of this review we have omitted extensive discussions on eDNA sampling (see Dickie et al., 2018), primer selection (see Schenekar et al., 2020) and bioinformatics (see Mathon et al., 2021), all of which have been reviewed elsewhere. Finally we explore future applications of eDNA-based monitoring, what components of agriculture are currently unexplored, and how to increase the accessibility of this technology to facilitate greater use in food production systems for both developed and emerging economies.

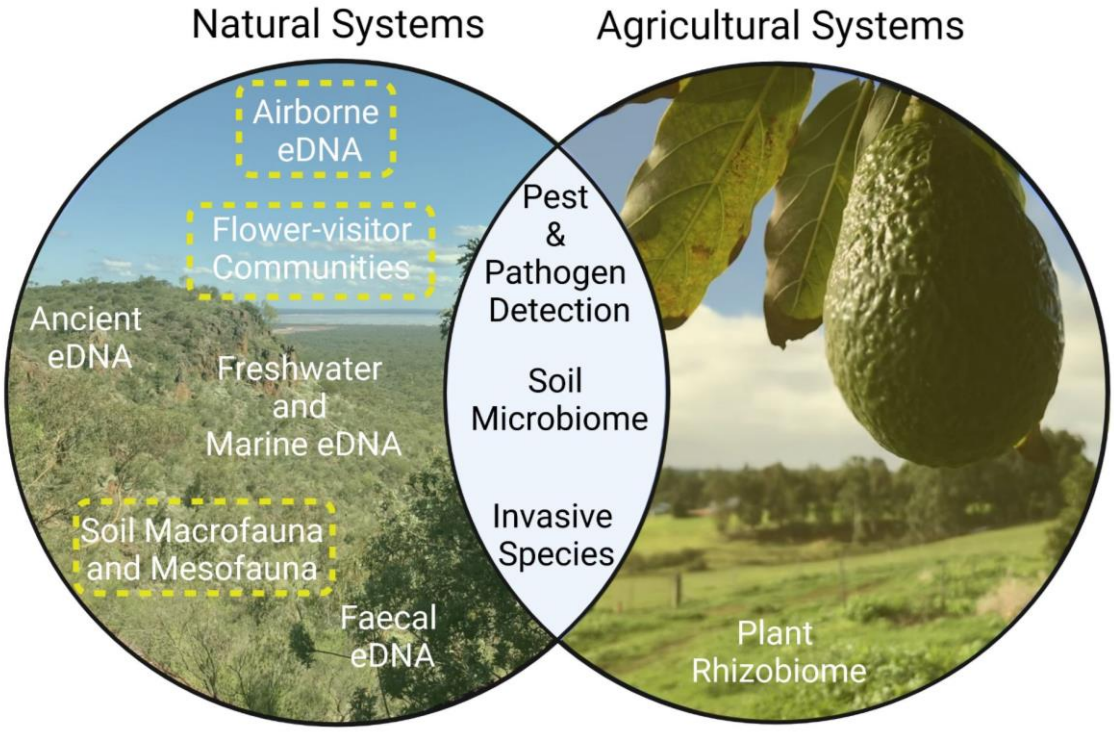

Figure. 2.2. Applications of eDNA-based surveys in natural and agricultural systems. Applications are based on the papers found during systematic review (Table 2.1). Yellow boxes designate applications of eDNA which are used in natural systems and are emerging in food production systems. Images captured by Joshua Kestel.

2.3 Methods

Literature searches were conducted on SCOPUS up to 6th of January 2022. The SCOPUS database was chosen because it offers greater coverage for the subjects relevant to eDNA, the life Sciences and biomedicine, when compared to the Web of Science (Mongeon & Paul-Hus, 2016; Vera-Baceta, Thelwall, & Kousha, 2019). The term 'eDNA' entered the mainstream scientific literature nearly a decade after DNA metabarcoding became commonplace for the soil sciences (see Buée et al., 2009), where inconsistent and changing terms are used for molecular studies of soil microbial communities (i.e. sed-eDNA, eDNA, metabarcoding, meta-barcoding, amplicon, tag-sequencing, total soil DNA, etc.). It was beyond the scope of this review to classify (e.g. geographic location, target taxa etc.) the many thousands of soil microbial community studies that utilise the eDNA workflow but not the terminology 'environmental DNA' or 'eDNA' as this is a review in itself, and has been done many times previously (e.g. Imfeld & Vuilleumier, 2012; Pankhurst et al., 1996; Rolf, 2005; Schloter et al., 2018; Trivedi et al., 2016). We therefore caution that this review is non-exhaustive in the context of soil microbial community analysis, but does provide a snapshot of the trends, emerging research and future directions for the field.

Three searches were undertaken, the first to determine the total number of eDNA studies, the second to specify the number of eDNA studies relevant to food production systems, and the third to identify the number of total soil DNA papers potentially missed from the first two searches. The first search used the terms; ('eDNA' OR 'environmental') AND 'DNA' AND ('barcode' OR 'barcoding' OR 'metabarcode' OR 'metabarcoding') to target all eDNA studies in the literature. For the second search, the terms ('eDNA' OR 'environmental') AND 'DNA' AND ('barcode' OR 'barcoding' OR 'metabarcode' OR 'metabarcoding') AND ('agriculture' OR 'agricultural' OR 'horticulture' OR 'horticultural') were used to target eDNA studies relevant to terrestrial food production systems, specifically agriculture and horticulture. Although not included in this review, we sought to quantify the number of soil microbial papers that use the eDNA workflow, but not necessarily the terms 'eDNA' or 'environmental DNA'. As such, a third search using the terms; ('extracellular' OR 'environmental') AND ('DNA' OR 'eDNA') AND 'soil' AND ('agriculture' OR 'agricultural' OR 'horticulture' OR 'horticultural') AND NOT 'metagenomics' was conducted in SCOPUS generating 1,022 results (Figure 2.4).

The first search for all eDNA studies generated 2,215 results, and the second search of eDNA studies relevant to food production systems generated 107 results. These results were then checked manually to determine relevance. Of the 2,215 results, 1076 (48 %) were deemed relevant for eDNA generally (i.e. studies which used eDNA-based surveys, either single species or community,

for taxonomic classification) and 45 (4 %) were deemed relevant for eDNA in agriculture (Figure 2.3 and Table 2.1). Papers were grouped according to year of publication, and papers specific to agriculture were graphed separately to the cumulative total of eDNA studies and total soil DNA studies (Figure 2.4).

2.4 Applications of eDNA in food production

The inclusion and further development of eDNA technology to complement species monitoring within food production systems can facilitate the timely detection of emerging pests and pathogens, and establish how new management strategies are affecting local biodiversity. Although, the use of eDNA for agriculture remains an emerging field (4 % of all eDNA studies) (Figure 2.3), with a geographical bias towards European countries (42 %). Relatively few records have been published for studies conducted in the Americas (18 %). China (13 %), and Oceania (16 %) despite two of these having the largest economies (America and China), and only one eDNA study was found for food production systems in Africa (Figure 2.3 and Table 2.1). Our systematic review highlights that relatively few food production systems appear to use eDNA in agriculture, especially those with developing economies (Figure 2.3). When used, plant material (36 %), and soil substrates (24 %) are most commonly sampled, while insects (33 %) and microorganisms (27 %) are the most targeted taxa. Further, where eDNA is implemented within food productions systems, there is an opportunity to survey more substrates (i.e. honey, faeces; 9 % of substrates measured) and to target a greater breadth of taxa (i.e. plants; 13 % of taxa targeted) than is currently measured. Clearly much research remains to be done across numerous agricultural and horticultural contexts for both broad and narrow ranges of geography in the future. Complementing traditional monitoring with eDNA-based tools is increasingly necessary as stakeholders require identifications and spatial distributions for mutualistic and antagonistic species, both to improve monitoring, and potentially food security (Weiss, Jacob, & Duveiller, 2020). One caveat to these findings is that they largely omit the extensive literature associated with total soil DNA (Figure 2.4). It is beyond the scope of this review to retrospectively disentangle the numerous eDNA and metagenomic soil studies relevant to agriculture. Instead, we direct readers to the following reviews for more discussion on this topic (Levy-Booth et al., 2007; Rolf, 2005; Taberlet et al., 2018). Below, we discuss recent studies where the benefits and limitations of eDNA in agricultural systems are highlighted.

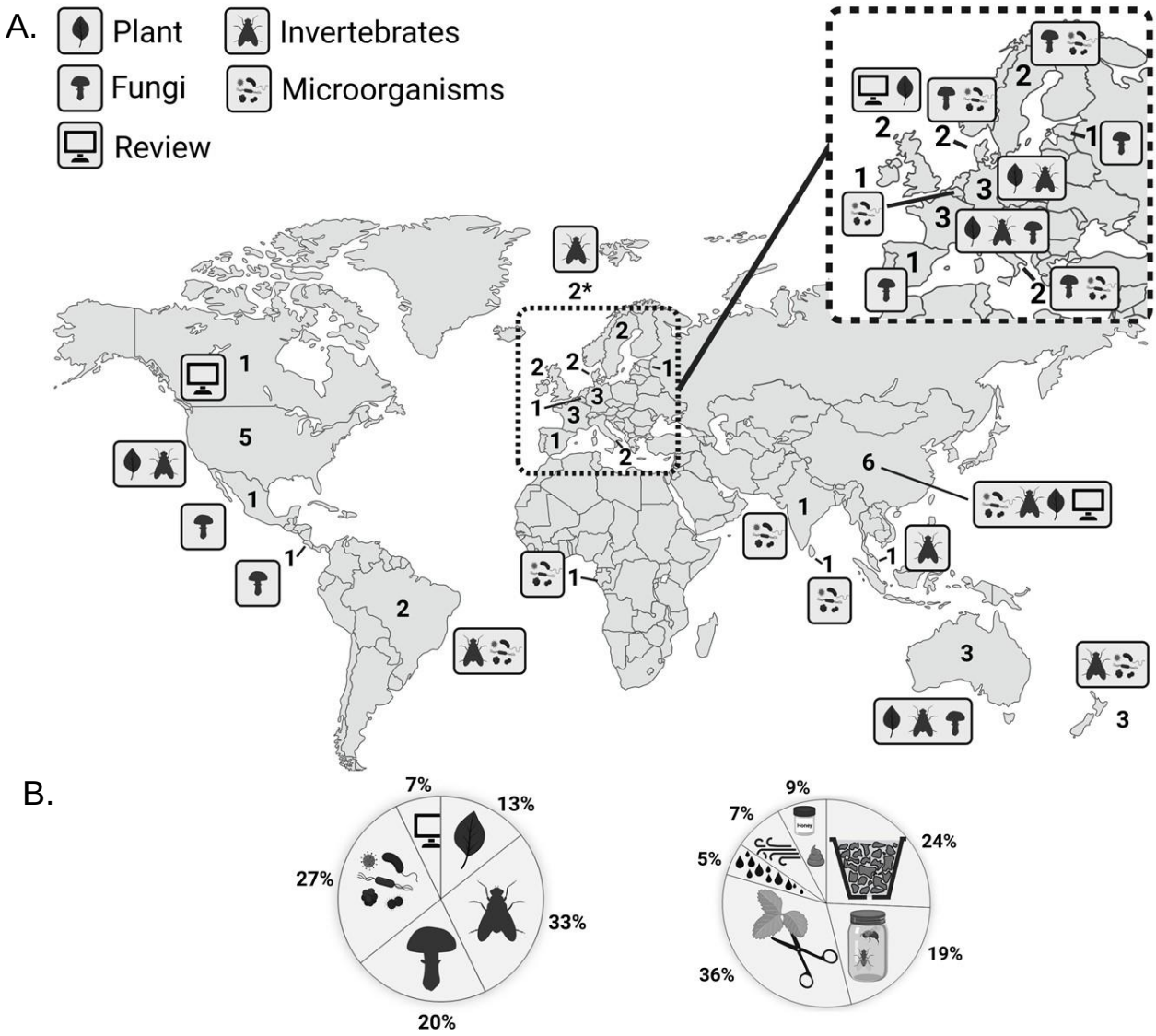

Figure. 2.3. Panel A; Global distribution of agricultural and horticultural eDNA studies (45). The taxa targeted are symbolised next to the number of studies in each country, not including duplicates. The 2* designates the two studies which used eDNA for agricultural purposes in various countries in Europe. Panel B; left; taxa targeted for each study, clockwise; plants (13 %), insects (33 %), fungi (20 %), microorganisms (27 %), and review (7 %). Right; substrates sampled for eDNA within agricultural and horticultural context, clockwise; soil (24 %), insects (19 %), plant material (36 %), water (5 %), air (7 %), and other (9 %). Graphic generated in BioRender.

Table 2.1. Forty-five studies found from SCOPUS. Search terms for SCOPUS included; 'eDNA' OR 'environmental' AND 'DNA' AND 'barcode' OR 'barcoding' OR 'metabarcode' OR 'metabarcoding' AND 'agriculture' OR 'agricultural' OR 'horticulture' OR 'horticultural'. Only studies that used the term 'eDNA' or 'environmental DNA' for the purposes of taxonomic identification were included, as well as studies that used bulk sampling combined with an eDNA workflow. Metagenomic and total soil DNA papers were not included as they were outside of the scope of this review. The literature search was conducted up to 6/01/2022 and generated 107 results, all results were checked manually to determine if they were relevant to applications of eDNA for agricultural practices.

Reference	Country	System	Substrate	Barcoding	Target taxa
Ashfaq et al. (2016)	Canada	Review	-	-	-
Wang et al. (2013)	China	Review	-	-	-
Littlefair & Clare (2016)	England	Review	-	-	-
Tordoni et al. (2021)	Italy	Various locations in two Italian regions	Air	Metabarcoding	Fungi
Karlsson et al. (2020)	Sweden	Various locations in two Swedish regions	Air	Metabarcoding	Bacteria and fungi
Redondo et al. (2020)	Sweden	Wheat fields	Air	Metabarcoding	Fungi
Rasmussen et al. (2021)	Germany	Vineyard	Bulk-insect samples	Metabarcoding	Insects
Zenker et al. (2020)	Brazil	Agricultural fields	Bulk-insect samples	Metabarcoding	Insects

Reference	Country	System	Substrate	Barcoding	Target taxa
Song & Huang (2016)	China	Farmland	Bulk-insect samples	Metabarcoding	Insects
Boetzl et al. (2021)	Germany	Flowering fields and calcareous grasslands	Bulk-insect samples	Metabarcoding	Insects
Edwards et al. (2014)	Malaysia	Oil palm plantations	Bulk-insect samples	Metabarcoding	Insects
Dopheide et al. (2020)	New Zealand	Perennial cropland	Bulk-insect samples	Metabarcoding	Various Metazoa
Chang et al. (2018)	China	Island habitat for long-distance migrating pest moth	Moths	Metabarcoding	Plants
Macgregor et al. (2019)	England	Farmland	Moths	Metabarcoding	Plants
Emenyeonu et al. (2018)	Australia	Laboratory	Flour, seed mixes and air	Species-specific	*Zea mays* and *Vigna unguiculata*
Latz et al. (2021)	Denmark	Greenhouse and field trial	Leaf, root, seed, and air	Metabarcoding	Fungi
Milazzo et al. (2021)	Australia	Barley fields	Leaf	Metabarcoding	Fungi
Barroso-Bergadà et al. (2021)	France	Vineyard	Leaf	Metabarcoding	Fungi
Loit et al. (2019)	Estonia	Laboratory	Leaf and tuber	Metabarcoding	Fungi
Smessaert et al. (2019)	Belgium	Apple and pear orchards	Nectar	Metabarcoding	Bacteria

Reference	Country	System	Substrate	Barcoding	Target taxa
Danner et al. (2017)	Germany	Agricultural landscapes	Pollen	Metabarcoding	Plants
Smart et al. (2017)	USA	Agricultural landscapes	Pollen	Metabarcoding	Plants
Michelot-Antalik et al. (2021)	France	Dairy Farms	Pollen	Metabarcoding	Plants
Sternhagen et al. (2020)	Costa Rica	Coffee fields	Root	Metabarcoding	Fungi
Mezzasalma et al. (2017)	Italy	Vineyards	Grapes	Metabarcoding	Bacteria and yeast
Zhou et al. (2020)	China	Rice Field	Seeds and roots	Metabarcoding	Bacteria
Makiola et al. (2019)	New Zealand	Perennial cropland	Soil, root and leaf	Metabarcoding	Bacteria, fungi and oomycetes
Caldwell et al. (2015)	Brazil	Coffee fields	Soil	Metabarcoding	Archaea and bacteria
Jiang et al. (2014)	China	Various crops	Soil	Metabarcoding	Archaea and bacteria
Wang et al. (2020)	China	Cropland soils	Soil	Metabarcoding	Bacteria and eukaryotes
Frøslev et al. (2021)	Denmark	Agricultural fields	Soil	Metabarcoding	Bacteria, fungi and eukaryotes
Meyer et al. (2019)	Gabon	Manioc and banana plantations	Soil	Metabarcoding	Bacteria

Reference	Country	System	Substrate	Barcoding	Target taxa
Navarro-Noya et al. (2021)	Mexico	Maize fields	Soil	Metabarcoding	Fungi
Todd et al. (2020)	New Zealand	Apple and kiwifruit orchards	Soil	Metabarcoding	Insects
Epelde et al. (2020)	Spain	Cultivated vegetable orchards	Soil	Metabarcoding	Fungi
Srivastava et al. (2021)	India	Field trial	Compost	Metabarcoding	Bacteria
Valentin et al. (2020)	USA	Various trees, shrubs and understorey vegetation	Spray aggregate and rollers from tree bark and leaf surfaces	Species-specific	*Lycorma delicatula*
Allen et al. (2021)	USA	Vineyards	Leaf and stem surfaces	Species-specific	*Lycorma delicatula*
Gamage et al. (2020)	Sri Lanka	Agricultural or rice fields	Water	Species-specific + Metabarcoding	*Leptospira* sp. and bacteria
Valentin et al. (2018)	USA	Apple Orchards	Water	Species-specific	*Halyomorpha halys*
Valentin et al. (2021)	USA	Laboratory	Fruit and leaf surfaces	Species-specific	*Halyomorpha halys*
Utzeri et al. (2018)	Various - Europe	Various orchards	Honey	Metabarcoding	Hemiptera species

Reference	Country	System	Substrate	Barcoding	Target taxa
Crisol-Martínez et al. (2016)	Australia	Macadamia orchards	Faecal	Metabarcoding	Insects
Aizpurua et al. (2017)	Various - Europe	Caves within agricultural landscape	Faecal	Metabarcoding	Insects
Tournayre et al. (2021)	France	Caves within agricultural landscape	Faecal	Metabarcoding	Insects

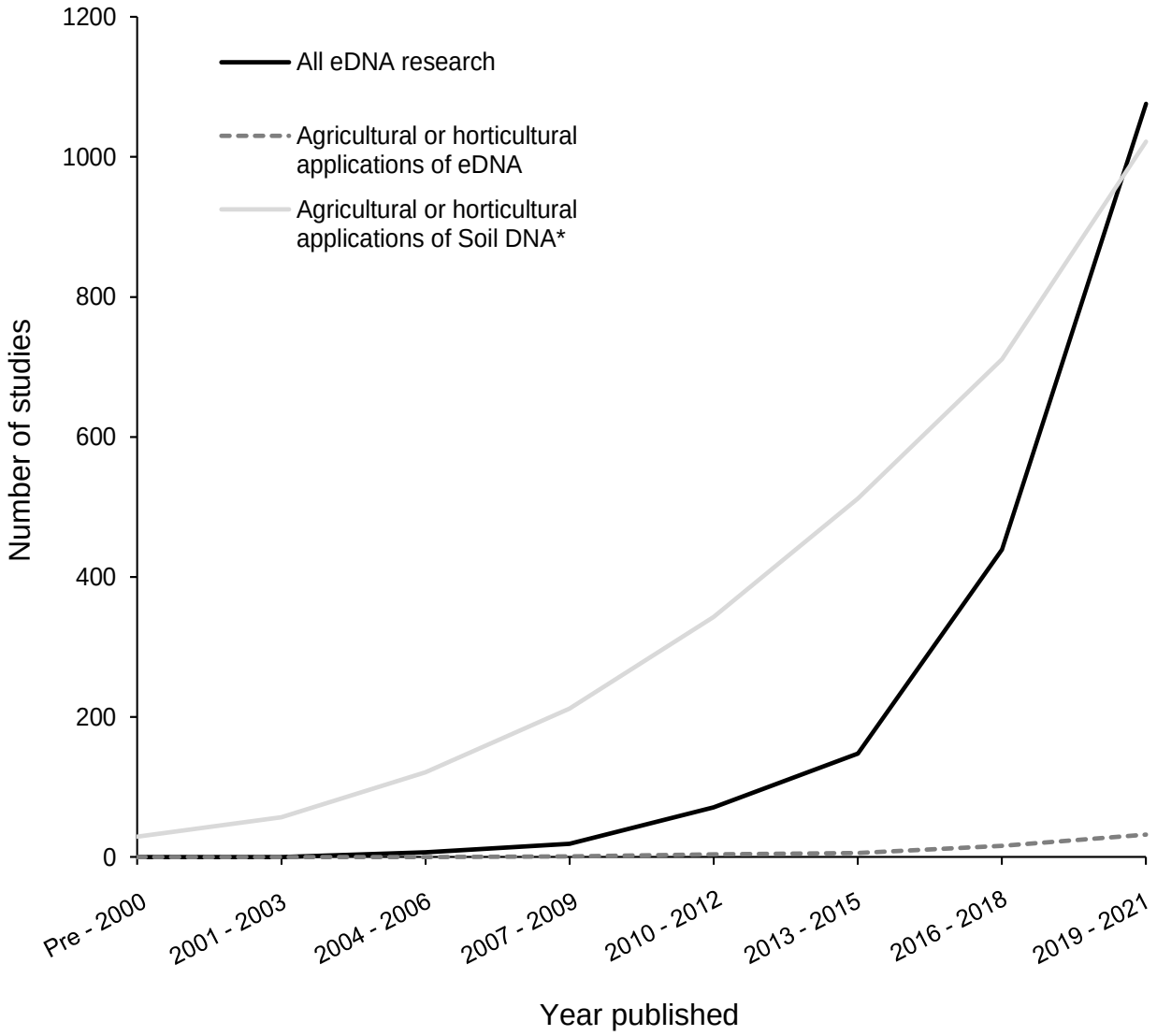

Figure. 2.4. Cumulative number of eDNA studies in both natural (terrestrial and aquatic) and food production systems (*N* = 1,076) (All eDNA research), agricultural eDNA studies (*N* = 42) (Agricultural or horticultural applications of eDNA), and total soil DNA studies (Agricultural and horticultural applications of soil DNA) (*N* = 1,022) generated from SCOPUS searches conducted up to 6/01/2022. Agricultural and horticultural applications of soil DNA* studies relevant to agriculture predominantly identified soil microorganisms (81% of soil studies).

2.4.1 Pest and pathogen surveillance - Cropping systems

Cultivated landscapes provide favourable conditions for the evolution, selection and spread of plant pests and pathogens (Brown & Hovmøll, 2002; Smith & Guégan, 2010). In the presence of a susceptible host and appropriate environmental conditions these pathogens and pests can threaten crop and pasture production, with global yields estimated to be reduced by 20 – 40 % annually

(see Flood, 2010) in the absence of effective control. Crop disease burdens increase with farming intensity and are predicted to increase as crop yields double to achieve food security by 2050, with the suite of disease-causing pathogens predicted to increase dramatically (Amari et al., 2021; Chaloner, Gurr, & Bebber, 2021). As a consequence, the viability of current farming systems may be threatened by the emergence of new plant pests and pathogens and/or changes in the virulence and distribution of known pests and pathogens, especially if new innovations and technologies are not harnessed to identify and monitor their emergence (Jones, 2009; Osunkoya, Lock, Dhileepan, & Buru, 2021; Wintermantel & Hladky, 2010). Two salient examples are seen in the global spread of wheat blast fungus (*Magnaporthe oryzae*) and Ramularia leaf spot in barley (*Ramularia* sp.). Both pathogenic fungi are difficult to detect/culture and have quickly spread between across international boundaries, where in some farms annual yield losses are being reported of up to 70 % (barely infected with *Ramularia* sp.; see Havis et al., 2015) and 100 % (wheat infected with *M. oryzae*; see Ceresini et al., 2019). eDNA-based identifications from agricultural substrates (i.e. leaf material, soil or air) offers a powerful and rapid method for pathogen detection (Figure 2.1). Tordoni et al., (2021) sampled fungal spores from air and were able to identify three times more fungal taxa with eDNA metabarcoding than from manual identifications, indicating that this technique is already helping detect and identify plant pathogens that may otherwise remain undiscovered within cultivated landscapes (Michael et al., 2020). Similarly, Redondo et al., (2020) measured spatio-temporal variation of airborne fungal spores within forest-agricultural mosaic landscapes using passive and active air samplers combined with eDNA metabarcoding. The results showed that fungal spores community compositions were consistently dominated by two potential agricultural pathogens, *Alternaria* spp. and *Ustilago* spp. With similar monitoring on smaller geographic scales, agricultural practitioners could use spatially focused fungicide applications, spraying only in areas where pathogen presence has actually been confirmed, maximising their effective lifespan and improving the return on investment. Further, by reducing and/or targeting fungicide usage, the risk of environmental damage may be minimised compared to more widespread application strategies (Sowunmi, Famuyiwa, Oluyole, Aroyeun, & Obasoro, 2019). We envisage that a comparable eDNA-based monitoring protocol could also have utility for biosecurity monitoring purposes, for example eDNA sampling (air, water-wash, crop surfaces, etc.) could be adopted at points of border control, complementing current techniques to help identify plant pests and pathogens and reduce instances of cross-border outbreaks (Boykin et al., 2019). These eDNA techniques are still emerging for agricultural systems, although their greater adoption holds promise to enhance current detection methods for plant pathogens such as *M. oryzae* and *Ramularia* sp., as well as helping to develop adaptive management solutions.

Spatially focused eDNA surveys can help detect emerging pests and pathogens with timely fine-scale geographical detections which allow for targeted sampling and decisions on control measures. Herbivorous pest insects typically feed on a defined range of host species or specialised groups of plants (Imms, 1947). The techniques traditionally used for identifying these potentially damaging species include; direct observation, microscopy work, rearing of pest insects, and feeding trials (Hamilton et al., 2005; Symondson, 2002; Vu et al., 2018). These traditional methods rely on detailed taxonomic expertise, and also require significant time commitments (Kudoh et al., 2020; Symondson, 2002). For instance, feeding trials can last up to 20 days, not including data analysis, and depending on the target species (Clay, Hardy, & Hammond, 1985; Dunse et al., 2010). Such extended time-periods will delay both detections and the subsequent targeted pesticide response, potentially resulting in major infestations and outbreaks (Kudoh et al., 2020; Simberloff et al., 2013; Valentin et al., 2018). Further, some traditional techniques such as direct observation by taxonomic specialists are simply not feasible given the extremely short generation times of some pest insects (i.e. aphids) and large scales that need to be surveyed in agriculture systems (Edwards et al., 2014; Rouland-Lefevre, 2010; Simberloff et al., 2013). This implies that practitioners are often left to adopt generalised/prophylactic pesticide applications, which are expensive, environmentally damaging and can increase the potential for pesticide resistance (Leskey, Lee, Short, & Wright, 2012; Morales, 2006; Rouland-Lefevre, 2010). As such, there exists a need to rapidly and accurately detect emerging plant pests within food production systems.

Barcoding and metabarcoding herbivorous insect DNA from plant material (i.e. leaves & fruit) or bulk insect traps (e.g. Vane traps & funnel traps) can be an effective means to rapidly assess the presence of pest and beneficial insects on crop and orchard species at large scales (Thomsen & Sigsgaard, 2019; Valentin et al., 2018; Young et al., 2021). Insects leave traces of DNA when they feed and/or excrete on, plant tissue, and this has allowed researchers to retrieve genetic insect identifications for flower-visitors, plant parasites, as well as insect prey (Bittleston, Baker, Strominger, Pringle, & Pierce, 2016; Derocles, Evans, Nichols, Evans, & Lunt, 2015; Kudoh et al., 2020). Such eDNA methods have also proven useful for the detection of pest taxa from plant material in croplands, viticulture and orchards. By using a species-specific assay and targeting rinse water collected after the harvested apples were cleaned, a cost-effective eDNA detection method for the highly invasive and destructive pest species the brown marmorated stink bug (*Halymorpha halys*) proved more efficient than traditional methods of pheromone traps and black lights (Valentin et al., 2018, 2016). Such accurate detection methods are not only important for treating crops post-harvest, but could also be extended to early pre-harvest detections, allowing for targeted pesticide applications before crops are widely damaged (Leskey et al., 2012; Sánchez-

Bayo & Wyckhuys, 2019; Valentin et al., 2018). Further, eDNA-based surveys could help detect co-occurring beneficial insects (i.e. native bees) to assess recovery following broad-spectrum insecticide applications, this approach could determine both the length of time it takes for the pest and beneficial species to return, thereby informing future spraying timings and strategies. Insect traps may also be considered as a complementary means to assess pest emergence which, if combined with traditional identification, can allow for abundance measures as well as molecular verification.

2.4.2 Pest and pathogen surveillance - Livestock

Of globally emerging pathogens, 75 % are estimated to be zoonotic (infect multiple host species including domesticated animals and humans) and twice as likely to be associated with emerging diseases as non-zoonotic pathogens (Taylor, Latham, & Woolhouse, 2001). Zoonotic pathogens in livestock can threaten animal welfare by increasing animal stress, inducing abortions, as well as decreasing overall herd productivity (Mohamed, 2020; Narrod, Zinsstag, & Tiongco, 2012; Saadiid et al., 2020). Such pathogens can also pose direct (i.e. human transmission) and indirect (i.e. economic losses) risks to human health (Alemayehu, Mamo, Desta, Alemu, & Wieland, 2021; Dorjsuren et al., 2020; Mohamed, 2020). Detecting and managing zoonotic pathogens remains challenging in many countries around the world, especially in emerging economies (FAO, 2020; Gebreyes et al., 2014; Paternoster et al., 2020; Thomas et al., 2020). For food production systems in developed economies, preventative measures such as surveillance are now a major focus (Narrod et al., 2012; Smith et al., 2017). Effective surveillance leading to early detection helps to circumvent mass livestock slaughter and quarantine necessary to prevent further spread of disease (Sobrino & Domino, 2001). Current surveillance methods for zoonotic pathogens include the collection of excretory products or blood, the detection of antibodies (either directly from the animals or from a mouse model), Polymerase Chain reaction (PCR) based detection of species-specific pathogens, or pathogen identification via microscopy (Abdel-Moein & Saeed, 2016; Delpietro, Larghi, & Russo, 2001; Rathinasamy et al., 2021; Sulaiman et al., 2003; Vasco, Graham, & Trueba, 2016). These techniques are sufficient for individual zoonotic species identification; however, a greater resolution may be needed given that bacterial, fungal, and viral infections are often made up of complex mutualistic interactions among multiple species (Roossinck, 2015; Roossinck & Bazán, 2017). For this, screening samples using eDNA metabarcoding based on primers with a broader multi-taxonomic detection spectrum could offer support for current surveillance methods.

The ideal mechanism for zoonotic pathogen surveillance is to use standardised individual based sampling, where blood, tissue, faecal, or swab samples are taken from individual animals and tested for an array of pathogens (Brunner, 2020). However, such tests are simply not feasible in either the live export trade or the domestic market, where the large number of samples required makes this financially unfeasible. For instance, the live export trade in Australia alone for 2019-2020 saw 1.3 million cattle and 1 million sheep exported (LiveCorp, 2020). Instead, eDNA analysis of pooled samples from animal consignments is providing a cost-effective alternative, to detect both common and rare zoonotic pathogens with relatively few non-invasive samples (Brunner, 2020; Trujillo-González, Edmunds, Becker, & Hutson, 2019). Indeed, the early use of eDNA-based tools provided health measures for individual animals by analysing diversity of prokaryotes and fungi from ruminal digesta (Fouts et al., 2012). Since then, eDNA measures have been extended (e.g. to detect zoonotic Leptospirosis causing bacteria (*Leptospira*) with universal and species-specific assays in agricultural irrigation water and determine which vertebrate animals act as reservoir hosts, concluding that cattle (*Bos indicus*) and water buffalo (*Bubalus bubalis*) showed a high correlation with the pathogenic bacteria; Gamage et al., 2020). If broadly adopted, eDNA-based monitoring for zoonotic pests and pathogens could provide detections for individual farms and at border control points. In theory, by pooling faecal, urine, or swab samples and using multiple assays, scientists would be able to detect a range of zoonotic pathogens, something not currently possible for large animal consignments (e.g. detection of SARS-Cov2 from sewerage; see Tran et al., 2021) (Brunner, 2020; Carroll et al., 2018). Further research is needed to test and develop this concept, and to establish the optimal baseline number of samples from different substrates (i.e. faecal, urine, or swab) which can be pooled and still provide accurate detections. With this knowledge, eDNA detections could help diagnose emerging zoonotic pests and pathogens with accurate and timely assessments, allowing for preventative measures that benefit both animal welfare and herd productivity.

2.4.3 Soil health – Soil Microbiome, Macrofauna, Mesofauna, and the Rhizobiome

Unlike most other agricultural monitoring efforts, DNA analyses have been the standard tool used to identify soil microorganisms for over two decades (Figure 2.4) (Hugenholtz & Pace, 1996; Rolf, 2005). Primarily because many soil microorganisms are difficult to cultivate and identify with traditional methods (i.e. only 0.1 - 1 % of bacteria are culturable using traditional cultivation methods; Rolf, 2005). The DNA methods used to identify soil microorganisms are analogous to those used for eDNA and metagenomic studies, although these terms have been inconsistently applied in the soil literature (Taberlet et al., 2018). Here, we focus on soil studies in food

production systems that use the term eDNA and measure taxonomic diversity of the soil microbiome (archaea, bacteria, fungi, and eukaryotes) (Figures 2.3, 2.4 and Table 2.1).

Soil microbiome biological and functional diversity are intrinsically linked with plant health and productivity (Barrios, 2007; Delgado-Baquerizo et al., 2017). Biologically diverse soils help suppress soil-borne pests and diseases through predation, competition, and parasitism that in turn benefit crop growth (Barrios, 2007; Susilo et al., 2004). Agricultural intensification practices (e.g. tillage regimes, grazing, and weed management) can however reduce the complexity of these soil food webs, driving parallel reductions in pest and disease-causing pathogen suppression qualities (Adhikari, Perwira, Araki, & Kubo, 2016; de Graaff, Hornslein, Throop, Kardol, & van Diepen, 2019; Tsiafouli et al., 2014). Practices which maintain and enhance soil biodiversity have therefore been identified as important elements of sustainable agriculture and global food security (FAO, 2020; Sarkar et al., 2020). Here, eDNA has enabled the classification of the major biotic components of soil microbiomes in agricultural systems, including archaea, bacteria, fungi and eukaryotes (Frøslev et al., 2021; Makiola et al., 2019; Wang et al., 2020). For example, Frøslev et al. (2021) collected bulk soil samples and amplified eDNA from bacteria, fungi and eukaryotes to determine if the tillage regimes associated with different agricultural practices changed soil biota composition and richness. Less intensive tillage regimes were found to only lead to minor compositional differences in soil microbiota, leading the authors to conclude that although reduced tillage can benefit soil diversity (see Brennan et al., 2006), this may not be the most appropriate strategy in all farming contexts. With greater adoption, eDNA-based monitoring of soil microbiome diversity could be a useful tool to identify soil biodiversity associated with different food production systems, which may ultimately help benefit crop productivity (de Graaff et al., 2019). Although currently there still remains a need to establish baselines for eDNA detections in soil (i.e. length of time eDNA is detected in soil; see (Guerrieri et al., 2021). Integration of these baselines will help develop eDNA datasets which include temporal ranges for detections in various soil substrates (e.g. relative abundance of added *Escherichia coli* eDNA decreased by 98 % after 30 days in control clay-loam soils; see Morrissey et al., 2015). Together, the detailed eDNA community identifications and temporal ranges for the taxa detected could provide a useful tool for agricultural practitioners to help monitor their own soil biodiversity.

Microorganisms only form part of the total biodiversity present in soil, their larger invertebrate counterparts, soil mesofauna (> 40 μm) and macrofauna (> 1 mm) also significantly contribute to soil health, although these taxa are relatively unexplored in agricultural eDNA monitoring (2 % of all studies) (Blouin et al., 2013; Menta & Remelli, 2020; Orgiazzi, Dunbar, Panagos, de Groot,

& Lemanceau, 2015). Current monitoring of soil meso- and macrofauna relies predominantly on morphological identification based on taxonomic keys (George et al., 2017; Gerlach et al., 2013), in contrast, eDNA-based detections offer a standardised high-throughput alternative to classify soil invertebrate diversity (Lanzén, Lekang, Jonassen, Thompson, & Troedsson, 2017; Taberlet et al., 2012a; Todd et al., 2020). An early example is the use of taxa-specific assays to identify extracellular earthworm DNA from soil enabling classification of species assemblages (Bienert et al., 2012). Compared to the time consuming manual detections and morphological identifications typically used, eDNA surveys allowed for the complete description of earthworm communities collected from less than 50 g of soil (Bartlett, Harris, James, & Ritz, 2006; Bienert et al., 2012; Čoja, Zehetner, Bruckner, Watzinger, & Meyer, 2008). More recently, eDNA biomonitoring has been trialled to detect differences in mesofauna communities associated with different horticultural crops. Here, universal and species-specific assays were compared with traditional monitoring in kiwifruit (*Actinidia* sp.) and apple (*Malus domestica*) orchards. Species-specific assays (100 % detection rate) and morphological analysis (40 - 100 % detection rate) performed significantly better than the universal assay (2.5 % detection rate) (Todd et al., 2020). These findings indicate that future meso- and macrofauna surveys may require universal assays which account for DNA degradation (see van der Heyde et al., 2020), or alternatively, morphological identifications can be combined with species-specific assays to survey both known and unknown diversity to increase the accuracy of eDNA biomonitoring. Further development of eDNA-based tools to detect soil invertebrate diversity will require testing in diverse agricultural and horticultural systems across broad and narrow geographic ranges to establish detection limits and verify assay specificity.

The complex microbial associations between plants and their immediate soil environment, the rhizobiome, are an essential component of plant health (Dessaux, Grandclément, & Faure, 2016). These interactions not only help to maintain crop vigour, they also contribute to nutrition and reduce crop stress levels in some instances (Meena et al., 2017; Olanrewaju, Oluwaseyi-Ayansina, Ayangbenro, Glick, & Babalola, 2018; Pandey et al., 2016). Thus, classifying the species composition of rhizobiomes associated with different agricultural and horticultural species has gained significant attention over the last decade (Berendsen, Pieterse, & Bakker, 2012; Castellano-Hinojosa & Strauss, 2021; Visioli, D'egidio, & Sanangelantoni, 2015). Although to date, monitoring rhizobiome diversity to inform management strategies for food production systems has remained relatively unexplored (Aloo et al., 2020; Caldwell et al., 2015). Recent studies have emerged demonstrating the potential of eDNA to identify these rhizospheres within agricultural ecosystems, with implications for developing new management strategies (Table 2.1). For instance, Sternhagen et al. (2020) used eDNA metabarcoding to show that the diversity of

rhizosphere fungi associated with coffee plants (*Coffea* sp.) was lower in conventionally managed fields compared to organic fields. While, Epelde et al. (2020) highlighted that inoculation of lettuce (*Lactuca sativa*) with naturally occurring arbuscular mycorrhizal fungi increased yield without influencing the composition of co-occurring soil fungi. More eDNA studies are now needed to measure rhizosphere diversity across a greater diversity of crop species in different farming contexts (e.g. different soil types, fertiliser inputs, etc.). This information is crucial in developing practices that enhance either overall diversity or the presence of specific beneficial taxa (Dessaux et al., 2016; Pandey et al., 2016; Schmidt, Mazza Rodrigues, Brisson, Kent, & Gaudin, 2020), ultimately increasing crop productivity.

2.4.4 Pollination - Monitoring flower visitors

Wild pollinator numbers have more than halved in some areas of Europe and managed pollinators -typically the European honey bee (*Apis mellifera*) - are starting to mirror these losses with colony collapse reaching 30 % annually both in European nations and North America (Biesmeijer et al., 2006; Gray et al., 2020; Steinhauer et al., 2021). In China, the demand for managed pollinators in 2018 was three times the stock available, a problem predicted to worsen for an ecosystem service valued at US$106 billion in 2010 (Mashilingi et al., 2021). Such pollinator declines are driven by a combination of habitat destruction, agro-chemicals, invasive species, climate change and disease, all of which place further pressure on future food security (Mbow & Rosenzweig, 2019; Potts et al., 2010, 2016; Sammataro, Gerson, & Needham, 2000). An accurate assessment of the health of plant-pollinator networks within cultivated food systems is a crucial first step to prevent further losses (IPBES, 2019; Ricketts et al., 2008; Tylianakis, Laliberté, Nielsen, & Bascompte, 2010; Van Zandt et al., 2020). Regrettably however, pollinator monitoring is insufficient in many areas because observing flower visitors and identifying pollen grains are time-consuming practices that require specialist taxonomic expertise which are becoming increasingly rare (Bell et al., 2016; Bosch, Martín González, Rodrigo, & Navarro, 2009; Howlett et al., 2018; Van Zandt et al., 2020). eDNA biomonitoring has the potential to greatly increase the capacity to study flower-visitor interactions through accurate analyses of large sample numbers, less need for taxonomic expertise, and an ability to detect rare plant-insect interactions (Evans & Kitson, 2020; Pornon, Andalo, Burrus, & Escaravage, 2017; Thomsen & Sigsgaard, 2019). Thomsen and Sigsgaard (2019) were the first to use this approach for biomonitoring in a diverse grassland ecosystem in Denmark. They used two assays to identify 135 arthropod species from more than 60 families, representing insect pollinators, parasitoids, and predators. This successful broad-scale community assessment based on a non-invasive approach supports the concept of using eDNA to identify flower-visiting insects (Evans & Kitson, 2020). This is especially true for the identification of unmanaged pollinators,

which are often less well-known, but, regarded as equally important pollinators for many crop species.

Unmanaged non-bee pollinating taxa have typically been omitted from crop pollination studies (Rader et al., 2016). Consequently, little is currently known about the services they provide or how they are impacted by anthropogenic stressors (Biesmeijer et al., 2006; Garibaldi et al., 2013; Potts et al., 2010; Rader et al., 2016). In an agricultural context, this means that pollination services are often increased only through greater hive numbers, rather than by encouraging native pollinators (Pardo & Borges, 2020; Potts et al., 2010; Rader et al., 2016). eDNA-based monitoring offers a means to help bridge this knowledge gap (Evans & Kitson, 2020). For example, eDNA metabarcoding data obtained from pollen collected by moth species has helped classify the often unobserved nocturnal pollen transport networks within a farmland site (see Macgregor et al., 2019). Metabarcoding analyses increased the number of known pollen types per moth species and resulted in the assembly of more complex flower-visitor networks than could be achieved by traditional microscopy techniques. Similarly, eDNA-based surveys have helped classify a broader range of host plant species and foraging resources for an economically damaging pest species, the turnip moth (*Agrotis segetum*), than had previously reported (see Chang et al., 2018). The use of eDNA to monitor pollinators and flower-visitors is still in its infancy for agriculture and horticulture, although the field is rich with open questions that could be answered with this technology. For example, vertical and horizontal stratification of unmanaged flower-visitors can significantly impact fruit production as a consequence of competition and predation (Cook & Power, 1996; Wyatt, 1983). Despite this, fine-scale variation is rarely measured during agricultural pollinator monitoring (Frimpong et al., 2011; but see Krishnan et al., 2014). Use of eDNA-based monitoring for flower samples collected at different horizontal and vertical stratification levels could help identify if variation exists for flower-visitor cohorts within cultivated tree canopies. This merits investigation because such information could be used to help develop new management practices, such as reducing canopy density, which may encourage more pollinator visitations (managed and unmanaged) and potentially increase yield.

2.5 Limitations, and how to overcome them

eDNA biomonitoring is already demonstrating potential to classify the biodiversity associated with plant, animal and soil health (e.g. classifying meso- and macrofauna diversity in orchard soils; Todd et al., 2020), and to aid in the early detection of invasive pests and pathogens (i.e. detecting Hemiptera pest species from honey; Utzeri et al., 2018) before large-scale outbreaks occur. Such information may enable improved accuracy of evidence-based decision making to inform orchard, farm and vineyard management practices. Despite these prospects, a number of potential pitfalls are associated with the collection, amplification and interpretation of data from environmental samples collected from agricultural systems (Ruppert et al., 2019; Taberlet et al., 2012a). The technical challenges of eDNA-based surveys include; contamination (Olds et al., 2016), false positives (Ficetola, Taberlet, & Coissac, 2016; Ficetola et al., 2015), false negatives (Ficetola et al., 2015), incomplete databases (Jackman et al., 2021), and degraded DNA (Deagle, Eveson, & Jarman, 2006; Goldberg, Strickler, & Fremier, 2018), each of which has been reviewed extensively. Below, we focus on some of the limitations that may currently prevent an efficient implementation of eDNA technology as a biomonitoring tool in agricultural systems, and a discussion of the possible solutions currently available or on the horizon.

2.5.1 DNA deposition and degradation

A better understanding is needed of the mechanisms by which DNA is released into the environment, and how its persistence is affected by various factors in order to take full advantage of eDNA-based biomonitoring technology. These factors include time, chemistry of the local environment (e.g. soil, gut contents, water), UV levels, temperature and microbial presence (Dejean et al., 2011; Levy-Booth et al., 2007; Nielsen, Johnsen, Bensasson, & Daffonchio, 2007). Fast DNA degradation has the potential to create false negative results (i.e. an apparent absence of taxa that are actually present) which can confound biodiversity assessments and lead to incorrect interpretations of community assemblages (Foote et al., 2012; Harrison, Sunday, & Rogers, 2019; Thomsen & Sigsgaard, 2019). For instance, Todd et al. (2020) attempted to analyse soil eDNA collected from two orchards using universal metabarcoding primers which amplified the entire COI gene (710 bp, Folmer primers; Folmer et al., 1994). Likely due to the deterioration of eDNA in the soil environment, this relatively large DNA fragment could not be PCR-amplified (see Jo et al., 2021), meaning that the diversity of ecologically important taxa captured from the metabarcoding results was significantly lower when compared to the results from species-specific PCR assays and manual surveys. Valentin et al. (2021) reported similar results on leaf surfaces with 3 μL of *Halyomorpha halys* eDNA added. Here, simulated rainfall events were found to reduce detection rates by 75 – 100 %, while exposure to high UV levels meant that extracellular

H. halys eDNA could not be detected after four days of full-sun treatment. Amelioration of such issues requires the use of assays which target a range of amplicon sizes to account for DNA degradation (e.g., Haile et al., 2009), or some adaptation of a shotgun sequencing approach where even very short DNA fragments can be sequenced. Further, if a specific taxon is of interest, then species-specific assays should be used for detections rather than relying on universal assays that may have low affinity for certain taxa and also amplify non-target DNA (Saccò et al., 2022). As well as tailoring assay design, establishing detection thresholds for target taxa can also aid in authenticating the taxon identifications generated from eDNA biomonitoring.

Detection thresholds established with pilot studies are occasionally used to determine how long eDNA remains detectable and what sized fragments amplify successfully after exposure to locally relevant factors (e.g. low and high UV levels) (Mächler, Deiner, Spahn, & Altermatt, 2016; Poudel et al., 2019). For eDNA-based tools in agriculture, such information helps provide a temporal range for the detected species or community of interest. For instance, eDNA is unstable in high moisture, high temperature, tiled soils where universal bacterial primers were unable to amplify added DNA (> 99 %) within 7 days because the fragments had degraded beyond the point of amplification for the chosen assay (Sirois & Buckley, 2019). With such information available, long term soil biomonitoring for agricultural regions with higher rates of DNA decay (e.g. tropical countries) could account for more degradation (and increased chances for false negatives) by sampling more frequently and using assays that target shorter DNA fragments (van der Heyde et al., 2020). Goldberg et al. (2018) has recommended that optimised eDNA sampling to account for degradation and dispersion requires data on eDNA production, the space covered by the taxon of interest, and the removal rate of DNA from the system under study (i.e. DNA degradation due to acidic conditions). Similar principles could be applied to eDNA-based monitoring in food production systems to increase the spatial sampling density when DNA degradation is significant or when a conservative approach is needed to capture a rare taxon. Furthermore, as modern agricultural production systems typically include high levels of mechanisation, there are opportunities to design high coverage and high frequency sampling methods that utilise or complement existing machinery and infrastructure. Together these approaches could enable greater accuracy and reproducibility of species detections for orchards, farms, and vineyards.

2.5.2 Assay development and biased amplification

To date, the assays used to target biological organisms within agricultural systems have generally provided broad-community, rather than taxon-specific monitoring (14 % of studies) (Table 2.1). Assay development is restricted when the target taxa are largely undescribed, known as the

Linnean shortfall (Lomolino, 2004). Such shortfalls are common in both microorganisms (i.e. only 3 – 8 % of all fungi described; Hawksworth & Lücking, 2017) and macroorganisms (i.e. only 20 % of all insects described; Stork, 2018). In the context of agricultural communities, microorganisms, unmanaged pollinators, and pests composed largely of unknown species may be missed if the assays used for such classification are too narrow, leading to incomplete community descriptions (Evans & Kitson, 2020). Instead, combinations of assays are needed to target the full variety of taxa present within these ecosystems. This approach is referred to as the 'needle vs haystack', where the 'haystack' metabarcoding (using universal assays) generates sequences from a broad range of taxa to assess complete diversity (generally at the genus or family level) for environmental samples (e.g. using fungal Internal Transcribed Spacer region 2 fragment to target airborne fungal spores; Tordoni et al., 2021). Although it should be noted that universal assays are not a 'silver bullet' and can show taxonomic biases (e.g. COI, a universal primer used for insects, has been shown to amplify only 62% of invertebrates; Horton, Kershner, & Blackwood, 2017) and therefore must be thoroughly tested (*in silico*, *in vitro*, and *in vivo*) prior to monitoring (Saccò et al., 2022). While, the 'needle' (using taxon-specific markers) approach generates sequences specific to individual species or group contained in the 'haystack', (e.g. use of species-specific assay that targets the marmorated stink bug (*H. halys*) from fruit wash water Valentin et al., 2018) (Saccò et al., 2022). This taxon-specific approach is, however, particularly prone to the knowledge gaps associated with Linnean shortfalls. Meaning that researchers may wish to use both the 'haystack' and the 'needle' to investigate a community of interest; specific taxonomic groups are targeted using universal assays that simultaneously capture the many unknown taxa present in environmental samples. The unknown organisms can then subsequently be described using traditional methods and targeted using the species-specific assays if they are of relevance to the orchard, field, or vineyard being surveyed (e.g. emerging pathogen or pollinator).

Biased amplification of specific sequences and the complete failure of other sequences to amplify can prevent effective detection of target species and communities from environmental samples. Although metabarcoding has the potential to detect multiple taxa from complex samples, the universal assays used for such broad assessments can often under-represent or entirely miss particular taxa (Clarke, Soubrier, Weyrich, & Cooper, 2014). In part, this issue arises when the homologous regions targeted and amplified by universal assays are not equally conserved across all taxonomic groups. The resulting sequence variation (i.e. base mismatches) can lead to the biased amplification of certain taxa or prevent amplification entirely (e.g. no bee taxa sequences amplified from vineyard insect traps despite visual confirmation of bees in the traps; Rasmussen et al., 2021) (Bellemain et al., 2010; Rodgers et al., 2017). These incomplete community

descriptions can, if not corrected by manual verification, then misinform management decisions for agricultural practitioners (e.g. unnecessarily increasing bee colony numbers; Ritten et al., 2018). The choice of which universal assay to use is therefore dependent on the presence and suitability of a conserved target locus as well as the availability of target reference sequences (Saccò et al., 2022). With the target locus chosen, the following should be considered for the design and validation stage of metabarcoding assays: i) desktop-based *in silico* validation - collect reference sequences and identify sympatric, and confounding taxa, then design an assay specific to the taxa of interest using tailored design software (e.g. Primer3 or Primer Premier); ii) lab-based *in-vitro* validation - synthetic or organic DNA for the taxa of interest at low concentrations to confirm high PCR sensitivity; and iii) field-based *in-situ* validation - consideration of assays with locally relevant degradation and inhibition found in environmental samples (Harrison et al., 2019; Langlois, Allison, Bergman, To, & Helbing, 2021; Saccò et al., 2022). Taking into account these considerations, assays can be developed which minimise the potential for biased amplification and generate reliable detections for informed management decisions.

2.5.3 Incomplete databases

Inferring taxonomic nomenclature using eDNA for agricultural ecosystems ideally requires the members from the community of interest to have assigned taxonomic ranks, voucher specimens identified and sequence data available (Saccò et al., 2022). The two most widely used databases which contain this information are GenBank and the Barcode of Life Data System (Meiklejohn, Damaso, & Robertson, 2019). Although given the Linnean shortfall and that new species are continually being discovered, direct or even closely related sequence data may not be available in the current databases for the organisms under study (Saccò et al., 2022). Indeed, Aizpurua et al. (2017), when monitoring pest insect species in agricultural landscapes using eDNA, were unable to assign species-level identifications to 53% of the samples collected. This limited the conclusions that could be made about shifts in dietary niche of pest-feeding bats in agricultural landscapes across Europe. For eDNA-based monitoring more broadly, the absence of pest and pathogen sequences could lead to false negatives and potentially fail to identify emerging pest/pathogen outbreaks (Jones, 2009; Valentin et al., 2016). Unfortunately, sequences available on public databases may still be subject to issues such as: incorrect taxonomy, sequence coverage variation (i.e. species barcoded for only one loci), or sequence data without species level taxonomic rank assignment (Saccò et al., 2022). Overcoming these knowledge gaps and inherent database issues can require *in silico* verification, the creation of custom databases, or the use of degenerate secondary assays.

Reference databases need to be assessed with *in silico* studies to determine if the taxa of interest (if known) have been sequenced for the chosen barcode *loci* (Bylemans, Furlan, Gleeson, Hardy, & Duncan, 2018). This desktop search helps identify if the taxa of interest are well represented in online databases or require the creation of a custom Barcode Reference Library (BRL) (Ruppert et al., 2019; Taberlet et al., 2012a). Custom BRLs are traditionally created by Sanger sequencing target barcode *loci* from voucher specimens, these custom barcodes are then incorporated into the chosen bioinformatic pipeline (e.g. OBITools, Barque or QIIME 2; see Mathon et al., 2021) with Basic Local Alignment Search Tool (BLAST) to provide taxonomic identifications (Kress, García-Robledo, Uriarte, & Erickson, 2015). Although a limitation to this approach is the significant expense and time commitment required in diverse agricultural ecosystems where large numbers of unknown taxa have to be sequenced. An innovative alternative is to use genome skimming to produce custom BRLs from many vouchered specimens (Nevill et al., 2020). If voucher specimens are not available however, or low-cost alternatives are needed, *in silico* studies and emerging web-based interfaces, like GAPeDNA, which provide an overview of genetic completeness for a given taxon (see Marques et al., 2021). With this information, a lower resolution secondary assay can be used to generate Family or Order level taxonomic assignments from eDNA samples (e.g. Leese et al., 2021). In the case of Aizpurua et al. (2017), the authors overcame the need to make a local reference database with a secondary low resolution assay to cross-reference species assignments and determine which taxa were missed. Verification may also be possible with traditional methods (i.e. Macgregor et al., 2019; Todd et al., 2020). With these approaches, eDNA-based monitoring for food production systems can generate community data without sequence data necessarily being available for all of the taxa present.

2.5.4 Abundance data

Multi-species reads generated from eDNA samples cannot currently be used to estimate taxonomic abundance or population size for complex environmental samples (Fonseca, 2018; Ruppert et al., 2019). Each PCR reaction in the metabarcoding workflow is unique (e.g. differences in chemistry, primer mismatch, see Cha & Thilly, 1993), meaning Operational Taxonomic Unit (OTU) reads cannot currently be compared quantitatively (Fonseca, 2018). Instead, the data generated from eDNA monitoring provides presence/absence measures for specific taxa and semi-quantitative results (e.g. weak versus strong interactions) (Ficetola et al., 2008; Pornon et al., 2017, 2016). These data can be used to infer relative abundance and commonality for the taxa of interest (e.g. universal fungi assay used to determine relative abundance of natural arbuscular mycorrhizal fungi in managed and unmanaged soils; Epelde et al., 2020). However, some have argued that quantitative counts for populations and taxonomic abundance still remain the gold standard

(Blanchet, Cazelles, & Gravel, 2020). New statistical methods are still being developed for eDNA presence/absence data to help derive ecologically meaningful conclusions, these include; occupancy models to account for imperfect detections of specific taxa (Dorazio & Erickson, 2017; Doser et al., 2022), multiview modelling for relative abundance estimation (Williamson, Hughes, & Willis, 2021), generalised dissimilarity modelling of zeta diversity (Latombe, Hui, & McGeoch, 2017), and joint species distribution modelling for inference of biotic interactions and conditional prediction (Poggiato et al., 2021). Although, as far as we are aware, none of these new statistical methods have been used in the eDNA studies for agriculture found in this review.

Integration of cross-validation techniques as well as alternative technologies to quantify DNA copy numbers may help to increase the robustness of eDNA surveys and generate abundance data. The first, and most relatively straight forward approach is to incorporate traditional surveys (e.g. visual observation) with eDNA surveys, thereby maximising the taxonomic breadth afforded by eDNA while also obtaining abundance data to inform on the strength of ecological interactions (Kelly et al., 2017; Schmidt, Kery, Ursenbacher, Hyman, & Collins, 2013). Alternatively, researches may wish to use multiple species-specific assays combined with droplet digital PCR (ddPCR) (Capo et al., 2021). This approach can be used to quantify the number of DNA sequences and estimate population abundance for the taxa of interest, although recent studies have shown considerable unexplained variation in these estimates (Capo et al., 2021; Mauvisseau et al., 2019). A third solution may be to add one or multiple generic internal standards (ISDs) (e.g. synthetically designed DNA molecules; see Harrison et al., 2020) to all samples prior to qPCR in known absolute abundance (i.e. number of moles of a DNA molecule) (Ushio et al., 2018). Through comparison to the ISD, the relative abundance of target eDNA can be converted into DNA copy numbers (see Harrison et al., 2020; Ushio et al., 2018), potentially allowing for more accurate population abundance estimates for the target taxa. Though, one of the main caveats of ISDs is primer bias causing different sequences to amplify differentially, meaning that pilot studies are essential to validate their effectiveness (Harrison et al., 2020; Ushio et al., 2018). We envisage a combined methodology, where eDNA could be used with universal assays to detect organisms of interest, which could then be counted using either traditional surveys, estimated using multiple species-specific assays with ddPCR or estimated by spiking in ISDs to samples prior to qPCR to estimate population sizes, allowing managers to determine the most appropriate management strategy for the taxa of interest in their orchard, farm or vineyard.

2.6 Future prospects

Applications of eDNA biomonitoring for agriculture are already aiding in the detection of pest and pathogenic species, as well as the classification of soil microbial biodiversity. More recent applications have emerged with biomonitoring of flower-visitors and soil meso- and macrofauna. The field of eDNA biomonitoring for food production systems is burgeoning, with new innovations and areas for future research (Figure 2.2). The topics of research listed below are nascent; however, their continued development holds exciting potential for eDNA-based monitoring to enable more sustainable cultivated food systems and aid global food security.

2.6.1 Air eDNA

Isolation of eDNA from air is a novel survey method capable of detecting and characterising taxa from airborne particles (Folloni et al., 2012; Johnson, Cox, & Barnes, 2019). Initial air eDNA studies targeted airborne pollen (Kraaijeveld et al., 2015; Longhi et al., 2009) and spores (Pashley, Fairs, Free, & Wardlaw, 2012; Williams, Ward, & McCartney, 2001) using aerobiological tape and vacuum pumps. Since then, eDNA has been used to characterise trace amounts of airborne DNA from microbes, plants, fungi, and animals in a variety of systems (Clare et al., 2021; Johnson et al., 2019; Tong et al., 2017; Tordoni et al., 2021). These detections may otherwise remain unknown given that some taxa cannot easily be identified from conventional monitoring or cultivating methods (Folloni et al., 2012; Tong et al., 2017; Tordoni et al., 2021). Although studies targeting air eDNA still remain relatively rare in both the eDNA literature more broadly and agriculture (Clare et al., 2021; Johnson et al., 2019). Namely, eDNA captured from the air accounted for only 7 % of substrates targeted in agricultural studies (Figure 2.3 and Table 2.1). These studies and those undertaken in other human-modified ecosystems do however provide a blueprint for future research to identify airborne DNA in the context of food production systems. For instance, Tong et al. (2017) illustrated that eDNA from archaea, bacteria, fungi and viruses could be collected from active air samplers indoors. This technique could be used to identify airborne microorganisms associated with zoonotic diseases within indoor livestock facilities, such as poultry markets, where disease-causing pathogens circulate but adequate surveillance remains an issue (Lu et al., 2021). Using eDNA to capture signals of emerging pathogens could provide an early warning system to identify the presence of pathogens and potentially isolate infected animals before widespread transmission occurs. Such techniques may also apply to cropping systems, where air eDNA could be used for timely detection of economically damaging weed species. Airborne plant material (vegetative fragments, pollen, etc.) can be captured and targeted with eDNA-based surveys to provide taxonomic classifications for local plants, without them necessarily being in flower (Johnson et al., 2019). In crop fields, air sampling could provide a fine-

scale presence/absence measure of weed species which are often difficult to detect in low numbers (Emenyeonu, Croxford, & Wilkinson, 2018). Such a resource may help inform managers where infestations are emerging and support targeted herbicide applications. Although for now, more studies are needed to determine the basic characteristics of air eDNA (i.e. fragment sizes and taxonomic identity) as well as the abiotic conditions which influence DNA molecule persistence in the atmosphere (Clare et al., 2021; Johnson et al., 2019). Thus, one of the primary questions for cultivated ecosystems is can air eDNA reliably provide taxonomic detections in farms, orchards and vineyards across a variety of different climates, which may have implications for how much eDNA can accumulate and persist in air (e.g. comparison of air eDNA composition in tropical and temperate farmlands).

2.6.2 Organic sentinel monitoring

Biological organisms harnessed as sampling units for the intermediary organisms that they interact with (organic sentinels) could provide an unparalleled ability to measure microcosms which make up agricultural systems (Bromenshenk, Carlson, Simpson, & Thomas, 1985; Bromenshenk et al., 2015; Gregorič et al., 2022; Halliday et al., 2007). Two examples which are relevant to food security include eDNA classifications obtained from within managed beehives and using trace amounts of DNA to detect plant pests and diseases (Sammataro et al., 2000; Tremblay et al., 2019; Utzeri et al., 2019). Managed bee species are currently the most important animal pollinators for cultivated plant species, and safeguarding their services is considered essential for food security (IPBES, 2019; Lautenbach, Seppelt, Liebscher, & Dormann, 2012). When foraging for pollen, bees incidentally collect pathogens that can subsequently be transmitted to the hive (e.g. chalkbrood disease caused by *Ascosphaera apis*) (Goulson & Hughes, 2015; Pereira, Meeus, & Smagghe, 2019). These pathogens are associated with conditions that range from declines in sexual reproductively to increased mortality rates, and can ultimately serve to reduce pollination services in surrounding crop species (Genersch et al., 2010; Lach, Kratz, & Baer, 2015; Pereira et al., 2019; Sammataro et al., 2000). Pathogen classifications using eDNA could help address this issue, potentially allowing for detections that may otherwise be difficult to achieve at the scale of food production systems.

At least 39 viruses and some fungal pathogens use pollen grains as an intermediary between host plants (Card, Pearson, & Clover, 2007). By collecting pollen, and foraging between flowering agricultural species, honeybees can inadvertently act as a vector for these plant pathogens, which can reduce yield and quality of produce (Card et al., 2007; Dodd, Jeger, & Plumbley, 1990; Tremblay et al., 2019). Given that the interactions between bees, and the plants that they pollinate

can have such a significant influence on plant productivity, and that they exhibit predictable and consistent behaviour, their use as organic sentinels merits investigation (Bromenshenk et al., 1985; Bromenshenk et al., 2015). By placing sterilised filter paper at the entrance of a beehive, where the paper would come into direct contact with the bees themselves, as well as the pollen on their bodies, researchers could amplify the trace amounts of parasite and pathogen eDNA collected during foraging in crop fields (Tremblay et al., 2019; Utzeri et al., 2019). With this approach, eDNA-based tools could provide early detections for both significant bee pathogens present in the hive and potentially plant diseases in the fields that the bees are servicing.

2.6.3 DNA sequencing in the field

Taking eDNA biomonitoring out of the laboratory and into farms, orchards and vineyards offers a rapid means to monitor organisms, while simultaneously reducing processing costs (Boykin et al., 2019; Loeza-Quintana, Abbot, Heath, Bernatchez, & Hanner, 2020). Significant expenses are associated with high-throughput laboratory-based sequencing platforms, especially with the input of skilled technicians required for successful data generation (Skinner, Murdoch, Loeza-Quintana, Crookes, & Hanner, 2020; Thomas et al., 2019). Not only are laboratory costs expensive, but the processing times for eDNA samples can also take weeks or sometimes even months depending on the number of samples and assays, often requiring refrigeration and taking samples back to the laboratory, potentially delaying opportunities for rapid detections (Loeza-Quintana et al., 2020; Nguyen et al., 2018). In agriculture, real-time monitoring is often critical for timely and informed management decisions, especially when monitoring disease-causing pathogens and pest outbreaks (Badial et al., 2018; Boykin et al., 2019; Valentin et al., 2018). Portable PCR machines (i.e. Field-portable quantitative PCR (qPCR)) and sequencers (i.e. Oxford MinION) were initially used as a human-point of care tool for disease diagnostics (Marx, 2015; Nguyen et al., 2018; Quick et al., 2016). Now these portable technologies are being utilised as a diagnostic tool for invasive species and pathogens with implications for food production. Badial et al. (2018) were the first authors to successfully detect crop pathogens from infected plant tissue and insect vectors using the portable Oxford MinION sequencer. In contrast to the standard immune assays and multiplex PCR used to detect plant pathogens, the Oxford MinION could detect a larger number of possible target pathogens in less than two hours. This technology has been trialled on small-scale cassava farms in sub-saharan Africa, where researchers were able to generate on-the-spot pest and disease diagnostics within one day (Boykin et al., 2019). Similarly, field-portable qPCR tools used in combination with species-specific primers, have been used to streamline a workflow that traditionally required three days on the laboratory bench into less than 60 minutes (Thomas et al., 2019). The information generated from these rapid tests could be used to help identify specific

pathogens at fine spatial scales, thereby enabling targeted pesticide applications while also reducing expenditure and minimising environmental harm (Badial et al., 2018). In the future, this technology could be extended to rapidly assess other taxa of economic value (e.g. wild pollinators, soil biodiversity, etc.) within food production systems when linked with appropriate sampling techniques (e.g. sampling air, honey or soil). Used in combination with improved bioinformatic pipelines (i.e. PEMA; see Pafilis et al., 2020, and eDNAFlow; see Mousavi-Derazmahalleh et al., 2021), portable PCR and sequencing technologies hold great potential for eDNA in food production systems, although much work is required to establish the protocols and limitations of these technologies.

2.6.4 Equitable eDNA monitoring – LAMP assays

eDNA-based monitoring requires precision equipment as well as ultra-clean laboratories (Ficetola et al., 2016; Yoccoz et al., 2012). Unfortunately, these equipment and facilities are typically underrepresented in developing countries where food security concerns are often greatest (FAO, 2020; Hamdi et al., 2021; Mbow & Rosenzweig, 2019). Likely this is also the reason for the low number of eDNA studies found in emerging economies (Figure 2.3). Low-cost equivalents for eDNA biomonitoring are therefore needed for the countries in greatest need of this technology, but who may lack the necessary infrastructure (Ibaba & Gubba, 2020). Loop-mediated isothermal amplification (LAMP) assays may provide one such alternative by allowing for the identification of individual species without the need for laboratories, PCR machines, or high-throughput sequencers (Ahuja et al., 2021; Davis et al., 2020; Notomi et al., 2000). Using only species-specific assays, DNA polymerase, a water bath/heating block, as well as a stain or dye, researchers have been able to identify the presence/absence of species of interest from eDNA samples (Davis et al., 2020; Notomi et al., 2000; Quyen, Ngo, Bang, Madsen, & Wolff, 2019). To date, LAMP assays have been used to detect a wide variety of plant and animal pathogens predominately in natural settings (Ahuja et al., 2021; Deng, Zhong, Kamolnetr, Limpanont, & Lv, 2019; Panno et al., 2020). While more recently, the technique has successfully been used within an agricultural context to measure the presence of an intermediary host species (*Galba truncatula*) for two trematodes (parasitic flatworm); *Fasciola hepatica* and *F. gigantica*, both of which cause the potentially fatal Fascioliasis disease in livestock (Davis et al., 2020; Deng et al., 2019). Although in the developmental stage, the potential to use LAMP assays in combination with multiple species-specific markers (one marker per reaction) could provide a low-cost counterpart for conventional eDNA-based monitoring in agricultural ecosystems and potentially aid in the timely detection of known plant and animal pathogens, hopefully helping prevent pest and disease outbreaks for food production systems in both developed and emerging economies.

2.7 Conclusion

Given the extensive use of eDNA in natural systems, biomonitoring using eDNA in agricultural systems is underutilised despite it being a potentially powerful tool to measure a wide variety of microcosms (Figure 2.5). Applications of this technology in food production systems are still in their infancy, with the exception of the soil sciences (Figure 2.4), and the field remains wide open for future eDNA applications for both cultivated plants and domesticated animals (Figures 2.4 and 2.3). Here, we have highlighted the growing number of studies that are now identifying specific species, monitoring communities, and rapidly detecting pests and pathogens in agriculture (Figure 2.4). We acknowledge that there are limitations to using eDNA for species identifications and detections within food production systems, and that as a consequence, applications of eDNA will not be equally effective in all settings and that current traditional and other molecular methods will still be the best practice in such cases (e.g. Todd et al., 2020). However, we argue that with further research into the locally relevant conditions for eDNA degradation, adequate pilot studies, and the development of local reference libraries, eDNA-based tools will offer a strong complement for current monitoring methods, and merits further integration into agricultural systems. In the future, eDNA is likely to include; unmeasured microcosms, sequencing in the field, as well as the wide-spread uptake of cost effective equivalent techniques. With such expansions, eDNA will offer a powerful tool to help maintain and increase food production with the ultimate goal of helping achieve more widespread food security for food production systems in developed and emerging economies alike.

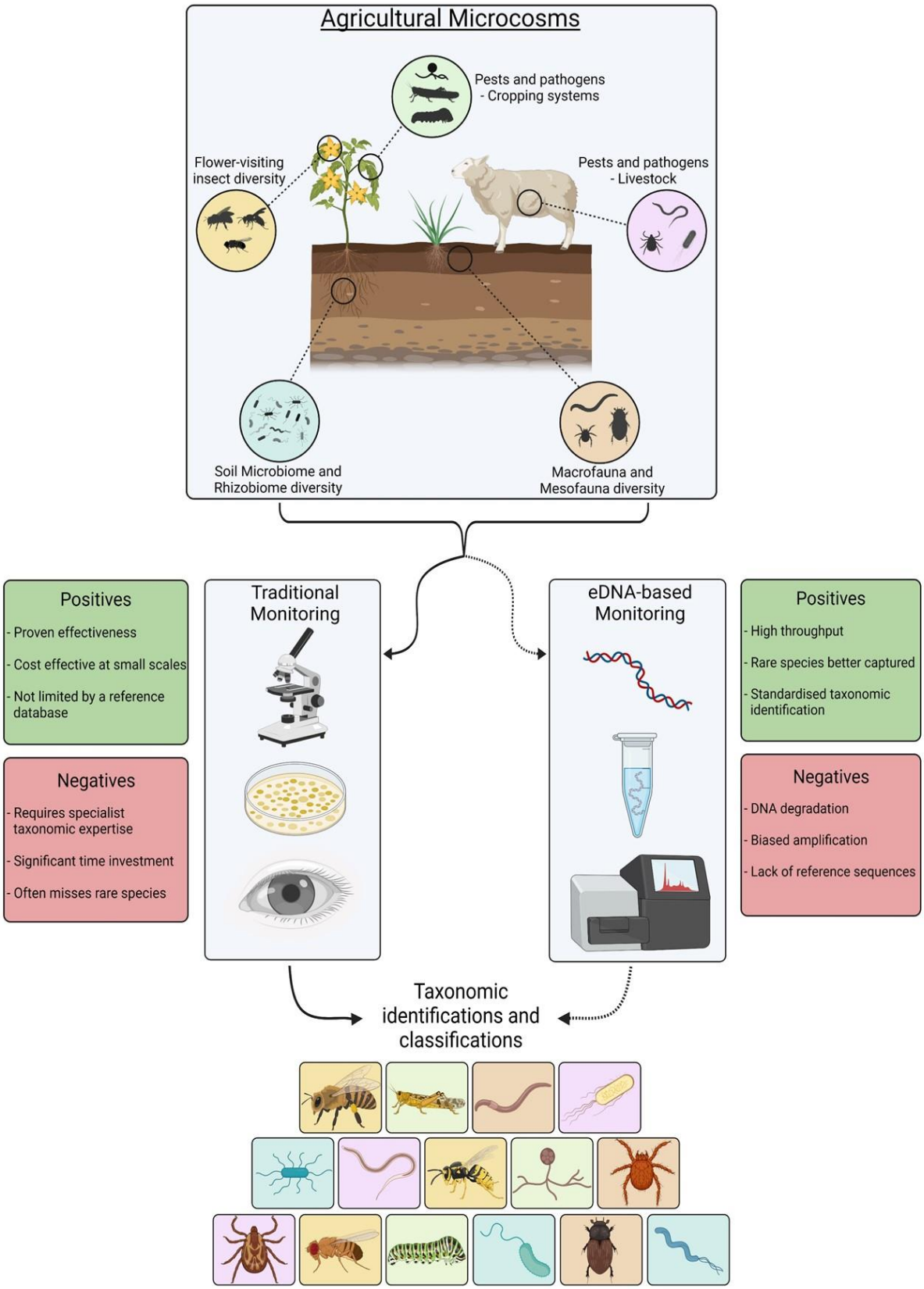

Figure. 2.5. Current and emerging monitoring techniques for agricultural microcosms. Substrates presented are currently being monitored using both traditional and eDNA-based monitoring to detect mutualistic (i.e. pollinators) and antagonistic species (i.e. pathogens). The potential to further incorporate eDNA biomonitoring (dashed line) to support current methods (solid line)

would greatly aid in taxonomic identifications and hopefully improve monitoring for food production systems. Both techniques are presented with some of the major pros and cons which have been identified here, and in other studies (Kelly et al., 2017; Pornon et al., 2017; Rasmussen et al., 2021; Todd et al., 2020). Graphic created using BioRender.

2.6 References

A thorough effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Chapter 3. eDNA metabarcoding of avocado flowers: ‘Hass’ it got potential to survey arthropods in food production systems?

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3.1 Abstract

In the face of global biodiversity declines, surveys of beneficial and antagonistic arthropod diversity as well as the ecological services that they provide are increasingly important in both natural and agro-ecosystems. Conventional survey methods used to monitor these communities often require extensive taxonomic expertise and are time-intensive, potentially limiting their application in industries such as agriculture, where arthropods often play a critical role in productivity (e.g. pollinators, pests and predators). Environmental DNA (eDNA) metabarcoding of a novel substrate, crop flowers, may offer an accurate and high throughput alternative to aid in the detection of these managed and unmanaged taxa. Here, we compared the arthropod communities detected with eDNA metabarcoding of flowers, from an agricultural species (*Persea americana* - 'Hass' avocado), with two conventional survey techniques; Digital Video Recording (DVR) devices and pan traps. In total, 80 eDNA flower samples, 96 hours of DVRs and 48 pan trap samples were collected. Across the three methods, 49 arthropod families were identified, of which 12 were unique to the eDNA dataset. Environmental DNA metabarcoding from flowers revealed potential arthropod pollinators, as well as plant pests and parasites. Alpha diversity levels did not differ across the three survey methods although taxonomic composition varied significantly, with only 12% of arthropod families common across all three methods. eDNA metabarcoding of flowers has the potential to revolutionise the way arthropod communities are monitored in natural and agro-ecosystems, potentially detecting the response of pollinators and pests to climate change, diseases, habitat loss and other disturbances.

3.2 Introduction

Effective management of food production systems requires detailed knowledge of both their abiotic (e.g. climate) and biotic features (e.g. ecosystem services), herein referred to as agroecosystems (Lippert, Feuerbacher, & Narjes, 2021; Savary et al., 2019; Wada et al., 2016). Of the many biotic features which make up agroecosystems, animal-mediated pollination is one of the most critical, with at least 75% of cultivated plant species relying on this ecosystem service to improve both the quantity and quality of crop yield (Aizen, Garibaldi, Cunningham, & Klein, 2009; Garibaldi et al., 2013; Ricketts et al., 2008). Consequently, pollination services have considerable value for countries with large agriculture sectors, including the United States ($US30 billion in 2012; Jordan, Patch, Grozinger, & Khanna, 2021), China (US$106 billion in 2010; Mashilingi et al., 2021) and Australia ($US1.1 billion in 2003; Hein, 2009). Unfortunately, the majority of pollination services within agroecosystems are largely reliant on pollinating taxa that are increasingly threatened by climate change and pathogens.

Currently, the majority of animal-mediated pollination services in agroecosystems are reliant on managed insects, primarily the European honeybee (*Apis mellifera*), to facilitate cross-pollination (Potts, Imperatriz-Fonseca, & Ngo, 2016). However, the focus is increasingly shifting towards unmanaged insects (e.g. native bees, flies and moths), which are now recognised as important contributors to global crop pollination (Cook et al., 2020; Garibaldi et al., 2013; Rader et al., 2016). This shift has become necessary as *A. mellifera* hives and the pollination services they provide are increasingly threatened by mites and fungal infections (e.g. *Varroa destructor* and *Ascosphaera apis*), and associated diseases (e.g. Varroosis and Chalkbrood disease) (see Goulson & Hughes, 2015; Sammataro, Gerson, & Needham, 2000). These biotic pressures weaken hives and increase the likelihood of colony collapse, a phenomenon which is currently estimated to affect over 30% of hives annually in the USA, Canada and many European nations (see Biesmeijer et al., 2006; Gray et al., 2020; Steinhauer et al., 2021). In response, practitioners and researchers alike are increasingly promoting the value of unmanaged pollinators, although surveys to detect such taxa remain relatively uncommon in agroecosystems.

Surveying arthropod diversity to determine the presence or absence of beneficial (e.g. pollinators, predators) and antagonistic species (e.g. herbivorous pests, arthropod vectors) is critical for managing the health of agroecosystems and increasing food security (Barrios, 2007; Kestel et al., 2022; Letourneau et al., 2011; Senapathi et al., 2021). To date, identifying these taxa has largely relied upon passive trapping (e.g. pan, Malaise and vane traps), visual observation, and active survey techniques (e.g. sweep netting) (Gervais, Chagnon, & Fournier, 2018; Kearns & Inouye,

1993; Prado, Ngo, Florez, & Collazo, 2017; Shi et al., 2022). Indeed, pan, Malaise and vane traps are some of the most commonly used methods to measure bee diversity in agroecosystems, largely because they provide a low-cost means to sample multiple sites simultaneously (McCravy, 2018; Prado et al., 2017; Spafford & Lortie, 2013). Studies using these passive survey methods have, for example, demonstrated the benefit of adjacent natural habitats for pollinator abundance and crop yield (see Klein et al., 2012; Morandin & Winston, 2006), and identified the inverse relationship between cultivated land use and wild pollinator diversity, particularly for wild bee species (see Bergholz, Sittel, Ristow, Jeltsch, & Weiss, 2022; Zou et al., 2017). Unfortunately, passive sampling techniques often require extensive time commitments and increasingly rare specialist taxonomic expertise to morphologically identify the arthropod taxa collected or observed (Brown, 2020; Pardo & Borges, 2020; Shi et al., 2022). Furthermore, even when morphological identifications are possible, passive sampling techniques often have intrinsic biases in the taxa that are captured. For instance, pan traps capture a range of taxa that share an attraction to the trap (e.g. bees attracted to blue pan traps; see Joshi et al., 2015), but are not necessarily ecologically relevant to the system under study (e.g. not all captured pan trap insects are flower-visitors or pollinators; see Popic, Davila, & Wardle, 2013). Pan traps also disproportionately capture small insect taxa (see Prado et al., 2017) and suffer from variable capture rates due to placement position (e.g. sampling under forest canopies can reduce capture rates for pan traps; see Abrahamczyk, Steudel, & Kessler, 2010). As a consequence of these limitations, passive sampling techniques are often complemented by visual observations and active surveys to provide more accurate measures of arthropod diversity (see Prendergast, Menz, Dixon, & Bateman, 2020) and overcome the biases of each individual technique.

Visual observations and interpretation of the relationships between arthropods and the plants they pollinate have been a part of scientific inquiry since insect pollination was first documented in the 18th Century (Baker, 1979; Kolreuter, 1761; Sprengel, 1793). Detailed observations of flower visits can be difficult to achieve however, as the process is generally time-consuming and often limited in sample size (Bosch, Martín González, Rodrigo, & Navarro, 2009; Waser, Chittka, Price, Williams, & Ollerton, 1996). Further, visual identification of each flower visitor requires specialist taxonomic expertise, which may become increasingly inaccurate as more species visit (Bosch et al., 2009; Ebeling, Klein, Schumacher, Weisser, & Tscharntke, 2008; Van Zandt et al., 2020). Observational-based studies may also fail to capture irregular movement patterns typically shown by floral-visiting insects, increasing the potential of misclassification of generalist and specialist relationships (Pornon, Andalo, Burrus, & Escaravage, 2017; Thomsen & Sigsgaard, 2019; Waser et al., 1996). In the context of agroecosystems, these issues can reduce the accuracy and

effectiveness of arthropod surveys. As a result, new survey methods are being used that complement visual observations, one of the most notable being digital video recording (DVR) devices. DVR devices have gained attention as a means to monitor flower-visitor interactions because they can capture multiple flower visits simultaneously across many plants, the recordings can then be watched to obtain taxonomic and behavioural data (e.g. animal identity, stigma contact; see Krauss et al., 2017). Previous studies have shown that DVRs of *A. mellifera* foraging on to *Lavandula angustifolia* provided significantly similar visit rates to visual observations, while also showing that this technology can capture over four times the number of interactions between Hymenoptera species and flowering plants than with visual observations alone (Gilpin, Denham, & Ayre, 2017; Naqvi, Wolff, Molano-Flores, & Sperry, 2022). Despite such promise, DVR devices are often limited by their resolution and the size of the visiting arthropod, both of which can limit the number of detections possible (although see Droissart et al., 2021; Steen, 2017), and prevent accurate taxonomic identifications below the family level (e.g. Bonelli et al., 2020). As such, DVR devices do not currently provide a 'silver bullet' for monitoring flower-visits and alternative technologies and methods are still required.

Recently, environmental DNA (eDNA) metabarcoding has been added to the biodiversity survey tool kit, whereby DNA barcodes from multiple organisms can be sequenced in parallel eDNA metabarcoding of environmental samples (e.g. soil, water and now air). It has been widely used to monitor aquatic and terrestrial systems (e.g. Capo et al., 2021; Clare et al., 2021; van der Heyde et al., 2020), but studies of plant-animal interactions using eDNA extracted from flowers are rare (Gomez, Sørensen, Chua, & Sigsgaard, 2023; Johnson et al., 2023; Newton et al., 2023; Thomsen & Sigsgaard, 2019), and few have systematically compared metabarcoding of arthropod DNA on flowers to other survey methods, despite alternative approaches potentially detecting different taxa (Gomez, Sørensen, Chua, & Sigsgaard, 2023; Newton et al., 2023). We compared two commonly used arthropod survey methods - pan traps and DVR devices - with two common eDNA barcoding assays, to detect a wide range of arthropods. We applied eDNA metabarcoding as it would be deployed in many agroecosystems around the world, by using existing arthropod metabarcoding assays (see Clarke, Soubrier, Weyrich, & Cooper, 2014; Vamos, Elbrecht, & Leese, 2017), and without comprehensive arthropod DNA barcode reference libraries for the study region (Rasmussen et al., 2021; Young, Milián-García, Yu, Bullas-Appleton, & Hanner, 2021). Our aim was to understand the extent to which different arthropod survey methods complement one another, and ultimately improve the monitoring of plant-animal interactions in agroecosystems.

3.3 Methods

3.3.1 Field Site

For this study, inflorescences were collected from a *Persea americana* ('Hass' Avocado) orchard, Marron Brook Farm (34°18′52 S, 116°08′36 E), located in the avocado production region of Manjimup-Pemberton in south-west Western Australia (SWWA) (Mccarthy & McCauley, 2020). DVRs and pan trap sampling were carried out at the same time that inflorescences were collected from the study orchard. In the Manjimup-Pemberton region, the dominant land uses are pasture and orchards, interspersed with remnants of native karri forest (*Eucalyptus diversicolor*). Orchards in this region are largely reliant on hiring managed *A. mellifera* hives to facilitate cross-pollination (Mccarthy & McCauley, 2020), although the importance of unmanaged arthropods to complement these services remains unclear (Ish-Am, 2005; Ish-Am & Eisikowitch, 1998; Mccarthy & McCauley, 2020). Marron Brook Farm sits ca. 200 m above sea level and is dominated by 'Hass' trees interspersed with the 'Fuerte' variety, which acts as a polliniser tree providing outcrossed pollen for 'Hass' fruit production. Unlike many other orchards in the region, Marron Brook Farm cultivates an understorey of wild radish (*Raphanus raphanistrum*), which grows to a height of 1 m, and aims to encourage avocado pollinator presence. We randomly selected eight 'Hass' trees between eight columns of 41 trees within this orchard, all of which were eight years old and of heights between 3 – 5 m. The final three columns and rows were excluded from sampling in both orchards to help reduce the impact of edge effects. For each sample tree, ten *P. americana* inflorescences were removed for eDNA analysis during the peak *P. americana* flowering season in 2020 (October 30th and 31st) (Figure 3.1A).

Figure 3.1 Three methods used to measure flower-visiting arthropods for *Persea americana* at Marron Brook Farm in Pemberton, Western Australia. (A) Inflorescences were removed from upper and lower storey of *P. americana* trees for eDNA metabarcoding. Lower understorey inflorescences were removed using sterilised hand secateurs (not pictured), while the upper storey inflorescences were removed using extended secateurs which were captured in net lined with a sterilised plastic bag (pictured). Inflorescences were then placed on ice until they could be stored at – 20°C. (B) Two inflorescences per tree were monitored for 6 hours over two days using GoPro Hero 7 Silver cameras. (C) Three pan traps (white, blue and yellow) were deployed for 16 hours over two days to capture flying insects. Images captured by Diana Adorno.

3.3.2 eDNA surveys

3.3.2.1 Sample collection and DNA extraction

Prior to sampling, a pilot study determined that more arthropod eDNA detections (fwhF2/fwhR2n assay; Vamos et al., 2017) were obtained for flowers ground in a mortar and pestle than metabarcoding MilliQ wash water from entire inflorescences (results not shown). For eDNA analysis, five inflorescences were collected from both the upper (> 2 m) and lower canopy (< 2 m) of each *P. americana* tree (*N* = 10 inflorescences per tree, *N* = 80 inflorescence total). Six inflorescences were collected from each tree on the 30th of October 2020 (three upper canopy, three lower canopy) and four inflorescences (two upper canopy and two lower canopy) were collected from each tree on the 31st of October 2020 (Figure 3.1A). Both days were sunny with low winds and no rain (Table S3.1). To minimise sampling bias, inflorescences were sampled randomly from both the upper and lower canopy while walking around the full circumference of each *P. americana* study tree (collection method adapted from Howlett et al. 2018). Inflorescences were removed from the lower canopy using sterilised hand secateurs, and inflorescences in the upper canopy were sampled using a net covered with a clean plastic bag replaced after each sample, and sterilised extended secateurs. To minimise cross-contamination, all equipment was sprayed with 10% bleach solution and wiped down after each inflorescence was collected. Once removed, each inflorescence was placed into a thick plastic bag, zip-tied and kept on ice until the samples could be stored at – 20°C. Frozen inflorescences were processed in the TrEnD laboratory at Curtin University. For inflorescence processing, open florets of each inflorescence were removed with doubled-gloves (changed after every inflorescence) and placed in a mortar and pestle where the plant material was ground into a fine paste. Mortars and pestles were soaked in 10% bleach solution, rinsed with reverse osmosis water, and placed in a UV oven for 15 mins to prevent cross contamination between samples. In total, 140 – 190 mg of ground material was weighed out and transferred into a 2 ml safe-lock Eppendorf tube with 540 µl ATL buffer and 60 µl Proteinase K (Qiagen, Venio, Limburg, Netherlands). Samples were digested in a slow-rotating hybridisation oven at 56°C overnight (~12 hrs). Following digestion, DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Venio, Limburg, Netherlands) using a QIAcube Connect automated DNA extraction platform (Qiagen, Venio, Limburg, Netherlands). The final elution volume was 100 µl, and extraction controls (blanks) were carried out for every batch of DNA extractions.

3.3.2.2 PCR amplification

Quantitative Polymerase Chain Reaction (qPCR) (Applied Biosystems, USA) was used to assess the quality of each eDNA sample targeting the Cytochrome Oxidase subunit 1 (CO1) and 16S ribosomal RNA subunit genes. Inhibitors in the PCR reactions and low copy number can impact

metabarcoding data (Murray, Coghlan, & Bunce, 2015; Murray, Bunce, Cannell, Oliver, & Houston, 2011), therefore each eDNA extract was assessed with a qPCR dilution series (neat, 1/10, 1/100) under the following conditions: 25 µl reaction volumes containing 2.5 µl of 10 × PCR Gold Buffer (Life Technologies, Massachusetts, USA), 2 µl of 2.5 mM $MgCl_2$ (Fisher Biotec, Australia), 1 µl of 0.4 mg/ml BSA (Fisher Biotec), 0.25 µl of dNTPs (Astral Scientific, Australia), 0.6 µl of 5x SYBR Green (Life Technologies), 0.4 µl of each primer (forward and reverse; 10 mM), 0.2 µl AmpliTaq Gold (Life Technologies), 2 µl of DNA and the remaining volume supplemented with DNase/RNase-Free Distilled Water. Two PCR assays were used: assay fwhF2/fwhR2n (Vamos et al., 2017), targeting the CO1 gene, herein referred to as CO1, and assay Ins_16S_shortF/Ins_16S_shortR (Clarke et al., 2014), targeting the 16S ribosomal RNA subunit gene, herein referred to as 16S. The forward primer sequence for CO1 was 5'—GGDACWGGWTGAACWGTWTAYCCHCC—3' and reverse primer sequence 5'—GTRATWGCHCCDGCTARWACWGG—3'. The forward sequence for 16S was 5'—TRRGACGAGAAGACCCTATA—3' and reverse sequence 5'—ACGCTGTTATCCCTAAGGTA—3'. Amplicons for each assay were ~205 bp and ~167 bp for CO1 and 16S, respectively. Extracts were amplified on a StepOnePlus Real-Time PCR System (Applied Biosystems, Massachusetts, USA) under the following conditions for CO1: initial denaturation at 95°C for 5 min, followed by 50 cycles of 30s at 95°C, 50°C for 30s and 2 min at 72°C, with a final extension for 10 min at 72°C. For 16S the conditions were as follows: initial denaturation at 95°C for 5 min, followed by 50 cycles of 30s at 95°C, 51°C for 30s and 45s at 72°C, with a final extension for 10 min at 72°C. Extraction and non-template controls were included in each qPCR assay. DNA extracts that showed inhibition were diluted using MilliQ water and the optimum quantity of DNA input was determined for fusion tagging.

Environmental DNA that were of sufficient quality and free of inhibition, as determined from the initial qPCR screen (qPCR dilution series), were assigned a unique (6 – 8 bp in length) multiplex identifier tag (MID-tag) for both the CO1 and 16S assays. To reduce the likelihood of contamination, chimera production and MID-tag jumping (Esling, Lejzerowicz, & Pawlowski, 2015), DNA was amplified in a single round of qPCR for each assay using MID-tag primers consisting of either the CO1 or 16S primers coupled to Illumina flow cell adaptors, custom sequencing primers and MID-tag combinations unique to this study. All fusion-tagged qPCR reactions were prepared in dedicated clean room facilities at the TrEnD Laboratory, Curtin University designed for ancient DNA work using an automated QIAgility robotics platform (Qiagen, Venio, Limburg, Netherlands) and were carried out in 25 µl reactions containing 2.5 µl of 10 × PCR Gold Buffer (Life Technologies), 2 µl of 2.5 mM $MgCl_2$ (Fisher Biotec), 1 µl of

0.4 mg/ml BSA (Fisher Biotec, Australia), 0.25 µl of dNTPs (Astral Scientific), 0.6 µl of 5x SYBR Green (Life Technologies), 0.5 µl of either forward primer (20 mM), 5 µl of either reverse primer (2 mM), 0.2 µl AmpliTaq Gold (Life Technologies), 4 – 8 µl of DNA and the remaining volume supplemented with DNase/RNase-Free Distilled Water. MID-tag PCR amplicons were carried out in duplicate reactions to control for PCR stochasticity and all fusion-tagged qPCRs were processed using the same parameters as the initial qPCR screens described above.

3.3.2.3 DNA library preparation and Sequencing

Replicate MID-tag amplicons were pooled at approximately equimolar concentrations (e.g. minipool) based on their respective qPCR DRn values and were measured under a high-resolution capillary electrophoresis system (QIAxcel; Qiagen Venio, Limburg, Netherlands) and the final library was size-selected (160 – 425 bp) using a PippinPrep (Millennium Science Pty Ltd, Australia) with a 2% ethidium bromide cassette (Sage Science, Beverly, USA) to remove any off-target amplicons and primer dimer. The final library was purified using the QIAquick PCR Purification Kit (Qiagen Venio, Limburg, Netherlands), quantified using a Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, USA) and diluted to 2 nM prior to sequencing. Sequencing by synthesis was performed on an Illumina MiSeq platform (Illumina, San Diego, USA) located in the Trace and Environmental DNA lab at Curtin University and as per Illumina's protocol for single-end sequencing with a 300 cycle MiSeq®V2 reagent kit and standard flow cell for environmental metabarcoding.

3.3.2.4 Data processing

Sequenced multiplex identifier-tagged amplicons were inputted to a containerised workflow (eDNAFlow; Mousavi-Derazmahalleh et al., 2021) and run through the Pawsey Supercomputing Centre in Kensington, Western Australia. Here, the sequences were filtered, formed into Zero-radius Operational Taxonomic Units (ZOTUs) and assigned taxonomic identifications. Sequences were quality checked using FASTQC (Andrews, 2010) and quality filtered (Phred quality score < 20), before the multiplex identifiers were trimmed from the sequence reads using AdapterRemoval v2 (Schubert, Lindgreen, & Orlando, 2016). Subsequently, the filtered reads were demultiplexed using OBITOOLS (Boyer et al., 2016) and sequences shorter than the minimum length of 120 bp were filtered out. Sequences were then dereplicated into ZOTUs with a minimum sequence abundance of 5 (see van der Heyde et al., 2020) using the USEARCH Unoise3 algorithm (Edgar, 2016). A database of ZOTUs was then generated and queried against the GenBank (NCBI) nucleotide database with 100% query coverage and 95% identity using BLASTN (Altschul, Gish, Miller, Myers, & Lipman, 1990). Erroneous ZOTUs with a sequence similarity below the 95% threshold were removed using the LULU post clustering curation method (Frøslev et al., 2017).

Finally, a custom Python script (eDNAFlow; Mousavi-Derazmahalleh et al., 2021) was used to assign taxonomic identifications to the curated ZOTUs using the Lowest Common Ancestor (LCA) approach. Taxonomic identification was assigned to a ZOTU when the percentage identity of two or more queried sequences with ≤ 1% difference had 100% query coverage and 97% sequence similarity. For the purposes of this study, we set the minimum threshold count of 5 reads for ZOTUs to classify a taxa as present within a sample.

3.3.2.5 DVR and pan trap surveys

Two GoPro cameras (Hero 7 Silver) were mounted on 1.5 m wooden stands to observe two of the ten sample inflorescences per study tree (Figure 3.1B). Due to the limited number of DVR devices and the complexity of building and transporting taller stands, only inflorescences in the lower canopy (< 2 m) were monitored. Each DVR device was set to Time-lapse mode (one image every 0.5 s) to maximise battery life. During video observations, three hours of arthropod visits per day for the two sample days were recorded and condensed into two 11 min 50 s videos (N = 64 DVRs). On the 30th of October DVRs commenced between 11:25 am – 12:25 pm and recorded a total three hours of footage, while on the 31st of October the DVRs commenced between 8:35 am – 9:23 am and recorded a total of three hours of footage. DVRs were deployed when temperatures were warm enough for bee activity (clear skies, wind speed <30 km/h, and >17°C; Prendergast et al., 2020). DVRs were downloaded at the end of each sampling day. One researcher watched each DVR on 0.4 x speed to allow for individual visitations to be classified (method adapted from Gilpin, et al. 2017). A visit was noted when an arthropod made contact with a flower on the inflorescence, subsequent flower contacts were not noted (method adapted from Sakamoto, Morinaga, Ito, & Kawakubo 2012). If an arthropod flew out of frame and then revisited the same inflorescence, this was counted as a new visit. Arthropod images were grouped into morphotypes and identified to the species level, where possible, using photographic reference material and descriptions from Zborowski & Storey (2017).

Pan traps offer a non-invasive, efficient and cost effective means to measure arthropod diversity without observer bias (Westphal et al., 2008; Wilson, Griswold, & Messingery, 2008). In contrast to other active sampling techniques, pan traps are effective at capturing arthropod communities independent of floral resource availability (see Popic et al., 2013), and may better capture beneficial (e.g. predators) and antagonistic (e.g. pests) taxa relevant to agricultural systems. In the present study, standard pan trapping procedures were followed, whereby three 4.8 cm x 10 cm polypropylene picnic bowls were painted either yellow, blue or white using waterproof enamel-based paint (Kearns & Inouye, 1993; Saunders, Luck, & Mayfield, 2013; Zou et al., 2017). Each

set of three bowls were deployed near the base of the eight chosen *P. americana* trees within the study orchard. Each bowl was filled with ~ 250 ml of ultrapure water and one drop of detergent. The three bowls were attached to a piece of chipboard with adhesive putty to prevent them from blowing over (Figure 3.1C). Pan traps were set up between 10:30 am – 12:40 pm on the 30th of October and remained in the orchard for 5 – 6 hours, while on the 31st of October, pan traps were set up between 9:00 am – 10:00 am and remained in the orchard for 5 – 6 hours. Pan traps were set up when temperatures were warm enough for bee activity (clear skies, wind speed <30 km/h, and >17°C; Prendergast et al., 2020). At the end of each sampling day, the arthropods collected in each pan trap were transferred using plastic tweezers into 15 ml falcon tubes (one per pan) filled with 20% Dimethyl sulfoxide (DMSO) with saturated salt (NaCl). Arthropod samples were identified morphologically by an entomologist, David Knowles, to provide taxonomic identifications to species-level where possible.

3.3.2.6 Statistical analysis

All statistical analyses were performed on R 3.5.1 (R Core Team 2018). For all three survey methods, taxa not resolved to the species level were grouped into morphotype at the family level (e.g. Chironomidae sp.) and these morphotypes were used as a proxy for species (method adapted from D'Souza et al., 2021). In the eDNA dataset, samples with low sequencing depth and ZOTUs with 5 or more reads found in the negative controls were removed. The arthropod ZOTUs obtained for CO1 or 16S were pooled for analysis. Read counts were transformed to presence-absence to reduce the effects of PCR amplification and primer biases (Elbrecht & Leese, 2015). Shapiro-Wilk and non-parametric correlation tests were used to verify that no correlation existed between arthropod species size and eDNA detection frequency. eDNA species counts per inflorescence were then calculated for all arthropods, as well as the two dominant flower-visiting cohorts determined by the DVRs: Diptera and Hymenoptera. The Diptera species cohort contained 7 families, representing 7 unique species: Drosophilidae sp., *Hydrellia tritici* (Ephydridae), Sciaridae sp., Chironomidae sp., *Simosyrphus grandicornis* (Syrphidae), *Aedes notoscriptus* (Culicidae), and *Musca domestica* (Muscidae). While the Hymenoptera species cohort was comprised of two families, representing two unique species: *Apis mellifera* (Apidae) and a Braconidae sp. Generalised linear models (GLMs) with Poisson distributions were then generated for each of the three eDNA datasets (all arthropods, Diptera, and Hymenoptera).

The co-variates included in these initial GLMs were: sample tree (1 – 8), inflorescence location (upper; sampled > 2 m in the canopy or lower; sampled < 2 m in the canopy), and sampling date (30/10/2020 or 31/10/2020), all three co-variates were listed as categorical. Dispersion statistics

were calculated for each GLM and 100,000 simulated datasets were run to confirm that the models could account for the high frequency of zeros, a common phenomenon in eDNA datasets (Song, Small, & Casman, 2017; Spear, Embke, Krysan, & Vander Zanden, 2021). Akaike information criterion (AIC) frequentist testing was then used to assess the quality of each model and select the most appropriate GLMs for each dataset. AIC frequentist values were then recalculated and the final models were rerun in the 100,000 dataset simulations. To determine the significance of the co-variates in the final models, we re-ran the final GLMs using robust standard errors and compared the output to the pan trap and DVR co-variate significance results. Cameron & Trivedi (2009) recommended using robust standard errors for estimating parameters derived from GLMs to control for instances when the distribution assumption that the variance equals the mean have minor violations.

For DVR and pan trap datasets the same taxa pooling procedures were followed to create three datasets for each method; all arthropods, Diptera and Hymenoptera. For the DVR dataset, the Diptera species cohort comprised seven families totalling 17 species: *Calliphora albifrontalis* (Calliphoridae), *Lucilia cuprina* (Calliphoridae), Calliphoridae spp., Chloropidae spp., Drosophilidae spp., Ephydridae sp., *Musca domestica* (Muscidae), *Musca vetustissima* (Muscidae), Sarcophagidae sp., Syrphidae sp., and unclassified Diptera sp. The Hymenoptera species cohort detected on DVRs comprised eight families representing seven species: *Apis mellifera* (Apidae), Bethylidae sp., Braconidae sp., Formicidae sp., Halictidae sp., Pompilidae sp., and *Polistes humilis* (Vespidae). For the pan trap dataset, all arthropods, Diptera and Hymenoptera species cohorts were pooled per set of three coloured pan traps (blue, yellow and white). The pan trap Diptera species cohort comprised 11 families representing 20 species: Agromyzidae sp., *Calliphora varifrons* (Calliphoridae), Chaemaeyiidae sp., Chironomidae spp., Chloropidae spp., Dolichopodidae sp., Drosophilidae spp., Phoridae sp., Sciaridae sp., *Melangyna viridiceps* (Syrphidae), and unclassified Diptera sp. The pan trap Hymenoptera species cohort comprised six families totalling ten species: *Apis mellifera* (Apidae), Bethylidae spp., Braconidae sp., Formicidae sp., *Lasioglossum hapsidium* (Halictidae), *L. castor* (Halictidae), *Lipotriches flavoviridis* (Halictidae), Mutillidae sp., and unclassified Hymenoptera sp. Both the DVR and pan trap datasets were tested for Skewness and Kurtosis values using the Skewness and Kurtosis function in the 'e1071' package. A Shapiro-Wilk normality test and a non-parametric Kruskal-Wallis test were used to examine if all arthropod, Diptera and Hymenoptera species counts for the DVR and pan traps datasets varied according to sample tree. Mann-Whitney tests were then used to see if DVR and pan trap Diptera and Hymenoptera species counts varied according to the date of collection.

Sampling effort was examined using rarefaction curves, in the package 'vegan' in R, to determine if the number of samples collected were enough to fully capture the diversity for all arthropod families and species for each survey method. The differences in richness and identity detected by all three methods were quantified using a Jaccard Index; this required all three datasets to be standardised into presence-absence data. The Jaccard index analysis was undertaken using the package 'Vegan' in R. Subsequently, non-metric multidimensional scaling (NMDS) ordination was used to visualise the similarity in species communities generated by each method using one dimension to minimise NMDS stress. An Analysis of Similarity (ANOSIM) was then used with the Jaccard dissimilarity matrix and 9999 permutations to test if the communities differed significantly between the methods. We compared alpha diversity between the three survey methods using Chao2 alpha diversity indices at both the family and species level with collection dates pooled, Chao2 was calculated using the package 'fossil' in R. The Chao2 index returns an estimate of richness based on incidence data (Chao, 1984). A Shapiro-Wilk normality test and a non-parametric Kruskal-Wallis test were used to examine if the Chao2 diversity indices for arthropod families and species differed between the three methods. A non-parametric Kruskal-Wallis test was used to compare the means for all arthropods, Diptera and Hymenoptera species counts per tree across the three survey methods. We tested the differences for family and species counts between survey methods for all arthropods, as well as for the dominant flower-visiting Diptera and Hymenoptera cohorts using Kruskal-Wallis tests and non-parametric post-hoc Dunn tests with the Benjamini-Hochberg adjustment method.

3.4 Results

3.4.1 eDNA surveys

We generated 15,366,374 raw sequence reads from the 80 inflorescence samples and 10 hybrid, extraction and PCR controls. In total, 13,392,006 quality-filtered sequences were generated with a mean sequencing depth of 148,800 per sample. One ZOTU, *Agrotis ipsilon*, was found in the hybrid control (5 reads) and was removed from the entire dataset. An additional 27 ZOTUS corresponding to fungi spp., *Homo sapiens* and *Canis* sp. were also removed from the eDNA dataset. Before the ZOTUs for both assays were pooled, the mean number of reads per sample was 2758 (± 556 SE) for CO1 and 3391 (± 1391 SE) for 16S, and between the two assays five samples failed to amplify.

3.4.2 Taxonomic composition

Overall, 24 families were represented in the eDNA dataset, of which Thripidae (*Thrips australis*, *T. tabaci*, *Frankliniella* sp. and *Megalurothrips* sp.: 80% of inflorescence samples), Apidae (*Apis mellifera*: 26% of inflorescence samples), as well as Sciaridae (*Lycoriella castanescens* and

Sciaridae sp.: 25% of inflorescence samples) were the most common. Between the two assays, 14 of the (58%) arthropod families detected unique to the CO1 assay, three families (12%) were unique to the 16S assay and 7 families (29%) were shared (Figure S3.5). In total, 38 taxa were identified by eDNA, with 10 (26%) resolved to genus level, 23 (61%) to species level, while 5 (13%) could not be resolved beyond family level. Taxa included potential pollinators (e.g. *Apis mellifera* and *Simosyrphus grandicornis*, pests (e.g. Helicidae and Limacidae sp.) and parasites (e.g. Eriophyidae sp.). Average arthropod size was not found to correlate with eDNA detection frequency (p = .98). For GLM testing, the species counts for all arthropods, Diptera and Hymenoptera returned dispersion values of 1.16, 0.98 and 0.87 respectively, potentially indicating overdispersion for all arthropods and underdispersion for Hymenoptera. The percentage of zeros was low for all arthropods (6%), but large for both the Diptera (57%) and Hymenoptera (72%) datasets. When simulated however, we found that the majority of the zeros for both Diptera and Hymenoptera GLMs could be explained (Figure S3.1) and concluded that our models were capable of accounting for the zeros in both groups. AIC testing indicated that sample tree was a non-significant co-variate for all three models and was removed (Table S3.2, S3.3 and S3.4). Subsequently, the dispersion values for all three models were close to 1 (Figure S3.2). With robust standard errors, we found that for all arthropods and Hymenoptera, inflorescence location was a significant explanatory co-variate (p = .05 in both instances) (Table S3.5). Indicating that samples collected from the understorey (< 2 m) yielded more arthropod species overall and more Hymenoptera species than samples collected from the upper storey (> 2 m), while date of collection was not a significant co-variate for either all arthropods or Hymenoptera (p = .35 and .92). For Diptera, neither inflorescence location (p = .76) nor sampling day (p = .25) were significant explanatory co-variates (Table S3.5).

3.4.3 DVR monitoring

Of the 14,032 flower visits observed across 96 hours of DVRs, 35 taxa were identified: 18 (52%) to family level, 12 (34%) to species level and 5 (14%) could not be resolved beyond the level of order. In total, the DVR dataset comprised 23 families, of which hoverflies (*Simosyrphus grandicornis* and *Melangyna viridiceps*) were the most numerous visitors (89% of all flower visits with 130 ± 15.5 SE visits per hour), followed by the European honeybee (*Apis mellifera*) (7 % of all flower visits with 10 ± 1.1 SE visits per hour) and non-syrphid Diptera species (Calliphoridae sp. and Muscidae sp.) (3 % of all flower visits with 4 ± 1.1 SE visits per hour). Flower visits by moth species (*Phrissogonus laticostata* and *Plutella xylostella*) and native wasp species (*Polistes humilis* and Bethylidae sp.) were rare (< 1 flower visit per hour). The percentage of zeros was zero for all arthropods, Diptera and Hymenoptera. The skewness values generated were 0.63 for all

arthropods, 0.54 for Diptera, and 0.76 for Hymenoptera, indicating a mild positive skews. The kurtosis values were -0.31 for all arthropods, -1.24 for Diptera and -0.24 for Hymenoptera, indicating platykurtic distributions. As with the pan trap dataset, we employed non-parametric testing and found that neither sample tree nor date of collection significantly influenced all arthropods, Diptera or Hymenoptera species counts (Table S3.5).

3.4.4 Pan traps

A total of 499 individual arthropods were collected from the pan traps, with 35 taxa identified of which 21 (60%) were resolved to family level, 6 (17%) to species level, and 8 (23%) could not be resolved beyond order level. In total, 28 families were represented in the pan trap dataset and among these the three most common taxa were all members of Diptera; Drosophilidae sp. (33%), Phoridae sp. (22%) and Dolichopodidae sp. (6%). Unlike the eDNA results, the pan traps also showed the presence of three native bee species: *Lipotriches flavoviridis* (Halictidae), *Lasioglossum hapsidum* (Halictidae), and *L. castor* (Halictidae). The data for all arthropods, Diptera, and Hymenoptera were non-normally distributed. The percentage of zeros was zero for all three datasets. The skewness values were 1.58 for all arthropods, -1.01 for Diptera and 1.02 for Hymenoptera, indicating a positive skew for both all arthropods and Hymenoptera and a negative skew for Diptera. All arthropods had a leptokurtic tail shape (kurtosis value = 2.99), while both Diptera and Hymenoptera had mesokurtic tail shapes (kurtosis values = 0.43 and 0.24, respectively). Using non-parametric testing, the species counts for all three pan trap datasets did not show significant variation between sample trees or collection date (Table S3.5).

3.4.5 Three method comparison

Overall, eDNA collected from flowers detected the greatest diversity of arthropod families of all three collection methods (Figure 3.2A and Table 3.1). Although none of the three methods alone appeared to capture the total arthropod diversity present within the orchard (Figure S3.3). Arthropod family composition recorded by each survey method showed clear partitioning (Figure 3.2B; ANOSIM, $p < .01$). Alpha diversity index values at both the family and species level did not differ significantly between the three survey methods (Figure 3.2C; Kruskal-Wallis test, $p = .10$). While the number of all arthropod species detected per tree did not vary significantly between the three survey methods, the number of Diptera and Hymenoptera species captured by eDNA were significantly lower when compared to pan traps and DVRs (Figure 3.2D; Dunn tests, $p < .01$). Collection method was also found to have a significant effect on the number of taxa recorded for both Diptera ($p < .01$) and Hymenoptera ($p < .01$), but not for all arthropod taxa ($p = .24$). Per sample, DVRs captured the greatest diversity of arthropod families (6 ± 0.35 SE), followed by pan traps (4 ± 0.27 SE), and eDNA (3 ± 0.19 SE) (Dunn tests, $p < .001$). Similarly at the level of

species, DVRs captured the greatest diversity (7 ± 0.39 SE), followed by pan traps (4 ± 0.27 SE), and eDNA (3 ± 0.21 SE) (Dunn tests, $p < .001$). A post-hoc test for Diptera showed that the three collection methods differed significantly for the number of species collected, with DVRs detecting the largest number of Diptera species (7 ± 0.39 SE), followed by pan traps (6 ± 0.33 SE), and eDNA (1 ± 0.35 SE). For Hymenoptera, the species counts between eDNA (1 ± 0.27 SE) and DVRs (2 ± 0.21 SE) as well as DVRs and pan traps (3 ± 0.30 SE) were comparable, with the only significant difference found between eDNA and pan traps (Figure 3.2D).

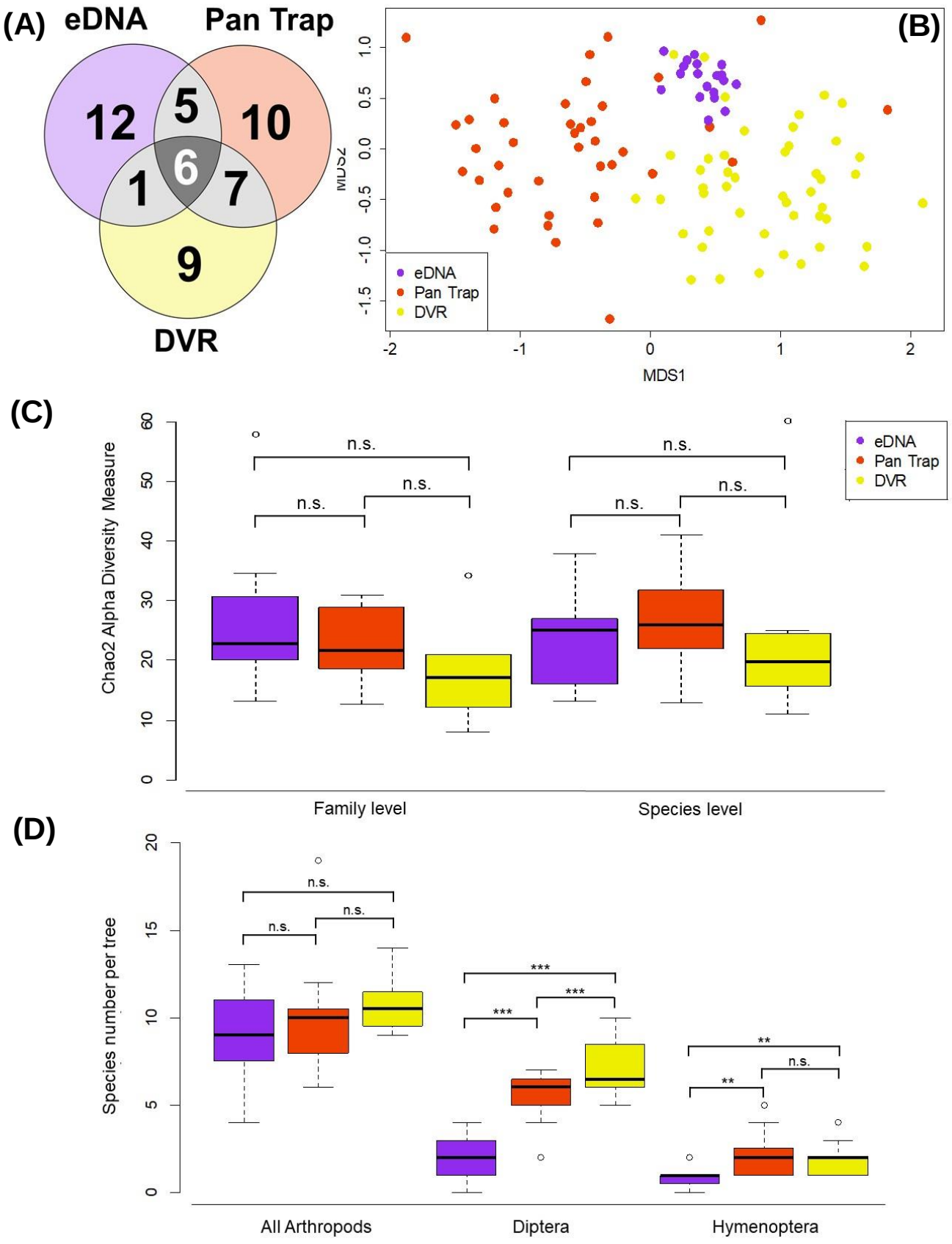

Figure 3.2 (A) Number of families identified for each survey method; eDNA (*N* = 24), DVR (*N* = 23) and Pan Trap (*N* = 28). **(B)** Non-metric multidimensional scaling ordination (Stress value = 0.1098) showing the relationship between arthropod family assemblage and survey method based

on a Jaccard dissimilarity matrix for factor method. **(C)** Chao2 alpha diversity measures based on presence-absence data for arthropod families and species. Chao2 values were calculated, per survey method, by pooling all samples over both collection dates for each tree (eDNA; $N = 10$ per tree, DVR; $N = 4$ per tree, pan trap; $N = 6$ per tree) and calculated using the package 'fossil' in R. **(D)** Dunn Tests generated for all arthropod species collected per tree and both major flower-visiting arthropod groups for *Persea americana* (Diptera and Hymenoptera) for the three methods (eDNA; $N = 80$; Pan trap $N = 48$; DVR $N = 32$). P-values were adjusted with the Benjamini-Hochberg method to correct for Type 1 errors. Significance values; n.s. = $p > .05$, * = $p \leq .05$, ** = $p \leq .01$ and *** = $p \leq .001$.

Table 3.1 Taxonomic identifications of the 49 arthropod families found between the three survey methods (eDNA, Pan Trap and DVR) at Marron Brook Farm between 30/10/2020 and 31/10/2020. The main flower-visiting orders, as determined by DVRs; Diptera and Hymenoptera, are highlighted. Shaded boxes indicate presence. The unknown families for Pan Traps were: Termite sp. (order Isoptera), elongated fly sp. (order Nematocera) and unclassified fly spp. (order Diptera). While the unknown families for DVR were: beetle spp. (order Coleoptera), unclassified fly spp. (order Diptera) and unclassified sp. (order unknown).

Order	Family	eDNA	Pan Trap	DVR
Diptera	Agromyzidae	☐	■	☐
	Calliphoridae	☐	■	■
	Chamaemyiidae	☐	■	☐
	Chironomidae	■	■	☐
	Chloropidae	■	■	■
	Culicidae	■	☐	☐
	Dolichopodidae	☐	■	☐
	Drosophilidae	■	■	■
	Ephydridae	☐	☐	■
	Muscidae	■	☐	■
	Phoridae	☐	■	☐
	Sarcophagidae	☐	☐	■
	Sciaridae	■	■	☐
	Syrphidae	■	■	■
Hymenoptera	Apidae	■	■	■
	Bethylidae	☐	■	■
	Braconidae	■	■	■
	Formicidae	☐	■	■
	Halictidae	☐	■	■
	Mutillidae	☐	■	☐
	Pompilidae	☐	☐	■
	Vespidae	☐	☐	■
Other	Acrididae	☐	■	■
	Bourletiellidae	■	■	☐
	Caeciliusidae	■	■	☐
	Chrysopidae	■	☐	☐
	Cicadellidae	☐	■	☐
	Coccinellidae	☐	☐	■
	Curculionidae	☐	■	☐
	Ectopsocidae	■	☐	☐
	Eriophyidae	■	☐	☐
	Geometridae	■	■	■
	Helicidae	■	☐	☐
	Latridiidae	■	☐	☐
	Limacidae	■	☐	☐
	Miridae	■	■	■
	Nitidulidae	■	☐	☐
	Noctuidae	■	☐	☐
	Phlaeothripidae	■	☐	☐
	Plutellidae	■	☐	■
	Staphylinidae	☐	■	☐
	Thomisidae	☐	☐	■
	Thripidae	■	■	☐
	Tydeidae	■	☐	☐
	Unknown families	☐	■	■
	(Number)	(NA)	(3)	(3)

3.5 Discussion

Globally, ~ 40% of all insect species, both managed and unmanaged, could be at risk of extinction in coming decades as a consequence of climate change, loss of habitat, pesticide use, as well as vulnerability to emerging pests and pathogens (Sánchez-Bayo & Wyckhuys, 2019). Improved arthropod survey methods are therefore increasingly necessary to complement current survey techniques and allow reliable and timely detections of both beneficial and antagonistic arthropod species (D'Souza et al., 2021; Evans & Kitson, 2020; Newton et al., 2023). Here, we show that when combined with traditional survey methods, metabarcoding of eDNA collected from flowers can increase the number of arthropod families detected by 25%. Consistent with previous studies, eDNA metabarcoding allowed efficient and reliable detections of potential pollinators, as well as plant pests and parasites without the need for extensive taxonomic expertise (Gomez et al., 2023; Johnson et al., 2023; Newton et al., 2023). The accuracy of this molecular method is, however, dependent on the quantity and quality of arthropod eDNA, the presence of non-target DNA contamination, the sequence analysis method chosen, and the availability of arthropod sequences in online databases (Evans & Kitson, 2020; Ficetola, Taberlet, & Coissac, 2016; Valentin, Kyle, Allen, Welbourne, & Lockwood, 2021). Importantly, this study demonstrates that no single survey method can capture the complete range of taxa foraging on the avocado orchards. For instance, eDNA detected the highest levels of arthropod family diversity overall, but family diversity levels for the main *P. americana* flower visitors – Diptera and Hymenoptera – were significantly higher in pan traps and DVR devices, respectively, compared to the eDNA results. While eDNA metabarcoding of flowers has the potential to revolutionise the way arthropod communities are monitored, as applications continue to diversify, it is crucial that novel uses are validated against longstanding survey methods, and that the relative strengths and weaknesses of each are evaluated.

3.5.1 Complementarity of three survey methods

Using three survey methods, we detected a wide range of arthropod taxa, and while alpha diversity was similar, each method characterised the arthropod community differently, and only represented a fraction of the total diversity. However, the most common flower-visiting insects - hoverflies (Syrphidae spp. 130 ± 15.5 SE visits per hour) and European honey bees (*Apis mellifera* 10 ± 1.1 SE visits per hour) - were detected by all three methods.

It is unclear why each survey method characterised the arthropod community differently, but it is likely that different detection biases are associated with each method. For example, Drosophilidae sp. were the most common insects found in the pan traps (62% of samples), but they were not observed by DVR devices (likely due to camera resolution), and only detected once by eDNA

metabarcoding (Figure S3.4). These findings complement those of Newton et al. (2023), where only two of the total 57 arthropod taxa were shared between eDNA metabarcoding of flowers and visual surveys. Indeed, compared to the arthropod communities described by DVRs and eDNA, the pan trap dataset likely represents a broader community of both Diptera and Hymenoptera than just flower-visiting arthropods (e.g. only pan traps detected members from Dolichopodidae, the adults and larvae of which are generally insectivorous; see Zborowski & Storey, 2017), thereby potentially increasing the dissimilarity between the three methods (Kelly et al., 2017; Leempoel, Hebert, & Hadly, 2020). Similarly, the presence of DNA from non-flower-visiting pest and parasite taxa likely increased the dissimilarity between the three survey methods. For instance, only eDNA detected herbivorous pests belonging to Helicidae, Limacidae and Eriophyidae (see Berlander & Baker, 2007), which, although not nectivorous, may have left traces of DNA while moving across floral tissue or during feeding (see Kudoh et al., 2020). As well as highlighting the differences within each method, the three-survey method approach also helped to identify the taxonomic gaps in the eDNA dataset.

The inclusion of DVRs highlighted the presence of false negatives in the eDNA metabarcoding dataset. For example, some families (e.g. Calliphoridae, Sarcophagidae, and Pompilidae), were observed by DVRs visiting flowers, but were absent in the eDNA dataset. A previous study by Thomsen & Sigsgaard (2019) also found that metabarcoding of arthropod eDNA on flowers failed to detect common flower visitors, possibly because of poor efficacy of available assays for some taxa. For instance, the Adenine-Thymine rich mitochondrial genomes of Hymenoptera and Hemiptera form homopolymer regions which are difficult to amplify and sequence (Hebert et al., 2016). Additionally, given that most crop flower visits are brief (e.g. Rader, Howlett, Cunningham, Westcott, & Edwards, 2012), the opportunities for arthropod DNA deposition that is detectable by metabarcoding may be limited. As a consequence, we stress that eDNA metabarcoding is currently unable to show which taxa are the most frequent flower-visitors, and potentially the most likely to affect pollination, due to the lack of abundance data (see Mathon et al., 2021), as well as the presence of incidental non-target DNA (e.g. herbivorous pest taxa; see Kudoh et al., 2020). We therefore recommend a combined approach of eDNA metabarcoding with DVR devices to utilise the significant taxonomic resolution afforded by eDNA with the abundance and behavioural data available from DVRs.

3.5.2 Assay, study design, and database considerations

Metabarcoding of arthropod DNA on flowers is a nascent method for measuring arthropod diversity, but one that holds great promise for the future. As an emerging monitoring tool however, we identify potential areas that can be incorporated into future studies to help improve its accuracy and reliability in agricultural systems. First, further development of eDNA metabarcoding assays that detect a broader range of agriculturally important flower-visiting taxa (e.g. Diptera, Lepidoptera and Hymenoptera sp.), or amplify shorter amplicons in regions that minimise sequencing errors (Leese et al., 2021; Marquina, Andersson, & Ronquist, 2019), would improve the robustness of eDNA-based surveys by reducing the frequency of false positive and negative species identifications. Second, incorporating additional complementary methods with eDNA surveys can help overcome the limitations of presence-absence data (Johnson et al., 2023; Newton et al., 2023). Third, we recommend that detection thresholds, established with pilot studies, are incorporated into eDNA studies to determine DNA deposition and degradation rates from different arthropod species, as well as quantifying the size range of fragments that successfully amplify (Valentin et al., 2021). These studies could incorporate locally relevant abiotic and biotic factors (e.g. UV light or microbial activity; see Strickler, Fremier, & Goldberg, 2015) to provide temporal caveats for species assemblages generated from eDNA detections (Macher et al., 2016). An *a priori* understanding of these caveats would help improve the accuracy of eDNA survey techniques because they could account for rapid DNA degradation with either more frequent sampling (see Krehenwinkel et al., 2018), or targeting of shorter amplicons (see Saito & Doi, 2021). By incorporating these recommendations, metabarcoding of arthropod DNA on flowers could generate reliable and accurate classifications of flower-visitor communities, while also accounting for the biotic (e.g. DNA deposition) and abiotic (UV levels) factors that can often influence the accuracy of eDNA-based surveys.

Robust eDNA metabarcoding based detection of arthropods important to agricultural systems have, to date, required combinations of assays targeting CO1 and 16S, paired with a custom reference library to achieve broad taxonomic coverage (Clarke et al., 2014; Magoga et al., 2022; Thomsen & Sigsgaard, 2019). This approach maximised the amount of recoverable taxa because ribosomal genes show less taxonomic bias (Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014), while the reference libraries developed for CO1 are more extensive and offer higher taxonomic resolution (Elbrecht et al., 2019). In the present study, we found that the addition of an assay targeting 16S helped to identify three insect families that were not identified with CO1 (Figure S3.5). Compared to CO1, the 16S region has a limited number of reference sequences for arthropods, meaning that many of the potentially flower-visiting taxa present in a given

agricultural system may erroneously appear as absent (Ficetola et al., 2015; Thomsen & Sigsgaard, 2019). In the context of monitoring agricultural systems, false negatives for flower-visitors could misinform management decisions and increase expenditure (e.g. unnecessarily hiring more managed bee hives to increase pollination services; see Ish-Am & Eisikowitch, 1998). This problem is notable because only ~ 20% of all insect taxa have been morphologically described (Stork, 2018). Hence, in countries like Australia that has more than 70,000 insect species, only a small proportion of specimens have been vouchered and barcoded (Rougerie et al., 2014; Zborowski & Storey, 2017), although globally the completeness of reference libraries is improving (Kjærandsen, 2022; Magoga et al., 2022; Taylor et al., 2018). While it remains possible to ignore this issue and analyse metabarcoding data without a custom reference library (see Aizpurua et al., 2017; Moran, Prosser, & Moran, 2019), we reason that, despite using two assays, the lack of barcode reference sequences in our study decreased the diversity of floral visitors detected by eDNA. In practice, without complementary surveys or prior verification (e.g. pilot surveys; see Goldberg, Strickler, & Fremier, 2018), eDNA surveys may underestimate the diversity of both beneficial and antagonistic agricultural arthropod taxa. Therefore, we recommend using active and/or passive survey techniques, such as sweep netting and pan traps, to capture local arthropod specimens (Saunders & Luck, 2013; Spafford & Lortie, 2013). These specimens can then be identified morphologically and compared against the NCBI and BOLD databases to determine if barcode regions from these species have already been sequenced.

3.5.3 Potential applications of eDNA metabarcoding of flowers

Either complementing long-standing survey approaches, or used on its own, eDNA metabarcoding offers many opportunities to improve the characterisation of plant-animal visitor networks within agroecosystems. For example, unmanaged non-bee flower-visitors have historically been omitted from crop pollination studies, in part because observing flower-visitors and identifying pollen grains are time-intensive and require specialist expertise that is not always readily available, and becoming rarer (Bell et al., 2016; Bosch et al., 2009; Rader et al., 2016). As a consequence, the pollination services offered by these unmanaged taxa as well as the food resources they require remains largely unexplored (Potts et al., 2016). By targeting pollen accumulated on the bodies of arthropods (arthropod-centric sampling), previous eDNA studies have been able to classify unobserved nocturnal pollen transport networks (Macgregor et al., 2019), as well as detecting broader foraging resources for an economically damaging pest species - the turnip moth (*Agrotis segetum*) - than formerly reported in the literature (see Chang et al., 2018). If combined with eDNA metabarcoding of flowers (plant-centric sampling), researchers could target arthropod DNA on floral structures significant for pollination (e.g. stigma or anthers) to help determine if these

unmanaged taxa are providing neutral (no effect), facilitative (e.g. pollination), or resource parasitism (e.g. only harvesting pollen) interactions (Evans & Kitson, 2020; Rathcke, 1983). Such targeted eDNA sampling could be incorporated with Single Visit Deposition (SVD) (see King, Ballantyne, & Willmer, 2013), often used for crop pollination studies, to help improve the accuracy of morphological classifications for both flower visitors and the pollen they deposit (Pornon et al., 2017). Furthermore, eDNA metabarcoding of flowers could be used to compare arthropod diversity at different flowering stages (e.g. dichogamy), or between flowering populations in separate geographical locations. The information generated from such studies could then be used by practitioners to encourage or suppress these unmanaged species, depending on their relationship to the crop under study, potentially helping increase the resilience of plant-pollinator networks within these agroecosystems (Kestel et al., 2022). The use of eDNA metabarcoding of flowers also holds potential in natural systems to both accurately identify flower-visiting species for previously undocumented plant species (see Newton et al., 2023) and assist in the detection of pest taxa before they become established in natural habitats, especially those in remote or difficult to access locations.

3.6 Conclusion

We compared the arthropod communities detected with eDNA metabarcoding of flowers from *P. americana* with two commonly used survey techniques: DVR devices and pan traps. In total, 80 eDNA flower samples, 96 hours of DVRs showing 14,032 flower visits, and 48 pan trap samples containing 499 arthropods were collected. This study confirms that eDNA metabarcoding of flowers can increase the number of arthropod families detected by 25% when combined with conventional DVR devices and pan traps. When comparing family diversity levels for the main *P. americana* flower visitors – Diptera and Hymenoptera – we found significantly higher diversity levels with pan traps and DVR devices, respectively, compared to eDNA. We suggest that the accuracy and reliability of eDNA metabarcoding of flowers could be improved by: i) further development of eDNA metabarcoding assays to target agriculturally important flower-visiting taxa, ii) including complementary methods to generate behavioural and count data for the flower-visiting species of interest (e.g. DVR devices), iii) pilot studies to establish locally relevant eDNA detection thresholds, and iv) sequencing of barcode regions from key taxa missing from the NCBI and BOLD databases. Overall, we have demonstrated that eDNA metabarcoding of flowers has the potential to transform the way arthropod communities are monitored in general, potentially detecting the response of pollinators and pests to climate change, diseases, habitat loss and other disturbances.

3.7 References

A thorough effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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3.8 Supplementary information

Table S3.1 Weather conditions taken from the Bureau of Meteorology for Manjimup (near Marron brook Farm) for 2020.

Date	Orchard	Maximum (°C)	Minimum (°C)	Rainfall (mm)
30th October	Marron Brook Farm	31.5	10.7	-
31st October	Marron Brook Farm	24.3	8.7	-

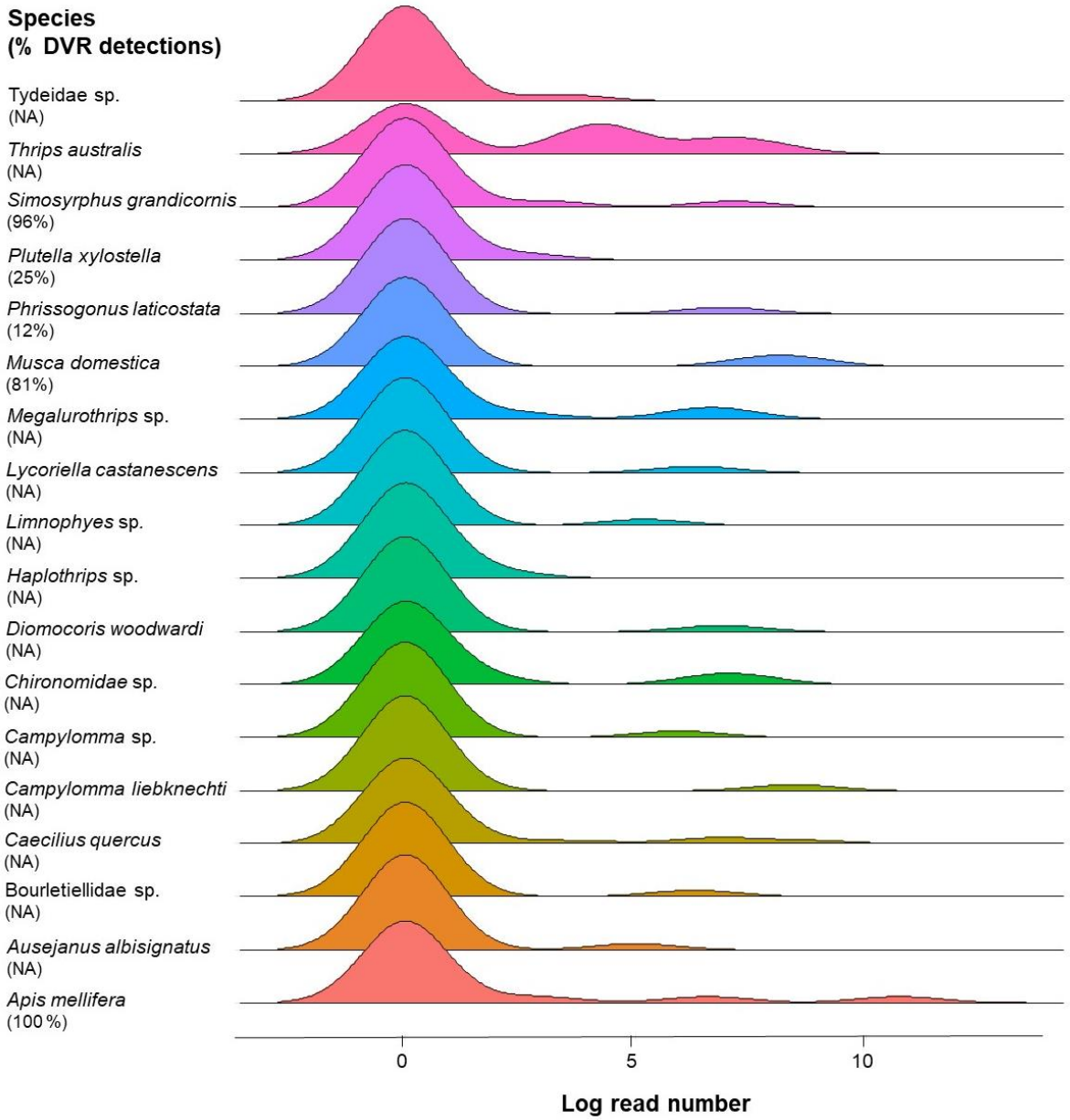

Figure S3.1 Ridgeline plot for log eDNA read number per arthropod species for the subset of inflorescences with DVR observations (N = 16). The percentage of detections per DVR (N = 16) are presented below each species, with species not detected on the DVRs represented as (NA). Ridgeline chart generated using the packages 'ggridges' and 'ggplot2' in R.

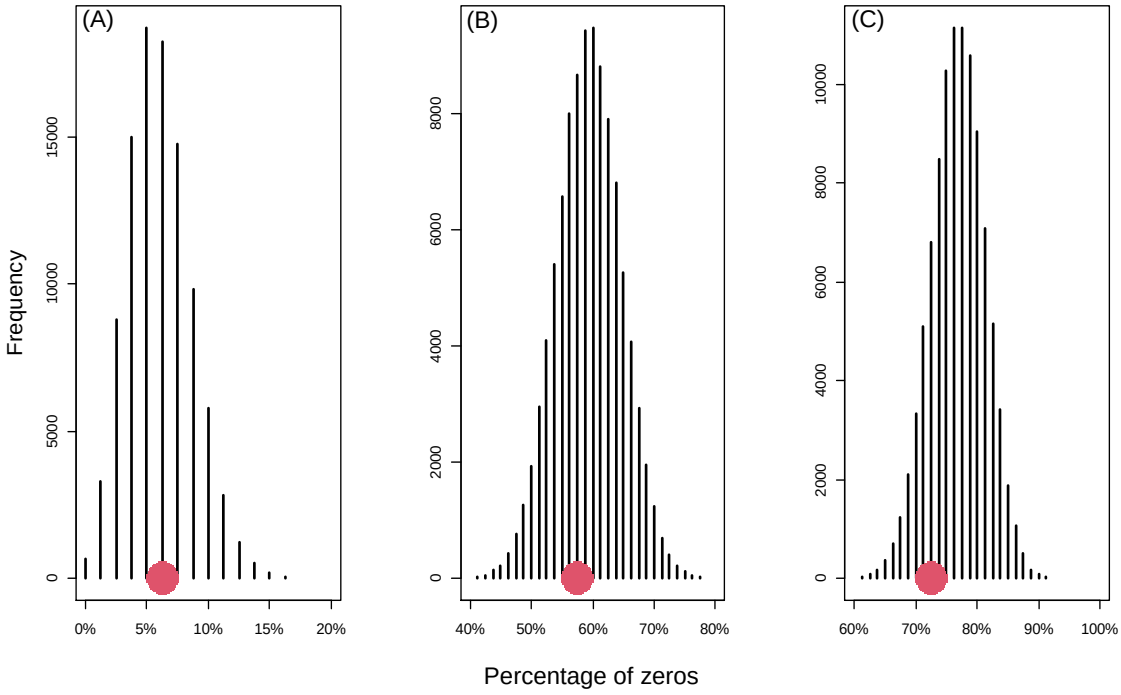

Figure S3.2 Frequency table for the percentage of zeros in 100,000 simulated datasets for Poisson GLM with all categorical covariates included (sample tree, inflorescence location and date sampled) for the three eDNA datasets; (A) All arthropods, (B) Diptera and (C) Hymenoptera. The red dots represents the percentage of zeros in the three datasets (A; 6.2%, B; 57% and C; 72%).

Table S3.2 eDNA all arthropods generalised linear model using AIC frequentist selection. The 'x' denotes removal of the covariate from the model. The best AIC model is bolded.

Model Number	Sample Tree (1-8)	Inflorescence location (U/L)	Date sampled (30/10/2020 or 31/10/2020)	AIC frequentist
1				328.42
2	x			**320.21**
3		x		331.25
4			x	327.28

Table S3.3 eDNA Diptera generalised linear model using AIC frequentist selection. The 'x' denotes removal of the covariate from the model. The best AIC model is bolded.

Model Number	Sample Tree (1-8)	Inflorescence location (U/L)	Date sampled (30/10/2020 or 31/10/2020)	AIC frequentist
1				166.14
2	x			**159.12**
3		x		164.23
4			x	165.40

Table S3.4 eDNA Hymenoptera generalised linear model using AIC frequentist selection. The 'x' denotes removal of the covariate from the model. The best AIC model is bolded.

Model Number	Sample Tree (1-8)	Inflorescence location (U/L)	Date sampled (30/10/2020 or 31/10/2020)	AIC frequentist
1				111.63
2	x			**107.10**
3		x		113.25
4			x	109.64

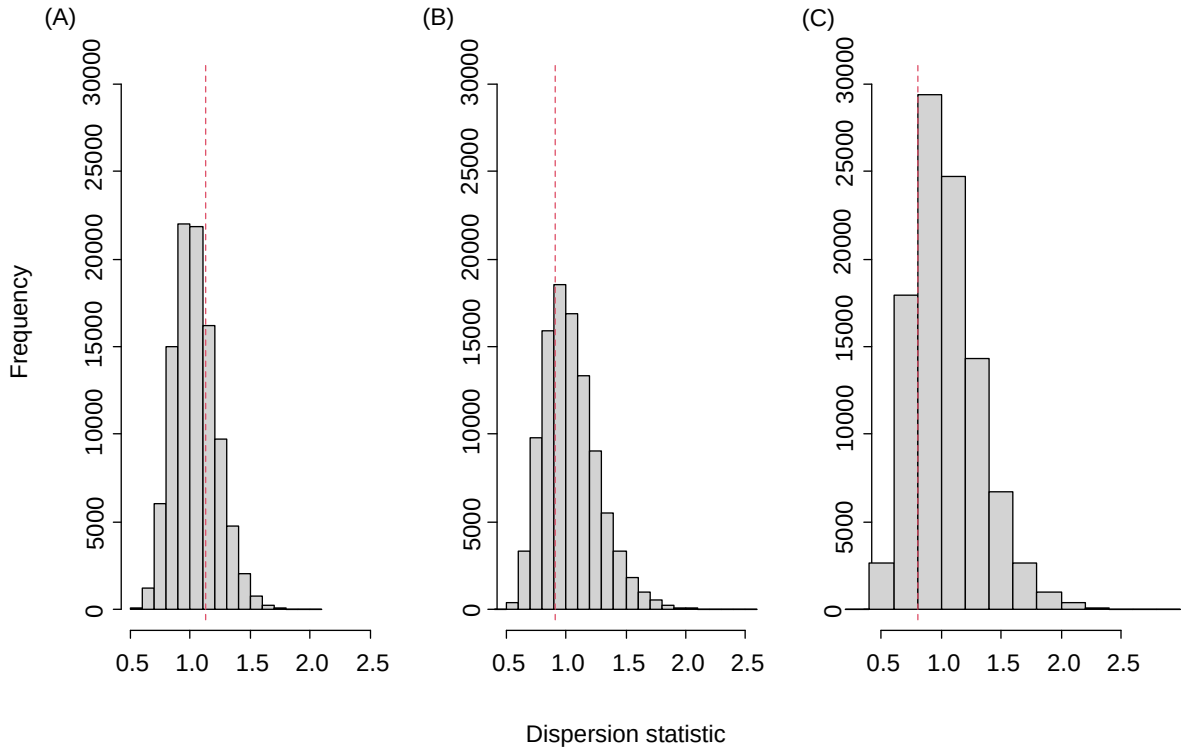

Figure S3.3 Dispersion statistics for the 100,000 simulated datasets. The dotted redline represents the dispersion statistic for the fitted models for the eDNA species detections for; (A) All arthropods, (B) Diptera and (C) Hymenoptera.

Table S3.5 Comparison of covariate significance for eDNA, Pan Trap, and DVR datasets. Sample tree was not significant (*NS*) for the eDNA generalised linear models and was not included. Inflorescence location was neither a feature of pan trap sampling nor DVRs**.**

Covariate	eDNA			Pan Trap			DVR		
	All	Diptera	Hymenoptera	All	Diptera	Hymenoptera	All	Diptera	Hymenoptera
Sample tree (1-8)	*NS*	*NS*	*NS*	.12	.34	.16	.34	.24	.46
Date of collection (Day 1 or 2)	.35	.25	.92	.28	.23	.31	.96	.19	.61
Inflorescence location (upper or lower)	.05*	.76	.05*	NA	NA	NA	NA	NA	NA

**Indicates significant p-value at the 95% confidence interval*

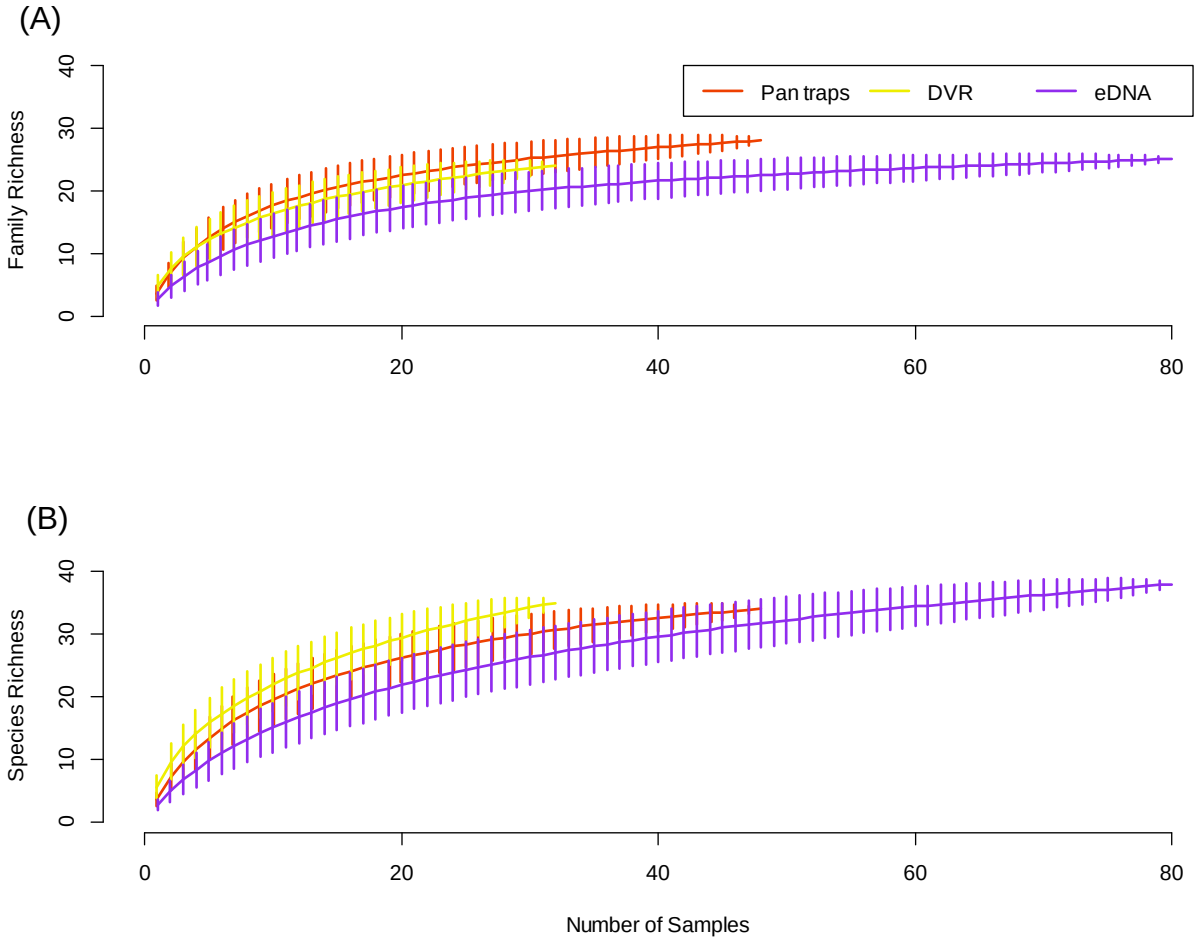

Figure S3.4 Rarefaction curves with standard error bars for; (A) arthropod families, and (B) arthropod species. Curves were generated for all three collection methods; Pan traps (*N* = 48), DVRs (*N* = 64), and eDNA (*N* = 80). Rarefaction curves were generated with 10,000 permutations and first order jack-knifing using the package 'Vegan' in R.

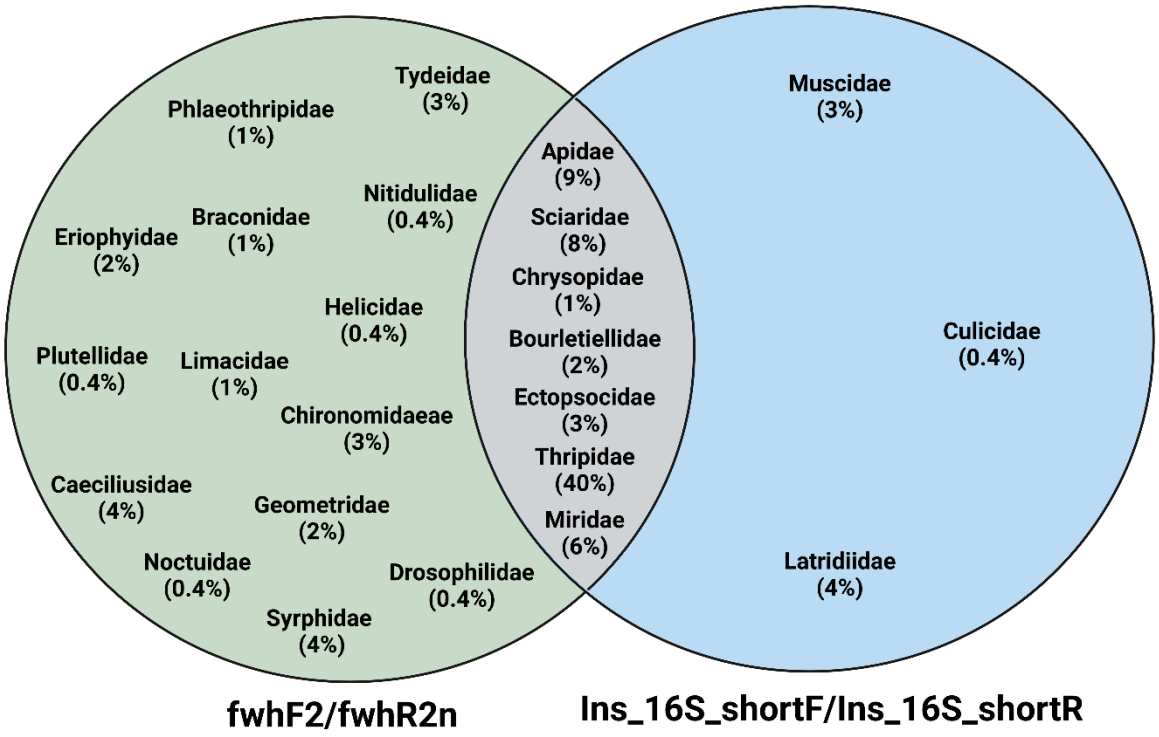

Figure S3.5 The 24 insect families identified from inflorescences using eDNA metabarcoding with two assays targeting the COI (fwhF2/R2n) and 16S (Ins_16S_shortF/R) regions. Each family is presented with the percentage of positive detections (N = 240 total). Inflorescences were sampled at Marron Brook Farm, Middlesex, Western Australia.

Chapter 4. Spatio-temporal variation in arthropod-plant interactions identified using eDNA and digital video recordings

The study presented in this chapter is in review within the peer-reviewed literature.

Kestel, J. K., Bateman, P.W., Field, D.L., White, N.E., & Nevill, P. Spatio-temporal variation in arthropod-plant interactions identified using eDNA and digital video recordings. *Agriculture, Ecosystems & Environment*, (In review).

4.1 Abstract

Collating data about natural capital and the ecological services that underpin agricultural productivity is critical as beneficial arthropod taxa (e.g. pollinators and predators) are increasingly threatened by biotic and anthropogenic stressors. Further, the global expansions of agricultural plant pests, as well as the pathogens they transmit necessitates greater implementation of timely and reliable monitoring efforts to detect and perturb further infestations. To date, these monitoring efforts have largely relied upon conventional surveys (e.g. sweep netting and morphological identifications), which are difficult to implement at the large scale of agriculture. Environmental DNA (eDNA) metabarcoding is an emerging molecular method that amplifies trace amounts of DNA deposited by organisms from diverse substrates ranging from soil, plant tissue and even air. When combined with digital video recording (DVR) devices, eDNA surveys can be used to identify and monitor shifts in arthropod taxa that facilitate key ecosystem services, as well as emerging crop pests. In this study, we used eDNA metabarcoding of crop flowers, complemented with DVRs, to detect temporal, fine- and large-scale arthropod community changes across two *Persea americana* ('Hass' avocado) orchards. In total, we detected 42 arthropod families with eDNA metabarcoding, representing 66 unique taxa. The number of arthropod eDNA detections increased by 14% during peak flowering and included species from different functional groups including known arthropod pollinators, pests, parasites and predators. At fine-spatial scales, inflorescence samples collected in the upper and lower canopy show that Hymenoptera taxa were 13% more likely to be detected in the upper canopy. While at large-spatial scales, eDNA metabarcoding showed that the arthropod communities in both orchards shared less than 50% similarity at low flowering and became more similar towards peak flowering. By comparing eDNA detections with those from DVRs, we determined that arthropods that visited flowers more frequently had a higher eDNA detection probability. Though, we also found that eDNA metabarcoding detections were biased towards smaller arthropods, emphasising the need to complement this molecular method with additional surveys approaches. Our findings highlight the value of eDNA-based monitoring of arthropods and show that this approach can complement current methods that identify beneficial and antagonistic species in agricultural systems. This will be particularly important in the future with growing awareness that agriculture does not end at a farm or orchard boundary, and that the goods and services that ecosystems provide need to be included on the balance sheet.

4.2 Introduction

Global agricultural productivity is intrinsically linked with arthropods, both by the beneficial ecosystem services they provide (e.g. pollination and predation), as well as the antagonistic interactions (e.g. herbivory and pathogen transmission) that reduce crop yields (FAO, 2020; Potts et al., 2016). Unfortunately, at a time when agricultural productivity needs to increase dramatically to keep pace with global population growth (FAO, 2017; Hunter et al., 2017), beneficial arthropod species are increasingly threatened by a combination of biotic and anthropogenic stressors (Wagner, 2020; Wagner et al., 2021). Some of the most significant threats include habitat loss, pollution (e.g. pesticides), biological factors (e.g. invasive species) and climate change (e.g. drought) (IPBES, 2019; Sánchez-Bayo & Wyckhuys, 2019; Williams et al., 2021). Whilst beneficial arthropod diversity is threatened, agricultural plant pests, as well as the pathogens they transmit, continue to expand globally and increase crop stress (DAWE, 2021; de Benedetta et al., 2022; Skendži´c et al., 2021).

Monitoring beneficial and antagonistic arthropods is fundamental for understanding the arthropod-plant dynamics influencing crop yields (Furlong, 2015; Kestel et al., 2022). Arthropods represent the most significant crop pollinators, particularly the managed honey bee *Apis mellifera*, which is regarded as the most important crop pollinator globally (Klein et al., 2007; Potts et al., 2016). Though increasingly, the pollination services delivered by unmanaged taxa, such as wild bees and flies, are recognised as significant contributors to reliable agricultural yields (Kleijn et al., 2015; Rader et al., 2016). Arthropod predators are another potentially beneficial cohort that provide a regulating ecosystem service within agroecosystems and offer an alternative to pesticide applications as a means of reducing the presence of agricultural pests and pathogens (Chaplin-Kramer et al., 2011; Furlong, 2015). Although, much remains to be discovered about these pollinator and predator taxa, such as how their communities change with fluctuations in crop resources (i.e. flowering intensity) and across different scales (i.e. within a tree and between orchards), which to-date have been challenging to answer using conventional survey methods (Furlong, 2015; Martínez-Sastre et al., 2020; Rader et al., 2016).

Plant pests, as well as the pathogens they transmit, represent the other extreme, as these taxa harm agricultural productivity and are responsible for global crop losses of ca. 20 – 40% (Flood, 2010), though monitoring these taxa and generating early detections before they spread is difficult to achieve (Chaplin-Kramer et al., 2011; Derocles et al., 2015). Principally, because arthropod pests and pathogens emerge in low numbers across large areas (see Edwards et al., 2014), and that these

taxa may not be equally well-detected by conventional survey methods (Chang et al., 2018; Valentin et al., 2016). Accurate information on spatio-temporal variation in arthropod communities is critical for natural capital accounting and the valuation of ecosystem services (e.g. pollination) that underpins agricultural production, and also to provide evidence for alternative management strategies that encourage beneficial taxa and supress antagonistic taxa (Dardonville et al., 2022; Smith et al., 2017; Statistics Canada, 2023). Therefore, the development of new survey methods that work at different spatial scales and provide timely detections of these ecologically significant arthropods may prove useful for informing agricultural management options.

Biodiversity surveys and monitoring of arthropods in agriculture have relied upon a mixture of passive (e.g. Malaise traps) and active sampling (e.g. sweep netting) methods (Kestel et al., 2022; Prendergast et al., 2020). Subsequent to capture, arthropod specimens are often identified by entomologists to provide taxonomic identifications (Joshi et al., 2015; Westphal et al., 2008). Unfortunately, at the large scale of agriculture these conventional surveys have proven difficult to implement, especially as the specialist expertise required is not always readily available (O'Connor et al., 2019). As a consequence, a number of complementary methods have emerged to help detect and monitor these ecologically significant arthropods within agroecosystems (Ratnayake et al., 2023; Valentin et al., 2020). Digital video recording (DVR) devices are one of the most notable new methods being used in agroecosystems (Gilpin et al., 2017; Kestel et al., 2023). DVR devices have proven equally or more effective than visual observations of flower-visiting arthropods (see Gilpin et al., 2017; Naqvi, Wolff, Molano-Flores, & Sperry, 2022), and have the additional benefit of capturing multiple flower visits simultaneously (depending on the number of DVRs deployed) (Sakamoto et al., 2012). In agriculture, these devices have been used to monitor both potential pollinating taxa (e.g. Sritongchuay, Hughes, Memmott, & Bumrungsri, 2019), as well as crop pests (e.g. Filho, Heldens, Kong, & de Lange, 2019). DVR devices are not appropriate in all research contexts, however. Often limited by resolution, the identification of arthropod taxa by DVRs generally cannot be taken beyond the level of family, especially for small taxa (although see Droissart et al., 2021) (e.g. Ratnayake, Amarathunga, Zaman, Dyer, & Dorin, 2023). Additional complementary methods are therefore still needed to allow for accurate monitoring of arthropod-plant interactions.

Trace amounts of DNA (e.g. saliva, faeces, scales) deposited by organisms on substrates (e.g. plant material, soil, spider webs, air) are known as environmental DNA (eDNA). Small fragments of this preserved, but often degraded, single- or multi-source DNA, referred to as 'barcodes', can be

amplified and sequenced, a process described as ‘metabarcoding’, to characterise biodiversity, establish diversity thresholds and monitor community change (Taberlet et al., 2012; Takahashi et al., 2023). When used in combination with High-Throughput Sequencing (HTS) technologies, eDNA can detect, for example, flower-visiting arthropods in orchards (Kestel et al., 2023), invasive crop pests from fruit (Valentin et al., 2018), and known beneficial predatory arthropods (Newton et al., 2023; Thomsen & Sigsgaard, 2019). Despite such diverse applications, eDNA metabarcoding remains an emerging technology in agriculture (see Kestel et al., 2022). For instance, only two studies have examined if the size of flower-visiting arthropods, correlates with their detectability using eDNA metabarcoding, although no consistent trends have emerged (Johnson et al., 2023; Kestel et al., 2023). The body size and abundance of flower-visiting arthropods can significantly affect the quality of pollination services (Solís-Montero et al., 2015), and may influence the likelihood of detection by molecular methods, such as eDNA (Johnson et al., 2023; Thomsen & Sigsgaard, 2019). Thus, it remains critical to identify potential sources of biases for eDNA detections and ultimately increase the accuracy and reliability of this molecular method.

We used two emerging arthropod survey methods – DVR devices and eDNA metabarcoding– in an agroecosystem to detect arthropods providing beneficial ecosystem services (i.e. potential pollinators and predators), as well as those antagonistic species which may affect crop yields (i.e. pests and pathogens). With this approach, we aimed to answer the following questions to improve monitoring of arthropod-plant interactions in agroecosystems:

1) How does the abundance and diversity of flower-visiting arthropods detected with eDNA and DVRs vary over time (i.e. crop flowering intensity) and space (i.e. inflorescence location in the canopy and between orchards)?
2) Do larger or more abundant flower-visiting arthropods have a higher detection rate using eDNA metabarcoding?

4.3 Methods

4.3.1 Field Site

For this study, inflorescences were collected from two *Persea americana* (‘Hass’ Avocado) orchards, Marron Brook Farm (34°18′52 S, 116°08′36 E), hereafter MB, and Bendotti Avocados (34°25'38 S 116°02'01 E), hereafter BA. Both orchards are located in the second largest avocado production area in Australia, the Manjimup-Pemberton region in south west Western Australia (SWWA) (Mccarthy & McCauley, 2020). Locally, the region is dominated by agricultural enterprises, primarily pasture lands and orchards, interspersed with remnants of secondary growth native karri forest (*Eucalyptus diversicolor*). As in other regions around the world, *Apis mellifera* hives are commonly hired in SWWA during *P. americana* flowering to facilitate cross-pollination (Dymond et al., 2021; Mccarthy & McCauley, 2020), although the reliability and efficiency of these services remains an area of ongoing research (Ish-Am & Eisikowitch, 1998; Perez-Balam et al., 2012; Sagwe et al., 2022). Orchard MB sits ca. 200 m asl and is dominated by ‘Hass’ *P. americana* trees interspersed with ‘Fuerte’ pollinisers, while orchard BA is located ca. 16 km SSW of MB at 138 m asl and cultivates only ‘Hass’ trees. In each orchard, we randomly selected eight ‘Hass’ trees across eight rows of 41 trees. All trees were of a similar age (~ eight years old) and height (between 3 – 5 m). The final three rows on all edges were excluded from sampling in both orchards to help reduce the impact of edge effects. For each sample tree, ten *P. americana* inflorescences were removed for eDNA analysis during low and peak *P. americana* flowering in 2020 (low; 3rd – 6th October, peak; October 30th – 3rd November). Digital video recordings were captured on the same trees as the eDNA inflorescence collections for both study orchards

4.3.2 eDNA surveys

4.3.2.1 Sample collection

For eDNA analysis, five inflorescences were collected from both the upper (> 2 m) and lower canopy (< 2 m) of each *P. americana* tree during low and peak flowering times (N = 10 inflorescences per tree, N = 320 inflorescence total). To sample evenly both the upper and lower canopy at each flowering intensity, six inflorescences were collected on the first day of sampling (three upper canopy, three lower canopy) and four inflorescences (two upper canopy and two lower canopy) were collected on the second day of sampling. All sampling days were sunny with low winds and no rain. To minimise sampling bias, inflorescences were sampled as per Kestel et al. (2023) from both the upper and lower canopy while walking around the full circumference of each *P. americana* study tree (collection method adapted from Howlett et al. 2018). To reduce cross-contamination, all equipment was sprayed with 10% bleach solution and wiped down after each

inflorescence was collected. Once removed, each inflorescence was placed in a plastic bag, zip-tied and kept on ice until the samples could be stored at – 20°C.

4.3.2.2 Laboratory processing

DNA was extracted from 140 –190 mg of ground florets, processed as per Kestel et al. (2023), using a DNeasy Blood and Tissue kit (Qiagen, Venio, Limburg, Netherlands) with the following modifications: 540 µl ATL lysis buffer and 60 µl Proteinase K (Qiagen, Venio, Limburg, Netherlands) during the cell digestion phase. Negative controls containing no ground material were digested alongside all samples to detect cross-contamination. Samples were digested in a slow-rotating hybridisation oven at 56°C overnight (~12 hrs). Following digestion, DNA was extracted using a QIAcube Connect automated DNA extraction platform (Qiagen, Venio, Limburg, Netherlands). The final elution volume was 100 µl, and extraction controls (blanks) were carried out for every batch of 30 DNA extracts (N = 10).

Quantitative Polymerase Chain Reaction (qPCR) (Applied Biosystems, USA) was used to assess the quality of each eDNA sample targeting the Cytochrome Oxidase subunit 1 (CO1) gene in the arthropod mitochondrial genome. Inhibitors in the PCR reactions and low copy number can impact metabarcoding data (Murray et al., 2015, 2011), therefore each eDNA extract was assessed with a qPCR dilution series (neat, 1/10, 1/100) under the following conditions: 25 µl reaction volumes containing 2.5 µl of 10 × PCR Gold Buffer (Life Technologies, Massachusetts, USA), 2 µl of 2.5 mM $MgCl_2$ (Fisher Biotec, Australia), 1 µl of 0.4 mg/ml BSA (Fisher Biotec), 0.25 µl of dNTPs (Astral Scientific, Australia), 0.6 µl of 5x SYBR Green (Life Technologies), 0.4 µl of each CO1 primer (forward and reverse; 10mM), 0.2 µl AmpliTaq Gold (Life Technologies), 2 µl of DNA and the remaining volume supplemented with DNase/RNase-Free Distilled Water. One PCR assays was used: assay fwhF2/fwhR2n (Vamos et al., 2017), targeting the CO1 gene, hereafter referred to as CO1. The forward primer sequence for CO1 was 5'—GGDACWGGWTGAACWGTWTAYCCHCC—3' and reverse primer sequence 5'—GTRATWGCHCCDGCTARWACWGG—3'. Amplicons for the CO1 assay were ~205 bp. Extracts were amplified on a StepOnePlus Real-Time PCR System (Applied Biosystems, Massachusetts, USA) under the following conditions for CO1: initial denaturation at 95°C for 5 min, followed by 50 cycles of 30 s at 95°C, 50°C for 30 s and 2 min at 72°C, with a final extension for 10 min at 72°C. Extraction and non-template controls were included in each qPCR assay. DNA extracts that showed inhibition were diluted using MilliQ water and the optimum quantity of DNA input was determined for fusion tagging.

Environmental DNA that were of sufficient quality and free of inhibition, as determined from the initial qPCR screen (qPCR dilution series), were assigned a unique (6 – 8 bp in length) multiplex identifier tag (MID-tag) for CO1. To reduce the likelihood of contamination, chimera production and MID-tag jumping (Esling et al., 2015), DNA was amplified in a single round of qPCR for each assay using MID-tag primers consisting of CO1 primers coupled to Illumina flow cell adaptors, custom sequencing primers and MID-tag combinations unique to this study. All fusion-tagged qPCR reactions were prepared in dedicated clean room facilities at the TrEnD Laboratory, Curtin University designed for ancient DNA work using an automated QIAgility robotics platform (Qiagen, Venio, Limburg, Netherlands) and were carried out in 25 µl reactions containing 2.5 µl of 10 × PCR Gold Buffer (Life Technologies), 2 µl of 2.5 mM $MgCl_2$ (Fisher Biotec), 1 µl of 0.4 mg/ml BSA (Fisher Biotec), 0.25 µl of dNTPs (Astral Scientific), 0.6 µl of 5x SYBR Green (Life Technologies), 0.5 µl of CO1 forward primer (20 mM), 5 µl of CO1 reverse primer (2 mM), 0.2 µl AmpliTaq Gold (Life Technologies), 4 – 8 µl of DNA and the remaining volume supplemented with DNase/RNase-Free Distilled Water. MID-tag PCR amplicons were carried out in duplicate reactions to control for PCR stochasticity and all fusion-tagged qPCRs were processed using the same parameters as the initial qPCR screens described above.

Replicate MID-tag amplicons were pooled at approximately equimolar concentrations (e.g. minipool) based on their respective qPCR DRn values and were measured under a high-resolution capillary electrophoresis system (QIAxcel; Qiagen Venio, Limburg, Netherlands) and the final library was size-selected (160 – 425 bp) using a PippinPrep (Millennium Science Pty Ltd, Australia) with a 2% ethidium bromide cassette (Sage Science, Beverly, USA) to remove any off-target amplicons and primer dimer. The final library was purified using the QIAquick PCR Purification Kit (Qiagen Venio, Limburg, Netherlands), quantified using a Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, USA) and diluted to 2 nM prior to sequencing. Sequencing by synthesis was performed on an Illumina MiSeq platform (Illumina, San Diego, USA) located in the Trace and Environmental DNA lab at Curtin University and as per Illumina's protocol for single-end sequencing with a 300 cycle MiSeq®V2 reagent kit and standard flow cell for environmental metabarcoding.

4.3.2.3 Data processing and bioinformatics

Sequenced multiplex identifier-tagged amplicons were inputted to a containerised workflow (eDNAFlow; Mousavi-Derazmahalleh et al., 2021) and run through the Pawsey Supercomputing Centre in Kensington, Western Australia. Here, the sequences were filtered, formed into Zero-radius Operational Taxonomic Units (ZOTUs) and assigned taxonomic identifications. Sequences

were quality checked using FASTQC (Andrews, 2010) and quality filtered (Phred quality score < 20), before the multiplex identifiers were trimmed from the sequence reads using AdapterRemoval v2 (Schubert et al., 2016). Subsequently, the filtered reads were demultiplexed using OBITOOLS (Boyer et al., 2016) and sequences shorter than the minimum length of 120 bp were filtered out. Sequences were then dereplicated into ZOTUs with a minimum sequence abundance of 5 (see Drake et al., 2022; Kestel et al., 2023) using the USEARCH Unoise3 algorithm (Edgar, 2016). A database of ZOTUs was then generated and queried against the GenBank (NCBI) nucleotide database with 100% query coverage and 97% identity using BLASTN (Altschul et al., 1990). Erroneous ZOTUs with a sequence similarity below the 97% threshold were removed using the LULU post clustering curation method (Frøslev et al., 2017). Finally, a custom Python script (eDNAFlow; Mousavi-Derazmahalleh et al., 2021) was used to assign taxonomic identifications to the curated ZOTUs using the Lowest Common Ancestor (LCA) approach. Taxonomic identification was assigned to a ZOTU when the percentage identity of two or more queried sequences with ≤ 0.5% difference had 100% query coverage and 97% sequence similarity. For the purposes of this study, we set the minimum threshold count of 5 reads for ZOTUs to classify a taxa as present. A custom reference database of locally occurring arthropod species that are known to visit *P. americana*, was developed to reduce false positives within the eDNA dataset (see Ficetola et al., 2016).

4.3.2.4 DVR surveys

Two GoPro cameras (Hero 7 Silver) were mounted on 1.5 m wooden stands to observe two of the ten sample inflorescences per study tree. Due to the limited number of DVR devices available and the complexity of building and transporting taller stands, only inflorescences in the lower canopy (< 2 m) were monitored. Each DVR device was set to Time-lapse mode (one image every 0.5 s) to maximise battery life. During video observations, three hours of arthropod visits per day for each tree were recorded and condensed into two 11 min 50 s videos per flowering intensity (N = 128 DVRs). DVR start times were staggered (Table S4.1). DVRs were deployed when temperatures were warm enough for bee activity (clear skies, wind speed <30 km/h, and >17°C; Prendergast et al., 2020). DVRs were downloaded at the end of each sampling day. One researcher watched each DVR on 0.4 x speed to allow for individual visits to be classified (method adapted from Gilpin, et al. 2017). As we sampled the whole *P. americana* inflorescence for eDNA analysis, we noted a visit when an arthropod species made contact with a flower on the inflorescence, any subsequent flower contacts within that same visit were not noted (method adapted from Sakamoto, Morinaga, Ito, & Kawakubo 2012). If an arthropod flew out of frame and then revisited the same inflorescence, this was counted as a new visit. Arthropod images were grouped into morphotypes

and identified to the species level where possible, using photographic reference material and descriptions from Zborowski & Storey (2017).

4.3.2.5 Statistical analysis

All statistical analyses were performed on R 4.3.1 (R Core Team, 2023). For both survey methods, taxa not resolved to the species level were grouped into morphotype at the family level (e.g. Syrphidae sp.) and these morphotypes were used as a proxy for species (D'Souza et al., 2021). Shapiro-Wilk and a paired Wilcoxon test were used to assess if the number of species detected per inflorescence sample differed significantly between low and high *P. americana* flowering intensity. We examined sampling effort with rarefaction curves, using the package 'vegan' in R, to verify if the number of DVRs and eDNA samples collected were sufficient to fully capture the total species diversity present within the two sample orchards.

To determine how the diversity and abundance of arthropods varied with *P. americana* flowering intensity and inflorescence location within tree canopies, as well as between orchards, we calculated arthropod detections per inflorescence for functional groups, whereby similar species (i.e. same Order) were pooled to capture novel flower visit patterns (see Fenster et al., 2004; Johnson et al., 2020). These functional groups included: all taxa (All), the dominant flower-visiting orders (Diptera and Hymenoptera), and the non-dipteran and non-hymenopteran taxa ('Ancillary'). For DVR detections, we calculated flower visits per species per inflorescence for the same functional groups used in the eDNA analysis. Skewness and Kurtosis values were generated for both the eDNA and DVR species cohorts using the Skewness and Kurtosis function in the 'e1071' package. Generalized linear models (GLMs) with binomial distributions were then generated for each of the four eDNA datasets (All, Diptera, Hymenoptera and 'Ancillary'). While for the DVRs, GLMs with negative binomial distributions were used for three of the All, Diptera, Hymenoptera species cohorts and a Poisson distribution was used for the 'Ancillary' species cohort to account for zero-inflation.

The co-variates included in the initial eDNA GLMs were: inflorescence location in the canopy (upper; sampled >2 m in the canopy or lower; sampled <2 m in the canopy), sample orchard (BA or MB) and flowering intensity (low or peak). All three co-variates were listed as categorical and species counts for all taxa, dipterans and hymenopterans were pooled across trees and sampling period. Sample tree and the date of sampling were not included in the GLM analysis for either eDNA or DVRs due to underdispersion. For DVRs the co-variates included in the initial GLMs were: sample orchard (BA or MB), flowering intensity (low or peak), and deployment time.

Dispersion statistics were calculated for each model with inflated zero counts (> 50% of samples with zeros) and run in 100,000 dataset simulations. To determine the significance of the co-variates in the final models, we re-ran the final GLMs using robust standard errors and compared the output to the DVR co-variate significance results. Cameron and Trivedi (2009) recommended using robust standard errors for estimating parameters derived from GLMs to control for instances when the distribution assumption that the variance equals the mean have minor violations. We used Kruskal-Wallis and Paired-sample Wilcoxon tests for family and species counts for all taxa, dipterans and hymenopterans with samples pooled across trees and sampling period to determine if inflorescence location in the canopy (upper canopy > 2m or lower canopy < 2m) influenced the number of detections.

For the eDNA detections, a Permutational Multivariate Analysis of Variance (PERMANOVA) based on Jaccard distances (eDNA presence-absence detections) and 9999 permutations was used to assess which significant explanatory variables (study orchard; BA or MB, flowering intensity; low flowering or peak flowering, or sample tree; trees 1 - 8) accounted for the most variation. The ordination for eDNA species composition detected between the study orchards at low and peak *P. americana* flowering intensities was calculated based on binary Jaccard distances (eDNA presence-absence detections). Species contributing to the most variation in community composition were overlayed on this ordination with vector length proportional to the strength of the correlation as determined by the goodness of fit statistic (squared Pearson correlation coefficient) using 'Vegan' in R. These findings were confirmed using a Similarity percentage analysis (SIMPER) (Clarke, 1993) between orchards and flowering intensities. Following the above procedure for the DVR dataset, we performed a PERMANOVA with the Bray-Curtis distances (DVR flower visits) and 9999 permutations to assess which explanatory variables (study orchard; BA or MB, flowering intensity; low flowering or peak flowering, or sample tree; trees 1 - 8) explained the most variation in the DVR flower visits. Significant explanatory variables were then included in a PCoA based on Bray-Curtis dissimilarity distances (DVR flower visits) to visually represent the difference in species composition between the study orchards at low and peak *P. americana* flowering intensities. Species contributing to the most variation in community composition were overlayed on this ordination with vector length proportional to the strength of the correlation as determined by the goodness of fit statistic (squared Pearson correlation coefficient) using 'Vegan' in R. These findings were confirmed using a SIMPER analysis (Clarke, 1993).

To determine if larger or more abundant arthropods had a higher detection rate for eDNA metabarcoding, we used generalised linear models with binomial or log-normal distributions to test whether the length of arthropods (mm) or the number of flower visits observed by DVRs affected the eDNA detections or read number of the observed arthropod taxon. Length measurements for 21 species observed visiting by DVRs and detected by eDNA surveys were obtained from the BOLD (www.boldsystems.org) and Atlas of Living Australia databases (www.ala.org.au). The first of these models included only observations from the DVRs and the binomial eDNA detection outcome of 1 – detection, or 0 – no detection. The variables arthropod length (mm) and the number of flower visits were log-transformed to normalise the distribution. Species-level associations between the DVRs and eDNA data were selected to allow for the most accurate length measurements for each arthropod taxa. Where taxa could not be identified to species-level on the DVRs (i.e. Braconidae sp.), we averaged several length measurements of known taxa within the study region to obtain a length estimate. For the second model, we also included only observations from the DVRs, although here we used a log-normal distribution for the number of reads generated for each inflorescence under DVR observation. As above, we used log-transformed arthropod length (mm) and log-transformed flower visits to normalise the distribution and included species-level associations.

4.4 Results

4.4.1 eDNA Surveys

We generated 29,608,114 raw sequence reads from 320 inflorescence samples and 10 extraction and PCR controls. Overall, 26,220,018 quality-filtered reads were generated with a mean sequencing depth of 79,214 per sample. No ZOTUS above the minimum threshold of five reads (see Drake et al., 2022; Kestel et al., 2023) were found in the extraction or PCR controls. We removed 36 ZOTUS corresponding to fungi spp. (e.g. Ascomycota, Basidiomycota and Oomycota), *Homo sapiens* and *Canis* sp. After removal, the mean number of reads per sample was 3914 (±625 SE) and 46 samples (14% of all samples) failed to amplify 5 or more reads. Neither eDNA nor DVRs alone captured the total species richness present within the sample orchards (Figure S4.1).

4.4.2 Taxonomic composition – eDNA and DVRs

Overall, we detected 42 arthropod families in the eDNA dataset, of which, potential pest Thripidae species (*Thrips australis*, *T. tabaci* and *Frankliniella* sp.: 54% of inflorescence samples), pollinator Apidae species (*Apis mellifera*: 31% of inflorescence samples) and a potential plant parasite, Tydeidae species, (Unknown Tydeidae sp.: 22% of inflorescence samples) were the most common. In total, 66 taxa were identified by eDNA, with 22 (33%) resolved to the genus level, 35 (53%) resolved to the species level, while 8 (12%) could not be resolved beyond family level and 1 (2%) could not be resolved beyond order level. Overall, the average number of arthropod families detected per inflorescence sample was 2 (± 0.09 SE) and the average number of species detected per sample was 2 (± 0.08 SE) and the number of species detected per sample did not differ significantly between low and peak flowering (p = .14). For the DVR dataset, we detected 22 families in total, of which Apidae (*A. mellifera:* 91% of DVRs), potential predator and pollinator Syrphidae species (*Melangyna viridiceps* and *Simosyrphus grandicornis*: 65% of DVRs) and potential pollinator Calliphoridae species (*Calliphora* spp.: 48% of DVRs) were the most commonly detected flower visitors per sample, while the most abundant flower visitors of the 15,138 directly observed visits were: Syrphidae spp. (*Melangyna viridiceps* and *Simosyrphus grandicornis*: 84% of flower visits), followed by Apidae (*Apis mellifera*: 11% of flower visits) and Calliphoridae (*Calliphora* spp.: 2% of flower visits).

4.4.3 eDNA-detected arthropod diversity changes significantly with flowering intensity, within canopies and across orchards

The eDNA Diptera cohort represented seven families representing 12 species (Table S4.2). The Hymenoptera cohort was comprised of five families representing six species (Table S4.2). The 'Ancillary' cohort was made up of 27 families, representing 44 species (Table S4.2). For the eDNA GLM analysis, the species counts for All, Diptera, Hymenoptera and 'Ancillary' generated dispersion values of 1.04, 1.18, 1.10 and 1.02, respectively, potentially indicating overdispersion for Diptera and Hymenoptera. Skewness values showed left-skews for All (-2.06) and 'Ancillary' (-1.33), while both Diptera (1.17) and Hymenoptera (0.77) showed right skews. Kurtosis values showed a positive excess for All (2.24) and negative excess for Diptera (-0.64), Hymenoptera (-1.40) and 'Ancillary' (-0.22). The percentage of zeros was low for All (14%) and 'Ancillary' (22%), but large for both the Diptera (75%) and Hymenoptera (68%) datasets. Although when simulated, the majority of zeros for both the Diptera and Hymenoptera GLMs could be explained (Figure S4.2). Flowering intensity and orchard were significant co-variates for All (flowering; $p < .001$, orchard; $p = .04$), Diptera (flowering; $p = .001$, orchard; $p < .001$) and Hymenoptera (flowering; $p < .001$, orchard; $p < .001$) species cohorts, while only flowering intensity was significant for 'Ancillary' (flowering; $p = .001$, orchard; $p = .2$) (Figure 4.1 and Table S4.4). Inflorescence location in the canopy was a significant explanatory co-variate for both Hymenoptera and 'Ancillary' ($p = .03$ for Hymenoptera and $p = .02$ for 'Ancillary') (Figure 4.1 and Table S4.4). Using non-parametric testing, we determined that the probability of detecting Hymenoptera species was greater in the upper canopy (> 2 m) compared to the lower canopy (< 2 m) (upper; 0.36 ± 0.04 SE, lower; 0.25 ± 0.03 SE; $p = .03$) (Figure 4.2). Similarly, for the 'Ancillary' species cohort, the probability of detecting species was greater in the upper canopy compared to the lower canopy (upper; 0.85 ± 0.03 SE, lower; 0.74 ± 0.04 SE; $p = .02$) (Figure 4.2).

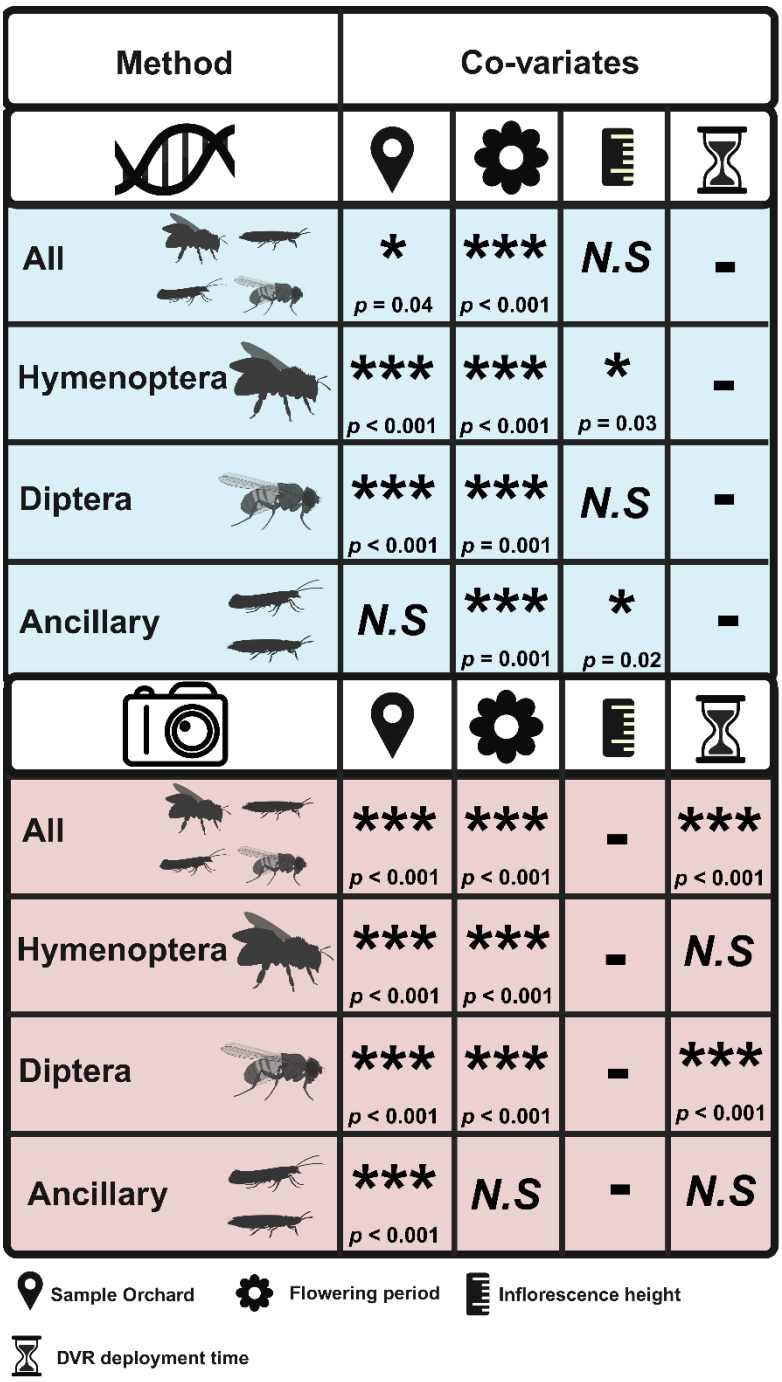

Figure 4.1 Comparison of covariate significance for eDNA and DVR datasets. *N.S* = not significant for GLMs. Deployment time was not a feature of the eDNA dataset and inflorescence height was not a feature of DVR dataset. Table created in Biorender.

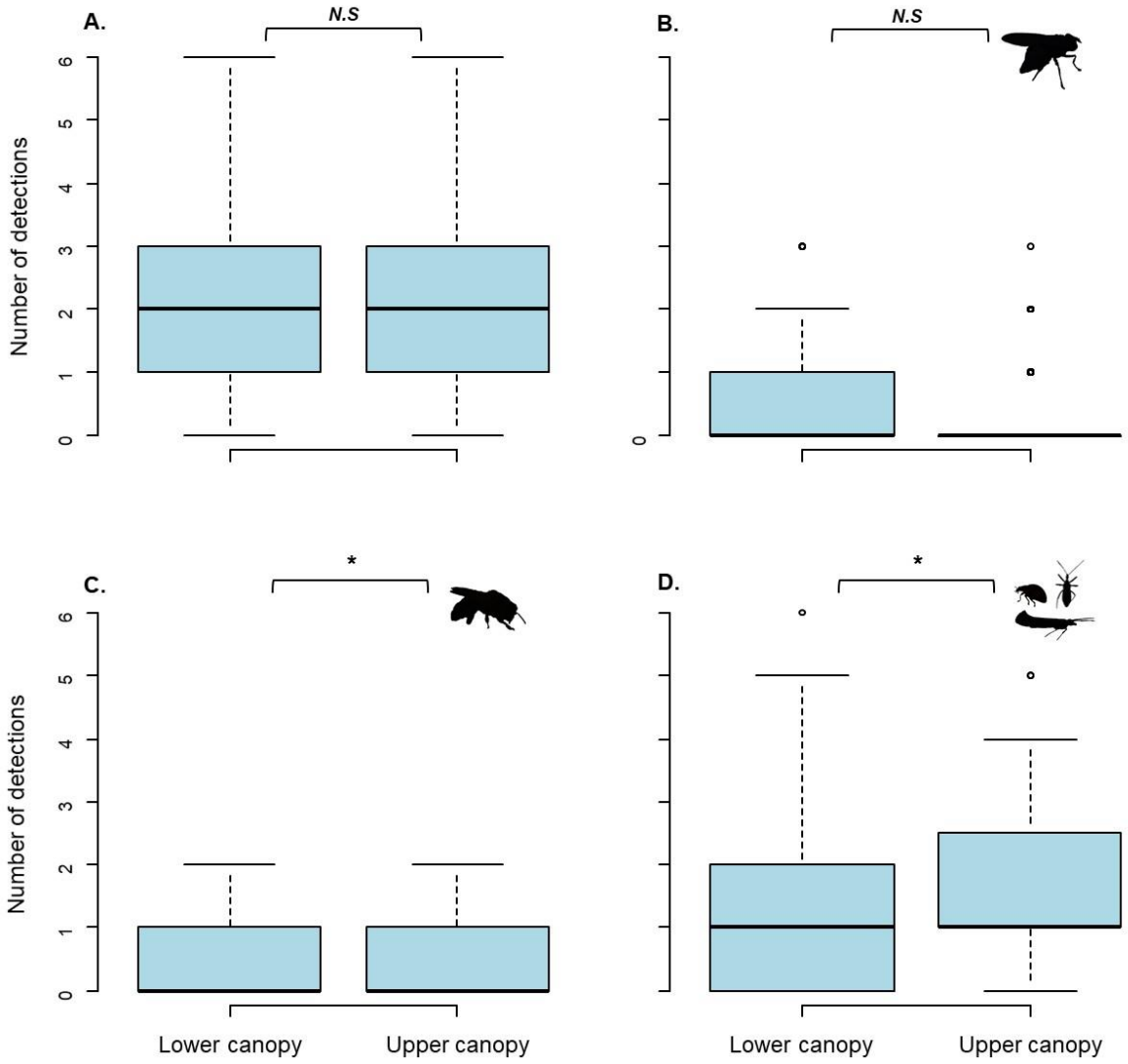

Figure 4.2 The number of eDNA detections within the lower canopy (≤ 2 m) and upper canopy (≥ 2 m) of each sampled *P. americana* tree. Vertical stratification for arthropod functional groups (A) all arthropods, (B) Diptera, (C) Hymenoptera, and (D) 'Ancillary' detected using eDNA metabarcoding. N.S = $p > .05$ and * = $p \leq .05$.

4.4.4 DVR-detected diversity shifts both temporally and spatially

The DVR Diptera cohort represented six families containing six species (Table S4.3). The DVR Hymenoptera cohort was comprised of seven families representing seven species (Table S4.3). While, the DVR 'Ancillary' cohort was made up of 9 families, representing 9 species (Table S4.3). For the DVR GLM analysis, the flower visit counts for All, Diptera, Hymenoptera and 'Ancillary' generated dispersion values of 1.35, 1.48, 1.26 and 1.08, respectively, potentially indicating overdispersion for All, Diptera and Hymenoptera. Skewness values showed right-skews for all

four species cohorts: All (2.32), Diptera (2.45), Hymenoptera (2.16), and 'Ancillary' (3). Kurtosis values showed a positive excess for all four species cohorts: All (4.96), Diptera (5.68), Hymenoptera (5.67) and 'Ancillary' (9.25). The percentage of zeros was low for three species cohorts: All (0%), Diptera (25%), Hymenoptera (4%), although the 'Ancillary' species cohort showed zero-inflation (75%). When simulated with a Poisson distribution however, we determined that the majority of zeros in the 'Ancillary' cohort could be explained (Figure S4.2). Flowering intensity and orchard were significant co-variates for All (flowering; $p < .001$, orchard; $p < .001$), Diptera (flowering; $p < .001$, orchard; $p < .001$) and Hymenoptera (flowering; $p < .001$, orchard; $p < .001$) species cohorts, while only sample orchard was significant for the 'Ancillary' species cohort (flowering; $p = .10$, orchard; $p < .001$) (Figure 4.1 and Table S4.4). Deployment time was a significant explanatory co-variate for both All and Diptera species cohorts ($p < .001$ in both instances) (Figure 4.1 and Table S4.4).

The DVR abundance data was analysed for five functional groups, these were: 1) All species, comprised of 22 families representing 22 species, 2) Syrphidae species cohort, comprised of one family (Syrphidae) representing two species (*Melangyna viridiceps* and *Simosyrphus grandicornis*), 3) Non-Syrphidae Diptera species cohort, comprising four families representing at least four species: *Calliphora* spp. (Calliphoridae), Sarcophagidae spp., *Poecilohetarus* sp. (Lauxaniidae), *Musca* spp. (Muscidae) and unclassified spp, 4), Hymenoptera species cohort, comprising seven families representing seven species: *Apis mellifera* (Apidae), *Pheidole megacephala* (Formicidae), Braconidae sp., Bethylidae sp., Vespidae sp., Pompilidae sp. and *Lasioglossum* sp. (Halictidae) and 5) 'Ancillary' species cohort, comprising nine families representing nine species: Thripidae spp., *Opisthoncus* sp. (Salticidae), Pentatomidae sp., Coccinellidae sp., unclassified Coleoptera sp., Acrididae sp., *Phrissogonus laticostata* (Geometridae), *Plutella xylostella* (Plutellidae) and *Diomocoris woodwardi* (Miridae).

Per tree, the number of visits for all species observed by DVRs increased significantly between low and peak flowering for orchard BA, where 25 visits per tree (± 3.04 SE) at low flowering increased to 81 visits per tree (± 12.75 SE) at peak flowering, while orchard MB also showed a significant increase from 39 visits per tree (± 6.28 SE) at low flowering to 1746 visits per tree (± 182.95 SE) at peak flowering ($p < .01$ for both orchards) (Figure 4.3, Tables S4.5 and S4.6). Of these, the most significant flower visitors for both orchards were Syrphidae spp. (*Melangyna viridiceps* and *Simosyrphus grandicornis*), which showed an increase in orchard BA from 3 visits per tree (± 0.77 SE) at low flowering to 28 visits per tree (± 6.61 SE) at peak flowering, while in

orchard MB, the number of Syrphidae spp. visits increased from 1 visit per tree (± 0.58) at low flowering to 1566 visits per tree (± 179.62 SE) at peak flowering ($p < .01$ for both orchards) (Figure S4.3 and Table S4.6). Between low and peak flowering, orchard MB also showed significant increases in Hymenoptera spp.; 33 visits per tree (± 6.59 SE) at low flowering to 128 (± 14.52 SE) visits per tree at peak flowering ($p < .01$), and non-Syrphidae Diptera spp.; where the number of visits increased from 2 per tree (± 1.02 SE) at low flowering to 48 visits per tree (± 15.18 SE) at peak flowering ($p < .01$). Whilst, no significant differences in visit number were found for orchard BA for either Hymenoptera spp. ($p = .19$) or non-Syrphidae Diptera spp. ($p = .06$) between flowering intensities (Figure S4.3, Tables S4.5 and S4.6).

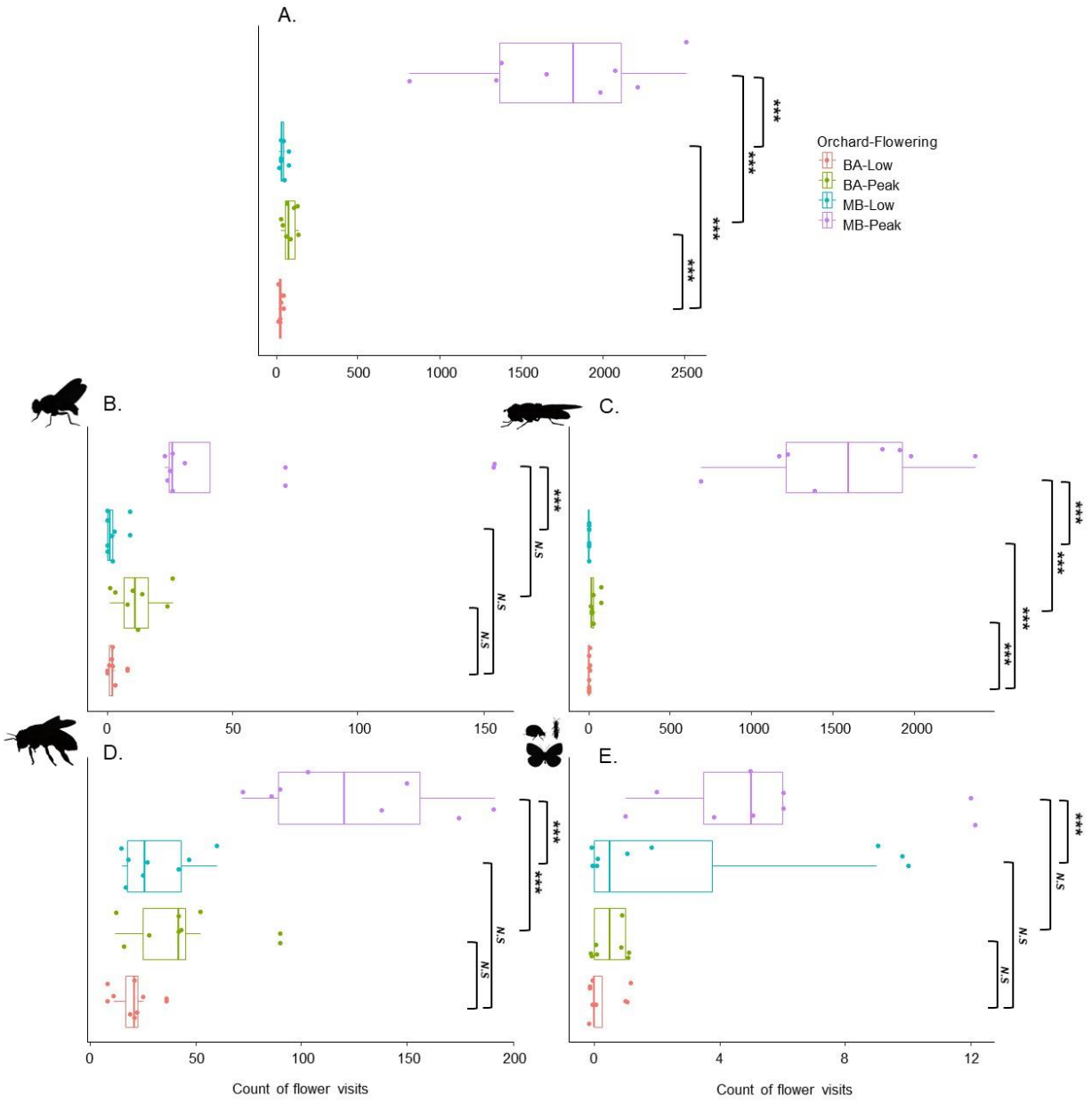

Figure 4.3 Flower visit counts, as determined by DVRs, for orchards BA and MB at low (1) and peak (2) flowering for: (A) all species, (B) Non-Syrphidae Diptera species, (C) Syrphidae species, (D) Hymenoptera species, and (E) 'Ancillary' species. Flower visits represent the total number of flower visits for two pooled inflorescences per tree observed per flowering period (N = 12 hours of observations per tree per flowering period, 192 hours of observation in total). *N.S* = $p > .05$ and *** = $p \leq .001$. Average and significance values for each boxplot are presented in Tables S4.8 and S4.9.

4.4.5 Potential pollinators, pests and predators drive divergence between low and peak flowering

A PERMANOVA analysis identified that both study orchard and flowering intensity were significant explanatory variables for the eDNA and DVR datasets ($p < .01$ for both study orchard and flowering intensity), while sample tree was not shown to be a significant explanatory variable for either survey method (eDNA; $p = .55$, DVR; $p = .99$). When visualised in a PCoA, eDNA detections showed stronger separation along the first axis when compared to the second axis, indicating that more variance in the eDNA dataset is explained by orchard location, rather than *P. americana* flowering (Figure 4.4). While DVR counts showed greater separation along the first axis when compared with the second, indicating that orchard location may account for more variance in the DVR dataset than *P. americana* flowering (Figure 4.4). There were 9 taxa significantly correlated with study orchard and flowering intensity within the DVR dataset, while 13 taxa were significantly correlated for the eDNA dataset (Figure 4.4 and Table S4.7). Similarity Percentage analysis (SIMPER) was used to identify prominent flower-visitors, pests and predators contributing to the most pairwise dissimilarity between orchards and flowering intensities for the DVR and eDNA datasets (Figures 4.5, 4.6 and Tables S4.8 and S4.9).

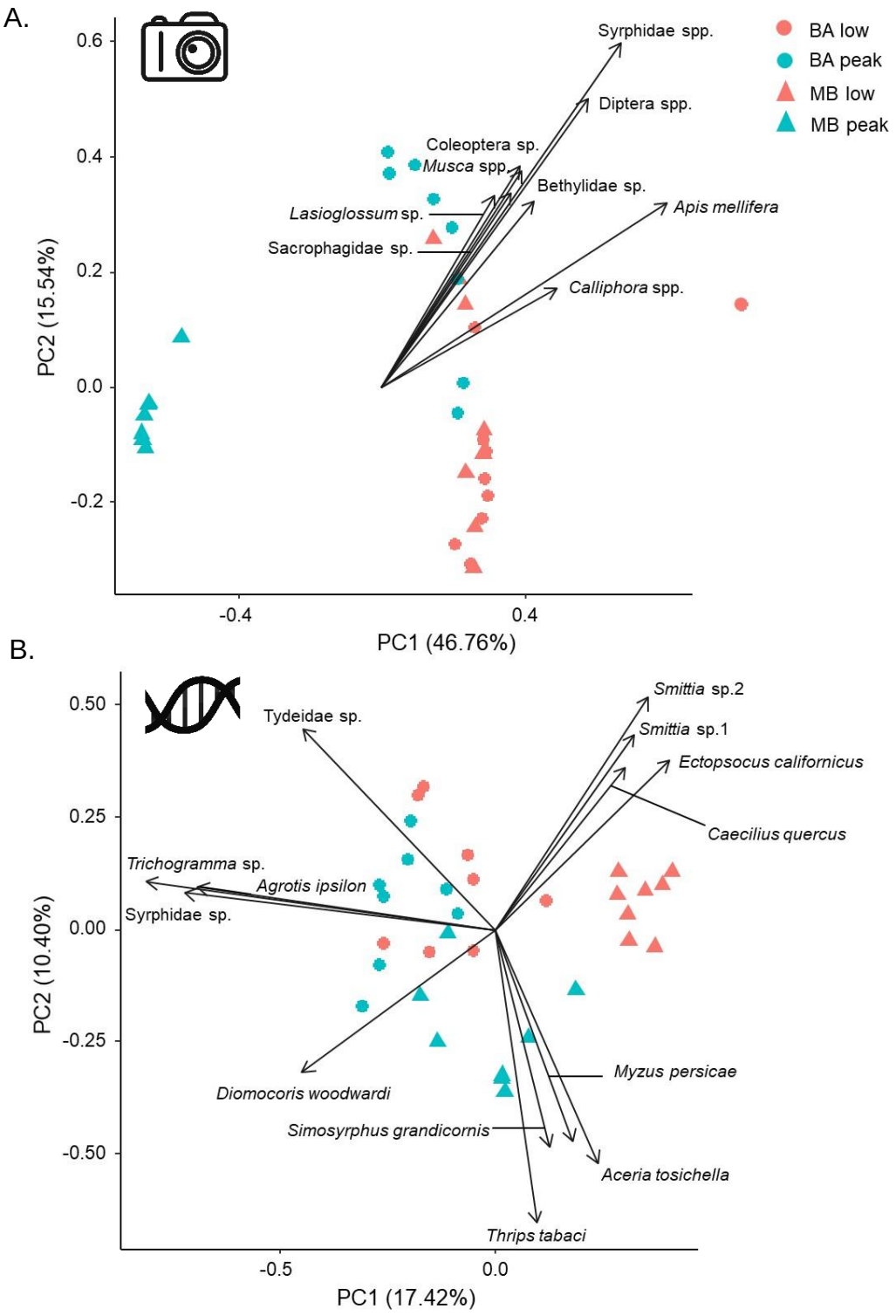

Figure 4.4 A. PCoA plot showing the grouping of samples between two study orchards at low and high *P. americana* flowering based on species community structures detected by DVRs. B. PCoA plot showing the grouping of samples between two study orchards at low and high *P. americana* flowering based on the species community structures detected by eDNA. Samples were obtained from two replicate orchards in Pemberton in south west Western Australia. Scaled arrows represent

species correlated with each study orchard and flowering intensity, all species plotted were significantly (Pearson correlation > 0.5).

A greater cumulative contribution of species was identified for the eDNA dataset compared to the DVRs (Figure 4.5). For the eDNA detections between two flowering intensities, the plant pest and potential pollinator moth *Phrissogonus laticostata*, the hoverfly *Simosyrphus grandicornis* had higher detection rates and the aphid plant pest species *Myzus persicae* had a lower detection rate at peak flowering in orchard BA (BA low vs. BA peak; 55% dissimilarity). Whereas for orchard MB, the chironomid *Smittia* sp. 2, Tydeidae sp. had a lower detection rate and *Simosyrphus grandicornis* had higher detection rate at peak flowering (MB low vs. MB peak; 61% dissimilarity) (Figure 4.6). Between the orchards at low flowering, the psocopteran *Caecilius quercus*, the ectopsocid *Ectopsocus californicus*, and *Smittia* sp. 2 were detected more frequently at orchard MB compared to orchard BA (BA low vs. MB low; 64% dissimilarity) (Figure 4.6). At peak flowering, Tydeidae sp., and the mirid *Diomocoris woodwardi* were more frequently detected at Orchard BA, while *Caecilius quercus* was only detected at orchard MB (BA peak vs. MB peak; 56% dissimilarity) (Figure 4.6 and Table S4.9). For DVRs between flowering intensities, this dissimilarity was primarily driven by a higher detection rate of *Apis mellifera*, potential pollinator *Calliphora* spp. and Coccinellidae sp. at peak flowering for BA (BA low vs. BA peak; 55% dissimilarity) and a higher detection rate of Syrphidae spp. (*Melangyna viridiceps* and *Simosyrphus grandicornis*), the arthropod predator Coleoptera sp. and the plant pest and potential pollinator moth *Plutella xylostella* at peak flowering for orchard MB (MB low vs. MB peak; 96% dissimilarity) (Figure 4.6). Between orchards at low flowering, the DVRs detected more *Apis mellifera*, Thripidae spp. and Diptera spp. at orchard MB compared to orchard BA (BA low vs. MB low; 39% dissimilarity) (Figure 4.6). While at peak flowering, DVRs at orchard MB showed the presence of more Syrphidae spp. (*Melangyna viridiceps* and *Simosyrphus grandicornis*), Coleoptera sp. and *Plutella xylostella* at Orchard MB in contrast to orchard BA (BA peak vs. MB peak; 90% dissimilarity) (Figure 4.6 and Table S4.8).

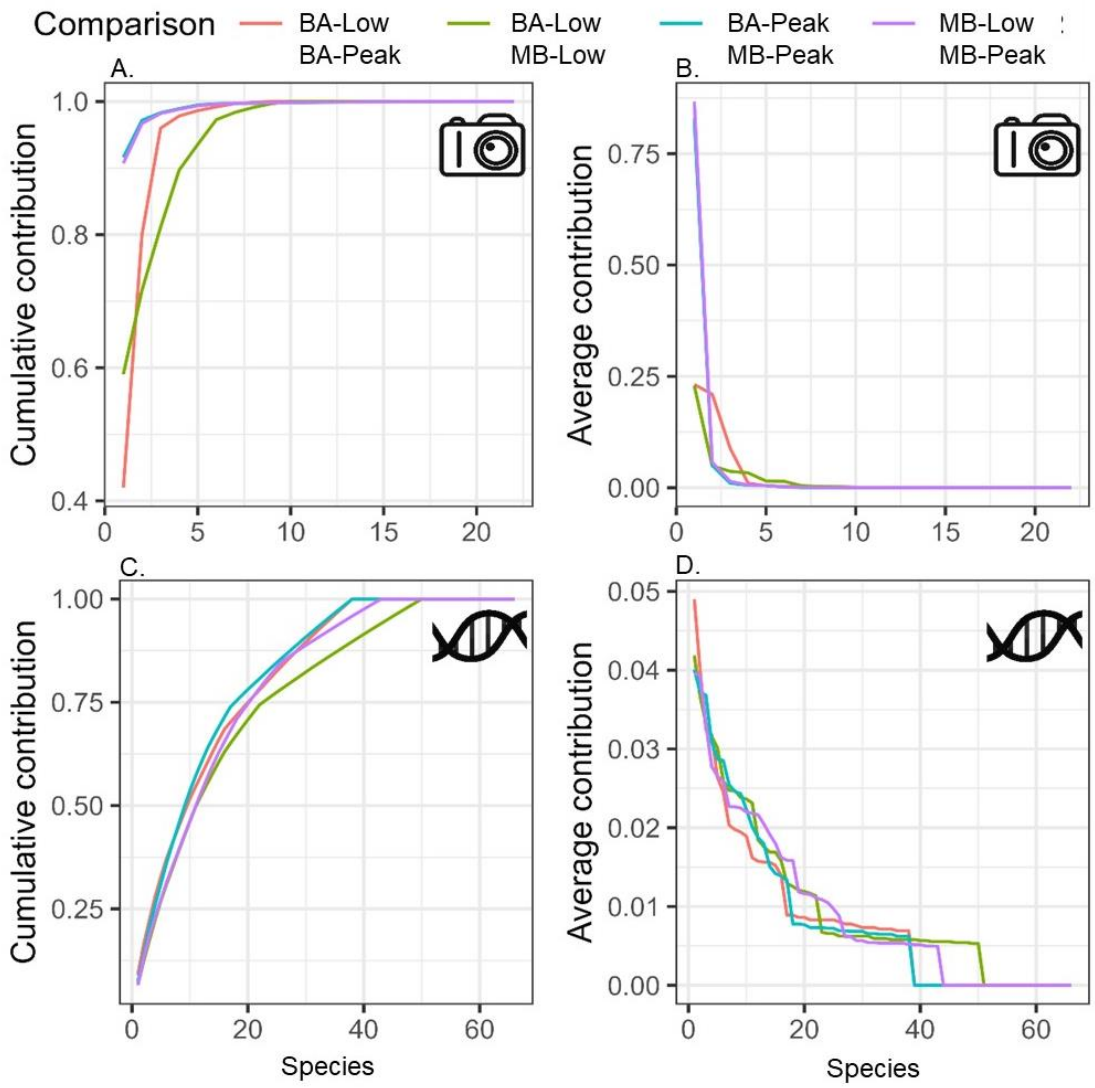

Figure 4.5 Cumulative and average contribution of species to the dissimilarity in the DVR (A, B) and eDNA (C, D) datasets for orchards BA and MB at low (1) and peak (2) flowering.

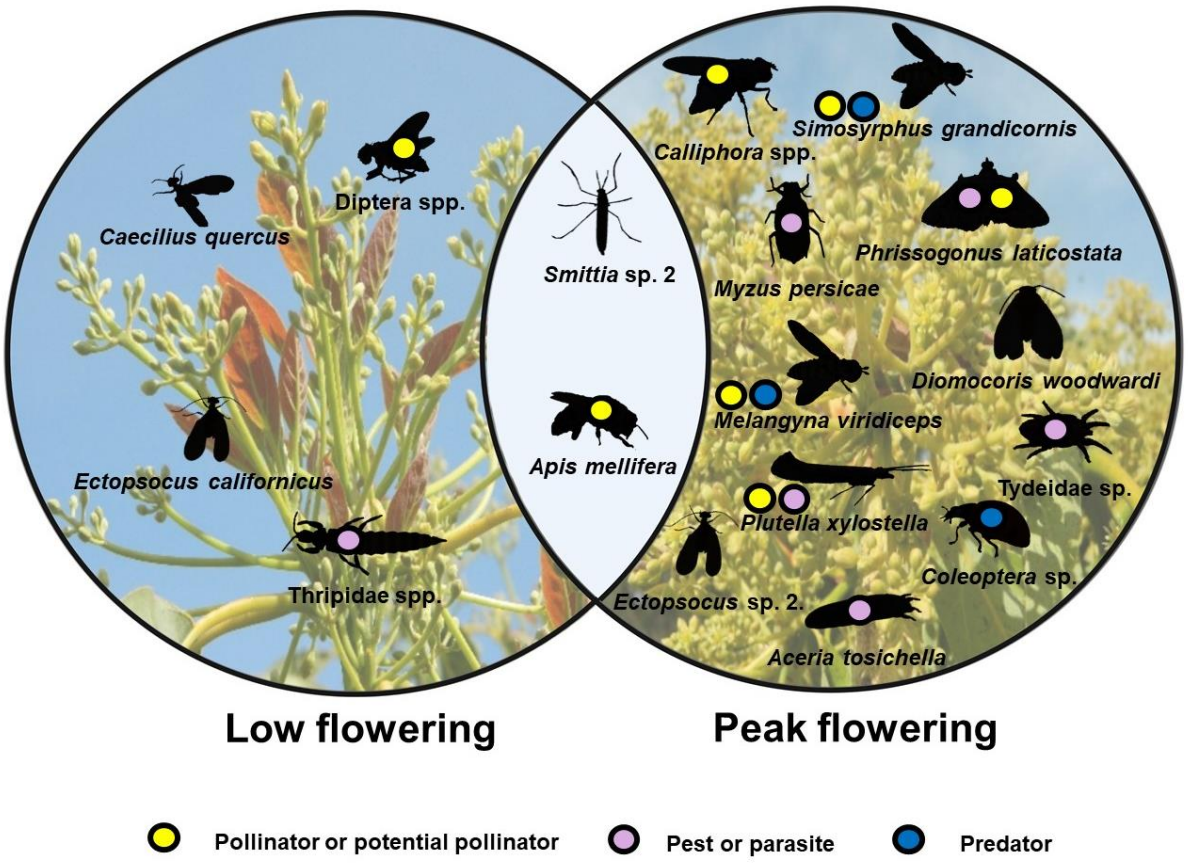

Figure 4.6 The key species driving dissimilarity identified in the eDNA and DVR SIMPER analyses at both sample orchards at low and high *P. americana* flowering. Where known, species have been colour-coded as either pollinator, pest or arthropod predators (see Table S4.10).

4.4.6 Arthropod length and abundance influence eDNA detection using eDNA metabarcoding

The majority (88%) of the arthropods detected by DVRs were 10 mm in length or smaller. The largest arthropod species observed were: *Apis mellifera* (mean length of 20 mm), *Phrissogonus laticostata* (mean length of 11 mm), *Melangyna viridiceps* (mean length of 10 mm) and *Simosyrphus grandicornis* (mean length of 10 mm). The generalised linear model (GLM) indicated that the length of the flower-visiting arthropod ($p < .01$) and the number of visits ($p < .01$) were significant factors for explaining eDNA detections. Smaller arthropods were more likely to be detected (Odds ratio = 0.44), as were those that visited the flowers multiple times (Odds ratio = 1.64) (Table 4.1). Similarly, the GLM for read number showed that both arthropod length ($p < .01$) and the number of visits ($p < .01$) were significant explanatory variables. Arthropod length was found to negatively correlate with read number (-0.28), while greater flower visits positively correlated with the read number for those same taxa (0.17) (Table 4.2).

Table 4.1 Results of a generalized linear model with binomial distribution showing the effect of arthropod length (mm), the number of arthropod visits on arthropod eDNA detections from flowers.

Predictors	Odds ratio	Lower Cl	Upper Cl	*p*
(Intercept)	0.16	0.10	0.25	< .001
Arthropod length (mm) + 1[Log10]	0.44	0.32	0.59	< .001
Arthropod visits + 1[Log10]	1.64	1.40	1.92	< .001

Table 4.2 Results of a generalized linear model with log-normal distribution showing the effect of arthropod length (mm) and the number of arthropod visits on log eDNA reads sequenced from flowers.

Predictors	Correlation coefficient	SE	*p*
(Intercept)	0.75	0.09	< .001
Arthropod length (mm) + 1[Log10]	0.17	0.04	< .001
Arthropod visits + 1[Log10]	-0.28	0.05	< .001

4.5 Discussion

Globally, crop yields are reliant on the ecosystem services from managed and unmanaged pollinators that are increasingly threatened by a mixture of biotic and anthropogenic stressors (Mashilingi et al., 2021; Potts et al., 2016; Wagner et al., 2021). Furthermore, annual yields are reduced by between 20 – 40% by plant pests and pathogens carried by arthropods (Flood, 2010). Unfortunately, detecting these complex arthropod-plant interactions is difficult to achieve at the large scale of many agricultural systems (Kestel et al., 2022; Maistrello et al., 2016; Valentin et al., 2020). Such circumstances necessitate the development of stand-alone or complementary high-throughput arthropod survey and monitoring methods. Here, we provide insight into the novel application of eDNA collected from tree flowers to detect spatio-temporal shifts of arthropod communities in an agroecosystem. We detected DNA from a wide array of functional groups, including potential pollinators (e.g. *Apis mellifera*, Muscidae sp., Syrphidae sp.), pests (e.g. *Thrips tabaci*, *Agrotis ipsilon*), potential parasites (e.g. Tydeidae sp.) and possible predator species (e.g. *Aphidius colemani*, *Simosyrphus grandicornis*, *Mallada signatus*). Additionally, we found that the relative abundance of many of these taxa changed in response to *P. americana* flowering intensity, inflorescence location in the canopy, and orchard, indicating that this molecular survey method can detect temporal, as well as fine and large scale arthropod community changes (however, see section *4.5.3 Complementarity of eDNA and DVRs*). Our findings support the growing recognition of the value of eDNA-based monitoring of biodiversity and show that this approach can support current methods that identify beneficial and antagonistic arthropods in agricultural systems.

4.5.1 Greater eDNA detections of potential pollinators, pest and predator taxa in response to crop flowering intensity

An increased availability of flowering resources (i.e. pollen and nectar) can positively affect both managed and unmanaged arthropod diversity and abundance (Bezerra da Silva Santos et al., 2022; Ebeling et al., 2008; Gilpin et al., 2022). In the present study, the number of arthropod eDNA detections increased by 14% during peak *P. americana* flowering and these detections included species from different functional groups including known pollinators, plant pests, and arthropod predators. For instance, the number of eDNA detections of *Apis mellifera*, one of the main managed pollinators of *P. americana* (see Dymond et al., 2021; Sagwe et al., 2022), doubled between low and peak flowering, a finding that was confirmed by DVR observations. It is likely that *A. mellifera* abundance and activity increased due to the presence of hired hives and warming seasonal temperatures (see Ish-Am, 2005; Ish-Am & Eisikowitch, 1998). For apple trees (*Malus domestica*), eDNA metabarcoding of flowers also consistently detected *A. mellifera*, which is the main managed pollinator for the crop (Gomez et al., 2023). Here, in addition to detecting known

pollinators for *P. americana*, eDNA metabarcoding also detected six unmanaged arthropod taxa, belonging to both Diptera and Lepidoptera, which may provide complementary pollination services (Buxton et al.,2023; Cook et al., 2020) (Table S4.10). We note, however, that eDNA detections alone do not prove successful pollination, as flower visits can be an inaccurate proxy for this ecological service (see King, Ballantyne, & Willmer, 2013), thus future eDNA surveys that aim to detect unmanaged pollinators should include exclusion experiments to confirm that the species detected are indeed pollinators (e.g. Webber et al., 2020). While the original aim of this study was to capture only potential pollinating species, we discovered that eDNA metabarcoding was also able to reliably detect the occurrence of small and often easily missed agricultural pest species.

Crop pests are often cryptic and difficult to detect at the large scale of agriculture (Kestel et al., 2022; Maistrello et al., 2016). Alarmingly, any delay in the capture and identification of these species can hinder management efforts and potentially increase crop losses (Hoebeke et al., 2003; Valentin et al., 2016). With the use of eDNA metabarcoding, we were able to detect 26 taxa (39% of all taxa detected), which are known agricultural pest species (Table S4.10). The inclusion of two sampling time points allowed us to examine if the relative abundance of these invasive species changed with flowering intensity. For instance, the number of detections for *Thrips tabaci*, a rapidly reproducing and largely insecticide-resistant crop pest (see Diaz-Montano, Fuchs, Nault, Fail, & Shelton, 2011; Kirk, 1987), increased by 70% between low and peak *P. americana* flowering. Species such as *T. tabaci* feed on pollen and reproduce inside flowers (Kirk, 1987), therefore, their increased presence likely reflects the larger abundance of flowers and floral resources at peak flowering (Atakan & Uygur, 2005). The ability to detect crop pests from flower samples opens up an array of potential research question; however, we stress that before such questions can be answered, more baseline studies are required. Specifically, numerous questions remain about the biases and temporal window of DNA deposition on plant tissue. For example, are certain arthropod behaviours, like walking over flowers, nectar/sap sucking or pollen collecting, associated with greater DNA deposition and subsequent detections (e.g. Kudoh et al., 2020). Second, does bacterial community diversity, UV levels or rainfall affect the persistence of arthropod DNA from plant tissue? (e.g. Valentin et al., 2021). Without such knowledge, we caution that farm managers may erroneously apply management options (e.g. pesticide applications) for crop species where the target pest is no longer present.

Biological control, achieved by maintaining and potentially increasing the presence of beneficial predatory arthropods, is becoming recognised as an intrinsic part of sustainable agriculture practices (Martínez-Sastre et al., 2020; Muñoz et al., 2021; Zhou et al., 2014). Unfortunately, identifying these species often relies upon microscopy and morphological-based methods, both of which require specialist expertise that may not always be readily available (Furlong, 2015; Gurr & You, 2016). Further, these beneficial predators are generally difficult to detect due to the ephemeral nature of their populations and the lack of easily identifiable prey from their gut contents to determine which taxa upon which they are predating (Furlong, 2015; Martínez-Sastre et al., 2020). With eDNA metabarcoding, we detected 12 taxa (18% of all taxa detected), known to provide predatory ecosystem services, and identified an increase in their presence during peak flowering (Table S4.10). One example of this was the increase from 0 to 15 detections of *S. grandicornis* between low and peak flowering. The larvae of this hoverfly species predates on plant pest aphids, which, if otherwise unchecked, can increase crop stress and reduce yields (Bowie et al., 2001; Robertson et al., 2020; Wotton et al., 2019). To further improve the ecological resolution of predator detections in eDNA flower surveys, we recommend the inclusion of either active (e.g. sweep netting) or passive sampling devices (e.g. Malaise traps) to capture predators and then use eDNA metabarcoding for their gut contents (see Furlong, 2015; Symondson, 2002) to determine which pest species they are consuming. We envisage that with timely implementation, these combined eDNA surveys could help inform appropriate pesticide applications, both timing and target area, to help reduce the loss of the ecosystem services provided by these beneficial predators. Although further studies are needed to determine how this molecular approach compares to conventional surveys to inform on predator abundance and location specificity within agricultural systems.

4.5.2 Fine and large spatial scale variability in arthropod communities identified with eDNA

Vertical and horizontal stratification of arthropod communities can be a source of variation for pollination success and crop production (Cook & Power, 1996; Frimpong et al., 2011), or reflect pest and predator microhabitat preference (Marler, 2013). Despite arthropod communities exhibiting such fine scale community changes, these measures are often omitted from agricultural surveys, largely due to the technical difficulties associated with conventional sampling in tree canopies (e.g. pan traps in tree canopies; see Yoshida et al., 2021). In the present study, by collecting inflorescences from both the upper (> 2m) and lower (< 2m) canopy, we were able to show a 12% greater probability of detecting Hymenoptera taxa (i.e. *A. mellifera* and the aphid predator, *Diaeretiella rapae*) in the upper canopy, a finding that was not easily achievable with the DVRs. This pattern may reflect the greater availability of flowering resources in the upper

compared to the lower canopy (Wellington & Cmiralova, 1979). We do note however, that this trend was not ubiquitous for all taxa belonging to the Hymenoptera species cohort (i.e. *Aphidius colemani*, a known aphid predator, was detected from one sample in the lower canopy). Our finding raises the possibility of using eDNA to help inform management strategies for crop species. For instance, with greater information about where pollinators may visit, farmers could employ tip-pruning and thinning strategies (see Olesen, 2005) to help promote flowering in areas of the canopy where they are more likely to be visited by the known pollinators for the particular growing region, thus potentially allowing for greater pollination success. As well as detecting fine scale variability in arthropod communities, eDNA metabarcoding also revealed significant spatial variability between the two sample orchards.

Arthropod communities can vary substantially within agricultural landscapes, even at relatively small distances between orchards in the same region (Willcox et al., 2019). Generally, these differences are driven by the presence of rare and/or site-specific species (Sutter et al., 2017; Willcox et al., 2019). In the present study, we found that with a separation of 16 km, the two sample orchards shared ca. 40% similarity in eDNA-detected arthropod communities at low *P. americana* flowering, a value that increased during peak flowering. Interestingly, both orchards became more similar towards peak flowering due to the decreased detections of rare species - here defined as singletons (arthropod taxa that were only detected once). Between low and peak flowering, the number of singletons declined by 23%, likely due to the overall increase in arthropod diversity and abundance during peak flowering (Howlett et al., 2009; Willcox et al., 2019). As well as fewer rare species, the number of site-specific species also declined between low and peak flowering. Here, we found that the number of site-specific eDNA detections declined by 37% at peak flowering, suggesting, that although both orchards had different baseline arthropod communities, the presence of a mass flowering crop species appeared to decrease this dissimilarity by attracting the same arthropod taxa from the surrounding landscape, although these findings were different in some aspects to those generated by the DVRs.

4.5.3 Complementarity of eDNA and DVRs

In contrast to the findings from eDNA metabarcoding, the DVRs identified a strong dissimilarity between arthropods visiting *P. americana* flowers at the two study sites. From low to peak flowering, the arthropod community similarity declined by 87% between the two orchards. Such a contrast between the two methods measuring the same communities has also been shown by Kestel et al. (2023), where only seven families (14% of arthropod families detected) were shared between eDNA collected from *P. americana* flowers and DVRs during peak flowering. Similar

patterns were also observed by Johnson et al. (2023), where DVRs and eDNA metabarcoding of flowers shared only ca. 5% of the same species and genera. The significant disparity between eDNA and DVRs is likely a reflection of methodological differences. Specifically, DVRs are biased towards detecting larger taxa, due to the resolution of the cameras (see Gomez et al., 2023; Kestel et al., 2023), whereas, eDNA from flowers is generally better at detecting smaller taxa (Gomez et al., 2023). Furthermore, unlike the eDNA results, DVRs were able to observe the enormous variation in Syrphidae abundance between both sample orchards at peak flowering. The number of flower visits observed for *S. grandicornis* and *M. viridiceps* was 56 times greater at orchard MB, compared to orchard BA at peak flowering, contributing 83% of the dissimilarity. At the relatively short distance between the orchards, the variation in Syrphidae abundance may have been the result of the species' ability to travel over large distances (400 – 1800 km; see Baldock et al., 2019; Finch & Cook, 2020) to reach resource rich areas, as well as the greater diversity of weed species at orchard MB (results not shown) (Robertson et al., 2020). These differences between eDNA and DVRs emphasise the need to include multiple methods to capture communities of interest (Gomez et al., 2023; Johnson et al., 2023; Newton et al., 2023). Here, both methods used in combination created a dataset that benefitted from the significant taxonomic resolution afforded by eDNA, while also capturing the species that were not detected by eDNA (e.g. *Calliphora* spp.) and abundance information captured by DVRs. Additionally, by using two methods, we were able to identify the traits of flower-visiting arthropods (e.g. body size) that allowed for greater eDNA detections.

4.5.4 Smaller and more frequent flower-visiting arthropods have a greater probability of being detected

We found that smaller arthropods and arthropods that visited flowers more frequently had a higher detection probability (Table 4.1). In other observation-based studies, smaller arthropods have been shown to interact with flowers over a longer time period compared to their larger counterparts (Liu & Pemberton, 2009; Vivarelli et al., 2011). Therefore, it is feasible that with more time spent on flowers, smaller arthropods may have a greater opportunity to deposit DNA, and thus be detected by eDNA metabarcoding (see Johnson et al., 2023). Another possibility is that some smaller taxa (e.g. potential pest Thripidae species) remained present within the samples after collection, although this is unlikely as all flowers were visually checked before DNA extraction. We note that several arthropod taxa were not included in this analysis as they were not seen on the DVRs and one metabarcoding assay was used to detect arthropods, which may be biased against certain taxonomic groups (e.g. Hymenoptera) (see Evans & Kitson, 2020). Thus, the conclusions

regarding arthropod size and abundance should be tested across other agricultural systems with a wider breadth of arthropod assays to determine if these conclusions are equally applicable.

4.5.5 Integrating eDNA metabarcoding of flowers into agricultural monitoring

Detection of arthropod taxa responsible for ecosystem services such as pollination, as well as those that negatively impact productivity through herbivory and pathogen transmission, are increasingly necessary to maintain or control in order to improve food security (FAO, 2020; Potts et al., 2016; Savary et al., 2019). We have demonstrated that eDNA metabarcoding of flowers, used in conjunction with DVRs, can identify temporal and spatial variation of known-pollinators, plant pests, parasites and arthropod predators. This approach offers many opportunities to complement long-standing survey methods and improve the detection of these critical taxa within agroecosystems. However, eDNA metabarcoding is an emerging tool in an agricultural context, and opportunities remain to improve the taxonomic resolution beyond that achieved in this study. If the goal is to identify a greater breadth of arthropod diversity, then we would recommend the inclusion of two or more assays, which would compensate for primer biases of any single assay (e.g. Newton et al., 2023; Thomsen & Sigsgaard, 2019). Finally, we envisage this technology will be harnessed in agroecosystems for natural capital accounting and the valuation of ecosystem services (e.g. pollination), and also guide management decisions (Ekins, 2003; Smith et al., 2017). For example, Dardonville et al. (2022) developed an approach for manually assessing ecosystem services (i.e. pollination, pest and disease control) which were synthesised into a metric that could be communicated to farmers to help improve agricultural production. With the inclusion of eDNA metabarcoding, these metrics could detect additional taxa, which may otherwise have been missed by conventional methods (see Gomez et al., 2023; Kestel et al., 2023). The inclusion of this molecular method for agricultural surveys could help generate more timely and reliable measures/metrics to encourage the use of farming practices which do not harm overall biodiversity (e.g. reduced generalised/prophylactic pesticide applications; see Leskey, Lee, Short, & Wright, 2012; Morales, 2006), while still allowing for the suppression of antagonistic taxa. In conclusion, the addition of eDNA metabarcoding as a molecular tool to support current survey methods will provide an unparalleled means to rapidly monitor economically important arthropod taxa in agroecosystems. However, this method, like any other, is most accurate when used in conjunction with supporting methods that can be used to cross-validate detections and provide ecological context.

4.6 References

A thorough effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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4.7 Supplementary information

Table S4.1 Deployment times for DVRs by date. Each DVR recorded three hours of time-lapse footage.

Date	Orchard	Deployment time window
3/10/2020	MB	10:26am – 12:45pm
4/10/2020	MB	8:58am – 10am
5/10/2020	BA	8:36am – 9:51am
6/10/2020	BA	8:28am – 9:23am
30/10/2020	MB	11:25am – 12:25am
31/20/2020	MB	8:35am – 9:23am
3/11/2020	BA	10:58am – 11:27am
4/11/2020	BA	7:56am – 8:26am

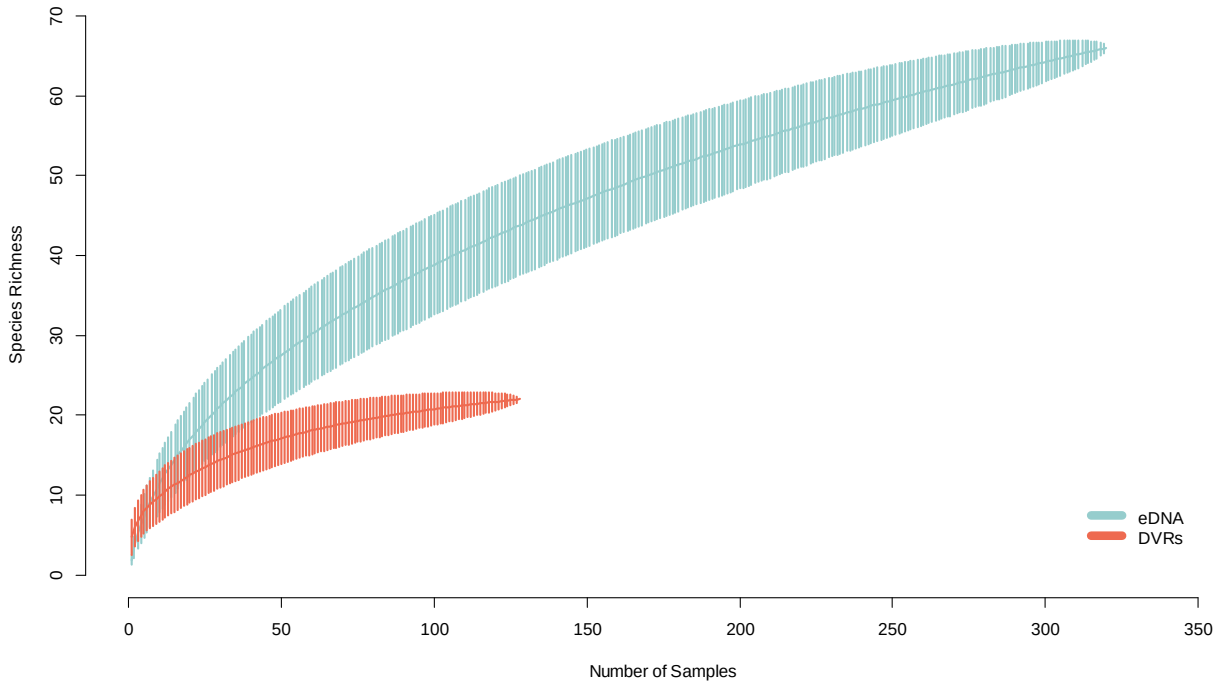

Figure S4.1 Rarefaction curves with standard error bars for arthropod species detected with eDNA and DVRs. DVRs (*N* = 128), and eDNA (*N* = 320). Rarefaction curves were generated with 10,000 permutations and first order jack-knifing using the package 'Vegan' in R.

Table S4.2 Species cohorts for eDNA GLM testing.

Cohort	Taxa
Diptera-eDNA	*Bradysia* sp.
	Dicranomyia sp.
	Hydrellia sp.
	Limnophyes sp.
	Muscidae sp.
	Psychoda albipennis
	Psychoda sp.
	Scaptomyza australis
	Sciaridae sp.
	Sciaridae sp. 2
	Simosyrphus grandicornis
	Smittia sp. 1
	Smittia sp. 2
	Smittia sp. 3
	Sphaeroceridae sp.
	Syrphidae sp.
Hymenoptera-eDNA	*Aphidius colemani*
	Apis mellifera
	Diaeretiella rapae
	Orthocentrus sp.
	Pheidole megacephala
	Trichogramma sp.
Ancillary-eDNA	*Arachnura higginsi*
	Aceria tosichella
	Aculops sp.
	Acyrthosiphon sp.
	Agrotis infusa
	Agrotis ipsilon
	Aphis craccivora
	Aphis spiraecola
	Ausejanus albisignatus
	Bourletiellidae sp.
	Caecilius quercus
	Campylomma liebknechti
	Carpophilus sp.
	Ceratophysella sp.
	Cicadellidae sp.
	Cicadellidae sp.
	Closterotomus norwegicus
	Coleoptera sp.
	Cornu aspersum
	Deroceras sp. 1
	Deroceras sp. 2
	Diomocoris woodwardi
	Ectopsocus californicus
	Ectopsocus sp. 1
	Ectopsocus sp. 2
	Frankliniella sp.
	Haplothrips sp.
	Lehmannia sp.
	Lepidocyrtus fimetarius
	Mallada signatus
	Micromus tasmaniae
	Myzus persicae
	Nysius sp.
	Opisthoncus sp.
	Orosius argentatus
	Persectania ewingii
	Phrissogonus laticostata
	Plutella xylostella
	Sminthurus sp.
	Steatoda grossa
	Thrips australis
	Thrips tabaci
	Tydeidae sp.
	Zygoribatula undulata

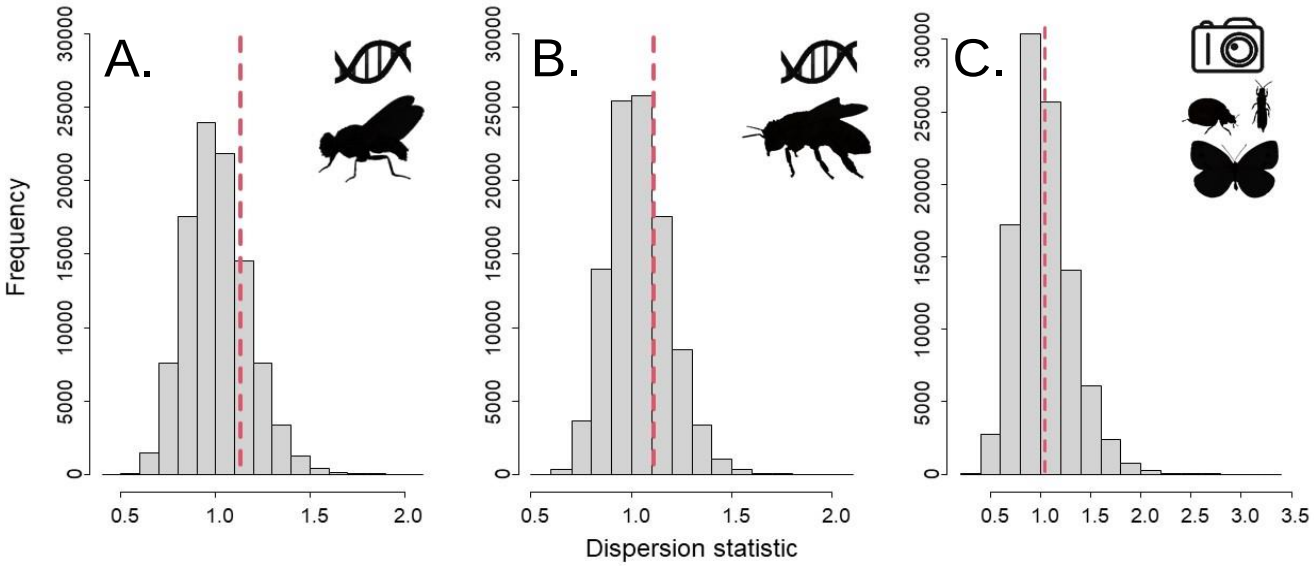

Figure S4.2 Dispersion statistics for species cohorts with > 50% zeros, presented in 100,000 simulated datasets. The dotted redline represent the fitted model dispersion statistics for; (A) eDNA-detected Diptera, (B) eDNA-detected Hymenoptera and (C) DVR-detected Ancillary.

Table S4.3 Species cohorts for DVR GLM testing.

Cohort	Taxa
Diptera-DVR	*Calliphora* spp.
	Musca spp.
	Poecilohetarus sp.
	Sacrophagidae sp.
	Syrphidae spp.
	Unclassified Diptera spp.
Hymenoptera-DVR	*Pheidole megacephala*
	Bethylidae sp.
	Braconidae sp.
	Lasioglossum sp.
	Pompilidae sp.
	Vespidae sp.
Ancillary-DVR	Acrididae sp.
	Coccinellidae sp.
	Coleoptera sp.
	Diomocoris woodwardi
	Opisthoncus sp.
	Pentatomidae sp.
	Phrissogonus laticostata
	Plutella xylostella
	Thripidae spp.

Table S4.4 Comparison of covariate significance for eDNA and DVR datasets. Inflorescence location was not a feature of DVRs, while deployment time was not a feature of the eDNA dataset. * denotes statistically significant p-values ≤ .05.

Covariate	eDNA				DVR			
	All	Diptera	Hymenoptera	Ancillary	All	Diptera	Hymenoptera	Ancillary
Inflorescence location (upper or lower)	.06	.67	.03*	.02*	NA	NA	NA	NA
Sample orchard (BA or MB)	.04*	< .01*	< .01*	*NS*	< .01*	< .01*	< .01*	< .01*
Flowering period (low or peak)	< .01*	< .01*	< .01*	< .01*	< .01*	< .01*	< .01*	.10
Deployment time	NA	NA	NA	NA	< .01*	< .01*	.30	.08

Table S4.5 Average (± SE) number of flower visits for each species cohort detected with DVRs for orchard BA and MB at low (1) and peak (2) flowering.

Orchard	All	Diptera*	Syrphidae	Hymenoptera	Ancillary
BA1	25 ± 3.04	2 ± 0.84	3 ± 0.77	20 ± 2.83	0 ± 0.15
BA2	81 ± 12.75	12 ± 2.97	28 ± 6.61	41 ± 8.06	1 ± 0.18
MB1	39 ± 6.28	2 ± 1.02	1 ± 0.58	33 ± 6.59	3 ± 1.40
MB2	1746 ± 182.95	48 ± 15.18	1566 ± 179.62	128 ± 14.52	5 ± 1.10

*Diptera species include non-Syrphids

Table S4.6 Dunn's test *p*-values for flower visits for species cohorts obtained from DVRs for orchard BA and MB at low (1) and peak (2) flowering. Dunn's tests were carried out using the package 'FSA' in R Studio.

Comparison	All	Diptera*	Syrphidae	Hymenoptera	Ancillary
BA1 – BA2	$p < .01$	.064	$p < .01$	.188	.537
BA1 – MB1	$p < .01$	.789	$p < .01$	.396	.228
BA2 – MB2	$p < .01$	.087	$p < .01$	.017	.010
MB1 – MB2	$p < .01$	$p < .01$	$p < .01$	.004	.066

* Diptera species include non-Syrphids

Table S4.7 Significant taxa ($p < .05$) as determined by the goodness of fit statistic (squared Pearson correlation coefficient) using 'Vegan' in R. Analyses were undertaken for both DVR counts ($N = 32$) and eDNA presence-absence detections ($N = 320$). ND = Not Detected and NS = Not Significant.

Species	DVR	eDNA
Aceria tosichella	ND	.01
Agrotis ipsilon	ND	.01
Apis mellifera	< .01	NS
Bethylidae sp.	< .01	NS
Caecilius quercus	ND	< .01
Calliphora spp.	.02	ND
Coleoptera sp.	.01	NS
Diomocoris woodwardi	NS	< .01
Unknown Diptera spp.	< .01	NS
Ectopsocus californicus	ND	.01
Lasioglossum sp.	.04	ND
Musca spp.	.04	NS
Myzus persicae	ND	< .01
Sacrophagidae sp.	.01	ND
Simosyrphus grandicornis	< .01	.01
Smitta sp. 1	ND	< .01
Smittia sp. 2	ND	< .01
Syrphidae spp.	< .01	< .01
Thrips tabaci	ND	< .01
Trichogramma sp.	ND	.03
Tydeidae sp.	ND	< .01

Table S4.8 Similarity percentage analysis (SIMPER) of species detected with DVRs between orchards BA and MB at low (1) and peak (2) flowering.

Species	Average contribution	Cumulative contribution	Group comparison	Position
Apis mellifera	0.210	0.800	BA1_BA2	2
Calliphora spp.	0.088	0.960	BA1_BA2	3
Coccinellidae sp.	0.003	0.997	BA1_BA2	7
Pentatomidae sp.	0.001	0.999	BA1_BA2	8
Syrphidae spp.	0.867	0.907	MB1_MB2	1
Coleoptera sp.	0.000	0.998	MB1_MB2	8
Plutella xylostella	0.000	0.999	MB1_MB2	11
Vespidae sp.	0.000	0.999	MB1_MB2	12
Bethylidae sp.	0.000	0.999	MB1_MB2	13
Phrissogonus laticostata	0.000	0.999	MB1_MB2	14
Lasioglossum sp.	0.000	1.000	MB1_MB2	15
Pompilidae sp.	0.000	1.000	MB1_MB2	16
Diomocoris woodwardi	0.000	1.000	MB1_MB2	18
Acrididae sp.	0.000	1.000	MB1_MB2	21
Apis mellifera	0.229	0.590	BA1_MB1	1
Thripidae spp.	0.049	0.716	BA1_MB1	2
Diptera spp.	0.015	0.935	BA1_MB1	5
Pheidole megacephala	0.014	0.973	BA1_MB1	6
Opisthoncus sp.	0.004	0.984	BA1_MB1	7
Poecilohetarus sp.	0.003	0.991	BA1_MB1	8
Braconidae sp.	0.001	1.000	BA1_MB1	10
Syrphidae spp.	0.829	0.916	BA2_MB2	1
Coleoptera sp.	0.000	0.997	BA2_MB2	7
Plutella xylostella	0.000	0.999	BA2_MB2	11
Vespidae sp.	0.000	0.999	BA2_MB2	12
Bethylidae sp.	0.000	0.999	BA2_MB2	13
Phrissogonus laticostata	0.000	0.999	BA2_MB2	15
Lasioglossum sp.	0.000	1.000	BA2_MB2	17
Pompilidae sp.	0.000	1.000	BA2_MB2	18
Diomocoris woodwardi	0.000	1.000	BA2_MB2	19
Acrididae sp.	0.000	1.000	BA2_MB2	21

Table S4.9 Similarity percentage analysis (SIMPER) of species detected with eDNA between orchards BA and MB at low (1) and peak (2) flowering.

Species	Average contribution	Cumulative contribution	Group comparison	Position
Phrissogonus laticostata	0.049	0.090	BA1_BA2	1
Simosyrphus grandicornis	0.040	0.163	BA1_BA2	2
Myzus persicae	0.026	0.329	BA1_BA2	5
Zygoribatula undulata	0.020	0.447	BA1_BA2	8
Micromus tasmaniae	0.019	0.517	BA1_BA2	10
Coleoptera sp.	0.016	0.575	BA1_BA2	12
Agrotis ipsilon	0.015	0.660	BA1_BA2	15
Opisthoncus sp.	0.009	0.702	BA1_BA2	17
Lepidocyrtus fimetarius	0.009	0.718	BA1_BA2	18
Orthocentrus sp.	0.009	0.734	BA1_BA2	19
Arachnura higginsi	0.008	0.765	BA1_BA2	21
Acyrthosiphon sp.	0.008	0.795	BA1_BA2	23
Trichogramma sp.	0.008	0.811	BA1_BA2	24
Apis mellifera	0.008	0.826	BA1_BA2	25
Cicadellidae sp. 1	0.008	0.869	BA1_BA2	28
Nysius sp.	0.008	0.883	BA1_BA2	29
Hydrellia sp.	0.007	0.896	BA1_BA2	30
Aphis spiraecola	0.007	0.923	BA1_BA2	32
Muscidae sp.	0.007	0.936	BA1_BA2	33
Orosius argentatus	0.007	0.962	BA1_BA2	35
Cicadellidae sp.	0.007	0.975	BA1_BA2	36
Ceratophysella sp.	0.007	0.987	BA1_BA2	37
Sminthurus sp.	0.007	1.000	BA1_BA2	38
Thrips australis	0.000	1.000	BA1_BA2	62
Smittia sp. 2	0.040	0.065	MB1_MB2	1
Tydeidae sp.	0.039	0.130	MB1_MB2	2
Simosyrphus grandicornis	0.033	0.184	MB1_MB2	3
Smittia sp. 3	0.028	0.230	MB1_MB2	4
Ectopsocus sp. 2	0.023	0.390	MB1_MB2	8
Bourletiellidae sp.	0.022	0.463	MB1_MB2	10
Cornu aspersum	0.022	0.535	MB1_MB2	12
Ectopsocus sp. 1	0.016	0.656	MB1_MB2	16
Thrips australis	0.000	1.000	MB1_MB2	65
Caecilius quercus	0.042	0.065	BA1_MB1	1
Smittia sp. 2	0.037	0.122	BA1_MB1	2
Ectopsocus californicus	0.033	0.174	BA1_MB1	3
Smittia sp. 3	0.030	0.270	BA1_MB1	5
Ectopsocus sp. 2	0.025	0.349	BA1_MB1	7
Bourletiellidae sp.	0.024	0.462	BA1_MB1	10
Cornu aspersum	0.023	0.498	BA1_MB1	11
Ectopsocus sp. 1	0.018	0.554	BA1_MB1	13
Closterotomus norwegicus	0.013	0.651	BA1_MB1	17
Sciaridae sp. 2	0.013	0.671	BA1_MB1	18
Dicranomyia sp.	0.012	0.689	BA1_MB1	19

Species	Average contribution	Cumulative contribution	Group comparison	Position
Psychoda albipennis	0.011	0.744	BA1_MB1	22
Aphidius colemani	0.007	0.754	BA1_MB1	23
Sciaridae sp.	0.006	0.879	BA1_MB1	36
Pheidole megacephala	0.006	0.888	BA1_MB1	37
Deroceras sp. 1	0.006	0.897	BA1_MB1	38
Sphaeroceridae sp.	0.006	0.906	BA1_MB1	39
Steatoda grossa	0.006	0.932	BA1_MB1	42
Deroceras sp. 2	0.006	0.958	BA1_MB1	45
Persectania ewingii	0.005	1.000	BA1_MB1	50
Thrips australis	0.000	1.000	BA1_MB1	62
Tydeidae sp.	0.040	0.072	BA2_MB2	1
Diomocoris woodwardi	0.037	0.139	BA2_MB2	2
Caecilius quercus	0.037	0.205	BA2_MB2	3
Aceria tosichella	0.022	0.536	BA2_MB2	10
Campylomma liebknechti	0.020	0.572	BA2_MB2	11
Mallada signatus	0.019	0.606	BA2_MB2	12
Agrotis infusa	0.018	0.638	BA2_MB2	13
Diaeretiella rapae	0.014	0.715	BA2_MB2	16
*Aculops*sp.	0.007	0.821	BA2_MB2	23
Frankliniella sp.	0.007	0.871	BA2_MB2	27
Carpophilus sp.	0.007	0.920	BA2_MB2	31
Scaptomyza australis	0.006	0.978	BA2_MB2	36
Plutella xylostella	0.006	1.000	BA2_MB2	38
Thrips australis	0.000	1.000	BA2_MB2	64

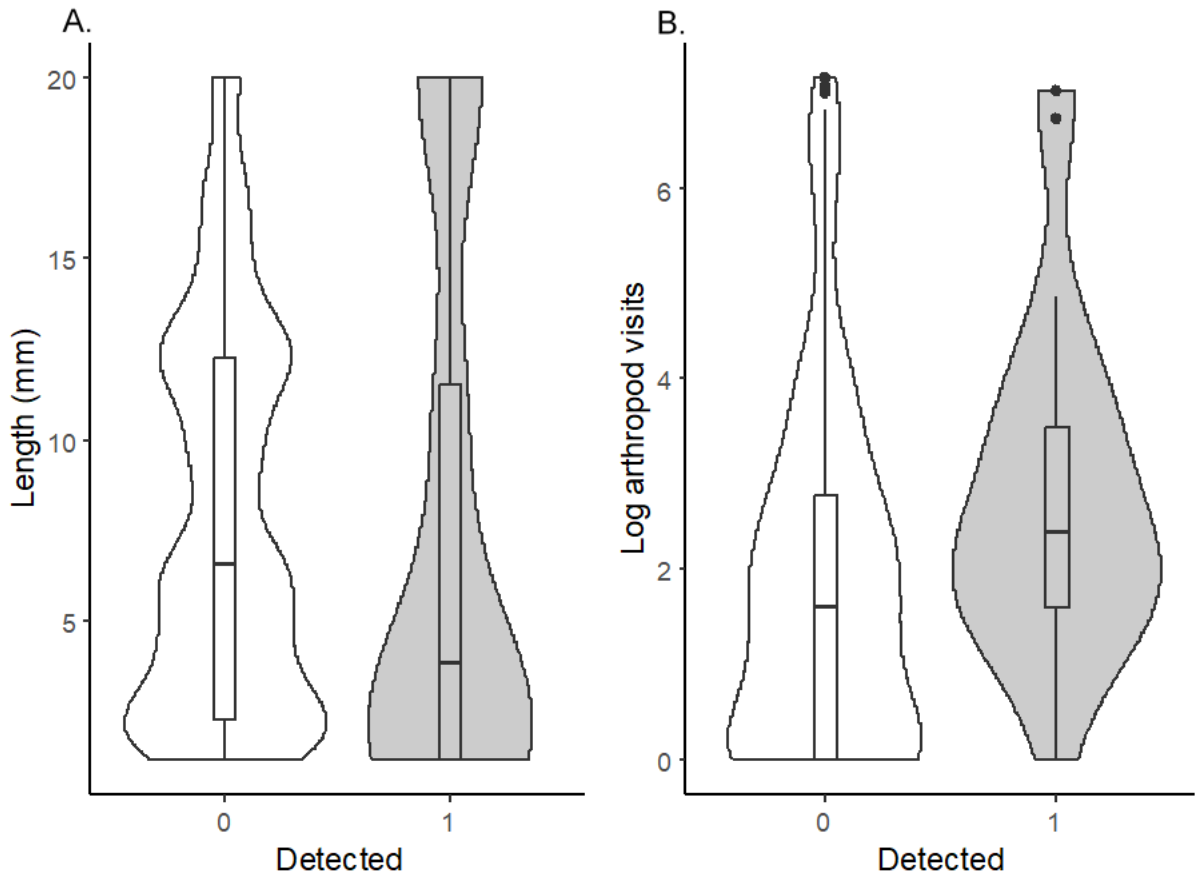

Figure S4.3 Arthropod length (mm) (A) and log arthropod visits (B) against the binary response variable of detected (1) or not detected (0). Violin plots are presented with interior boxplots to display the median and quartile range.

Table S4.10 Functional groups identified by eDNA metabarcoding.

Species	Functional group	Reference
Aceria tosichella	Pest	10.1007/s10493-012-9633-y
Aculops sp.	Pest	10.1007/s10493-009-9300-0
Acyrthosiphon sp.	Pest	10.1186/s43141-022-00442-0
Agrotis infusa	Potential pollinator	10.1016/j.gecco.2023.e02482
Agrotis ipsilon	Pest and potential pollinator	10.1002/ps.7029
Aphidius colemani	Predator	10.3390/insects6020538
Aphis craccivora	Pest	10.1007/s10340-009-0262-0
Aphis spiraecola	Pest	10.1016/j.sajb.2018.05.005
Apis mellifera	Known pollinator	10.1111/jen.12869
Arachnura higginsi	Predator	10.1080/00222938500770261
Ausejanus albisignatus	Pest	10.1111/afe.12374
Bourletiellidae sp.		
Bradysia sp.	Pest	10.1002/ps.1987
Caecilius quercus		
Campylomma liebknechti		
Carpophilus sp.	Pest	10.1038/s41598-022-23520-2
Ceratophysella sp.		
Cicadellidae sp.		
Cicadellidae sp.		
Closterotomus norwegicus	Pest	10.1016/j.cropro.2017.02.006
Coleoptera sp.	Predator	10.1007/s004420000476
Cornu aspersum	Pest	10.1371/journal.pone.0049674
Deroceras sp. 1	Pest	10.1007/s10340-019-01154-0
Deroceras sp. 2	Pest	10.1007/s10340-019-01154-0
Diaeretiella rapae	Predator	10.1017/S0007485317000657
Dicranomyia sp.		
Diomocoris woodwardi		
Ectopsocus californicus		
Ectopsocus sp. 1		
Ectopsocus sp. 2		
Frankliniella sp.	Pest	10.1002/ps.3389
Haplothrips sp.	Pest	10.1080/03235408.2013.796698
Hydrellia sp.		
Lehmannia sp.	Pest	10.1186/s41936-021-00214-1
Lepidocyrtus fimetarius		
Limnophyes sp.		
Mallada signatus	Predator	10.1080/09583157.2011.622036
Micromus tasmaniae	Predator	10.1016/j.biocontrol.2007.07.003
Muscidae sp.	Potential pollinator	10.3390/insects11060341
Myzus persicae	Pest	10.1016/j.ibmb.2014.05.003
Nysius sp.	Pest	10.1080/09670874.2019.1666174
Opisthoncus sp.	Predator	10.1002/ps.5477

Species	Functional group	Reference
Orosius argentatus	Pest	10.1071/AR9500144
Orthocentrus sp.	Predator	10.1111/jen.12843
Persectania ewingii	Pest	10.1017/S174275840002258X
Pheidole megacephala	Pest	10.1071/PC980250
Phrissogonus laticostata	Pest and Pollinator	10.1111/j.1365-2656.2012.01974.x
Plutella xylostella	Pest	10.1080/09583150500136956
Psychoda albipennis		
Psychoda sp.		
Scaptomyza australis		
Sciaridae sp. 1	Pest	10.11646/zootaxa.4415.2.1
Sciaridae sp. 2	Pest	10.11646/zootaxa.4415.2.1
Simosyrphus grandicornis	Potential pollinator and predator	10.3390/insects11060341
Sminthurus sp.	Pest	10.1016/j.biocontrol.2011.06.007
Smittia sp. 1		
Smittia sp. 2		
Smittia sp. 3		
Sphaeroceridae sp.		
Steatoda grossa	Predator	10.1016/j.jinsphys.2021.104267
Syrphidae sp.	Potential pollinator and predator	10.3390/insects11060341
Thrips australis		
Thrips tabaci	Pest	10.1016/S0261-2194(03)00092-9
Trichogramma sp.	Predator	10.1146/annurev.en.41.010196.002111
Tydeidae sp.		
Zygoribatula undulata		

Chapter 5. Environmental DNA metabarcoding of pan-trap water to monitor arthropod-plant interactions

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5.1 Abstract

Globally, the diversity of arthropods and the plants upon which they rely are under increasing pressure due to a combination of biotic and abiotic anthropogenic stressors. Unfortunately, conventional survey methods used to monitor ecosystems are often challenging to conduct on large scales. Pan traps are a commonly used pollinator survey method and environmental DNA (eDNA) metabarcoding of pan-trap water may offer a high-throughput alternative to aid in the detection of both arthropods and the plant resources they rely on. Here, we examined if eDNA metabarcoding can be used to identify arthropods and plant species from pan-trap water, and investigated the effect of different DNA extraction methods. We then compared plant species identified by metabarcoding with observation-based floral surveys and also assessed the contribution of airborne plant DNA (plant DNA not carried by arthropods) using marble traps to reduce putative false positives in the pan trap dataset. Arthropod eDNA was only detected in 17% of pan trap samples and there was minimal overlap between the eDNA results and morphological identifications. In contrast, for plants, we detected 64 taxa, of which 53 were unique to the eDNA dataset, and no differences were identified between the two extraction kits. We were able to significantly reduce the contribution of airborne plant DNA to the final dataset using marble traps. This study demonstrates that eDNA metabarcoding of pan-trap water can detect plant resources used by arthropods and highlights the potential for eDNA metabarcoding to be applied to investigations of arthropod-plant interactions.

5.2 Introduction

Global declines in arthropod taxa (e.g. bees, moths, flies and wasps) are resulting in the annual loss of ~1% of the 1 million morphologically described and the estimated 4.5 – 7 million undescribed terrestrial arthropod species (Stork, 2018; Wagner, 2020; Wagner, Grames, Forister, Berenbaum, & Stopak, 2021). These declines are, *inter alia*, the result of complex interactions between land use change (e.g. agricultural expansion and forest reduction), climate change (e.g. more frequent extreme climatic events such as drought), introduced species (e.g. *Varroa destructor* and *Ascosphaera apis*), light pollution, as well as generalised use of pesticides (Wagner et al., 2021). Taken together, these biotic and abiotic anthropogenic stressors can threaten arthropod-mediated ecosystem services, including pollination, decomposition and nutrient recycling (Potts et al., 2016; Wagner et al., 2021). In the face of such threats, scalable, accurate and cost-effective methods are needed to monitor arthropods across all environments.

Arthropod communities are often targeted using a mixture of active (e.g. sweep netting) and passive (e.g. pan traps) sampling techniques to capture the most accurate representation of taxonomic diversity for a habitat or landscape (Prendergast, Menz, Dixon, & Bateman, 2020; Spafford & Lortie, 2013). Pan traps, coloured plastic bowls filled with soap water, are often used to sample arthropod pollinators (Meissle et al., 2022; Popic et al., 2013; Saunders & Luck, 2013) and are an efficient and cost effective means to measure arthropod diversity without observer bias (Westphal et al., 2008; Wilson, Griswold, & Messinger, 2008). Increasingly, pan traps are used to survey in both agricultural and natural landscapes. For instance, 40% of all pan trap studies available through Scopus (357 studies total) have been published in the last five years alone (Table S5.1), probably due to the low cost and simple implementation of this sampling tool. Pan traps have proven to be most effective at capturing insect assemblages when flowering levels are low or fluctuating, primarily because pan trap capture rates are independent of floral resource availability (Popic et al., 2013). Although like other observation-based techniques, this method can be time-intensive (Saunders & Luck, 2013), requires taxonomic expertise (Joshi et al., 2015), and thus may be challenging to implement on a large scale. Despite these challenges, pan traps still remain a common sampling technique, in both natural and agricultural systems, to survey the taxonomic diversity of arthropod communities (Meissle et al., 2022; Joshi et al., 2015, Popic et al., 2013), although the potential plant taxonomic information incidentally captured by using this method remains largely unexplored.

Pollen accumulated on the bodies of arthropods can be used to catalogue which flowering species were visited during insect foraging and infer potential pollinators for plants with limited observational data (Ladd, Yates, Dillon, & Palmer, 2019; Macgregor et al., 2019). To date, these pollen-centric studies have favoured active sampling methods (e.g. sweep netting), followed by washing or swabbing arthropods to obtain animal-collected pollen (Evans & Kitson, 2020; Pornon, Andalo, Burrus, & Escaravage, 2017; Prendergast et al., 2020). Increasingly however, microscopy-based techniques are being combined with pan traps to identify foraging resources in both natural (Ladd et al., 2019) and agricultural systems (Bowie, Gurr, Hossain, Baggen, & Frampton, 1999; Campbell, Melles, Vaz, Parker, & Burgess, 2018; Gill & O'Neal, 2015). Although these identifications often require specialist expertise and may achieve low taxonomic resolution due to the lack of morphological characters for species-level identification (Bell et al., 2016; Khansari et al., 2012; Rahl, 2008). Moreover, even when plant taxa are detected from pan traps, they are rarely used to infer species-specific flower visits or pollen transport (Popic et al., 2013); the primary reasons being that 1) pollen collected from pan traps represents foraging from the entire community of arthropods captured, and; 2) pan trap plant detections may include non-target wind-pollinated species, which could result from contamination, either airborne pollen/plant material (Johnson et al., 2019b) or herbivorous regurgitant (Sword 2001), rather than flower visits (Campbell et al., 2018; Popic et al., 2013). Such contributions may be significant considering that wind-pollinated species produce copious quantities of pollen (see Harrington & Metzger, 1963). Despite this potential for contamination, ambient pollen (pollen dispersed by air, not invertebrates) is rarely quantified, with studies instead relying upon alternative approaches, such as artificial pollen (see Campbell et al., 2018), to circumvent this issue. Plant surveys using passive traps may therefore benefit from alternative approaches, like those afforded by environmental DNA (eDNA) metabarcoding, to detect both pan trap-specific and ambient pollen, in order to generate high throughput and high resolution pollen datasets, which can help form robust conclusions about the plant resources used by pollinator communities.

DNA-based approaches offer a reliable and efficient method for surveying and monitoring arthropod and plant community assemblages from a diverse array of substrates. For arthropods, eDNA metabarcoding has been used to survey community composition from bulk samples collected from passive traps (Banerjee et al., 2022; Rasmussen et al., 2021; Zizka, Leese, Peinert, & Geiger, 2018), detect mesofauna in soil (Todd et al., 2020), and identify plant-animal interactions (Kestel et al., 2023; Newton et al., 2023; Thomsen & Sigsgaard, 2019). For plants, eDNA metabarcoding was originally used to reconstruct ancient vegetation communities from

permafrost, ice cores and cave sediment (Sonstebo et al., 2010; Willerslev et al., 2003), while more recently, extant plant diversity has been classified from soil (van der Heyde et al., 2020; Yoccoz et al., 2012), scat (van der Heyde et al., 2020), honey (Bruni et al., 2015; Hawkins et al., 2015), air (Johnson, Fokar, Cox, & Barnes, 2021), as well as arthropod bodies' (Chang et al., 2018; Pornon et al., 2017, 2016). Compared to conventional microscopy and morphological identifications, which are limited by throughput (Stillman & Flenley, 1996) and taxonomic resolution (Lau et al., 2019), eDNA metabarcoding has been shown to provide reliable identifications from diverse sources of arthropod and plant DNA (Bell et al., 2022; Pornon et al., 2017; van der Heyde et al., 2020). The accuracy of this molecular method is, however, dependent on recognising and detecting possible sources of DNA contamination from non-target species.

eDNA metabarcoding of arthropod-collected plant material (i.e. pollen) often requires non-destructive sampling approaches (e.g. wash water or swabs from arthropod specimens – see Batuecas et al., 2022; Pornon et al., 2017) to reduce contamination between the gut contents of arthropods and the pollen collected on their bodies' (Chang et al., 2018; Pornon et al., 2017, 2016). Additionally, non-destructive sampling allows for the preservation of whole specimens for morphological identification and barcoding of species missing from the online sequence databases (e.g. GenBank (NCBI)) (Chang et al., 2018; Macgregor et al., 2019). Though, non-destructive samples should also include measures of possible contamination from non-target airborne material (i.e. airborne pollen from wind-pollinated species) (Evans & Kitson, 2020; Macgregor et al., 2019). These measures can be obtained in the field using either passive dust collectors (e.g. marble traps - see Johnson et al., 2019a) or active air filters (e.g. air particle filters – see Clare et al., 2021). Indeed, when used in tandem with eDNA metabarcoding, pan traps complemented with measures of airborne plant DNA, may generate higher resolution plant identifications, allowing more robust conclusions about the plant resources utilised by pollinator communities within cultivated ecosystems. To date, no studies have applied eDNA metabarcoding to pan-trap water.

Here, we use eDNA metabarcoding of pan-trap water, combined with marble trap detections of ambient plant DNA, to survey arthropod species and the plant resources they utilise in an agricultural landscape. We aim to improve eDNA based studies of arthropod-plant interactions by:

1) Determining if eDNA metabarcoding can be used to identify arthropod and plant species from pan-trap water, and examining the effect of different DNA extraction methods on species detections.

2) Comparing plant species identified using eDNA metabarcoding of pan-trap water with conventional surveys of the floral resources available to arthropods at the study sites, to understand the extent to which they complement one another

3) Determining the contribution of ambient plant DNA to the pan trap eDNA species dataset.

5.3 Methods

5.3.1 Study orchards and sample collection

Three *Persea americana* ('Hass' avocado) orchards located in the Manjimup – Pemberton region of south west Western Australia (SWWA) were chosen for this study. The orchards were separated by an average distance of 18 km (Orchard A - 34°18′52 S, 116°08′36 E; Orchard B - 34°25'30 S, 116°01'23 E; and Orchard C - 34°26'28 S, 115°54'02 E). *Persea americana* trees in each study orchard were similar in age (3 – 5 years) and trees in each orchard had less than 10% of flowers open. All three orchards were adjacent to pasture, dominated by *Arctotheca calendula*, *Trifolium subterraneum* and various grass species that were not bearing flowers at the time of sampling. All three orchards were located ~ 1 km away from secondary growth Eucalypt Forest. To survey arthropods and the pollen they collect on their bodies, in each orchard, five pan trap arrays (Figure 5.1A) were deployed at 10 m intervals, starting at 10 m, along a 50 m transect, located 50 m away from the edge of the orchard, between the 29th and 31st of October 2021. Pan traps were deployed when temperatures were warm enough for bee activity (clear skies, wind speed <30 km/h, and temperatures >17°C and <25°C; Prendergast et al., 2020). In each pan trap array, three coloured bowls (white, yellow and blue) were deployed to capture the broadest range of insect colour preference (Abrahamczyk, Steudel, & Kessler, 2010; Cane, Minckley, & Kervin, 2000; Saunders, Luck, & Mayfield, 2013). Each array was placed at a height of 1.2 m above the ground to match the height of the *P. americana* trees in the study orchards. All of the pan traps were filled with ~ 200 ml of sterile MilliQ water, and one drop of detergent to disrupt the surface tension (Campbell et al., 2018; Gervais, Chagnon, & Fournier, 2018). Pan trap evaporation was minimal during the sample period due to the mild temperatures (average of 19.7°C), while, the water temperature of the pan traps did not exceed 25°C. After eight hours, each pan trap was stirred using sterilised tweezers (washed in 10% bleach solution and placed under UV light for 15 mins prior to use), and 50 ml of liquid (presumed to contain pollen) was subsampled into a labelled 50 ml falcon tube (one tube per white, blue and yellow pan trap; 150 ml collected per pan trap array). Any captured insects captured in the pan traps were transferred into the same 50 ml of collected water using sterilised tweezers. The falcon tubes were then placed on ice until they could be transferred to a -20°C freezer at Curtin University.

Figure. 5.1 (A) Pan trap arrays were setup at 10 m distance intervals along a 50 m transect in each of the three study orchards. (B) eDNA results from the pan traps were compared with conventional floral surveys using a $1m^2$ quadrat to survey understorey and adjacent pasture flowering, method follows Fisher et al. (2017). (C) One marble trap was placed in the centre (25 m) of each orchard transect to collect ambient pollen and plant material in the atmosphere, method follows Reheis and Kihl (1995). Images captured by Diana Adorno.

Potential contamination of pan traps with plant DNA carried in the atmosphere (e.g. pollen from predominantly wind-pollinated plant species, and plant DNA attached to dust particles), which is

not necessarily representative of insect flower visits, was assessed by using marble traps to capture this ambient DNA. Marble traps were made following the design of Reheis and Kihl (1995); a sterilised cake pan is lined with metal mesh and filled with sterilised marbles (washed in 10% bleach solution and placed under UV light for 15 mins prior to deployment in the field) (Figure 5.1C). This design allows for vertical flow of air through the marbles and hence ambient DNA is captured in the cake pan. The marble traps were inexpensive to build and perform equally well as commercially available alternatives (see Johnson et al., 2019a). In each orchard, one marble trap was deployed in the middle of the 50 m pan trap transect. Marble traps were placed 1.2 m above the ground to match the height of the pan trap arrays. Marble traps were deployed for the same amount of time as the pan trap arrays (eight hours). During collection, the marble traps were wrapped in Clingfilm and stored at room temperature (below 25°C) until they could be transferred to a - 20°C freezer at Curtin University.

5.3.2 Floral surveys

Flowering plant species present in the *P. americana* orchards and adjacent pasture were quantified using observation-based floral surveys to cross-validate the eDNA results. On the same day as pan traps were deployed, floral surveys were carried out following Fisher et al. (2017). Here, four 1 m^2 quadrats were deployed along the 50 m pan trap transect in each orchard and along a parallel 50 m transect in the adjacent pasture, located 100 m from the orchard transect (Figure 5.1B). Four replicates were undertaken per floral survey due to the low diversity of plant taxa and the fact that no new species were being detected after the fourth quadrat (Figure S5.1). Within each quadrat, flowering species were identified and a field herbarium created with leaf material from each species. Field identifications of flowering species were confirmed using relevant taxonomic keys and reference materials.

5.3.3 Sample processing and DNA extraction

Our approach prioritised the collection of pollen from the external surface of captured arthropods without causing disruption to their internal organs and maintaining body integrity for morphological identification, and use of specimens in subsequent studies. To achieve this, once the pan-trap water samples were defrosted, the arthropods were separated from the pan-trap water using a sterilised stainless steel tea strainer (changed between samples). Each arthropod specimen was subsequently transferred into labelled 2 ml Eppendorf tubes containing 99% ethanol. Arthropods were identified morphologically by two entomologists, Dr Terry Huston and Christopher Swinstead, to provide taxonomic identifications to species-level, where possible (Table S5.2). The pan-trap water was filtered in preparation for DNA extraction.

To understand how different DNA extraction methods may influence the quantity of arthropod and plant reads, as well as species composition identified, we compared two of the most commonly used DNA extraction kits in eDNA metabarcoding studies; the DNeasy Blood and Tissue kit (Qiagen; Qiagen Inc., Valencia, CA, USA), hereafter referred to as the Blood and Tissue kit, and the Plant Pro kit (Qiagen; Qiagen Inc., Valencia, CA, USA) (Hawkins et al., 2015; Johnson et al., 2021; McFrederick & Rehan, 2016). Pan-trap water samples were pooled per array (N = 15) and filtered using two Sentino peristaltic microbiology pumps (Pall Life Sciences, New York, USA), through sterile 47 mm cellulose filters with a pore size of 0.22 µm (Pall Life Sciences, New York, USA). Filters were transferred into sterile zip-lock bags and placed in a - 20°C freezer until processed for DNA extraction. For the marble traps (N = 3), 1000 ml of MilliQ water was added (in 2x 500 ml batches) to the traps and agitated periodically for 5 mins (10 mins total); before being filtered through sterile 47 mm cellulose filters with a pore size of 0.22 µm (Pall Life Sciences, New York, USA). The marble trap filter papers were then transferred into sterile zip-lock bags and frozen at - 20°C until further processing.

For DNA extraction, each filter was halved, finely chopped using sterilised scissors (pre-washed with 10 % bleach solution) and placed in two separate tubes for each of the DNA extraction methods explored in this study. The Blood and Tissue kit extractions were followed as per the kit instructions with a modified protocol (see West et al., 2020), while the Plant Pro kit extractions were followed as per the kit instructions with a modified protocol from Oliver et al. (2021). For the Blood and Tissue kit, the samples were extracted with the following modifications: a total of 540 µl ATL Buffer and 60 µl Proteinase K (Qiagen, Venio, Limburg, Netherlands) were added to the tube containing half of the finely chopped membrane to ensure that the filter membranes were adequately exposed to the lysis solution, to optimise DNA yield. The combined filter and lysis solution (600 µl) were incubated overnight at 56°C in a slow rotating hybridization oven. The following morning, 400 µl of the DNA digest supernatant was then transferred to a labelled 2 ml tube and loaded into an automated sample preparation system for DNA extraction (QIAcube; Qiagen Inc., Valencia, CA, USA). The final DNA extracts were eluted from the silica column in 100 µl AE buffer and frozen until further assessment. Pan and marble trap samples (N = 18) and a DNA extraction control (N = 1) for the Blood and Tissue extraction were DNA extracted using Blood and Tissue kit (Qiagen; Qiagen Inc., Valencia, CA, USA), with the same modifications as stated above.

Similarly, the other half of the finely chopped filter membrane was processed, in addition to the pan and marble trap samples (N = 18) and a DNA extraction control (N = 1) with the modified Plant Pro kit. These modifications were: 500 µl of CD1 buffer, 10 µl of Proteinase K and 0.25 g of ≤ 106 µm acid etched beads (Merck, Darmstadt, Germany) added to the tissue lysis tube containing the filter membrane as per Oliver et al. (2021). The samples were then placed in a Tissuelyser (Qiagen; Qiagen Inc., Valencia, CA, USA) and run for 2 mins at 500 rpm. Afterwards, the combined filter and lysis solution were incubated overnight at 56°C in a slow rotating hybridization oven. The following morning, 400 µl of the DNA digest supernatant was then transferred to a labelled 2 ml tube and loaded into an automated sample preparation system for DNA extraction following the kit protocol (QIAcube; Qiagen Inc., Valencia, CA, USA). The final DNA extracts were eluted off the silica column in 100 µl AE buffer and frozen until further assessment.

The quality and quantity of DNA extracted from each membrane for each extraction method described above was measured by quantitative PCR (qPCR), using two common assays targeting Ribulose-1,5-bisphosphate carboxylase-oxygenase (rbcL) region and *trnL* (UAA) intron region (trnL), and one arthropod assay targeting cytochrome c oxidase subunit I (COI). Low copy number and PCR inhibitors from eDNA extracts can impact metabarcoding data (Murray et al., 2015), therefore each eDNA extract was assessed with a qPCR dilution series (neat, 1:10, 1:100) based on the following conditions, 25 µl reaction volumes consisting of 2.5 µl of 10 × PCR Gold Buffer (Life Technologies, Massachusetts, USA), 2 µl of 2 mM $MgCl_2$ (Fisher Biotec, Australia), 0.25 µl of dNTPs (Astral Scientific, Australia), 1 µl of 0.4 mg/ml BSA (Fisher Biotec), 0.4 µl of each primer; (rbcLh2aF: 5'—GGCAGCATTCCGAGTAACTCCTC—3': rbclh2aR: 5'—CGTCCTTTGTAACGATCAAG—3'; Poinar, et al., 1998), (trnL_c: 5'—CGAAATCGGTAGACGCTACG—3': trnL_h: 5'— CCATTGAGTCTCTGCACCTATC—3'; Taberlet et al., 1991, 2007), (fwhF2: 5'— GGDACWGGWTGAACWGTWTAYCCHCC— 3': fwhR2n: 5'—GTRATWGCHCCDGCTARWACWGG—3'; Vamos et al., 2017), 0.2 µl of AmpliTaq Gold (Life Technologies), 0.6 µl 5x SYBR Green (Life Technologies), 2 µl of template eDNA (3x qPCR reactions per eDNA extract) and the remaining volume supplemented with DNase/RNase-Free Distilled Water. The cycling conditions for rbcL, trnL and COI were initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30s, 52°C for 30s, 72°C for 45s, and a final extension at 72°C for 10 min. For rbcL and trnL only 2 samples were identified to contain PCR inhibition and these samples were carried forward through the metabarcoding workflow using a 1:10 dilution of the extract. For COI, 8 samples were identified to contain PCR

inhibition and were carried forward through the metabarcoding workflow using a 1:10 dilution of the extract.

Environmental DNA samples that were of sufficient quality, as determined by the initial qPCR screen (qPCR dilution series), were fusion tagged with a unique 7 – 9 bp multiplex identifier tag (MID-tag) for the trnL, rbcL and COI primer sets. To reduce the likelihood of cross-contamination, chimera production, and MID-tag jumping (Esling et al., 2015), DNA amplification was performed in a single round of amplification using MID-tag primers consisting of the Illumina flow cell adaptors (P5 and P7), a custom sequencing primer binding site, the unique MID-tag combination and the gene specific primer, either rbcL, trnL, or CO1. All qPCR reactions were prepared in a physically separate ultra-clean laboratory at Curtin University designed for ancient DNA work using an automated QIAgility robotics platform (Qiagen Inc., Valencia, CA, USA) and were carried out in duplicate reactions of 25 µl which contained 2.5 µl of 10 x PCR Gold Buffer (Life Technologies), 2 µl of 2.5 mM $MgCl_2$ (Fisher Biotec), 1 µl of 0.4 mg/ml BSA (Fisher Biotec), 0.25 µl of dNTPs (Astral Scientific), 0.5 µl of either forward primer (20 mM), 5 µl of either reverse primer (2 mM), 0.25 µL AmpliTaq Gold (Life Technologies), 2 – 6 µl DNA and the remaining volume supplemented with DNase/RNase-Free Distilled Water. The cycling conditions for the fusion tagged qPCR using the MID-tag primer sets were the same as described above. Replicate MID-tagged amplicons were pooled at approximately equimolar concentrations (e.g. minipool) based on their respective qPCR DRn values (West et al., 2020). The minipool concentrations were measured using a high-resolution capillary electrophoresis system (QIAxcel; Qiagen Inc., Valencia, CA, USA) and the final library was blended equimolar ratio based on the minipool concentrations and size selected (180 – 350 bp) using a PippinPrep (Millennium Science Pty Ltd., Australia) to remove any off-target amplicons and primer dimer. The size selected final library was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Australia) prior to high-throughput sequencing (HTS). Sequencing by synthesis was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) located in the Trace and Environmental DNA lab at Curtin University and as per Illumina's protocol for single-end sequencing with a 300 cycle V2 reagent kit and standard flow cell for environmental metabarcoding with a mean sequencing depth of 178,021 per sample.

5.3.4 Data processing

Sequenced multiplex identifier-tagged amplicons were input to a containerised workflow and run through the Pawsey Supercomputing Centre in Kensington, Western Australia. Here, the sequences were filtered, formed into Zero-radius Operational Taxonomic Units (ZOTUs) and

assigned taxonomic identifications. Sequences were quality checked using FASTQC (Andrews, 2010) and quality filtered (Phred quality score < 20), before the multiplex identifiers were trimmed from the sequence reads using AdapterRemoval v2 (Schubert, Lindgreen, & Orlando, 2016). Subsequently, the filtered reads were demultiplexed using OBITOOLS (Boyer et al., 2016) and sequences shorter than the minimum length of 94 bp for plants and 120 bp for arthropods were filtered out. Sequences were then dereplicated into ZOTUs with a minimum sequence abundance of 5 (Drake et al., 2022) using the USEARCH Unoise3 algorithm (Edgar, 2016). Thresholds for clustering and LULU curation between plant and arthropod taxa were tailored for each group based on their unique mutation and hybridisation rates (Smith & Keeling, 2015). A database of ZOTUs was then generated and queried against the GenBank (NCBI) nucleotide database with 100% query coverage for both plants and arthropods and 98% identity for plants and a 90% identity for arthropods using BLASTN (Altschul, Gish, Miller, Myers, & Lipman, 1990). Erroneous ZOTUs with a sequence similarity below 98% for plants and 96% for arthropods were removed using the LULU post clustering curation method (Frøslev et al., 2017). Finally, a custom Python script (eDNAFlow; Mousavi-Derazmahalleh et al., 2021) was used to assign taxonomic identifications to the curated ZOTUs using the Lowest Common Ancestor (LCA) approach. Taxonomic identification was assigned to a ZOTU when the percentage identity of two or more queried sequences with ≤ 0.5% difference had 99% query coverage and 95% sequence similarity. Taxonomic identifications for plants and arthropods below 97% identity were retained at the family level. For the purposes of this study, we set the minimum threshold count of 10 reads for plant ZOTUs (see Drake et al., 2022) to classify a taxon as present within a sample. While for arthropod ZOTUs, due to the low number of detections, we only removed singletons. All plant and arthropod taxa detected using eDNA were compared with historical records using the Atlas of Living Australia (https://www.ala.org.au/), a collaborative, digital, open infrastructure that pulls together Australian biodiversity data from multiple sources, to confirm that they occurred within the study region.

5.3.5 Statistical analysis

All statistical analyses were performed on R 3.5.1 (R Core Team, 2018). Due to the low amplification success of arthropod eDNA from the pan-trap water samples, subsequent analyses were only undertaken for pollen data. The plant ZOTUs obtained for rbcL and trnL in the pan and marble traps were pooled for analysis. Plant sequence counts for taxa detected in the marble traps were used as minimum sequence copy thresholds for those same taxa in the pan traps (Bell et al., 2017; Drake et al., 2022). This filtering procedure was included to reduce the presence of artefacts (i.e. inflated read counts and false positives; see Ficetola et al., 2016) from contaminant ambient

pollen captured in pan traps. Subsequently, pollen read counts were converted to presence-absence data. To compare between eDNA and floral surveys, the floral survey flowering percentages were also converted into presence-absence data.

To verify if the eDNA method was successful for amplifying pollen eDNA (objective 1) and could provide results comparable to floral surveys (objective 2), we visualised the overlap in species composition according to survey method. Differences in species richness between the survey methods was assessed using an ANOVA. Subsequently, a Principal Coordinate Analysis (PCoA) was created using Jaccard similarity matrices to visually represent the differences in taxonomic composition between the survey methods using the package 'vegan' in R Studio. The significance of any apparent differences between the two survey methods was then tested using an Analysis of Similarity (ANOSIM) with the Jaccard similarity matrices and 9999 permutations. Sampling effort was examined using rarefaction curves with 9999 permutations and first order jack-knifing using the package 'vegan' in R. Chao2 alpha diversity indices at the species level were then calculated for the observation-based floral surveys at each orchard ($N = 3$) and for the combined observation-based floral surveys and eDNA-based pan trap detections at each orchard ($N = 3$). We calculated Chao2 using the package 'fossil' in R. The Chao2 index returns an estimate of richness based on incidence data (Chao, 1984). Secondly, to establish if there was a significant difference between the two extraction methods (objective 1), we compared the reads generated per sample using a paired two-tailed *t*-test. We then used a Kruskal-Wallis test to determine if the number of species detected per sample differed significantly between the two extraction kits. Finally, to assess potential contamination from ambient pollen to the pan trap dataset (objective 3), we calculated the average percentage reduction in read numbers and plant taxa per pan trap sample following the removal of putative false positives, as per Bell et al. (2017). Lastly, as described above, we generated stacked histograms for the log read count for each genus (pre- and post-false positive removal) to visualise read reduction for the pan trap samples.

5.4 Results

5.4.1 eDNA metabarcoding can be used to amplify arthropod and plant DNA from pan-trap water.

We generated 14,241,740 raw sequences from the 15 pan trap array samples, 3 marble trap samples, and 8 extraction and PCR controls - amplified using two plant specific assays and one arthropod assay. In total, 12,828,012 quality-filtered sequences were generated with a mean sequencing depth of 178,021 per sample. For COI, only five pan trap samples (extracted with the Blood and Tissue kit) were successfully amplified and sequenced, the average number of reads per sample was 2,880 (± 1,842 SE) and no reads were generated from the marble traps. For rbcL and trnL, the average number of reads per pan trap array sample was 289,534 (± 125,431 SE), while the average number of reads per marble trap was 284,069 (± 71,383 SE). One marble trap (extracted with the Blood and Tissue kit) and one pan trap sample (extracted with the Plant Pro kit) failed to amplify for rbcL and both rbcL and trnL respectively. Four plant ZOTUs, *Calamagrostis scotica* (9 reads), *T. subterraneum* (34 reads), *Helianthus* sp. (8 reads) and Poaceae (8 reads) showed amplification in one of the PCR controls. The DNA reads corresponding to the plant ZOTUs identified in the controls were removed from the pan trap sample dataset (method following Bell et al., 2017) prior to further analyses.

5.4.2 Arthropod eDNA was not consistently detected from pan-trap water

Only 17% of pan-trap water samples, all extracted with the Blood and Tissue extraction kit, showed successful amplification for arthropod DNA, while no amplification was achieved for samples extracted with the Plant Pro kit. The amplification success of arthropod eDNA was low despite all of the pan traps showing the presence of arthropod taxa during the morphological identifications (Figure 5.2 and Table S5.2). In total, 13 arthropod families were detected using eDNA metabarcoding of pan-trap water of which Chironomidae (35% of detections), Sphaeroceridae (16% of detections) and Phoridae (11% of detections) were the most common families (Figure 5.2). Morphological identifications of the pan trap samples yielded 17 arthropod families, although here the most common taxa belonged to Apidae (15% of detections), Coelopidae (20% of detections), and Tenebrionidae (13% of detections) (Figure 5.2 and Table S5.2).

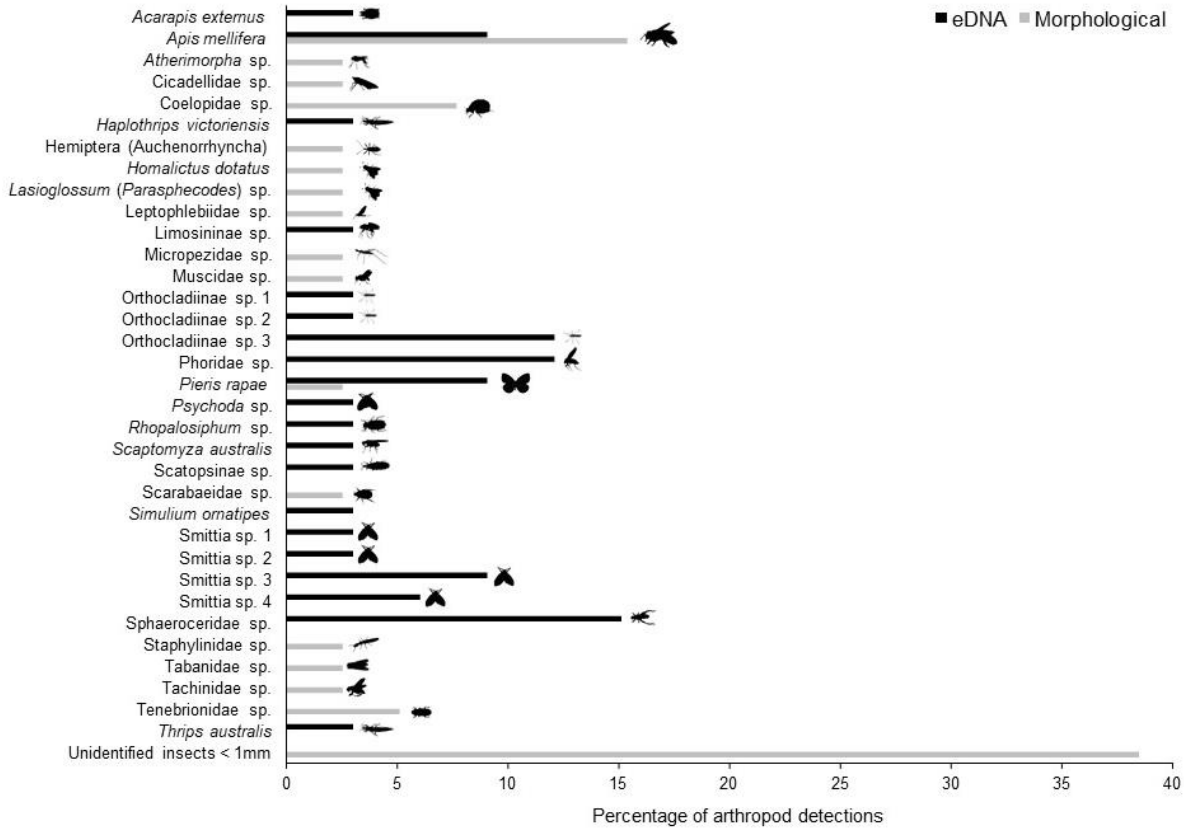

Figure. 5.2 Percentage of taxa detected for arthropods classified by eDNA metabarcoding of pan-trap water (*N* = 20 taxa total) and morphological identification (*N* = 17 taxa total). For the morphological identifications, arthropods that were less than 1mm in size were not identified, due to the focal orchard species, *Persea americana*, typically requiring pollinators with larger body sizes (Ish-Am, 2005).

5.4.3 Extraction method did not influence the number of plant species detected using pan traps

The average number of plant reads generated per sample did not differ significantly (p = .13) between the Blood and Tissue extraction kit - average reads = 129,658 (± 18,269 SE), and the Plant Pro kit - average reads = 158,965 (± 16,123 SE). There was no significant difference in the number of plant taxa detected between the DNA extraction protocols used (p = .61); both methods on average generated 17 (± 0.78 SE) plant taxa per pan trap array (Figure 5.3A). Taxonomic composition differed between the extraction kits, with three plant taxa only detected in the samples processed with the Blood and Tissue kit and five plant taxa detected in the samples processed with the Plant Pro kit, these species were classified as rare and represented less than 0.5% of the overall detections (Figure 5.3B). Given the almost identical plant detections and similar read counts in pan traps between the Blood and Tissue and Plant Pro kits, the two datasets were combined for subsequent analyses.

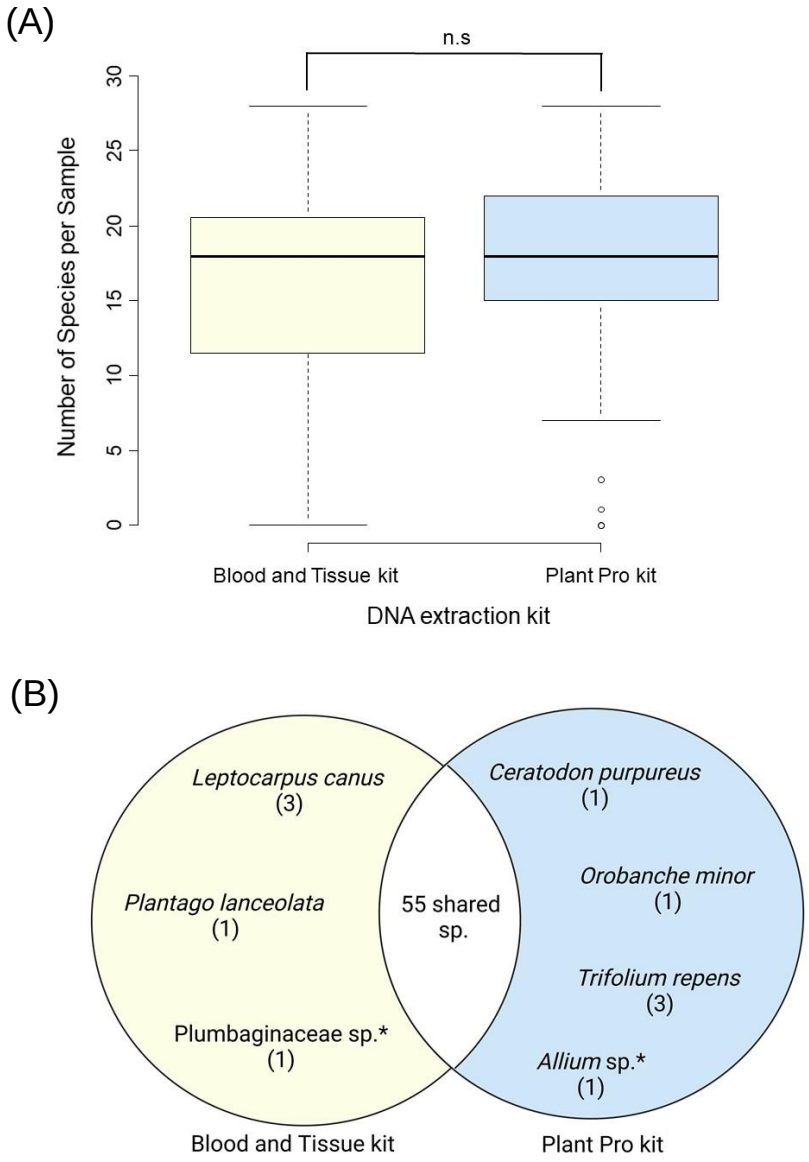

Figure. 5.3 (A) The number of plant species detected per sample for the Blood and Tissue (*N* = 18) and Plant Pro extraction kits (*N* = 18) for pan trap array and marble trap samples. (B) Plant species unique each extraction kit (Blood and Tissue; *N* = 3, Plant Pro; *N* = 5). n.s = not significant. *Species unique to the marble traps.

5.4.4 eDNA detects more plant diversity than conventional floral surveys

Overall, 30 plant families (33% animal-pollinated, 30% wind-pollinated and 37% both animal- and wind-pollinated) were represented in the pan trap eDNA dataset, of which Asteraceae (*A. calendula*, *Gamochaeta calviceps*, *Helianthus* sp., *Sonchus* sp.; detected in 100% of pan trap samples), Myrtaceae (*Callistemon* sp., *Eucalyptus* spp. and *Leptospermum* sp.; detected in 100% of pan trap samples), and Fabaceae (*Acacia* sp., *Bossiaea aquifolium*, *Dilwynia* sp., *Goodia* sp., *Lotus* sp., *Mirbelia* sp., *Paraserianthes lophantha*, *Trifolium repens* and *T. subterraneum*; detected

in 93% of pan trap samples) were the most common (Table 5.1 and Figure 5.4). Many of these plant taxa detected using eDNA were not present within the orchard or adjacent pasture and may represent flowering species beyond the survey area (e.g. Eucalypt Forest located less than 1 km away from each survey orchard). In contrast to the eDNA results, the floral surveys documented six plant families, of which Fabaceae (*T. subterraneum* and *T. repens*; 18% of surveys), Asteraceae (*Arctotheca calendula* and *Sonchus* sp.; 16% of surveys), Brassicaceae (*Raphanus raphanistrum*; 7% of surveys) and Poaceae (*Bromus catharticus*, *Poa annua*; 7% of surveys) were the most common (Table S5.3). Four out of the six families documented with the floral surveys were detected in the eDNA pan trap dataset, while only Geraniaceae and Caryophyllaceae were unique to the floral surveys. At the level of species, 64 taxa were found between eDNA and the floral surveys, of which 53 (83%) were unique to eDNA, 3 (5%) were unique to the floral surveys and 8 (12%) were shared between both survey methods (Table 5.1). The three species that were unique to the floral surveys (*Bromus catharticus*, *Cerastium glomeratum* and *Erodium moschatum*) had an average percentage cover of less than 0.01% per $1m^2$ quadrat. Predictably, species composition differed significantly between the eDNA and floral survey methods ($R = 0.365$, $p < .001$) (Figure 5.5). Although neither method alone appeared to capture the total plant diversity present within the orchards (Figure S5.1). eDNA metabarcoding detected an average of 25 plant species per pan trap array sample (± 1.5 SE), while the floral surveys detected an average of 2 species (± 0.2 SE) per quadrat. When floral surveys were combined with eDNA metabarcoding, the average alpha diversity per orchard increased from 7.3 (± 1.5 SE) to 56.3 (± 1 SE).

Table 5.1 Plant taxa (*N* = 64) detected using eDNA metabarcoding of pan-trap water (after putative false positives were removed) and conventional floral surveys at three *Persea americana* orchards visited between the 29th – 31st of October 2021. Number of plant species identified by each method; eDNA (*N* = 60), floral survey (*N* = 10) and shared (*N* = 8). Some plant taxa rely on both wind- and animal-pollination (Regal, 1982), therefore, we conducted a literature search using Google Scholar to categorise the pollination syndromes of each plant taxa detected. In total, we categorised three pollination syndromes for the plant taxa detected by both survey methods: animal-pollinated (*N* = 22), wind-pollinated (*N* = 27) and mixed animal- and wind-pollinated (*N* = 15). Although not captured in the quadrats (and therefore not included in the statistical analysis), *P. americana* was counted as present for the floral surveys based on visual observation of the trees flowering.

Species	eDNA	Floral Survey	Both
Acacia sp.	◑		
Aphelia cyperoides	○		
Arctotheca calendula			◑
Aristida behriana	○		
Asteraceae	◑		
Bossiaea aquifolium	●		
Bromus catharticus		○	
Calamagrostis scotica	○		
Callistemon sp.	●		
Callitropsis nootkatensis	○		
Casuarinaceae sp.	○		
Catapodium rigidum	○		
Cenchrus sp.	○		
Cerastium glomeratum		●	
Ceratodon purpureus	○		
Citrus sp.	●		
Colchicaceae	●		
Cotoneaster sp.	●		
Cyperaceae	◑		
Dillwynia sp.	●		
Eleocharis sp.	○		
Erodium moschatum		●	
Eucalyptus sp.	◑		
Festuca sp.	○		
Gamochaeta calviceps	●		
Goodia sp.	●		
Helianthus sp.	◑		
Heliotropium europaeum	●		
Holcus lanatus	○		
Hopkinsia adscendens	○		
Juglans regia	○		
Juncus oxycarpus	○		
Juncus sp.	○		
Juniperus sp.	○		
Leptocarpus canus	○		
Leptocarpus sp.	○		
Leptodermis sp.	●		
Leptospermum sp.	◑		
Ligustrum ovalifolium	●		
Lotus sp.	●		
Lysimachia arvensis	●		
Macrozamia riedlei	●		
Mirbelia sp.	●		
Orobanche minor	●		
Paraserianthes lophantha	◑		
Persea sp.			◑
Pimelea sp.	●		
Pinus sp.	○		
Plantago lanceolata	○		
Poa annua			○
Poaceae			○
Pyracantha sp.	●		
Quercus sp.	○		
Raphanus raphanistrum			◑
Restionaceae	○		
Ribes sp.	◑		
Rubus sp.	◑		
Rumex sp.	○		
Sonchus sp.			◑
Sporobolus africanus	○		
Streptophyta	◑		
Trifolium repens			●
Trifolium subterraneum			●
Trymalium odoratissimum	◑		

● Animal-pollinated

○ Wind-pollinated

◑ Both animal- and wind-pollinated

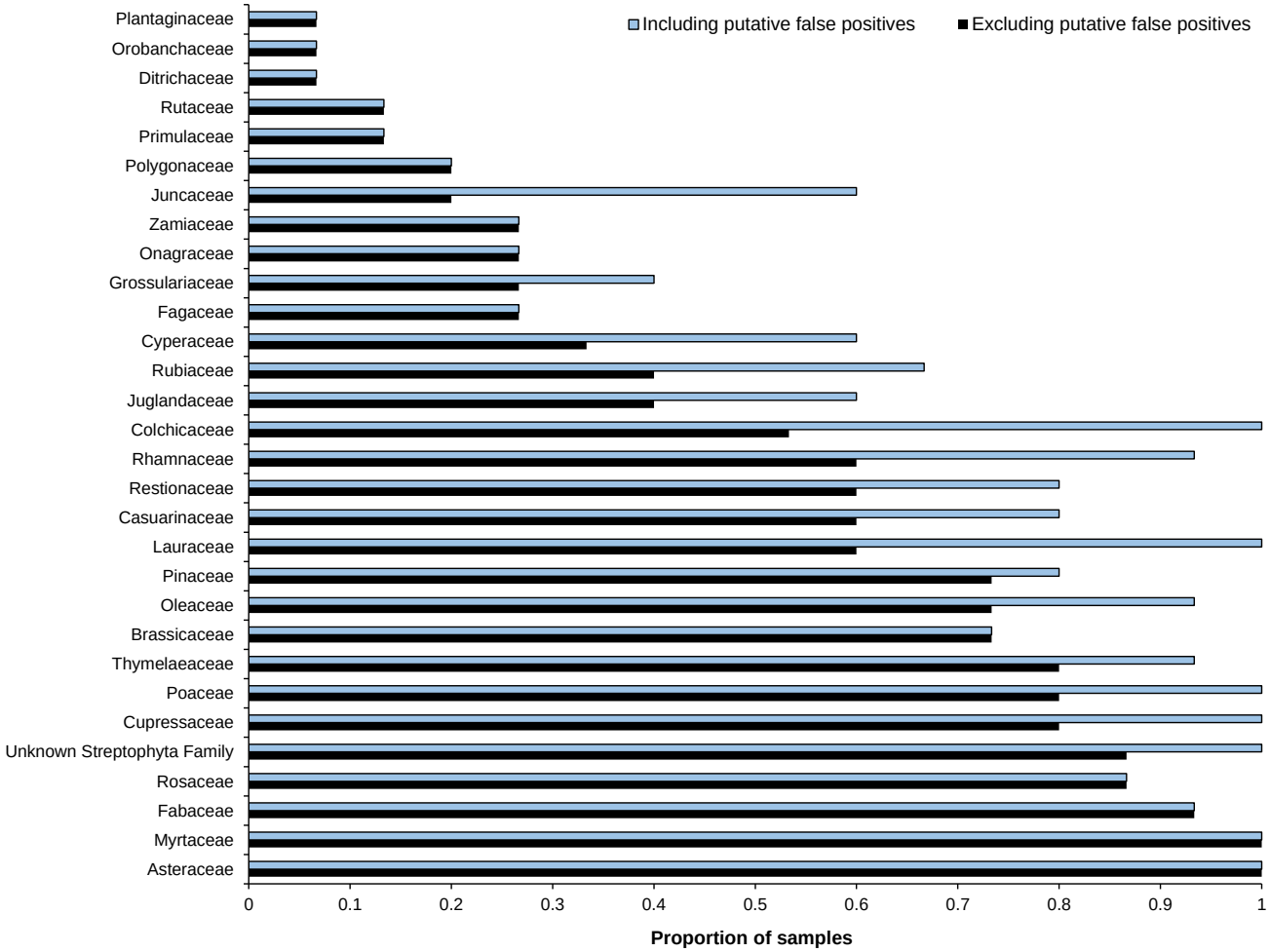

Figure 5.4 Major plant families represented in the eDNA pan trap samples (*N* = 30 families), before and after putative false positives were removed from the pan trap array samples following Bell et al. (2017).

5.4.5 Ambient plant DNA is a significant contribution to pan traps

Sequence counts generated from the marble trap samples were used as minimum sequence copy thresholds for the pan traps when plant taxa were found in both (Figure S5.2). With this method, the average reads per sample were reduced by 60% (± 6.7 SE), 55% (± 7.3 SE) and 13% (± 4.2 SE) for three survey orchards, respectively. One wind-pollinated species (*Rumex acetosa*) was removed from the pan trap dataset with this filtering procedure. Per sample, the removal of putative false positives reduced the average number of plant taxa detected in the pan traps by 53% (± 5.5 SE), 51% (± 3.8 SE) and 10% (± 1.9 SE) for the three survey orchards, respectively.

Figure 5.5 Principal Co-ordinate analysis showing the grouping of samples between the two survey methods (eDNA and Floral surveys) based on Jaccard similarity matrices (species presence-absence).

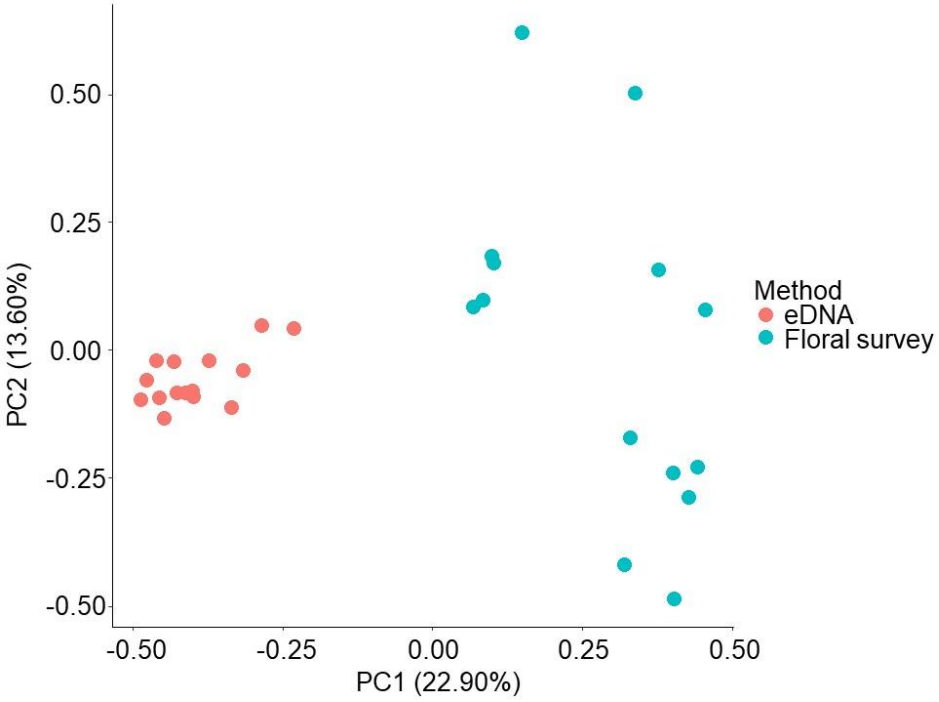

Figure 5.5 Principal Co-ordinate analysis showing the grouping of samples between the two survey methods (eDNA and Floral surveys) based on Jaccard similarity matrices (species presence-absence).

5.5 Discussion

Terrestrial arthropod biomass and species diversity are threatened globally, with some monitored populations showing mean abundance declines of ~ 45% in the past 40 years alone (Dirzo et al., 2014; Wagner 2020). Despite such threats however, much remains unknown about wild pollinators in both natural and agro-ecosystems (Biesmeijer et al., 2006; Potts et al., 2016). New survey methods are therefore needed which can help monitor arthropod pollinators, as well as the plant resources upon which they rely (Bell et al., 2019, 2022; Evans & Kitson, 2020). Here, we provide new insights on a novel approach to detect plant resources used by arthropods within agricultural landscapes. The three most commonly detected plant families from metabarcoding of pan-trap water (Asteraceae, Myrtaceae and Fabaceae) are all known to require arthropod pollination to facilitate or enhance fruit set. eDNA metabarcoding also identified species that were not detected using the conventional floral surveys, indicating the detection of plant species beyond the orchard boundaries (however, see section *5.5.3 Pan trap pollen – ambient or animal-mediated?* below). Our results provide compelling evidence that metabarcoding can be combined with pan trapping techniques to identify potential plant resources used by arthropods in agricultural systems.

5.5.1 Extracting arthropod and plant DNA from pan traps

Arthropod taxa were not consistently detected in pan traps by eDNA metabarcoding, using either DNA extraction method, and little overlap was found between the eDNA and morphological datasets. Previous studies comparing eDNA metabarcoding of homogenised bulk-tissue macroinvertebrate samples and ethanol from preserved non-homogenised bulk-insect samples detected similar low read counts and reduced species detections (Persaud, Cottenie, & Gleason, 2021; Zizka et al., 2018). For instance, Persaud et al. (2021) showed that less than 10% of the OTUs from non-homogenised ethanol eDNA were retained in the final dataset and that there was minimal overlap (0 – 1.4% OTU overlap) between the ethanol eDNA and paired homogenised bulk-tissue samples. In the present study, the lack of consistent arthropod detections (only 17% of pan trap samples) may be caused by the presence of an exoskeleton reducing the release of DNA (see Shokralla et al., 2010; Zizka et al., 2018). The choice of destructive versus non-destructive sampling will depend on the study aims. Our aim was to minimise the contribution of plant material from the guts of herbivorous arthropods by preserving whole specimens, however, if future studies wish to detect a greater number of arthropod taxa from pan-trap water, then we would recommend suspending bulk arthropod samples in a lysis buffer as an initial step in DNA extraction. This non-destructive approach has previously generated comparable arthropod detections to homogenised bulk samples (see Kirse et al., 2022), while also retaining whole specimens for morphological

identification and abundance data. Additionally, this approach may reduce the contribution of ingested material from captured arthropods (although regurgitant can contain ingested material from flower visits, see Bowie et al., 1999). However, further testing remains necessary to verify if of the transfer of arthropods to a lysis buffer could compromise 'true' insect-mediated pollen detections from pan trap water.

In contrast to the low number of arthropod species detections, metabarcoding of pan-trap water enabled consistent plant identification across all pan trap arrays. Pan traps provide a passive and cost-effective means to capture arthropods without observer bias, which is probably a contributing factor to their growing use in terrestrial surveys (Prendergast et al., 2020; Westphal et al., 2008; Wilson et al., 2008). So far, however, the use of pan traps to detect animal-mediated pollen has relied largely upon microscopy-based techniques (Ladd et al., 2019; Popic et al., 2013) and few studies have quantified the contribution of ambient pollen to total plant diversity. Our combined approach using eDNA metabarcoding of pan and marble traps allowed for the identification of known arthropod-pollinated plant species, while minimising artefacts from non-target ambient pollen. In contrast to approaches based on pollen loads of individual arthropod specimens (e.g. Pornon et al., 2017), eDNA metabarcoding of pan-trap water provided a measure of the total plant diversity supporting the captured arthropod community, but cannot be used to infer individual arthropod-plant interactions due to the mixed species pool in the pan traps. Although, the total plant diversity captured from pan traps may provide arthropod community-level foraging measures, which could be used to make generalisations about fluctuations in key floral resources through time (i.e. relative abundance of plant taxa). Such measures may help capture the dynamic and often generalised nature of arthropod-plant interactions (Waser et al., 1996). In total, eDNA metabarcoding of pan-trap water detected 30 plant families, representing at least 60 species, of which 53 species were unique to the eDNA dataset. Our findings also echo those of Campbell et al. (2018), who found that 30 pan traps could collect over 15,000 pollen grains, suggesting that this passive sampling technique can provide a rich source of information for local arthropod foraging resources. Further, many of the plant species in the eDNA dataset were expected, for instance, we consistently detected annual weed species (e.g. *Arctotheca calendula* and *Raphanus raphanistrum*) in all of our field sites, which was not surprising given that we sampled in spring and all of the sample orchards were adjacent to pasture.

Both DNA extraction methods detected similar plant communities. Nevertheless, each extraction kit identified a few unique taxa (Blood and Tissue; $N = 3$, Plant Pro; $N = 5$), although these taxa

were all uncommon (< 0.5% of detections) and probably reflect stochasticity in sampling (Jensen et al., 2022). We stress that eDNA metabarcoding of pan-trap water, like any new substrate, will require further testing of additional DNA extraction kits used for pollen metabarcoding (e.g. DNeasy PowerSoil Pro kit; see Johnson et al. 2019a) and plant assays (i.e. ITS2; see Cheng et al. 2016) to determine the optimal procedure for this method.

5.5.2 Complementarity of eDNA metabarcoding and conventional floral surveys

A major short-coming of floral surveys is that they are limited in the area that they can encompass, meaning multiple survey trips are often necessary to produce robust data (Uniyal & Singh, 2014). Also, at locations with high plant species diversity, broad taxonomic expertise required to identify plant species may not be available (O'Connor et al., 2019). Consequently, floral surveys may be appropriate for some systems (e.g. small-scale farms with low plant species diversity), although at the typically large scales of many food production systems, these surveys may benefit from the addition of eDNA metabarcoding of pan-trap water (Kestel et al., 2022).

In our study, only eight species (12%) were shared between the eDNA metabarcoding and flora surveys, which may reflect arthropod foraging beyond the pasture area surveyed. Floral surveys conducted at a larger spatial scale may have detected more plant taxa and reduced the disparity between the two methods. Differences in taxa identified with eDNA metabarcoding-based surveys of biodiversity and those using other methods are not uncommon (Johnson et al., 2021; Kestel et al., 2023; Ryan, Bateman, Fernandes, van der Heyde, & Nevill, 2022). For example, when comparing plant communities detected with air eDNA to those identified from conventional transect surveys, Johnson et al. (2021) detected more rare flowering forb species (e.g. *Berlandiera lyrata*) with transect surveys than with eDNA. Indeed, for our study, conventional floral surveys detected *Bromus catharticus* (wind-pollinated), *Cerastium glomeratum* (animal-pollinated) and *Erodium moschatum* (animal-pollinated), none of which were identified using eDNA metabarcoding, although sequences for the three species for both trnL and rbcL are present on NCBI and an *in silico* analyses confirmed that primers would bind to target DNA sections. However, all three species were rare (< 0.01% flowering per 1 m^2 quadrat) and potentially occurred at levels below the detection thresholds possible for pollen metabarcoding of pan traps. An additional possibility is that there were no specific flower-visiting arthropods that visited these three species and the pan traps at the time of sampling. If the intention of surveys is to detect all plant species in a given area, we stress the need for complementing eDNA metabarcoding of pan-trap water with conventional floral surveys with a more intensive survey effort than what was used

in the present study, to capture and identify potential false negatives that could arise in the eDNA metabarcoding dataset. Additionally, by including these surveys using other approaches, measures of species abundance and percentage flowering can also be generated, information which is otherwise difficult to infer from the presence-absence values generated from eDNA (see Bell et al., 2019).

5.5.3 Pan trap pollen – ambient or animal-mediated?

In our study, we used plant DNA collected in marble traps to filter out ambient pollen and airborne plant sequencing reads from the pan trap dataset (Figure 5.4 and Figure S5.2). With this method, we were able to significantly reduce the contribution of airborne plant DNA to the final data (Figure S5.2), however, we acknowledge that the use of marble traps only minimises the contribution of ambient plant DNA (e.g. airborne pollen and plant tissue) and does not eliminate it completely. For instance, species belonging to Poaceae and Cupressaceae were still found in 80% of the pan trap samples, even though both plant families primarily rely on wind-pollination (García-Mozo, 2017; Takaso & Owens, 2008). Similar results have been shown with HTS approaches used to measure trophic interactions for insects in agricultural ecosystems (see Batuecas et al., 2022). Here, metabarcoding the wash water from external surfaces of *Rhagonycha fulva* individuals showed the presence of 11 plant taxa, including wind-pollinated Poaceae spp. and *Pinus* spp. Thus, although marble traps can reduce the contribution of ambient plant DNA to the pan trap detections, the incidental collection of wind-pollinated pollen (either during feeding or while flying; see Batuecas et al., 2022) is more challenging to ascertain. Indeed, the water collected from pan traps will likely contain plant DNA originating from the digestive tracts of captured arthropods, which may represent non-pollen material (i.e. leaf and stem tissues) collected during foraging or nest building (Batuecas et al., 2022; Evans & Kitson, 2020; McPherson, Avanesyan, & Lamp, 2022). Thus, an understanding of the presumed pollination syndrome of each plant taxa detected remains essential to help determine which plant detections represent potential floral resources and which represent non-target airborne plant material.

The recent application of eDNA metabarcoding to air samples (e.g. Clare et al., 2021; Johnson et al., 2021) presents a critical innovation for this molecular survey method, one which could further improve the resolution of studies of arthropod-plant interactions using pan traps. In the present study, we addressed the presence of ambient pollen and plant material using marble traps to generate relative quantifications of non-target eDNA (see Johnson et al., 2019b), which could otherwise add artefacts, such as inflated read counts and false positives (see Ficetola et al., 2016).

The marble traps proved to be a scalable, cost-effective and simple method to produce minimum sequence copy threshold values (Bell et al., 2017; Drake et al., 2022). Despite this however, we acknowledge that incorporating higher minimum sequence copy thresholds to complement the marble traps could help further increase the reliability of floral resources detected with pan traps, although at the risk of increasing false negatives (Drake et al., 2022). These minimum sequence copy thresholds could be improved by incorporating novel techniques developed in air eDNA studies to accurately determine false positives due to ambient pollen. Since Longhi et al. (2009) first characterised DNA from airborne pollen, numerous authors have targeted airborne pollen and plant material (Emenyeonu et al., 2018; Johnson et al., 2019a,b; Kraaijeveld et al., 2015). Emenyeonu et al. (2018) provided a proof-of-concept study to detect trace amounts of aerosolised 'seed dust' DNA by using a purpose-built air-sampler able to filter air at 620 L/min. Increasingly, these high-volume active air samplers are being combined with autonomous technologies to allow for long-term pollen monitoring (see Khan et al., 2022). Such technologies present an opportunity to improve the accuracy of thresholds by using ambient plant eDNA counts that are derived by a combination of methods, rather than relying on one method alone. In this way, future studies could potentially remove a greater proportion of artefacts and increase the reliability of the floral detections; without removing too much data and generating false negatives (see Hänfling et al., 2016). Together, this combined approach integrated with internal standards (see Harrison et al., 2020), could also help generate absolute quantification values for pollen and allow for the standardisation of read numbers across samples.

While the marble trap minimum sequence copy thresholds proved effective at reducing the contribution of ambient pollen and plant material in the pan traps, this filtering procedure may have also generated false negatives in the eDNA dataset. False negatives arise when species actually present are missed during detection, potentially leading to underestimates of species richness and, in the case of the present study, reducing the diversity of flowering and habitat plants detected for captured arthropods (Ficetola et al., 2015). In the eDNA dataset, two plant families (Colchicaceae and Lauraceae), both of which are largely animal-pollinated (Barrett & Case, 2006; Ish-Am, 2005), detections were reduced by 47% and 40%, respectively, from the pan traps by using minimum sequence copy thresholds set by the marble traps. Although these taxa may have been correctly removed as non-target ambient eDNA (e.g. Lauraceae species can be wind-pollinated; see Ish-Am, 2005), there is a possibility that they represent false negatives, generated by similar sequence reads between the marble traps and the pollen and plant material actually carried by the arthropods captured in the pan traps; however, in the present study, for the purposes

of assessing the viability of eDNA metabarcoding of pan-trap water, an over-cautious approach was deemed preferable to reduce the opportunities for inflated foraging species diversity measures.

5.5.4 Incorporating pan trap eDNA into agricultural monitoring

The plant resources supporting arthropods in natural and agricultural landscapes are of increasing interest, largely because of the increasing threats to global arthropod abundance and species diversity, as well as the increasing recognition of wild native pollinators as important contributors to crop yields. We have demonstrated that eDNA metabarcoding of pan-trap water can be used to identify the plant resources arthropods rely on. This approach complements long-standing survey methods and may potentially improve the characterisation of arthropod-plant interactions in agro-ecosystems (Kestel et al., 2022). Such an approach also lends itself to modification depending on the research context and questions. For instance, pan traps could be deployed during crop flowering to capture potential pollinators and cross-validated with the inclusion of cameras to monitor flower visits for the crop species of interest. Such information is needed for identifying which floral resources are critical to managing arthropod communities in food production areas (e.g. encouraging non-bee pollinators to remain in orchards and improve crop production; see Cook et al., 2020; Rader et al., 2016; Senapathi et al., 2021). Surveys and monitoring using eDNA metabarcoding of pan-trap water could easily be tailored to different studies by, for example; 1) varying the number of biological replicates to accurately represent arthropod communities at orchards of different spatial scales, and; 2) pooling samples across pan trap arrays depending on the available budget. As application of this method is in its infancy, we acknowledge that more studies are required to generate reliable data. Controlled experiments are needed to address the 'incidental' collection of eDNA by arthropods, such as the accumulation of plant eDNA from neutral arthropod-plant interactions (e.g. an arthropod taxa resting on a leaf) (though see Kudoh et al., 2020; Thomsen & Sigsgaard, 2019). Such baseline studies will help further unravel which eDNA detections likely arise from animal-mediated pollen transport, thereby allowing for more detailed conclusions concerning pollinator foraging within agro-ecosystems. In conclusion, the availability of this approach will modernize floral resource surveys by reducing constraints on survey effort and enabling rapid identification of arthropod-plant interactions. However, the limitations of the method must be carefully considered to allow for robust management decisions based on data from this new molecular tool.

5.6 References

A thorough effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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5.7 Supplementary information

Table S5.1 357 results from Scopus search from 02/03/2023 using the search terms: ‘pan trap’ OR ‘pan trap’ OR ‘bowl trap’ OR ‘bowl trap’ AND ‘insect’ OR ‘arthropod’. Articles in Medicine, Immunology and Microbiology, Earth and Planetary Science, Social Sciences and Energy were excluded.

DOI	Year	Cited by
10.1016/j.agee.2023.108415	2023	0
10.1016/j.actao.2022.103873	2023	0
10.1016/j.ecolind.2023.109980	2023	0
10.1111/afe.12538	2023	0
10.1007/s10841-023-00460-4	2023	0
10.3390/agronomy13010084	2023	0
10.1007/s12595-023-00465-6	2023	0
10.3390/insects13121152	2022	0
10.1007/s10841-022-00437-9	2022	0
10.1007/s10841-022-00436-w	2022	0
10.1186/s40693-022-00108-8	2022	1
10.1017/S0007485322000104	2022	0
10.3390/plants11192596	2022	1
10.1111/eea.13218	2022	0
10.1007/s10841-022-00402-6	2022	1
10.1007/s10457-022-00744-9	2022	1
10.3390/plants11131734	2022	1
10.1016/j.agee.2022.107930	2022	2
NM	2022	0
10.1111/fwb.13902	2022	1
10.1016/j.foreco.2022.120127	2022	0
10.18805/AG.D-332	2022	1
10.3390/plants11070898	2022	1
10.1007/s10841-022-00387-2	2022	6
10.5958/0974-4576.2022.00005.6	2022	0
10.1002/ece3.8708	2022	1
10.1002/ece3.8653	2022	0
10.1088/1755-1315/985/1/012051	2022	0
10.1088/1755-1315/974/1/012134	2022	1
10.14720/aas.2022.118.4.2441	2022	0
10.1088/1755-1315/1107/1/012061	2022	0
NM	2022	0
10.1088/1755-1315/1083/1/012021	2022	0
10.13057/biodiv/d230738	2022	0
10.21475/ajcs.22.16.04.p3551	2022	0
10.1016/j.gecco.2021.e01988	2022	3
10.1016/j.jksus.2021.101686	2022	1

DOI	Year	Cited by
10.1007/s10841-021-00346-3	2021	2
10.1016/j.actao.2021.103790	2021	0
10.1111/1365-2664.13990	2021	4
10.1111/icad.12517	2021	2
10.4039/tce.2021.17	2021	1
10.1111/rec.13407	2021	2
10.13102/SOCIOBIOLOGY.V68I3.5783	2021	0
10.3390/su13179612	2021	2
10.3390/plants10091788	2021	5
10.1007/s00436-021-07270-0	2021	3
10.1016/j.agee.2021.107470	2021	4
10.1007/s13355-021-00748-4	2021	2
10.1016/j.agee.2021.107383	2021	20
10.3389/ffgc.2021.672601	2021	1
10.1016/j.agee.2021.107391	2021	1
10.33307/entomon.v46i2.597	2021	0
10.1016/j.ecolind.2021.107573	2021	9
10.1007/s10457-020-00568-5	2021	3
10.1371/journal.pone.0251572	2021	2
10.3390/insects12050404	2021	3
10.1002/ece3.7252	2021	6
10.1016/j.biocontrol.2021.104535	2021	3
10.7717/peerj.11132	2021	1
10.17520/biods.2020034	2021	1
10.17660/ActaHortic.2021.1306.13	2021	0
10.1111/eea.13008	2021	3
10.1016/j.agee.2020.107255	2021	3
10.1007/s42690-020-00255-z	2021	3
10.1007/s42690-020-00252-2	2021	2
10.1016/j.ecolind.2020.107132	2021	14
NM	2021	0
10.1155/2021/8876388	2021	0
10.24425/jppr.2021.137953	2021	2
10.1590/1806-9665-RBENT-2021-0057	2021	0
10.3897/abs.7.e62610	2021	0
10.1080/00218839.2021.1944569	2021	0
10.14411/EJE.2021.019	2021	2
10.3390/biology10050445	2021	3
10.3390/d13040148	2021	0
NM	2021	4
10.1080/00218839.2021.1888524	2021	1
10.24189/ncr.2021.013	2021	8
10.3897/BDJ.9.E60665	2021	1
10.3390/insects12010054	2021	4
10.3923/pjbs.2021.25.34	2021	1
10.1111/1365-2664.13755	2021	32

DOI	Year	Cited by
10.13057/biodiv/d211249	2020	2
10.1038/s41598-020-75566-9	2020	19
10.1007/s10841-020-00274-8	2020	1
10.1038/s41598-020-70518-9	2020	10
10.1007/978-3-030-53226-0_7	2020	2
10.3390/insects11110795	2020	6
10.18474/0749-8004-55.4.547	2020	4
10.1371/journal.pone.0240138	2020	9
10.13057/biodiv/d210937	2020	1
10.1093/jme/tjaa057	2020	2
10.1111/aje.12718	2020	0
10.1371/journal.pone.0235054	2020	7
10.1088/1755-1315/515/1/012006	2020	1
10.1093/aesa/saaa010	2020	59
10.1093/ee/nvaa022	2020	3
10.3390/insects11060351	2020	4
10.1007/s10886-020-01188-0	2020	1
10.1007/s10841-020-00224-4	2020	12
10.1016/j.dib.2020.105132	2020	3
10.1007/s10841-019-00201-6	2020	3
10.1007/978-981-15-0794-6_8	2020	4
10.13057/biodiv/d210323	2020	4
10.11646/zootaxa.4731.2.7	2020	0
10.3390/insects11020094	2020	4
10.1016/j.agee.2019.106706	2020	11
10.1088/1755-1315/418/1/012017	2020	3
NM	2020	0
10.21315/tlsr2020.31.3.6	2020	1
10.31018/jans.v12i3.2316	2020	0
10.3390/insects11080488	2020	3
10.3390/insects11010026	2020	10
10.3390/insects11010046	2020	10
10.1111/1755-0998.13056	2020	25
10.1088/1755-1315/399/1/012103	2019	2
10.1088/1755-1315/394/1/012013	2019	0
10.1088/1755-1315/388/1/012004	2019	4
10.1007/s10764-019-00111-z	2019	8
10.1002/ece3.5756	2019	11
10.1111/2041-210X.13292	2019	58
10.3390/su11226399	2019	14
10.1111/ddi.12972	2019	35
10.1016/j.sjbs.2018.02.007	2019	14
10.1016/j.gecco.2019.e00781	2019	18
10.1016/j.gecco.2019.e00711	2019	6
10.1088/1755-1315/325/1/012002	2019	1
10.1002/ecs2.2849	2019	4

DOI	Year	Cited by
10.1080/00779962.2019.1660450	2019	7
NM	2019	1
10.2478/jas-2019-0014	2019	14
10.1016/j.biombioe.2019.04.023	2019	8
10.4039/tce.2019.15	2019	3
10.1007/s10980-018-0757-2	2019	17
10.1111/1748-5967.12348	2019	4
10.1093/jee/toy309	2019	2
10.3390/insects10020040	2019	21
10.14411/EJE.2019.043	2019	3
10.1127/entomologia/2019/0726	2019	1
NM	2019	0
10.1071/BT18249	2019	6
10.3390/insects9040170	2018	53
NM	2018	2
10.1016/j.foreco.2018.07.022	2018	7
10.1088/1755-1315/197/1/012012	2018	0
10.1016/j.agee.2018.07.020	2018	38
10.1016/j.ecolind.2018.06.008	2018	6
10.1063/1.5061891	2018	1
10.1371/journal.pone.0206387	2018	14
10.3390/insects9030114	2018	16
10.1007/s00442-018-4169-4	2018	22
10.1016/j.agee.2018.04.001	2018	4
10.3390/insects9020046	2018	3
10.1093/forsci/fxx008	2018	24
10.1007/s13355-018-0546-z	2018	3
10.1007/s11356-018-1818-4	2018	8
10.1111/afe.12247	2018	1
10.1007/s10841-018-0056-x	2018	3
10.1016/j.agee.2017.11.019	2018	33
10.1371/journal.pone.0191567	2018	20
10.1007/s10841-017-0038-4	2018	12
NM	2018	0
10.17503/agrivita.v39i2.1038	2018	5
10.1002/aps3.1020	2018	0
10.1016/j.foreco.2017.06.010	2017	4
10.1653/024.100.0408	2017	4
10.1111/icad.12249	2017	8
10.1111/1744-7917.12377	2017	18
10.1007/s10841-017-0013-0	2017	35
10.1016/j.agee.2017.05.035	2017	34
10.3897/natureconservation.18.12314	2017	22
10.1007/s00027-017-0529-0	2017	16
10.4039/tce.2017.6	2017	5
10.1093/ee/nvx046	2017	4

DOI	Year	Cited by
10.13057/biodiv/d180253	2017	1
10.1007/s10750-016-3039-5	2017	5
10.23960/j.hptt.11737-44	2017	1
10.1186/s12898-017-0116-1	2017	34
10.1016/j.agee.2017.01.032	2017	22
10.3923/JE.2017.228.233	2017	0
10.7717/peerj.3620	2017	34
NM	2017	0
10.18474/JES16-13.1	2017	4
10.5846/stxb201511292392	2017	2
10.32800/abc.2017.40.0193	2017	7
10.1093/ee/nvw131	2016	23
10.1653/024.099.0428	2016	10
10.1016/j.cropro.2016.06.020	2016	13
10.1016/j.jaridenv.2016.06.009	2016	11
10.1093/jee/tow195	2016	5
10.1093/ee/nvw087	2016	24
10.1111/1365-2664.12693	2016	6
10.23960/j.hptt.216165-174	2016	0
10.23960/j.hptt.216138-146	2016	4
10.1007/s10841-016-9890-x	2016	33
10.1016/j.agee.2016.05.010	2016	36
10.1371/journal.pone.0143479	2016	19
10.13287/j.1001-9332.201607.020	2016	5
10.2984/70.3.3	2016	8
10.1653/024.099.0230	2016	16
10.3897/BDJ.4.e7607	2016	5
10.3897/BDJ.4.e8830	2016	5
NM	2016	2
10.3897/BDJ.4.e9977	2016	2
10.4289/0013-8797.118.1.37	2016	19
10.18520/cs/v110/i3/438-443	2016	6
10.1111/icad.12143	2016	11
10.3897/BDJ.3.e6832	2015	14
10.1007/s10980-015-0224-2	2015	16
10.1371/journal.pone.0140600	2015	2
10.4039/tce.2014.55	2015	13
10.1016/j.foreco.2015.03.044	2015	57
10.1016/j.rbe.2015.07.001	2015	9
10.1111/icad.12117	2015	32
10.1093/ee/nvv001	2015	45
10.1093/ee/nvv058	2015	6
10.1098/rstb.2014.0125	2015	78
10.1111/icad.12105	2015	17
10.23960/j.hptt.11553-64	2015	2
NM	2015	2

DOI	Year	Cited by
10.1007/s10841-014-9730-9	2014	15
10.1007/s10841-014-9723-8	2014	38
10.1016/j.meegid.2014.03.023	2014	9
10.1007/s10841-014-9698-5	2014	2
10.1371/journal.pone.0104679	2014	201
10.1073/pnas.1407773111	2014	84
10.1007/s10980-014-0024-0	2014	27
10.11646/zootaxa.3779.1.9	2014	8
10.1186/1472-6785-14-1	2014	15
10.1371/journal.pone.0085635	2014	52
10.1673/031.014.15	2014	0
10.1656/058.013.0213	2014	26
10.1093/jisesa/ieu112	2014	9
10.1016/j.biombioe.2014.05.021	2014	20
10.11118/actaun201462010167	2014	4
10.1007/s11273-013-9325-3	2014	19
10.1303/jjaez.2014.32	2014	6
10.2987/13-6349.1	2014	3
10.1002/ece3.688	2013	33
10.1007/s13355-013-0219-x	2013	224
10.1016/j.biocon.2013.09.001	2013	21
10.1371/journal.pone.0077999	2013	74
NM	2013	10
10.1371/journal.pone.0071030	2013	13
10.1371/journal.pone.0066665	2013	80
10.1111/aen.12008	2013	53
10.1371/journal.pone.0062484	2013	7
10.4039/tce.2012.111	2013	12
10.4039/tce.2013.4	2013	44
10.3390/d5010001	2013	13
10.1371/journal.pone.0054819	2013	44
NM	2013	10
NM	2013	0
10.2317/JKES121126.1	2013	9
10.1673/031.013.0401	2013	5
10.1007/s10329-012-0329-z	2013	33
10.1603/EC12085	2012	43
10.1590/S1519-69842012000500010	2012	5
10.1603/EC12179	2012	19
10.1016/j.landurbplan.2012.07.008	2012	47
10.4039/tce.2012.59	2012	3
10.4039/tce.2012.45	2012	2
10.3958/059.037.0205	2012	11
10.1603/EC11102	2012	2
10.1007/s10841-011-9420-9	2012	103
10.1111/j.1752-4598.2011.00154.x	2012	14

DOI	Year	Cited by
10.1080/10440046.2011.611586	2012	6
10.4039/tce.2012.6	2012	8
NM	2012	2
NM	2011	2
NM	2011	2
10.1111/j.1752-4598.2011.00131.x	2011	53
10.2317/JKES110319.1	2011	23
10.1111/j.1752-4598.2010.00123.x	2011	14
10.1371/journal.pone.0021079	2011	28
10.1016/j.biocon.2011.03.014	2011	90
10.1017/S0007485310000398	2011	19
10.1603/EN10229	2011	12
10.1080/03235400903145244	2011	1
10.1653/024.093.0418	2010	7
10.1111/j.1570-7458.2010.01063.x	2010	32
10.1016/j.ecoleng.2010.07.006	2010	13
10.1111/j.1365-2915.2010.00857.x	2010	16
10.1007/s10841-009-9246-x	2010	36
10.1603/EN09087	2010	9
10.1603/EC09360	2010	36
10.1111/j.1439-0418.2009.01464.x	2010	4
10.1111/j.1570-7458.2009.00922.x	2009	162
10.1603/022.038.0501	2009	7
NM	2009	2
10.1007/s10841-007-9130-5	2009	64
10.1007/BF03055288	2008	2
10.1672/08-83.1	2008	36
NM	2008	17
10.3157/0013-872X-119.4.361	2008	19
NM	2008	4
10.1603/0013-8746(2008)101[362:ADOATP]2.0.CO;2	2008	19
10.14411/eje.2008.116	2008	39
10.1590/s1676-06032008000400004	2008	23
10.1007/s10980-007-9153-z	2007	71
10.1007/s10841-006-9055-4	2007	189
10.3923/pjbs.2007.2562.2568	2007	7
10.23960/j.hptt.1710-20	2007	6
10.3923/pjbs.2007.682.691	2007	5
10.1007/978-1-4020-6059-5_35	2007	24
10.1016/j.biocon.2006.08.029	2007	114
10.1556/APhyt.41.2006.3-4.9	2006	10
10.3923/pjbs.2006.1839.1851	2006	25
NM	2006	11
10.3159/1095-5674(2006)133[403:LTFASP]2.0.CO;2	2006	5
10.1303/jjaez.2006.123	2006	15
10.3376/1081-1710(2006)31[152:VTFCAM]2.0.CO;2	2006	19

DOI	Year	Cited by
10.1007/s10886-006-9051-x	2006	132
10.1111/j.1744-7348.2006.00046.x	2006	52
NM	2006	15
10.22621/cfn.v120i1.239	2006	13
10.1303/jjaez.2005.99	2005	21
NM	2005	23
10.1111/j.1366-9516.2005.00199.x	2005	13
10.1093/ee/34.5.1325	2005	52
10.1603/0046-225X-34.4.857	2005	51
10.1111/j.0013-8703.2004.00208.x	2004	62
10.1111/j.1366-9516.2004.00104.x	2004	65
10.1023/B:BIOC.0000011722.44714.a4	2004	61
10.1111/j.1365-2915.2003.00460.x	2003	103
10.1046/j.1439-0418.2003.00737.x	2003	25
10.1603/0046-225X-32.6.1343	2003	31
10.1590/S1519-69842003000200021	2003	5
10.7202/007815ar	2003	4
NM	2002	4
NM	2002	55
10.1556/APhyt.36.2001.1-2.14	2001	6
10.1046/j.1461-9563.2001.00087.x	2001	56
10.1046/j.1461-9563.2001.00095.x	2001	82
10.1023/A:1006447813882	2000	36
NM	2000	14
10.1007/s002679910035	2000	29
10.1046/j.1365-2427.2000.00578.x	2000	103
10.1139/z99-254	2000	59
NM	1999	4
10.1046/j.1365-2311.1999.00196.x	1999	139
10.1080/096708799228076	1999	46
10.1023/A:1009602513263	1999	43
10.1577/1548-8659(1998)127<0876:EOCROI>2.0.CO;2	1998	31
10.1080/09583159830063	1998	108
10.1016/S0022-474X(97)00016-7	1997	5
10.4039/entm126169221-1	1994	32
10.1080/0021213X.1990.10677131	1990	156
10.1007/BF00377195	1989	6
10.1093/ee/17.5.872	1988	2
10.4039/Ent1191101-12	1987	21
10.1007/BF00299433	1985	101
10.3382/ps.0632368	1984	54
10.1111/j.1439-0418.1984.tb03784.x	1984	8
10.4039/Ent115847-7	1983	12
10.1303/aez.3.37	1968	12

Table S5.2 Arthropod taxa captured in pan trap arrays setup across three orchards in SWWA between the 29th and 31st of October 2021. Small arthropods less than 1mm in size were not identified due to the focal orchard species, *Persea americana*, typically requiring pollinators with larger body sizes (Ish-Am, 2005)

Orchard	Pan trap array distance interval	Arthropod taxa
A		
	10m	*Atherimorpha* sp. and unidentified insects < 1mm
	20m	*Apis mellifera,* Tenebrionidae sp., Micropezidae sp. and unidentified insects < 1mm
	30m	Tenebrionidae sp. and unidentified insects < 1mm
	40m	Tachinidae sp., Scarabaeidae sp. and unidentified insects < 1mm
	50m	*Homalictus dotatus,* Muscidae sp., Tabanidae sp., Leptophlebiidae sp. and unidentified insects < 1mm
B		
	10m	Cicadellidae sp. and unidentified insects < 1mm
	20m	*Apis mellifera, Lasioglossum* (*Parasphecodes*), Staphylinidae sp., Hemiptera (Auchenorrhyncha) and unidentified insects < 1mm
	30m	unidentified insects < 1mm
	40m	unidentified insects < 1mm
	50m	unidentified insects < 1mm
C		
	10m	*Apis mellifera* and unidentified insects < 1mm
	20m	*Apis mellifera* and unidentified insects < 1mm
	30m	*Pieris rapae*, Coelopidae sp. and unidentified insects < 1mm
	40m	*Apis mellifera,* Coelopidae sp. and unidentified insects < 1mm
	50m	*Apis mellifera,* Coelopidae sp. and unidentified insects < 1mm

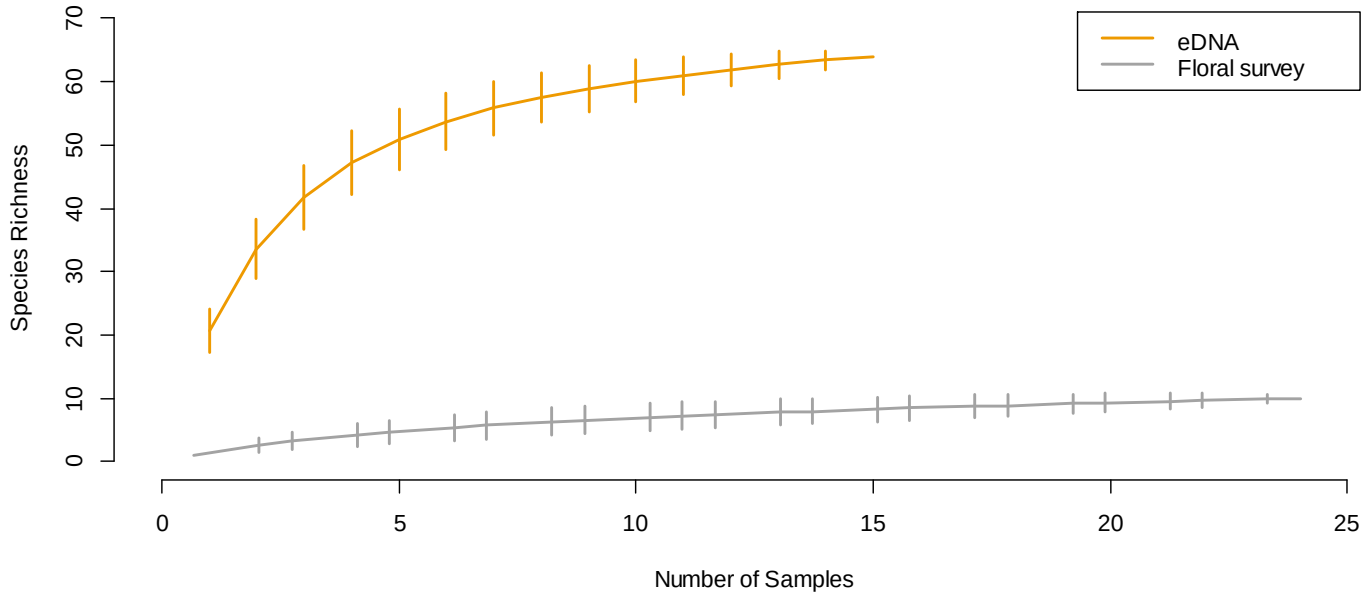


Figure S5.1 Rarefaction curves with standard error bars for plant species detected from eDNA metabarcoding of pan traps and conventional floral surveys. Curves were generated for both survey methods; eDNA (N = 15) and Floral survey (N = 24). Rarefaction curves were generated with 10,000 permutations and first order jack-knifing using the package 'vegan' in R.

Table S5.3 Floral survey results from 50 m transects in three *Persea americana* orchards (Orchard A – OA; Orchard B – OB; and Orchard C – OC) and adjacent pasture in SWWA, surveyed between the 29th and 31st of October 2021. Floral surveys were carried out following the methods of Fisher, Gonthier, Ennis, & Perfecto (2017). Only flowering species were recorded for the purposes of this study.

Date	Site	Flowering species	Average % cover
31/10/2021	OA - pasture	*Arctoteca calenolula*	0.005
31/10/2021	OA - pasture	*Raphanus raphanistrum*	0.025
31/10/2021	OA - pasture	*Trifolium subterraneum*	0.055
31/10/2021	OA - pasture	*Hordeum leporinum*	0.000
31/10/2021	OA - pasture	*Taraxacum officinale*	0.003
31/10/2021	OA - orchard	*Arctoteca calenolula*	0.040
31/10/2021	OA - orchard	*Raphanus raphanistrum*	0.075
31/10/2021	OA - orchard	*Trifolium subterraneum*	0.003
31/10/2021	OA - orchard	*Hordeum leporinum*	0.003
30/10/2021	OB - pasture	*Arctoteca calenolula*	0.040
30/10/2021	OB - pasture	*Trifolium subterraneum*	0.013
30/10/2021	OB - pasture	*Erodium moschatum*	0.003
30/10/2021	OB - pasture	*Taraxacum officinale*	0.003
30/10/2021	OB - orchard	*Raphanus raphanistrum*	0.013
29/10/2021	OC - pasture	*Cerastium glomeratum*	0.003
29/10/2021	OC - pasture	*Poa annua*	0.003
29/10/2021	OC - pasture	*Arctoteca calenolula*	0.003
29/10/2021	OC - pasture	*Trifolium repens*	0.175
29/10/2021	OC - pasture	*Trifolium subterraneum*	0.015
29/10/2021	OC - orchard	*Bromus catharticus*	0.055

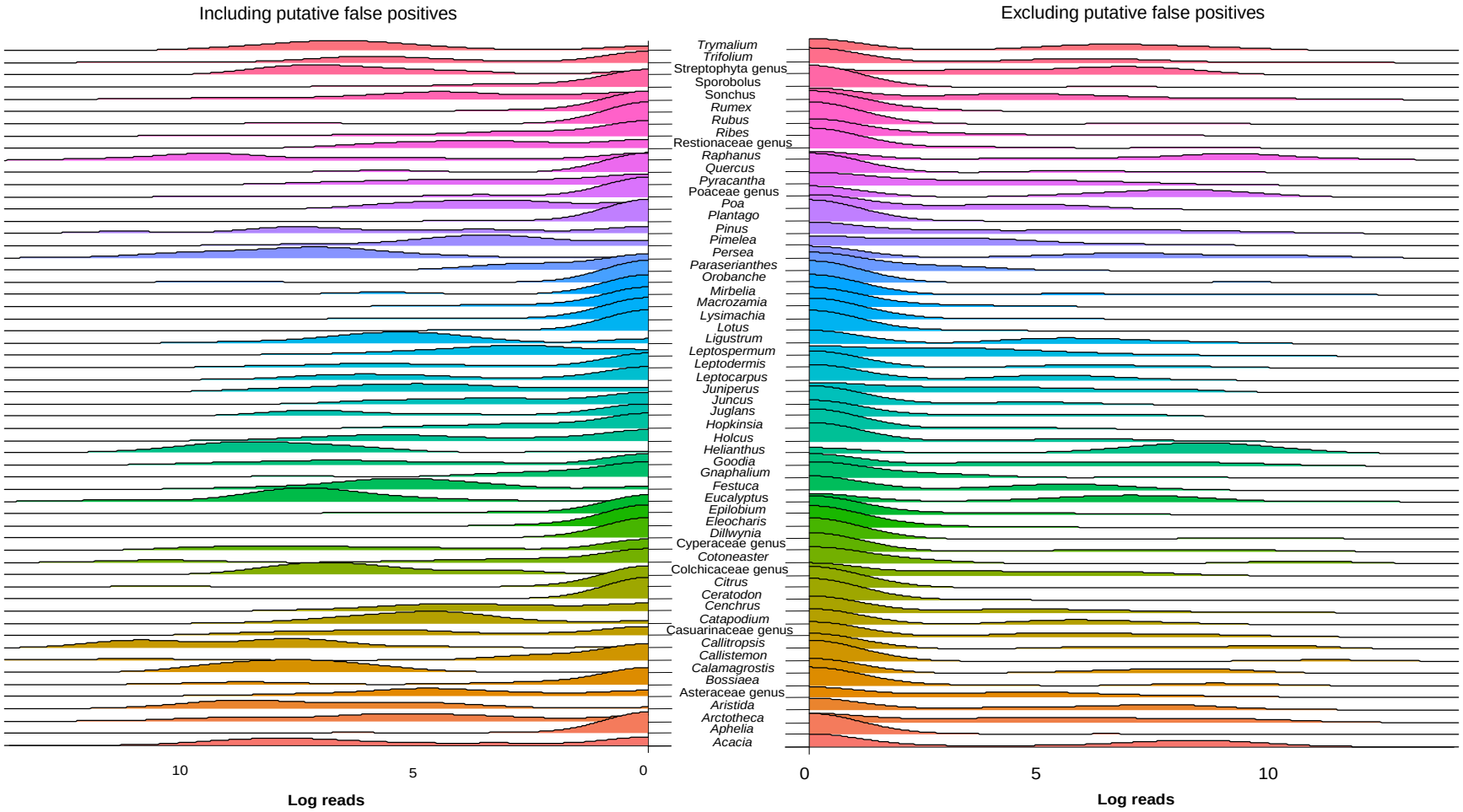


Figure S5.2 Stacked histogram for pan trap log reads per plant genus - including putative false positives (left) and excluding putative false positives removed (right), following Bell et al. (2017).

5.7.1 Supplementary references

Bell, K. L., Fowler, J., Burgess, K., Dobbs, E., Gruenewald, D., Lawley, B., … Brosi, B. J. (2017). Applying pollen DNA metabarcoding to the study of plant-pollinator interactions. *Applications in Plant Sciences*, *6*, 1600124. doi: 10.3732/apps.1600124

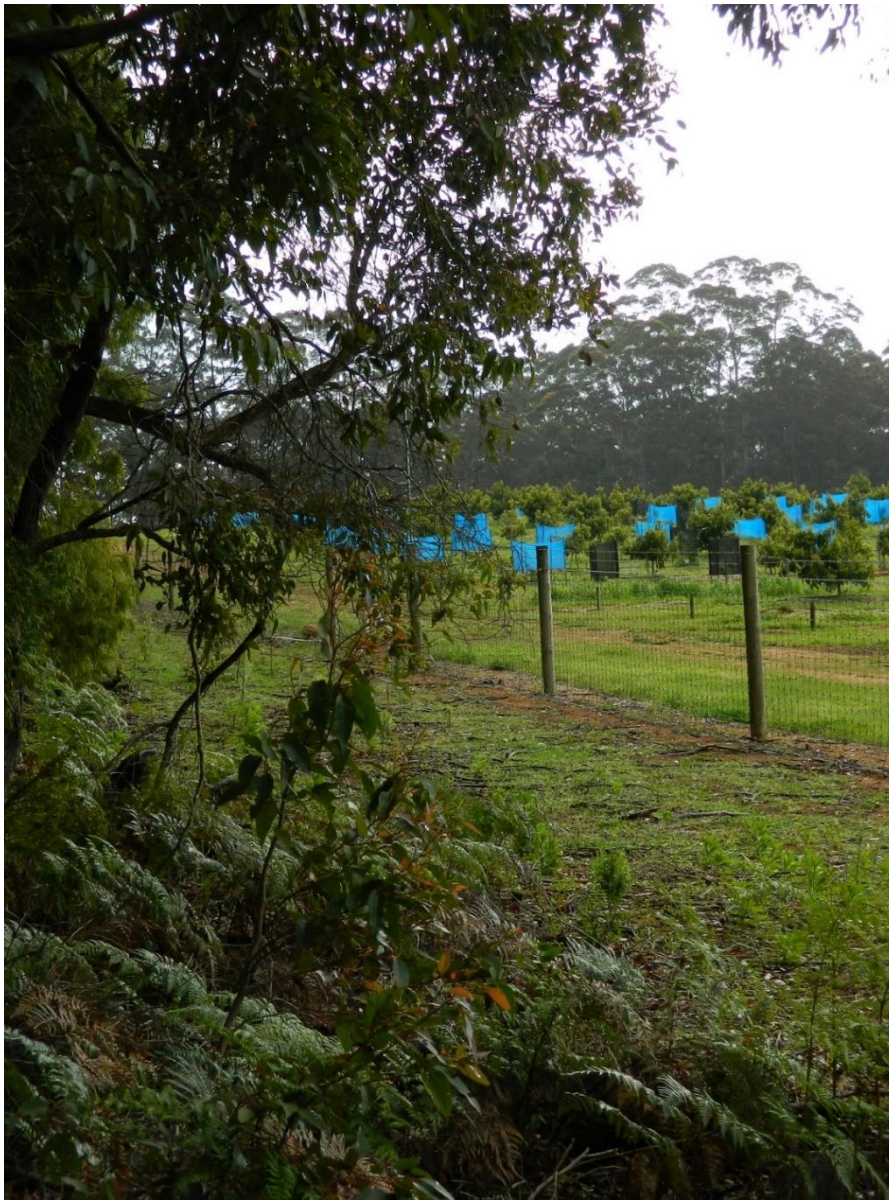
Fisher, K., Gonthier, D. J., Ennis, K. K., & Perfecto, I. (2017). Floral resource availability from groundcover promotes bee abundance in coffee agroecosystems. *Ecological Applications*, *27*(6), 1815–1826. doi: 10.1002/eap.1568

Ish-Am, G. (2005). Avocado pollination: A review. *New Zealand and Australia Avocado Grower's Conference '05*, 9. Tauranga, New Zealand: Avocado Source.

Chapter 6. Come Together: Adjacent natural vegetation affects arthropod community composition and alters arthropod-plant foraging diversity in ‘Hass’ avocado orchards

The study presented in this chapter is in review within the peer-reviewed literature.

Kestel, J. K., Bateman, P.W., Field, D.L., White, N.E., & Nevill, P. Come Together: How adjacent natural vegetation increases arthropod foraging diversity in ‘Hass’ avocado orchards, *Agriculture, Ecosystems & Environment*, (In preparation).



6.1 Abstract

Agricultural intensification has a significant and often negative impact on areas of natural vegetation, which may provide natural capital for local farms and orchards. Areas of natural and semi-natural vegetation in agroecosystems often support diverse arthropod communities, as well as the ecosystem services they deliver (e.g. pollination and predation), by providing consistent sources of nectar, pollen and nesting materials. However, not all arthropod taxa benefit equally from the presence of natural vegetation in agroecosystems, and for many arthropod taxa, their role within agroecosystems, and the plant taxa upon which they rely, are yet to be documented. Thus, the generalisations about which agricultural management practices support diverse arthropod communities and arthropod-plant foraging patterns remain on-going areas of research. To date, the conventional approaches used to survey arthropod communities and the foraging resources upon which they rely (e.g. sweep netting and microscopy) have been difficult to implement at the large scale of many agriculture enterprises. In this study, we aimed to examine if the presence of adjacent natural vegetation and crop flowering intensity affected arthropod community diversity by using pan traps to capture flying arthropods in six *Persea americana* orchards adjacent to either pasture habitat or natural vegetation. We complemented this approach with environmental DNA (eDNA) metabarcoding (trnL and rbcL) of pan-trap water, a novel method for targeting the pollen and plant tissue carried by arthropods, to determine if the presence of adjacent natural vegetation enhanced arthropod-plant foraging with different *P. americana* flowering intensities. In total, we collected more than 2,000 arthropod specimens, which represented 62 families and 141 predominantly wild species, including native bees, flies, beetles and butterflies. We found that the presence of natural vegetation adjacent to orchards only affected arthropod community composition and did not enhance species richness or abundance. However, within the adjacent habitats themselves, we found that arthropod species richness was positively correlated with habitat complexity and was significantly greater in natural vegetation compared to pasture habitats. We identified over 250 foraging plant taxa, including a wide variety of crop species, understorey weeds and native taxa using eDNA metabarcoding. Unlike the arthropods, the diversity of arthropod-plant foraging in orchards fluctuated depending on the type of adjacent habitat, as well as the level of *P. americana* flowering. Together, these findings indicate that adjacent natural vegetation may enhance the presence of particular arthropod taxa in orchards, while also providing reliable foraging resources when pasture habitats are grazed or when crops are not in flower. Overall, eDNA metabarcoding of pan-trap water provided a scalable method capable of evaluating how natural capital enhances arthropod foraging diversity in agroecosystems.

6.2 Introduction

Globally, nearly 10 billion people will require access to safe and nutritious food by 2050, and to achieve this, agricultural productivity will have to increase by 25 – 75% (Hunter, Smith, Schipanski, Atwood, & Mortensen, 2017). Agricultural intensification, the process whereby food production is increased with the expansion of farming systems and a corresponding increase in inputs (e.g. fertilisers, pesticides and herbicides), has been the overriding strategy, thus far, for increasing agricultural productivity and improving crop yields (FAO, 2020; Tscharntke et al., 2012). Unfortunately, agricultural intensification often occurs at the expense of natural capital, which comprises areas of both natural and semi-natural vegetation, as well as life-supporting ecosystem services and the organisms that mediate them (e.g. pollinators, decomposers and predators) (Han, Lavoir, Rodriguez-Saona, & Desneux, 2022; Monfreda, Wackernagel, & Deumling, 2004; Wagner, 2020). For instance, in Australia nearly 57% of all plant and animal taxa are directly affected by agricultural activities, the dominant land use for approximately half of Australia's landmass (Australian Bureau of Statistics, 2021; Kearney et al., 2019). The most vulnerable taxa to agricultural expansion are plant species, which are either directly lost through broad scale habitat clearing, or by livestock grazing (Beckmann et al., 2019; Cresswell, Janke, & Johnston, 2021). Plants are intrinsic to ecosystem functioning (see Pornon, Andalo, Burrus, & Escaravage, 2017; Sutter, Jeanneret, Bartual, Bocci, & Albrecht, 2017), and their losses are significant for arthropod taxa which rely upon these species for pollen, nectar, nesting materials, as well as the habitat they provide.

Arthropod-mediated pollination is critical for global agricultural productivity and has been valued between US$235 - $577 billion annually (Lautenbach, Seppelt, Liebscher, & Dormann, 2012; Rader et al., 2016). Similarly, arthropod predators, which remove pests and reduce pathogen transmission (see Furlong, 2015) have been estimated at annual worth of US$4.49 billion in the United States alone (Losey & Vaughan, 2006). Principally, these beneficial services are maintained and/or enhanced through retaining natural and semi-natural vegetation in agricultural ecosystems, which are externalities not typically accounted for in farm management (Allasino, Haedo, Lázaro, Torretta, & Marrero, 2023; Aviron, Berry, Leroy, Savary, & Alignier, 2023; Kremen, Albrecht, & Ponisio, 2019). In these areas, natural and semi-natural vegetation provide a dual function: initially, they support general ecosystem functioning (e.g. primary productivity, nutrient cycling and soil fertility), through maintaining biodiversity and the subsequent delivery of these services (Kremen et al., 2019; Vitousek & Hooper, 1994). Then, through the maintenance of this ecosystem functioning, they directly support the health and productivity of local farms and orchards (e.g. delivery of beneficial ecosystem services contributing to greater yields)

(Timberlake, Vaughan, & Memmott, 2019; Wratten, Gillespie, Decourtye, Mader, & Desneux, 2012). Preserving these unmanaged areas may benefit from adopting a valuation-based approach (e.g. an index or monetary amount; see Dardonville et al., 2022), whereby areas of natural or semi-natural vegetation are recognised as a source of natural capital, which contribute to the productivity and yields of local farms and orchards (Marais et al., 2019; Monfreda et al., 2004).

Crop flowering intensity can enhance the abundance and diversity of both beneficial (e.g. pollinators and predators) and antagonistic (e.g. crop pests) arthropod taxa, which may have significant implications for crop yields (Atakan & Uygur, 2005; Bezerra da Silva Santos, Frost, Samnegård, Saunders, & Rader, 2022; Gilpin et al., 2022). Further, these effects may be magnified by the presence of a reservoir for arthropod diversity, in the form of adjacent natural and semi-natural vegetation fragments (Chaperon et al., 2022; Klein et al., 2012). However, all arthropod taxa do not benefit equally from greater crop flowering or the presence of natural and semi-natural vegetation in agroecosystems (Hannon & Sisk, 2009; Wang et al., 2021; Willcox et al., 2019), and for many arthropod taxa, their role within agroecosystems, and the resources upon which they rely, are yet to be documented (Cresswell et al., 2021; Rader et al., 2016). For instance, in Australia there are an estimated 320,465 arthropod species, of which only 35% have been described (Cresswell et al., 2021). Thus, the generalisations about which circumstances (e.g. presence or absence of unmanaged areas) support diverse arthropod-plant foraging and arthropod community diversity, and the documentation of preferences for unmanaged wild taxa, which have historically been omitted from agricultural studies (see Rader et al., 2016), remain an area of on-going research. It has proven challenging however, to measure these arthropod-plant interactions with conventional methods at the large scale of agriculture.

Arthropod foraging preferences have conventionally been assessed using active (e.g. sweep netting), passive (e.g. pan traps) and observation-based (e.g. visual observations of flower visits) methods, followed by swabbing and visual microscopy-based morphological identification of pollen (Ladd, Yates, Dillon, & Palmer, 2019; Macgregor et al., 2019; Smart et al., 2017). Though these approaches have proven useful for detecting fine-scale arthropod foraging patterns (e.g. Bowie, Gurr, Hossain, Baggen, & Frampton, 1999; Silberbauer et al., 2004), they are often restricted by; 1) the lack of morphological characters for species-level identification (Hawkins et al., 2015); 2) availability of specialist expertise (Khansari et al., 2012), and 3) significant time-investments with large-scale applications, like those in agriculture (Bell et al., 2016). These circumstances have necessitated the development of new high-throughput methods to reliably identify pollen grains and provide greater taxonomic depth for arthropod foraging preferences.

Environmental DNA (eDNA) metabarcoding is a molecular method that harnesses high-throughput sequencing to amplify trace quantities of DNA from substrates ranging from soil to air (Clare et al., 2021; Kestel et al., 2023; van der Heyde et al., 2020). Taxonomically informative 'barcode' regions are then amplified using various assays, in a process known as metabarcoding. To date, arthropod-plant interactions have been detected using eDNA metabarcoding of flowers to detect potential pollinators, pests and predators (Gomez, Sørensen, Chua, & Sigsgaard, 2023; Kestel et al., 2023; Newton et al., 2023), pollen swabs from individual arthropods to detect foraging resources (Macgregor et al., 2019; Pornon et al., 2017) and crop surfaces to detect herbivory (Kudoh, Minamoto, & Yamamoto, 2020; Valentin, Fonseca, Nielsen, Leskey, & Lockwood, 2018). Recently, Kestel et al. (2024) combined eDNA metabarcoding with pan traps to passively sample arthropods and the plants upon which they forage in orchards. In that study, eDNA metabarcoding of pan-trap water presumed to contain pollen, identified 60 foraging plant species, including crops, weeds and native species. Given the need to monitor arthropod communities and the plant taxa upon which they rely in agroecosystems (see Kestel et al., 2022), we used eDNA metabarcoding of pan traps to evaluate if the presence of natural vegetation affected arthropod community composition and enhanced arthropod-plant foraging diversity in orchards.

In the present study, we used pan traps and an emerging survey method in agricultural systems – eDNA metabarcoding – to examine the effect of adjacent vegetation type (natural vegetation and exotic pasture species), and flowering intensity of an orchard tree species, on the richness, abundance and community composition of arthropods, and the diversity of foraging resources they use. We hypothesised that 1) the presence of adjacent natural vegetation would enhance arthropod abundance and diversity in orchards, 2) the presence of adjacent natural vegetation would increase the diversity of plant resources utilised for orchard arthropods, and 3) adjacent natural vegetation would support greater arthropod diversity and abundance compared to the pasture habitat.

6.3 Methods

6.3.1 Study orchards and sample collection

Six *Persea americana* (‘Hass’ avocado) orchards located in the Manjimup – Pemberton region of south west Western Australia (SWWA) were chosen for this study. The orchards were separated by an average distance of 15.55 km, the greatest distance between orchards was 28.24 km and the least distance was 7.19 km (Orchard MB - 34°18′52 S, 116°08′36 E; Orchard BA - 34°25'30 S, 116°01'23 E; and Orchard BD - 34°26'28 S, 115°54'02 E, Orchard SD - 34°22′55 S, 115°57′47 E, Orchard PB 34°22′29 S, 116°12′00 E, Orchard DC 34°18′19 S, 116°03′10 E). *Persea americana* trees in each study orchard were similar in age (2 – 7 years). Three of the sample orchards were situated ca. 1 km away from natural vegetation and situated in area dominated by exotic groundcover species *Arctotheca calendula* and *Trifolium subterraneum* (Figure 6.1A). While the remaining three sample orchards were situated ca. 1 km away from pasture habitats in areas dominated by secondary growth Eucalypt forest, where *Eucalyptus diversicolor* was the most common species (Figure 6.1B).

Figure 6.1 *Persea americana* orchards (*N* = 6) were sampled in the Pemberton-Manjimup adjacent to either (A) pasture or natural vegetation (B), (*N* = 3 for both adjacent habitats). Orchards and the adjacent pasture or natural vegetation were sampled over four periods, each of which corresponded with a different flowering phase for *P. americana*. Bottom row, left to right: sampling period A (10th – 11th of October; pre-flowering), sampling period B (29th – 31st of October; low flowering), sampling period C (27th – 29th November; moderate flowering), and sampling period D (11th – 13th December; post-flowering).

To survey arthropods and the plants they forage upon in each orchard and adjacent habitat, five pan trap arrays were deployed at 10 m intervals along a 50 m transect, located 50 m into the orchard and 50 m into the adjacent habitat. Each orchard and adjacent habitat were sampled over four periods between the 10th of October and the 13th of December 2021. The sampling periods corresponded with different flowering phases for *P. americana* – sampling period A (10th – 11th of October; pre-flowering), sampling period B (29th – 31st of October; low flowering), sampling period C (27th – 29th November; moderate flowering), and sampling period D (11th – 13th December; post-flowering) (Figure 6.1). Pan traps were deployed when temperatures were warm enough for bee activity (clear skies, wind speed <30 km/h, and temperatures >15°C ; Prendergast et al., 2020). In each pan trap array, three coloured bowls (white, yellow and blue) were deployed to capture the broadest range of arthropod colour preference (Abrahamczyk, Steudel, & Kessler, 2010; Cane, Minckley, & Kervin, 2000; Saunders, Luck, & Mayfield, 2013). Each array was placed at a height of 1.2 m above the ground to match the height of the *P. americana* trees in the study orchards. All of the pan traps were filled with ~ 200 ml of sterile MilliQ water, and one drop of detergent to disrupt the surface tension (Campbell, Melles, Vaz, Parker, & Burgess, 2018; Gervais, Chagnon, & Fournier, 2018). After eight hours, each pan trap was stirred using sterilised tweezers (washed in 10% bleach solution and placed under UV light for 15 min prior to use), and 50 ml of liquid (presumed to contain pollen) was subsampled into a labelled 50 ml falcon tube (one tube per white, blue and yellow pan trap; 150 ml collected per pan trap array). Any arthropods captured in the pan traps were transferred into the same 50 ml of collected water using sterilised tweezers. The falcon tubes were then placed on ice until they could be transferred to a -20°C freezer at Curtin University. At Curtin University, falcon tubes and their contents were defrosted and each arthropod specimen was subsequently transferred into a labelled 2 ml Eppendorf tube containing 99% ethanol. Arthropods were identified morphologically by two entomologists, Dr Terry Huston at the West Australia Museum and Christopher Swinstead at Curtin University, to provide taxonomic identifications to species-level, where possible. Where identifications to the species-level were not possible, specimens were assigned a morphotype number at the family level, method adapted from D'Souza et al. (2021).

Potential contamination from airborne pollen and plant material was minimised with the use of marble traps to capture ambient DNA (method following Kestel et al., 2024). Marble traps were made following Reheis & Kihl (1995) and placed in each orchard at 1.2 m above the ground to match the height of the pan trap arrays. Marble traps were deployed at the same times as the pan traps and collected after eight hours. During collection, the marble traps were wrapped in Clingfilm

and stored at room temperature (below 25°C) until they could be transferred to a -20°C freezer at Curtin University.

6.3.2 Sample processing and DNA extraction

Pan-trap water samples (*N* = 120) were filtered using two Sentino peristaltic microbiology pumps (Pall Life Sciences, New York, USA), through sterile 47 mm cellulose filters with a pore size of 0.22 µm (Pall Life Sciences, New York, USA). Filters were transferred into sterile zip-lock bags and placed in a - 20°C freezer until processed for DNA extraction. For the marble traps (*N* = 24), 1000 ml of MilliQ water was added (in 2x 500 ml batches) to the traps and agitated periodically for 5 min (10 min total); before being filtered through sterile 47 mm cellulose filters with a pore size of 0.22 µm (Pall Life Sciences, New York, USA). The marble trap filter papers were then transferred into sterile zip-lock bags and frozen at - 20°C until further processing.

For DNA extraction, each filter was halved, with one half placed back into the - 20°C freezer and the other finely chopped with sterilised scissors (pre-washed with 10 % bleach solution) and placed into a labelled 2 ml tube. Pollen DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen; Qiagen Inc., Valencia, CA, USA), hereafter referred to as the Blood and Tissue kit, which has previously been validated for detecting pollen from pan-trap water (Kestel et al., 2024). The Blood and Tissue kit extractions were followed as per the kit instructions with a modified protocol (see West et al., 2020). The following modifications were used: a total of 540 µl ATL Buffer and 60 µl Proteinase K (Qiagen; Qiagen Inc., Valencia, CA, USA) were added to the tube containing half of the finely chopped membrane to ensure that the filter membranes were adequately exposed to the lysis solution, to optimise DNA yield. The combined filter and lysis solution (600 µl) were incubated overnight at 56°C in a slow rotating hybridization oven. The following morning, 400 µl of the DNA digest supernatant was then transferred to a labelled 2 ml tube and loaded into an automated sample preparation system for DNA extraction (QIAcube; Qiagen Inc., Valencia, CA, USA). The final DNA extracts were eluted from the silica column in 100 µl AE buffer and frozen until further assessment. DNA was extracted from marble trap samples (*N* = 24), DNA extraction controls (*N* = 12) and wash water controls (*N* = 11) using the Blood and Tissue kit (Qiagen; Qiagen Inc., Valencia, CA, USA), with the same modifications as stated above.

The quality and quantity of DNA extracted from each membrane for each extraction method described above was measured by quantitative PCR (qPCR), using two assays targeting the Ribulose-1,5-bisphosphate carboxylase-oxygenase (rbcL) region and the *trnL* (UAA) intron region (trnL) (see below) in plants. Amplicons for each assay were ca. 140 bp and ca. 250 bp for

rbcL and trnL, respectively. Low copy number and PCR inhibitors from eDNA extracts can impact metabarcoding data (Murray, Coghlan, & Bunce, 2015), therefore each eDNA extract was assessed with a qPCR dilution series (neat, 1:10, 1:100) based on the following conditions, 25 µl reaction volumes consisting of 2 µl of 2 mM $MgCl_2$ (Fisher Biotec, Australia), 2.5 µl of 10 × PCR Gold Buffer (Life Technologies, Massachusetts, USA), 0.25 µl of dNTPs (Astral Scientific, Australia), 1 µl of 0.4 mg/ml BSA (Fisher Biotec, Australia), 0.4 µl of each primer (10 mM); (rbcLh2aF: 5'—GGCAGCATTCCGAGTAACTCCTC—3': rbclh2aR: 5'—CGTCCTTTGTAACGATCAAG—3'; Poinar et al., 1998), (trnL_c: 5'—CGAAATCGGTAGACGCTACG—3': trnL_h: 5'—CCATTGAGTCTCTGCACCTATC—3'; Taberlet et al., 2007; Taberlet, Gielly, Pautou, & Bouvet, 1991), 0.2 µl of AmpliTaq Gold (Life Technologies), 0.6 µl of 5x SYBR Green (Life Technologies), 2 µl of template eDNA (3x qPCR reactions per eDNA extract) and the remaining volume supplemented with DNase/RNase-Free Distilled Water. The cycling conditions for rbcL and trnL were: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 2 min. For rbcL and trnL, 15 samples were identified to contain PCR inhibition and eight of these samples were carried forward through the metabarcoding workflow using a 1:10 dilution of the extract, while the remaining seven were carried forward using a 1:100 dilution of the extract.

Environmental DNA samples that were of sufficient quality, as determined by the initial qPCR screen (qPCR dilution series), were fusion tagged with a unique 7 – 9 bp multiplex identifier tag (MID-tag) for the trnL and rbcL primer sets. To reduce the likelihood of cross-contamination, chimera production, and MID-tag jumping (Esling, Lejzerowicz, & Pawlowski, 2015), DNA amplification was performed in a single round of amplification using MID-tag primers consisting of the Illumina flow cell adaptors (P5 and P7), a custom sequencing primer binding site, the unique MID-tag combination and the gene specific primer, either rbcL or trnL. All PCR reactions were prepared in a physically separate ultra-clean laboratory at Curtin University designed for ancient DNA work using an automated QIAgility robotics platform (Qiagen Inc., Valencia, CA, USA) and were carried out in duplicate reactions of 25 µl which contained 2.5 µl of 10 × PCR Gold Buffer (Life Technologies), 2 µl of 2.5 mM $MgCl_2$ (Fisher Biotec), 1 µl of 0.4 mg/ml BSA (Fisher Biotec), 0.25 µl of dNTPs (Astral Scientific), 0.5 µL of either forward primer (20 mM), 5 µL of either reverse primer (2 mM), 0.25 µL AmpliTaq Gold (Life Technologies), 2 - 6 µl DNA and the remaining volume supplemented with DNase/RNase-Free Distilled Water. MID-tag PCR amplicons were carried out in duplicate reactions to control for PCR stochasticity and all fusion-tagged qPCRs were processed using the same parameters as the initial qPCR screens described

above. Technical replicate MID-tagged amplicons were pooled at approximately equimolar concentrations (e.g. minipool) based on their respective qPCR DRn values (West et al., 2020). The minipool concentrations were measured using a high-resolution capillary electrophoresis system (QIAxcel; Qiagen Inc., Valencia, CA, USA) and the final library was blended equimolar ratio based on the minipool concentrations and size selected (180 – 350 bp) using a PippinPrep (Millennium Science Pty Ltd., Australia) to remove any off-target amplicons and primer dimer. The size selected final library was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Australia) prior to high-throughput sequencing (HTS). Sequencing by synthesis was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) located in the Trace and Environmental DNA labortory at Curtin University and as per Illumina's protocol for single-end sequencing with a 300 cycle V2 reagent kit and standard flow cell for environmental metabarcoding.

6.3.3 Data processing

Sequenced multiplex identifier-tagged amplicons were input to a containerised workflow and run through the Pawsey Supercomputing Centre in Kensington, Western Australia. Here, the sequences were filtered, formed into Zero-radius Operational Taxonomic Units (ZOTUs) and assigned taxonomic identifications. Sequences were quality checked using FASTQC (Andrews, 2010) and quality filtered (Phred quality score < 20), before the multiplex identifiers were trimmed from the sequence reads using AdapterRemoval v2 (Schubert, Lindgreen, & Orlando, 2016). Subsequently, the filtered reads were demultiplexed using OBITOOLS (Boyer et al., 2016) and sequences shorter than the minimum length of 90 bp were filtered out. Sequences were then dereplicated into ZOTUs with a minimum sequence abundance of 5 (Drake et al., 2022) using the USEARCH Unoise3 algorithm (Edgar, 2016). A database of ZOTUs was then generated and queried against the GenBank (NCBI) nucleotide database and a custom reference database (see section *6.3.4 Avocado flowering and co-flowering floral surveys*) with 100% query coverage and 97% identity using BLASTN (Altschul, Gish, Miller, Myers, & Lipman, 1990). Erroneous ZOTUs with a sequence similarity below 97% were removed using the LULU post clustering curation method (Frøslev et al., 2017). Finally, a custom Python script (eDNAFlow; Mousavi-Derazmahalleh et al., 2021) was used to assign taxonomic identifications to the curated ZOTUs using the Lowest Common Ancestor (LCA) approach. Taxonomic identification was assigned to a ZOTU when the percentage identity of two or more queried sequences with ≤ 0.5% difference had 99% query coverage and 95% sequence similarity. Taxonomic identifications for plants below 97% identity were retained at the family level. For the purposes of this study, we set the minimum threshold count of 10 reads for plant ZOTUs (see Drake et al., 2022) to classify a taxon as present

within a sample. All plant taxa detected using eDNA metabarcoding were compared with historical records using the Atlas of Living Australia (https://www.ala.org.au/), a collaborative, digital, open infrastructure that pulls together Australian biodiversity data from multiple sources, to confirm that they occurred within the study region.

6.3.4 Avocado flowering and co-flowering floral surveys

Arthropod community richness and abundance may fluctuate in relation to both crop flowering and the flowering of co-occurring weed and native species (Gilpin et al., 2022; Kline & Joshi, 2020; Saunders et al., 2013). The percentage of *P. americana* flowering was quantified during each sampling period by counting the ratio of open to closed florets for nine 'Hass' trees along each 50 m orchard transect, as per Howlett et al. (2018). These nine trees per orchard were selected randomly in each sampling period to account for the different development of inflorescences for each tree, method adapted as per Carabalí-Banguero, Montoya-Lerma, & Carabalí (2021). To capture the influence of co-flower species, we quantified the diversity and percentage cover of co-flowering weeds (orchard understorey and adjacent pasture) and native species (adjacent natural vegetation) with observation-based floral surveys. On the same day as pan traps were deployed, floral surveys were carried out following Fisher et al. (2017). Here, four 1 m^2 quadrats were deployed along the 50 m pan trap transect in each orchard and along a parallel 50 m transect in the adjacent pasture or natural vegetation, located 100 m away from the orchard transect. Canopy cover (%), a proxy for habitat complexity (see Lassau et al., 2005), was measured four times along each adjacent pasture and natural vegetation transect using a spherical convex desiometer. Within each quadrat, flowering species were morphologically identified, percentage flower cover calculated and a field herbarium created with leaf material collected from each species present. Field identifications of flowering species were confirmed using relevant taxonomic keys and reference materials at the Western Australia Herbarium. Once identified, these were cross-referenced with GenBank to obtain reference barcode sequences. Any plant species that were not available on the online reference databases for the rbcL or trnL assays were extracted using the Plant Pro kit (Qiagen; Qiagen Inc., Valencia, CA, USA) as per the kit instructions.

The quality and quantity of DNA extracted from each plant species was measured by qPCR using two assays targeting the entire *rbcL* gene region (rbcL_F1 and rbcL_R634; Fazekas et al., 2008; Kress & Erickson, 2007) and the whole chloroplast *trn*L (UAA) intron region (trnL_C and trnL_D; Taberlet et al., 1991). Each DNA extracted plant species was assessed in duplicates based on the following conditions, 25 µl reaction volumes consisting of: 2.5 µl of 10 × PCR Gold Buffer (Life Technologies), 2 µl of 2 mM $MgCl_2$ (Fisher Biotec), 0.25 µl of dNTPs (Astral Scientific), 1 µl of

0.4 mg/ml BSA (Fisher Biotec) 1 µl of each primer (forward and reverse; 10 mM), 0.2 µl of AmpliTaq Gold (Life Technologies), 0.6 µl 5x SYBR Green (Life Technologies), 2 – 4 µl of template eDNA and the remaining volume supplemented with DNase/RNase-Free Distilled Water. The cycling conditions for the *rbcL,* gene were initial denaturation at 95°C for 2.5 min, followed by 35 cycles of 95°C for 30s, 48°C for 30s, 72°C for 45s, and a final extension at 72°C for 10 min. Whereas for the *trn*L intro region, the cycling conditions were initial denaturation at 96°C for 5 min, followed by 35 cycles of 96°C for 1 min, 48.5°C for 30s, 72°C for 45s, and a final extension at 72°C for 10 min. Amplicons of sufficient quality, as determined by gel electrophoresis, were then taken through to Sanger sequencing by the Australian Genome Research Facility. In total, 11 plant species were sequenced for both *rbcL* and *trn*L, nine species were sequenced for *trn*L and two species were sequenced for *rbc*L, making a custom reference database of 22 sequences (S6.7.1). All sequences were aligned and edited on Geneious (Kearse et al., 2012).

6.3.5 Statistical analysis

All statistical analyses were performed on R 4.3.1 (R Core Team 2023). To assess if the presence of adjacent natural vegetation supported greater orchard arthropod diversity and abundance, we generated estimates of alpha diversity and tested if *P. americana* flowering, weed species co-flowering, maximum daily temperature and adjacent habitat were significantly correlated using Spearman's correlation analysis. The estimates of alpha diversity generated were; Shannon's H' Index, species richness and Pielou's Eveness, a measure that reflects how evenly distributed the abundances of all species are at each site (Pielou, 1966). These metrics were estimated using the 'vegan' package (Oksanen et al., 2019). Similarly, to establish of arthropod diversity and abundance was greater within adjacent pasture or natural vegetation habitats, we generated the estimates of alpha diversity mentioned above for arthropods captured in adjacent pasture and natural vegetation habitats and tested if: habitat type (canopy cover %), adjacent habitat flowering or maximum daily temperature were significantly correlated with arthropod alpha diversity.

To examine the effect of adjacent vegetation type on arthropod assemblages, arthropod community assemblages captured in orchards adjacent to either natural vegetation or exotic pasture species were visualised using a Principal Component Analysis (PCoA). Subsequently, we performed PERMANOVA analyses based on Bray-Curtis distances with 9999 permutations to assess if adjacent habitat and sample period explained a significant amount of variation in the orchard pan trap dataset. Once confirmed, these significant explanatory variables were then included in a PCoA based on Bray-Curtis dissimilarity distances (pan trap arthropod communities) to visually represent the difference between orchards adjacent to pasture or natural vegetation across the four

sample periods. Significant species contributing to the most variation in community composition were identified using a SIMPER analysis (Clarke, 1993). To establish if arthropod diversity and abundance was greater within adjacent pasture or natural vegetation habitats, we repeated the above PERMANOVA analyses and PCoA ordination for arthropod communities captured in the adjacent pasture and natural vegetation, including both sampling period and habitat type (pasture or natural vegetation) as significant explanatory variables.

We then used Generalised Linear Mixed Effect Models (GLMMs) with either Poisson or Negative Binomial distributions for the six main arthropod orders captured: all arthropod species (All), Diptera, Hymenoptera (excluding *Apis mellifera*), Coleoptera, Lepidoptera and Other. Arthropods were grouped by order based on ecological significance. Diptera, Hymenoptera and Lepidoptera represented potential unmanaged pollinators for *P. americana* (see Carabalí-Banguero et al., 2021; Cook et al., 2020; Dymond et al., 2021), whilst Coleoptera represented potential beneficial predators and antagonistic herbivores (see Furlong, 2015). The taxonomic composition of these cohorts are detailed in Table S6.1. A GLMM analysis approach was chosen to account for the non-normal distribution and overdispersion in the species richness and abundance data, as well as the non-independence associated with sampling the same six orchards across four time points. Thus, to avoid the potential effects of spatial autocorrelation, we considered sample orchard as random factor nested within adjacent habitat in all GLMMs, we also included sampling period and adjacent habitat as fixed factors. Model analysis was undertaken using the package 'lme4' (Bates, Mächler, Bolker, & Walker, 2015), and the significance of fixed factors were assessed using a Type II Wald χ^2 test in the 'car' package (Fox & Weisberg, 2019). When factors and factor interactions were significant, we used the 'lsmeans' package (Lenth, 2016) to undertake multiple pairwise comparisons using Bonferroni adjustments to assess for differences between the groups. To establish if arthropod diversity and abundance was greater within adjacent pasture or natural vegetation habitats, we repeated the above GLMM analyses with the six main arthropod orders captured (see Table S6.1) and the same random and fixed factors.

In the eDNA dataset, marble and pan trap array samples with ZOTUs with 10 or more reads found in the negative controls were removed. Subsequently, plant sequence counts for genera detected in the marble traps were used as Minimum Sequence Copy Thresholds (MSCTs) for those same taxa detected in the pan trap arrays (Bell et al., 2017; Drake et al., 2022; Kestel et al., 2024). We included this filtering procedure to reduce inflated read counts and the presence of false positives (see Ficetola, Taberlet, & Coissac, 2016), which could arise from contaminant ambient pollen and

plant material (Johnson, Cox, & Barnes, 2019). Following filtering with marble traps MSCTs, the pollen read counts in the pan trap arrays were converted to presence-absence data.

To determine if the presence of natural vegetation supported greater foraging plant diversity for orchard arthropods, Non-metric multidimensional scaling (NMDS) ordination with a Jaccard dissimilarity matrix, undertaken using the package 'Vegan' in R, was used to visualise the similarity in foraging plant community composition between sampling periods and orchard adjacent habitats (pasture or natural vegetation). Following the ordination, an analysis of similarity (ANOSIM) with 9999 permutations, also with a Jaccard dissimilarity matrix, was used to test if the plant foraging communities differed significantly between sampling periods and orchard adjacent habitats. Afterwards, two linear models were generated, the first for Chao2 alpha diversity per pan trap array and the second for the total plant genera detected per pan trap array. For both of these linear models, sampling period and adjacent habitat were included as fixed factors, with an interaction effect also included between sampling period and adjacent habitat. The Chao2 index, which generates richness based on presence-absence data (see Chao, 1984), was calculated at the genera level using the package 'fossil' in R. We then generated the least-squares means for each fixed factor and the interaction between them using the package 'emmeans' in R (Lenth, 2021). The 'bipartite' package in R (Dormann, Frund, Bluthgen, & Gruber, 2009) was then used to visualise foraging plant diversity networks for arthropods captured within orchards adjacent to either pasture or natural vegetation (method adapted from Padrón et al., 2021). Plants were retained at the family level due to the large number of taxa detected. Bipartite plots were generated for each sampling period, with relative abundance measures for each plant family included. Measures of network complexity (linkage density) and species shared between the two orchard adjacent habitats were generated using the 'bipartite' package in R.

6.4 Results

6.4.1 Arthropod taxonomic diversity

Overall, 2,078 arthropod specimens representing 62 families and 141 species were collected from the pan trap arrays deployed in the six study orchards and in the adjacent natural vegetation and pasture habitats. The most commonly detected families across the four sampling periods were: Muscidae (*Musca domestica* and Muscidae spp.: 23% of detections), Tachinidae (Tachinidae spp.: 17% of detections), Halictidae (*Homalictus urbanus*, *H. dotatus*, *Lasioglossum castor*, *L.* (*Chilalictus*), *L. erythrurum*, *L. lanarium* and *L.* (*Parasphecodes*): 12% of detections) and Apidae (*Amegilla chlorocyanea*, *Apis mellifera* and *Exoneurella* sp.: 10% of pan trap detections). Per pan trap array, we captured an average of nine (± 0.81 SE) individuals, representing three (± 0.12 SE) families and three (± 0.14 SE) species.

6.4.2 Arthropod community composition significantly alters irrespective of adjacent habitat

When visualised in the PCoA, arthropods captured in the pan traps showed a stronger separation along the first axis in comparison to the second, suggesting that more variance was explained by where the samples were collected (orchards or adjacent habitat), than when they were sampled (sampling periods A – D) (Figure 6.2A). Within orchards, arthropod detections also showed a stronger separation along the first axis when compared to the second, indicating greater variance was explained by adjacent habitat, rather than sampling period (Figure 6.2B). Similarly, arthropod detections in the adjacent pasture and natural vegetation also showed a stronger separation along the first axis compared to the second, potentially highlighting that habitat type explained more variance than sampling period (Figure 6.2C). These findings were confirmed with a PERMANOVA analysis which showed that for arthropod communities captured in orchards, both adjacent habitat type ($DF = 1$, $p < .01$) and sampling period ($DF = 3$, $p < .01$) were significant. While for arthropod communities captured in adjacent pasture or natural vegetation, both habitat type ($DF = 1$, $p < .01$) and sampling period ($DF = 1$, $p < .01$) were significant explanatory co-variates.

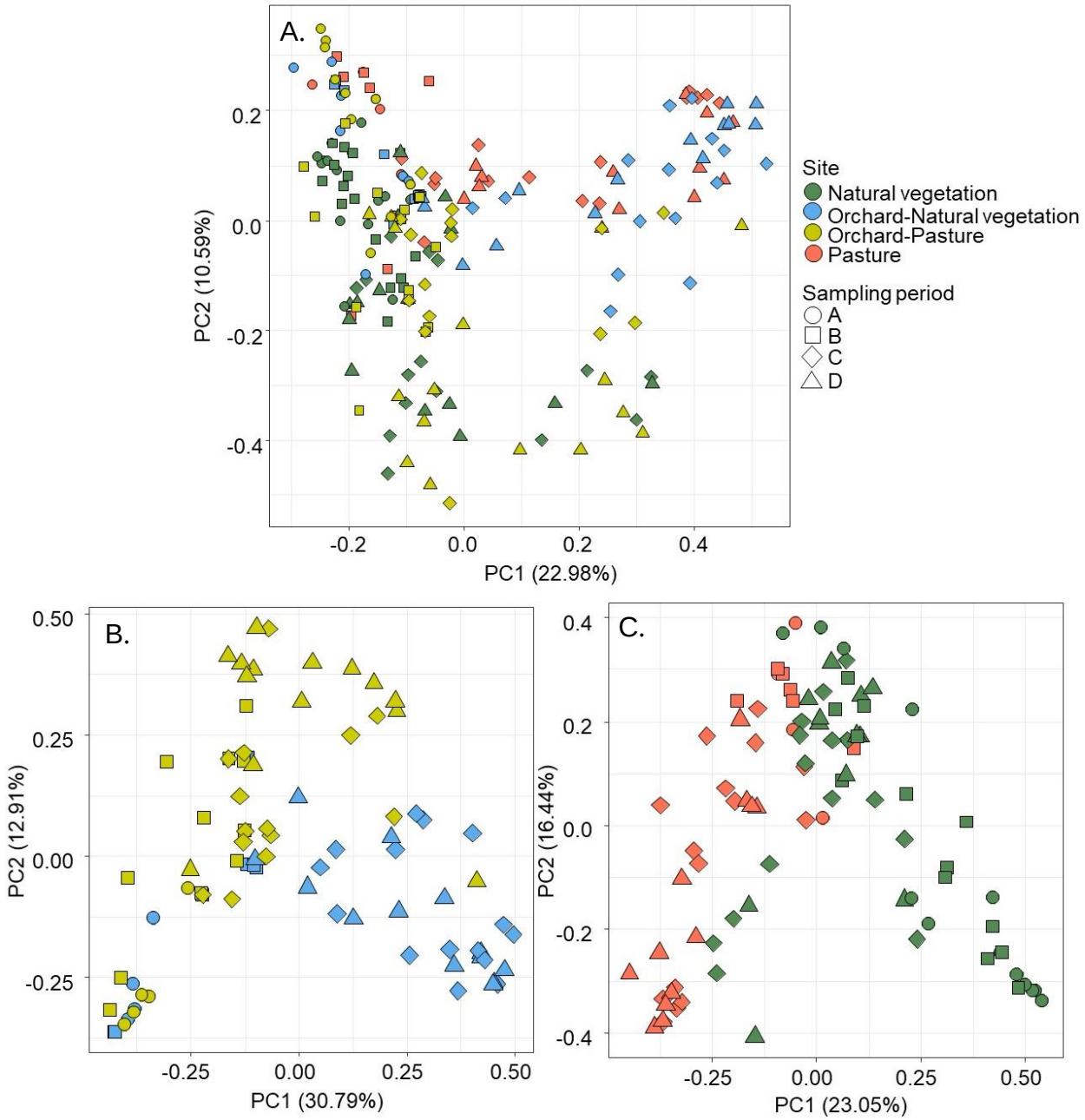


Figure 6.2 A. PCoA plot produced from the arthropod species community captured in pan traps in: native vegetation (*N* = 3), orchards adjacent to native vegetation (*N* = 3), pasture (*N* = 3) and orchards adjacent to pasture (*N* = 3). B. PCoA plot generated from the species community structure of pan trap samples collected in the six orchards adjacent to pasture (*N* = 3) and natural vegetation (*N* = 3). C. PCoA plot generated from the species community showing the grouping of pan trap samples in adjacent pasture and natural vegetation. Orchard and adjacent habitat pan trap samples were collected over four sampling points (A; 10th – 11th of October, B; 29th – 31st of October, C; 27th – 29th November; and D; 11th – 13th December).

The alpha diversity of arthropods captured in orchard pan trap samples showed no correlations with flowering resource availability within orchards or adjacent habitat. The alpha diversity of arthropods (Shannon's H' index) was not correlated with *P. americana* flowering (Spearman's rho = -0.02, p = .80) nor the flowering of co-occurring understorey weed species (Spearman's rho = 0.07, p = .41). Neither the presence of adjacent pasture nor natural vegetation were correlated with arthropod alpha diversity (Spearman's rho = -0.15, p = .09). These findings were confirmed with non-parametric testing, which showed no significant differences for alpha diversity (H'; p = .09), species richness (p = .21), or evenness (Pielou's Eveness; p = .07) between orchards adjacent to pasture or natural vegetation. The only significant correlations were found for maximum daily temperature, which showed a positive correlation with arthropod alpha diversity (Spearman's rho = 0.27, p < .01). The average temperatures were lowest pre-*P. americana* flowering (sampling period A; 18°C ± 1.3 SE) and highest during moderate *P. americana* flowering (sampling period C; 29°C ± 2.2 SE). Alpha diversity for arthropods captured in adjacent pasture and natural vegetation habitats were not correlated with co-flowering resources (Spearman's rho = 0.05, p = .61). Though, habitat complexity (canopy cover %) and maximum daily temperature both showed positive correlations with arthropod diversity (canopy cover; Spearman's rho = 0.38, p < .01, maximum daily temperature; Spearman's rho = 0.41, p < .01).

The arthropod taxa contributing to dissimilarities between orchards adjacent to pasture and natural vegetation habitats were compared using a SIMPER analysis. Here, arthropods captured in orchards adjacent to natural vegetation showed a greater cumulative contribution of species dissimilarity compared to orchards adjacent to pasture (Tables S6.2, S6.3 and S6.4). Though despite this, orchard arthropod communities changed significantly (> 85% dissimilarity) between *P. americana* flowering (sampling periods B and C) and non-flowering (sampling periods A and D), irrespective of adjacent habitat. In orchards adjacent to pasture, the dissimilarity between arthropods collected during non-flowering and flowering was 90%, primarily resulting from the higher capture rates of *Ditrysia* sp. during the non-flowering sampling periods and higher capture rates of Tachinidae spp. and Muscidae sp. during crop flowering (Table S6.2). While in orchards adjacent to natural vegetation, arthropod communities captured during flowering were 92% dissimilar with those captured during non-flowering, primarily due to the increased capture rates of; Formicidae sp., *Melangyna viridiceps*, Buprestidae sp and *Lasioglossum* sp. during *P. americana* flowering (Table S6.3). When orchards adjacent to pasture and natural vegetation were compared during *P. americana* flowering, we found the highest dissimilarity (95%), principally driven by the higher capture rates of Formicidae sp., Buprestidae sp in orchards adjacent to natural

vegetation habitat and higher capture rates of *Podalonia* sp. in orchards adjacent to pasture compared to orchards adjacent to natural vegetation (Table S6.4).

6.4.3 Diptera and Hymenoptera diversity and abundance fluctuate over time

Species richness of All arthropods captured in pan traps within orchards differed significantly among the sampling periods (Wald $\chi^2 = 53.28$, $DF = 3$, $p < .01$), although not between orchards adjacent to either pasture or natural vegetation (Wald $\chi^2 = 0.88$, $DF = 1$, $p = .35$) and there was a significant interaction effect between sampling period and adjacent habitat on arthropod species richness (Wald $\chi^2 = 11.50$, $DF = 3$, $p < .01$) (Figure 6.3A). The observed difference in species richness between sampling periods were primarily driven by Diptera (Wald $\chi^2 = 29.85$, $DF = 3$, $p < .01$) and Hymenoptera (Wald $\chi^2 = 24.83$, $DF = 3$, $p < .01$) (Figure 6.3A), although neither species cohort differed significantly in richness between the two orchard adjacent habitats (Diptera; Wald $\chi^2 = .24$, $DF = 1$, $p = .63$, Hymenoptera; Wald $\chi^2 = .39$, $DF = 1$, $p = .53$), nor was there any significant interaction effects between the two co-variates (Diptera; Wald $\chi^2 = 5.79$, $DF = 3$, $p = .12$, Hymenoptera; Wald $\chi^2 = 6.77$, $DF = 3$, $p = .08$). The species richness of Coleoptera, Lepidoptera and Other did not differ significantly between sampling periods or adjacent habitats, nor were there any significant interaction effects between the two co-variates (Figure 6.3A and Table S6.5). Pairwise comparisons with the Bonferroni adjustment indicated that the lowest species richness of All arthropods was in orchards pre-*P. americana* flowering (period A) and at low *P. americana* flowering (period B) for orchards adjacent to pasture ($DF = 1$, $p = <.01$). While in orchards adjacent to native vegetation, species richness of All arthropods was lowest during pre-*P. americana* flowering (period A) ($DF = 1$, $p = .05$) (Table S6.6). The species richness of Diptera and Hymenoptera in orchards was lowest, irrespective of adjacent habitat, pre-*P. americana* flowering (period A) and at low *P. americana* flowering (period B) and highest during moderate flowering (period C) and post-flowering (period D) (Figure 6.3A and Table S6.6).

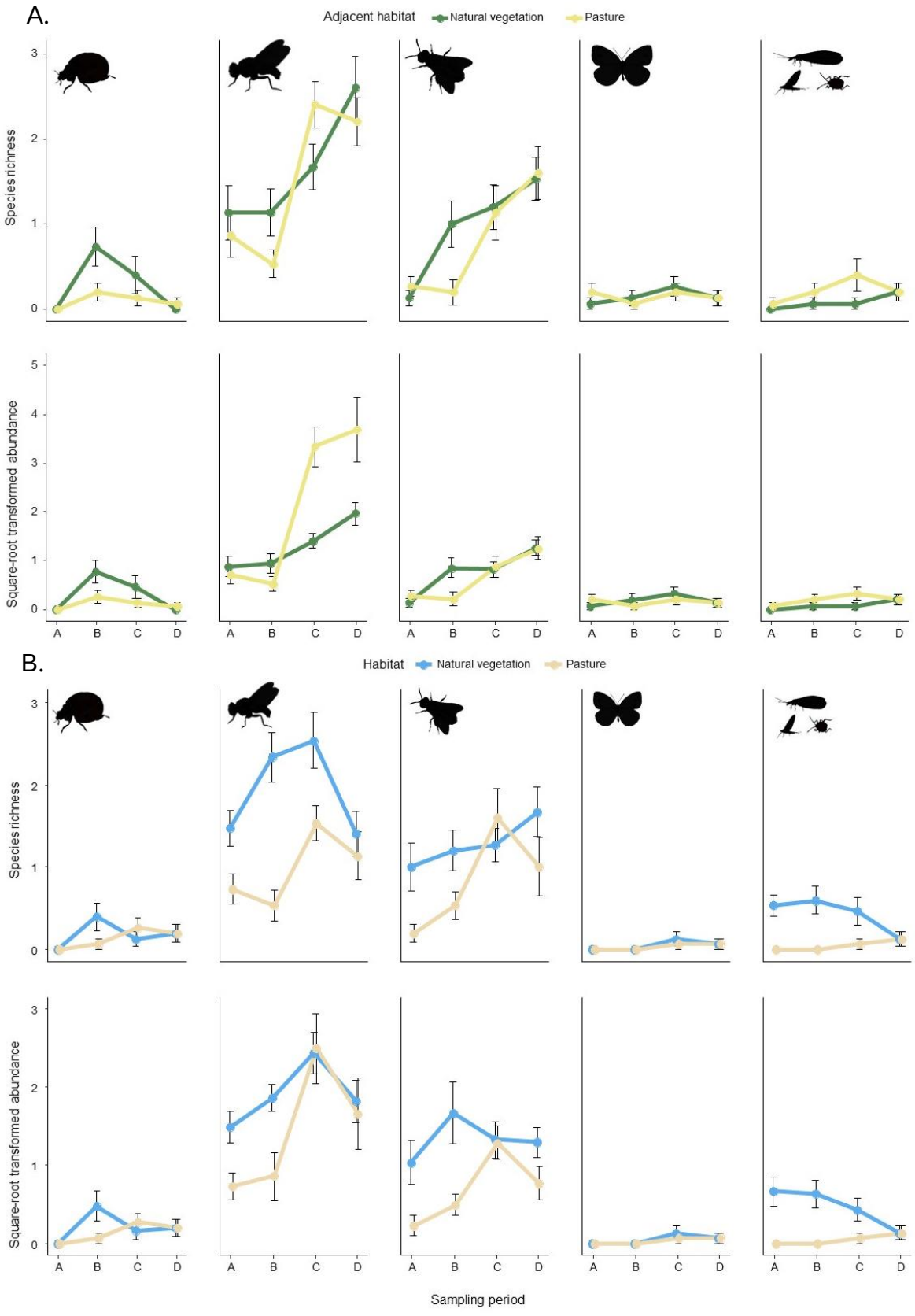


Figure 6.3. A. Orchard measures of species richness and square-root transformed abundance data, with standard error bars, for the five dominant orders collected in six orchards adjacent to either natural vegetation or pasture in the Manjimup-Pemberton region of SWWA. B. Adjacent habitat

measures of species richness and square-root transformed abundance data, with standard error bars, for the five dominant orders collected in three natural vegetation and three pasture sites adjacent to *P. americana* orchards in the Manjimup-Pemberton region of SWWA. Orchards and adjacent habitats were sampled over four *P. americana* sampling periods (A – before flowering. B – low flowering, C – moderate flowering and D – after flowering). Orders displayed left to right are as follows: Coleoptera, Diptera, Hymenoptera, Lepidoptera and Other. The Hymenoptera order excludes both *Apis mellifera* and Formicidae sp. 26. The Other order includes species belonging to Arachnid, Ephemeroptera, Hemiptera, Neuroptera, Odonata and Orthoptera.

The abundance of All arthropods captured in pan traps within orchards did not differ significantly between adjacent habitats (Wald $\chi^2 = 1.59$, $DF = 1$, $p = .21$), although abundance did differ significantly between sampling periods (Wald $\chi^2 = 113.67$, $DF = 3$, $p < .01$) and there was a significant interaction effect between orchard adjacent habitat and sampling period (Wald $\chi^2 = 39.01$, $DF = 3$, $p < .01$). These significant interactions were primarily driven by Diptera, which although not significantly different between orchard adjacent habitats (see Figure 6.3A and Table S6.7), did differ significantly between sampling periods (Wald $\chi^2 = 99.12$, $DF = 3$, $p < .01$), and showed a significant interaction effect between sampling period and orchard adjacent habitat (Wald $\chi^2 = 36.00$, $DF = 3$, $p < .01$). Similarly, the abundance of Hymenoptera differed significantly only between sampling periods (Wald $\chi^2 = 30.09$, $DF = 3$, $p < .01$), and did not show a significant difference between orchard adjacent habitat, nor was there an interaction effect between the two co-variates (see Table S6.7). The abundance of Coleoptera, Lepidoptera and Other did not differ significantly between sampling periods or orchard adjacent habitats and no significant interactions were found between the two co-variates (Table S6.7). Pairwise comparisons with the Bonferroni adjustment indicated that the abundance of All arthropods was lowest in orchards adjacent to pasture during pre-*P. americana* flowering (period A) and low *P. americana* flowering (period B) ($DF = 1$, p-value $< .01$). While in orchards adjacent to natural vegetation, the abundance of All arthropods was lowest duing pre-*P. americana* flowering (period A) ($DF = 1$, $p = .03$). For orchards adjacent to pasture, the abundance of All arthropods was greatest during moderate *P. americana* flowering (period C) and post-*P. americana* flowering (period D) ($DF = 1$, p-value $< .01$) Whilst the abundance of All arthropods in orchards adjacent to natural vegetation was greatest during low *P. americana* flowering (period B), moderate *P. americana* flowering (period C) and post-*P. americana* flowering (period D), which did not differ significantly from one another (Table S6.8). The abundance of Hymenoptera and Diptera was lowest, irrespective of adjacent habitat, pre-*P. americana* flowering (periods A) and at low flowering (period B), and highest during moderate flowering (period C) and post-flowering (period D) (Table S6.8).

6.4.4 Adjacent natural vegetation provides a reservoir for species diversity and abundance

Species richness for All arthropods captured in pan traps within the adjacent habitats differed significantly between sampling periods (Wald $\chi^2 = 19.60$, $DF = 3$, $p < .01$) and between habitat types (Wald $\chi^2 = 22.99$, $DF = 1$, $p < .01$). We also found a significant interaction between the two co-variates (Wald $\chi^2 = 17.32$, $DF = 3$, $p < .01$). Diptera and Hymenoptera were the main drivers of these findings, whereby Diptera species richness differed significantly between pasture and natural vegetation habitats (Wald $\chi^2 = 15.17$, $DF = 1$, $p < .01$), while Hymenoptera species richness varied significantly between sampling periods (Wald $\chi^2 = 10.02$, $DF = 3$, $p = .02$) (Figure 6.3B). Sampling period was significant for Diptera species richness in adjacent habitats (Wald $\chi^2 = 9.65$, $DF = 3$, $p = .02$), though there was no significant interaction effect between habitat type and sampling period (Table S6.9). The type of adjacent habitat was not significant for Hymenoptera species richness, although there was a significant interaction effect between habitat type and sampling period (Wald $\chi^2 = 9.03$, $DF = 3$, $p = .03$) (Table S6.9). Neither sampling period nor adjacent habitat type were significant for Coleoptera, Lepidoptera or Other species richness, nor were there any significant interaction effects between the two co-variates (Figure 6.3B and Table S6.9). Pairwise comparisons with the Bonferroni adjustment indicated that species richness for All arthropods was significantly higher in adjacent natural vegetation compared to adjacent pasture (Table S6.10). Within both adjacent pasture and natural vegetation, the abudance of all arthropods was lowest during pre-*P. americana* flowering (period A) and during low *P. americana* flowering (period B) ($DF = 1$, $p < .01$) (Table S6.10). While species richness for All arthropods was highest during moderate and post-*P. americana* flowering (periods C and D), which did not differ significantly from one another (Table S6.10). The species richness for Diptera was highest in adjacent natural vegetation habitat compared to pasture ($DF = 1$, $p = .03$) (Table S6.10). For Hymenoptera, the lowest species richness in pasture was captured pre-*P. americana* flowering (period A) and highest during low, moderate and post-*P. americana* flowering (periods B, C and D), which did not vary significantly from one another (Table S6.10). While in adjacent natural vegetation, Hymenoptera species richness did not differ significantly between the sampling periods (Table S6.10).

The abundance of All arthropods captured in pan traps within adjacent habitats varied significantly with sampling period (Wald $\chi^2 = 32.42$, $DF = 3$, $p < .01$) and with habitat type (Wald $\chi^2 = 4.10$, $DF = 1$, $p = .04$). The interaction between sampling period and habitat type was also significant (Wald $\chi^2 = 23.93$, $DF = 3$, $p < .01$). Sample period was significant for the abundance of both Diptera (Wald $\chi^2 = 29.58$, $DF = 3$, $p < .01$) and Hymenoptera species cohorts (Wald $\chi^2 = 10.17$, $DF = 3$, $p = .02$). Although adjacent habitat type was not significant for the abundance of Diptera and Hymenoptera (Figure 6.3B and Table S6.11), there were significant interaction effects were

between sampling period and adjacent habitat type (Diptera; Wald χ^2 = 7.76, *DF* = 3, *p* = .05, Hymenoptera; Wald χ^2 = 16.96, *DF* = 3, *p* <.01). The abundance of Coleoptera, Lepidoptera and Other did not vary significantly with either sampling period or adjacent habitat type, nor were there any significant interactions between the two co-variates (Figure 6.3B and Table S6.11). Pairwise comparisons with the Bonferroni adjustment indicated that adjacent natural vegetation supported a greater abundance of arthropods compared to adjacent pasture (*DF* = 1, *p* = .01) (Table S6.12). Within adjacent pasture, the abundance of All arthropods was lowest during pre-*P. americana* flowering (period A) and low *P. americana* flowering (period B) (*DF* = 1, *p* < .01) and highest during moderate *P. americana* flowering (period C) and post-*P. americana* flowering (period D) (Table S6.12). While, the abundance of All arthropods in adjacent natural vegetation did not differ significantly from one another (Table S6.12). For Diptera and Hymenoptera in adjacent pasture, the lowest measures of arthropod abundance were captured pre-*P. americana* flowering (period A) and low *P. americana* flowering (period B), while the highest measures were captured during moderate *P. americana* flowering (period C) and post-flowering (period D) (Table S6.12). The abundance of Diptera and Hymenoptera in adjacent natural vegetation did not differ significantly between the sampling periods (Table S6.12).

6.4.5 eDNA surveys and marble trap MSCTs

We generated 32,248,187 raw sequence reads from two sequencing runs for 120 pan trap samples, 24 marble traps and 34 wash water, extraction and PCR controls – amplified using two plant specific assays. Overall, 29,448,603 quality-filtered reads were generated with a mean sequencing depth of 87,126 reads per sample. The average number of reads per pan trap array sample was 105,042 (± 5918 SE), while the average number of reads per marble trap was 95,966 (± 12,399 SE). One marble trap (4% of marble trap samples) and five pan traps (4% of pan trap array samples) failed to amplify for both trnL and rbcL. Ten plant ZOTUs (*Callistemon*, *Camellia*, *Citrus*, Desmidiaceae, *Eucalyptus*, *Lavandula*, *Sonchus*, *Pinus*, *Physalis*, Poaceae, and *Prunus*) showed amplification (> 10 reads) in the wash water used to clean equipment during filtering of pan trap water. While twelve plant ZOTUs (*Allium*, Colchicaceae, Desmidiaceae, *Helianthus*, Pedaliaceae, *Physalis*, *Prunus*, *Rubus*, *Solanum*, *Sonchus*, *Spinacia* and Zingiberales) showed amplification (> 10 reads) in the extraction controls, no plant ZOTUs showed amplification from the PCR controls. Any plant ZOTUs that were identified in the wash water or extraction controls were removed from the dataset prior to further analysis (method following van der Heyde et al., 2020). A minimum sequence copy threshold of ten reads was used as a cut-off, above which plant ZOTUs were retained (see Drake et al., 2022). With this filtering procedure, the average number of reads per pan trap array were reduced by 42% from 105,042 (± 5918 SE) to 61,304 (± 4431

SE). Marble trap reads were then used as MSCTs for the corresponding pan traps when plant taxa were detected in both samples (method following Kestel et al., 2024). With this approach, the average reads per pan trap array was reduced by a further 22% from 61,304 (± 5918 SE) to 48,050 (± 4154 SE). Ten plant genera (*Daucus*, *Petroselinum*, *Romulea*, *Heliotropium*, *Plumbago*, *Veronica*, *Tricostularia*, *Stirlingia*, Sapindaceae and Hamamelidaceae) were removed with this filtering procedure. All of the genera removed were rare (≤ 2 detections). Per pan trap array, the removal of putative false positives with marble trap MSCTs reduced the average number of plant taxa detected by 24% from 39 (± 1.6 SE) to 29 (± 1.4 SE).

6.4.6 Plant taxonomic diversity – eDNA and conventional floral surveys

In total, 113 plant families were detected in the eDNA dataset, of which Fabaceae (*Lotus*, *Trifolium* and *Acacia*; 10% of pan trap array samples), Asteraceae (*Arctotheca*, *Bidens* and *Hypochaeris*; 9% of pan trap array samples) and Poaceae (*Poa*, *Holcus* and *Avenella*; 6% of pan trap array samples) were the most common. Overall, 251 plant taxa were identified by eDNA metabarcoding, with 195 (78%) resolved to the genus level, while 41 (16%) could not be resolved beyond the family level, 13 (5%) could not be resolved beyond the order level and 2 (1%) were not resolved beyond the class level. The average number of families detected per pan trap array was 29 (±1.4 SE) and the average number of genera detected per pan trap array was 29 (±1.4 SE). For the conventional floral surveys, we identified 18 plant families, of which Fabaceae (*Trifolim*, *Bossiaea*, *Hovea*, *Hardenbergia*; 28% of quadrats), Poaceae (*Poa*, *Bromus*, *Avena*, *Tetrarrhena*, *Holcus*, *Hordeum* and *Lolium*; 23% of quadrats) and Asteraceae (*Arctotheca*, *Sonchus* and *Taraxacum*; 16% of quadrats) were the most common. In total, the conventional floral surveys identified 33 plant genera, 25 (76%) of which were also identified by eDNA metabarcoding, while 8 genera (24%) were unique to the conventional floral surveys. All 8 genera unique to the floral surveys (*Agrostocrinum*, *Banksia*, *Geranium*, *Hardenbergia*, *Lolium*, *Taraxacum*, *Tetrarrhena* and *Tremandra*) were rare and detected in 11% or fewer of the sample quadrats (Table S6.13).

6.4.7 Adjacent natural vegetation can support greater arthropod-plant foraging diversity in orchards

The number of plant genera detected by eDNA metabarcoding pan trap water in orchards adjacent to pasture (N = 215) and natural vegetation habitat (N = 221) were similar, with the majority of genera (74%) shared between the two adjacent habitat types (Figure 6.4A). Plant foraging genera composition recorded by eDNA showed some distinct partitioning for both adjacent habitat type and sampling period (Figure 6.4B, ANOSIM, $p < .01$ in both instances). With least-squares means testing, we determined that the Chao2 alpha diversity measures only differed significantly between orchards adjacent to pasture and natural vegetation during low *P. americana* flowering at sampling

period B (least-squares means; p = .02). Here, the alpha diversity values measured in orchards adjacent to natural vegetation (mean chao2 index of 98 ± 19 SE) were three times larger than those generated in orchards adjacent to pasture (mean chao2 index of 29 ± 19 SE) (Figure 6.4C; least-squares means; p = .02). There was also no significant differences among the orchards adjacent to pasture and natural vegetation for the other three sampling periods (p > .05). The number of plant genera detected from the pan traps differed only during low *P. americana* flowering at sampling period B, where pan trap arrays in orchards adjacent to natural vegetation detected over two times the number of genera (20 genera per array ± 3.4 SE) than were detected from pan trap arrays in orchards adjacent to pasture (8.6 genera per array ± 1.7 SE) (Figure 6.4D; least-squares means; p = .01). Whereas, orchards adjacent to pasture and natural vegetation during pre-, moderate and post-*P.americana* flowering (periods A, B and D) were not significantly different (p > .05). For each sampling period, we constructed quantitative arthropod-plant foraging resource networks for each orchard adjacent habitat based on the relative abundance of plant families (Figure 6.5). The complexity (linkage density) of these networks in this agricultural landscape was lowest during low *P. americana* flowering (period B; linkage density = 18.66) and highest at moderate *P. americana* flowering (period C; linkage density = 21.03) (Figure 6.6). The number of shared species between the orchards adjacent to pasture and natural vegetation was lowest during low *P. americana* flowering (period B; 22 shared species) and highest at moderate *P. americana* flowering (period C; 63 shared species).

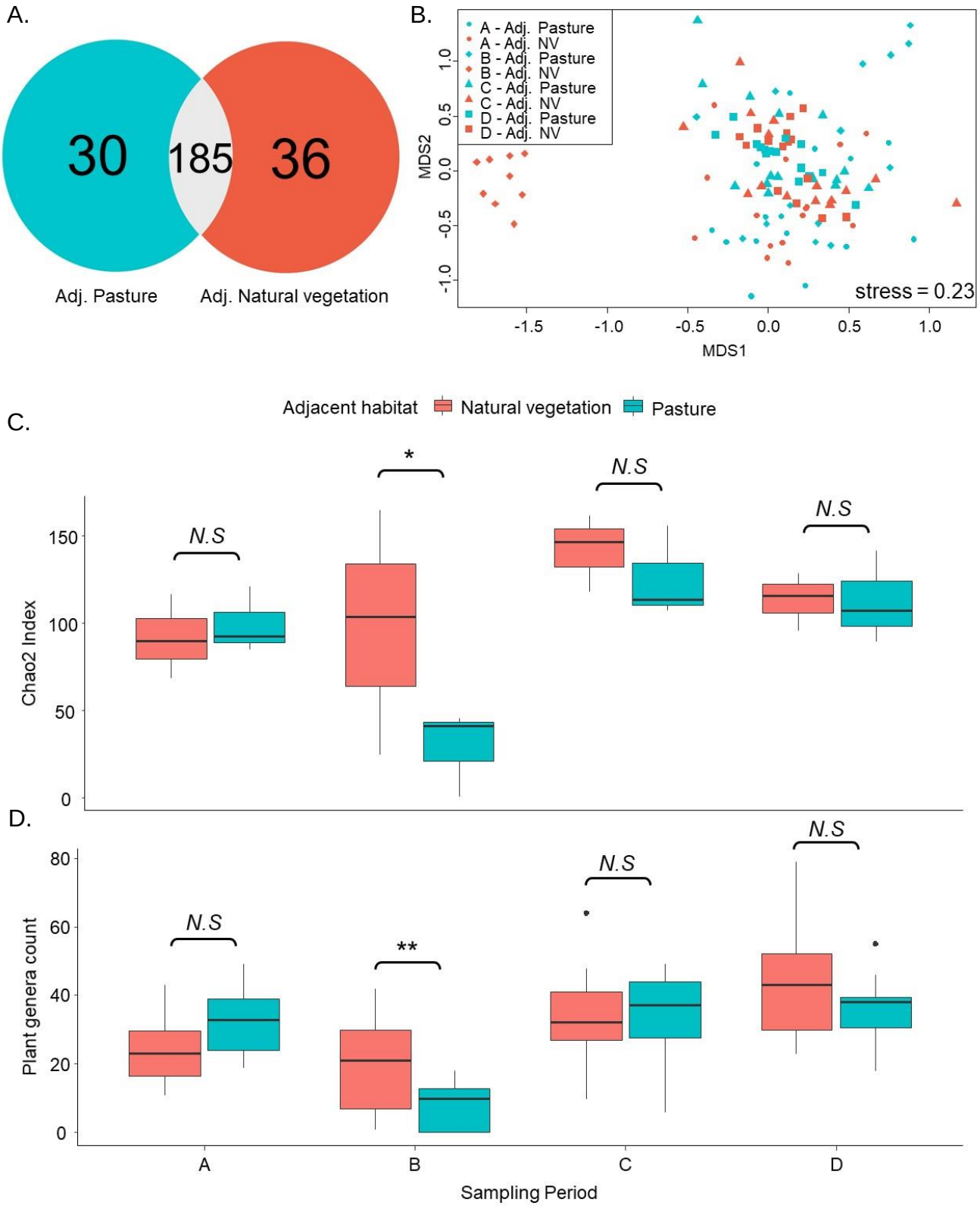


Figure 6.4. A. Number of plant genera identified in orchards adjacent to pasture and natural vegetation; Adj. Pasture (*N* = 215) and Adj. Natural vegetation (*N* = 221). B. Non-metric multidimensional scaling ordination based on a Jaccard dissimilarity matrix (Stress value = 0.23), showing the relationship between plant genera assemblage, orchard adjacent habitat (pasture and NV – native vegetation) and sampling period (A – D). C. Chao2 alpha diversity measures based

on presence-absence data for plant genera. Chao2 values were calculated by pooling pan trap arrays for each orchard adjacent habitat at each sampling point (pasture; N = 15 per sampling period, native vegetation; N = 15 per sampling period). D. Relative abundance of plant genera per pan trap array for orchard samples adjacent to either pasture or native vegetation. Least-squares means were calculated for each adjacent habitat at each sampling period to assess significance. N.S = Not significant, * = $p \leq .05$, ** = $p \leq .01$ and ***= $p \leq .001$.

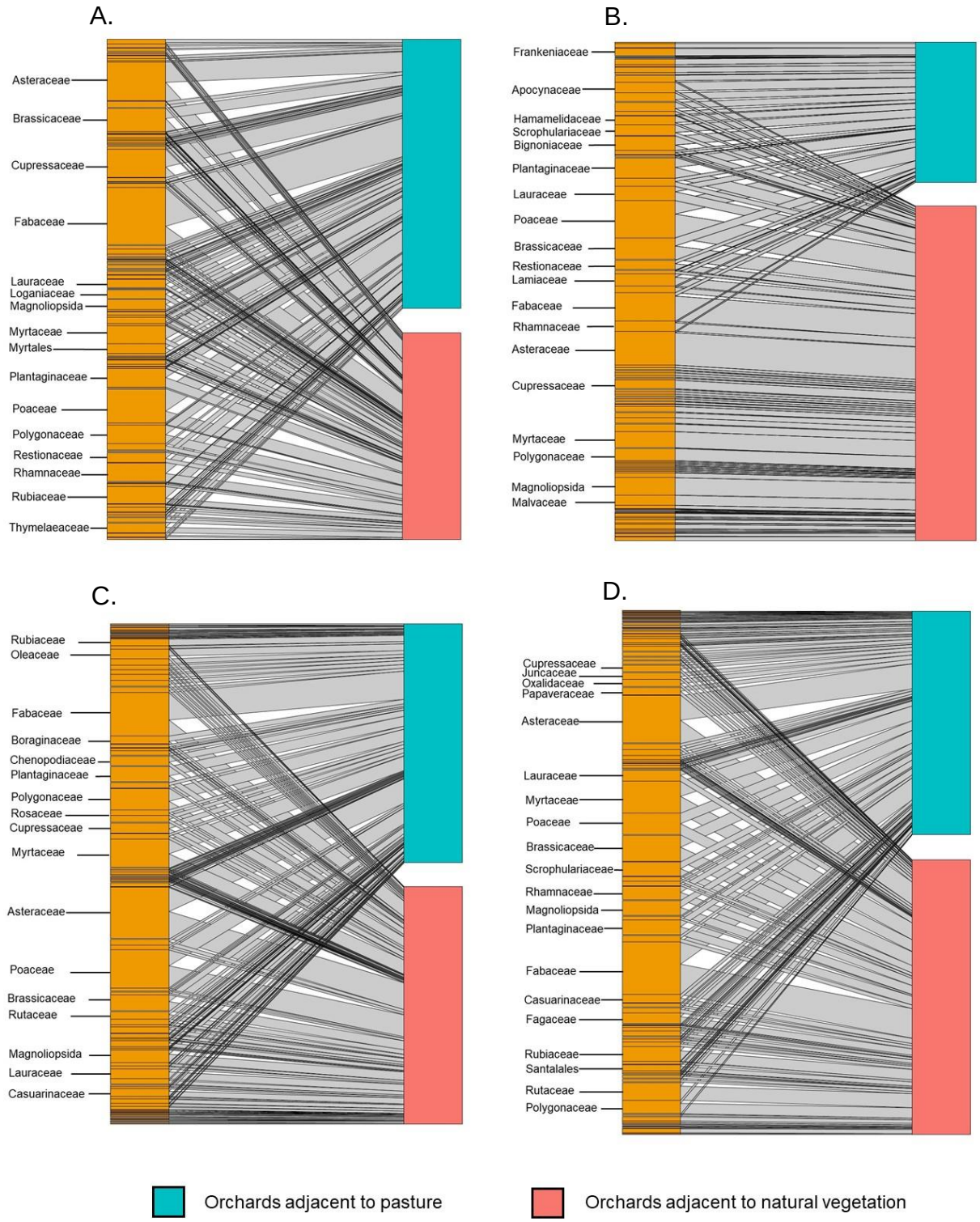


Figure 6.5. Bipartite network diagrams based on relative abundance of foraging plant families with five or more detections generated from eDNA metabarcoding of pan-trap water. Orchard sites were sampled over four *P. americana* sampling periods (A – pre-flowering, B– low flowering, C- moderate flowering and D – post-flowering). Plots were created using the package 'Bipartite' in R.

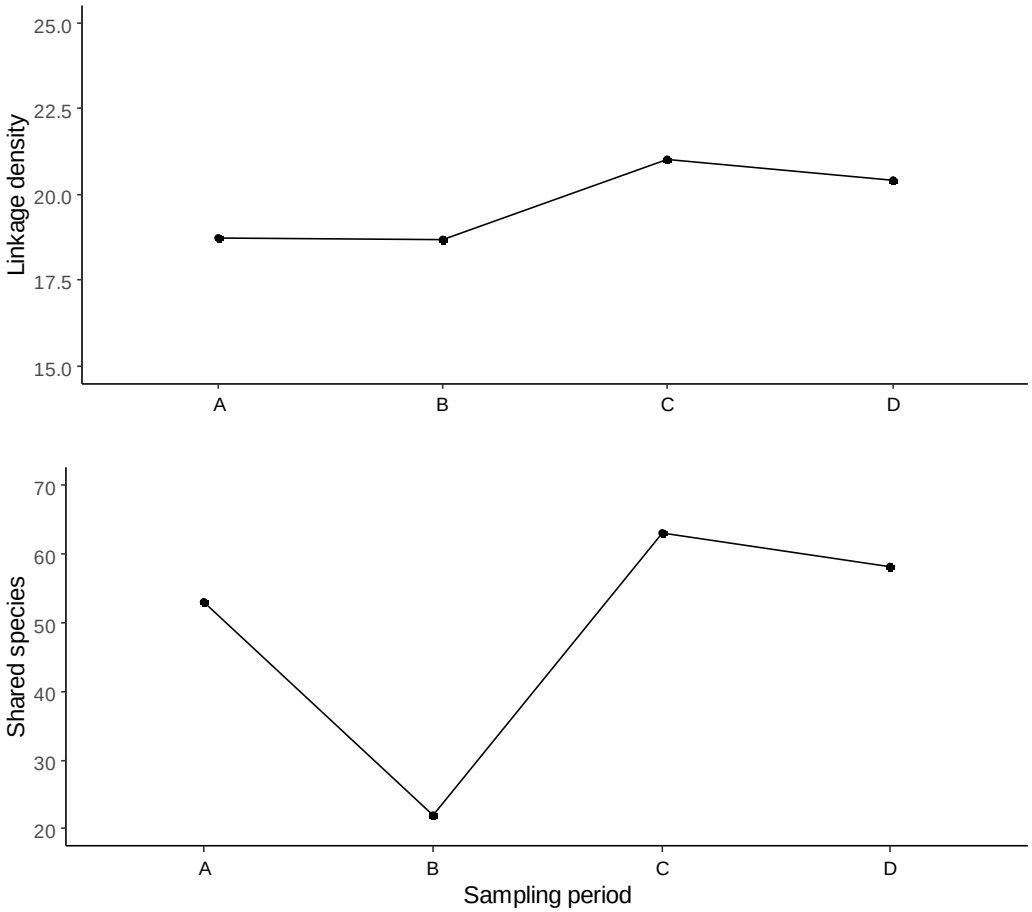


Figure 6.6. Linkage density and shared species between orchard adjacent habitats calculated over four *P. americana* sampling periods (A – pre-flowering, B– low flowering, C- moderate flowering and D – post-flowering). Metrics were calculated using the package 'Bipartite' in R.

6.5 Discussion

Globally, approximately five million hectares of natural vegetation is removed each year, with nearly a quarter of these losses being attributed to agricultural expansion (Curtis, Slay, Harris, Tyukavina, & Hansen, 2018). Unfortunately, the loss of natural (e.g. primary forests) and semi-natural (e.g. hedgerows) vegetation negatively affects biodiversity within these landscapes, especially the diversity of native plant taxa (Beckmann et al., 2019). Here, we used pan traps and eDNA metabarcoding of pan-trap water, to determine if arthropod community diversity and arthropod-plant foraging was enhanced with the presence of adjacent natural vegetation and greater *Persea americana* flowering intensity. Overall, we collected more than 2,000 arthropod specimens and by metabarcoding the pan trap water identified over 250 foraging plant taxa upon which these communities rely. The arthropod taxa detected were varied and included: potential pollinator species belonging to Hymenoptera (e.g. *Apis mellifera*, *Homalictus urbanus*, and *Lasioglossum castor*), Diptera (e.g. *Musca domestica*, *Melangyna viridiceps* and Tachinidae spp.), Coleoptera (e.g. Buprestidae, Coccinellidae and Scarabaeidae) and cryptic Lepidoptera species (e.g. *Pieris rapae* and *Neolveia agricola occidens*). We found that while arthropod community composition was affected by the presence of adjacent natural vegetation, species richness and abundance did not vary significantly. The eDNA detections of arthropod-plant foraging resources showed that arthropods in *P. americana* orchards rely upon a diverse array of species including: crops (e.g. *Malus*, *Olea*, *Persea* and *Vitis*), understorey weeds (e.g. *Arctotheca*, *Raphanus*, *Sonchus* and *Holcus*) and native taxa (e.g. *Acacia*, *Hibbertia*, *Hovea* and *Sowerbaea*). Further, we found that the diversity of these foraging preferences fluctuated depending on the type of adjacent habitat, as well as the level of *P. americana* flowering (however, see section *6.5.4 Benchmarking pan-trap eDNA metabarcoding*). Our results highlight that eDNA-based surveys can be used to monitor the foraging preferences for entire arthropod communities, while also showing the significance of natural capital for sustainable agroecosystem management.

6.5.1 Adjacent natural vegetation benefits specific arthropod taxa in orchards

Generally, arthropod biodiversity and the beneficial ecosystem services some of these taxa provide (e.g. pollination and pest reduction) can be enhanced when natural habitats or semi-natural habitats are maintained adjacent to agricultural areas (Dainese, Montecchiari, Sitzia, Sigura, & Marini, 2017; Wratten et al., 2012), although not all arthropod groups benefit equally from these areas of natural capital (Esquivel et al., 2021; Hannon & Sisk, 2009; Wang et al., 2021). In our study, the presence of adjacent natural vegetation affected arthropod community composition, though not arthropod species richness or abundance. Our findings indicate that particular species, rather than entire arthropod orders, respond positively to adjacent natural vegetation (Meyer, Jauker, &

Steffan-Dewenter, 2009; Rader et al., 2016). For instance, although overall Diptera abundance did not differ between orchards adjacent pasture or natural vegetation, the abundance of *Melangyna viridiceps* (Syrphidae), a known crop pollinator and potential predator of pests (see Bowie, Gurr, & Frampton, 2001; Cook et al., 2020), was significantly greater in orchards adjacent to natural vegetation. Such findings echo those from other studies, where Syrphidae species richness and community composition have been significantly correlated with forage plant richness in adjacent hedgerows (Ahmed et al., 2021; Meyer et al., 2009). Thus, areas of natural vegetation do not significantly increase the diversity and abundance of all arthropods, although these areas can help encourage the persistence of particular beneficial arthropod taxa within orchards.

6.5.2 Natural vegetation provides a reservoir for arthropod diversity in agroecosystems

Natural habitat fragments within agroecosystems support arthropod diversity by providing diverse foraging resources, microhabitats, as well as refuge from predators (Kovalenko, Thomaz, & Warfe, 2012; Marja, Tscharntke, & Batáry, 2022). When arthropod detections between pasture habitats and natural vegetation were compared, species richness positively correlated with habitat type and the species richness and abundance of overall captured arthropods was significantly greater in natural vegetation compared to pasture habitats. These patterns were primarily driven by the greater species richness and abundance of Diptera (e.g. Tipulidae sp. and Dolichopodidae sp.) and of specialist native bees (e.g. *Lasioglossum* (*Chilalictus*) and *L. occidens*) in natural vegetation. Our findings are in line with M'Gonigle, Ponisio, Cutler & Kremen (2015), where the species richness and between-season persistence of specialist bee species and syrphid (Diptera) pollinators changed positively over time for restored hedgerows. In the present study, natural vegetation appears to be particularly beneficial to specialised bee species because the unique foraging resources upon which they rely may be rare or absent from the adjacent orchards and surrounding pasture habitats (Brown & Cunningham, 2022). Together, these findings demonstrate that natural vegetation can enhance arthropod biodiversity within agroecosystems and may act as reservoirs of diversity, which may spread into other areas where their preferred foraging resources are present.

6.5.3 Adjacent native vegetation increases foraging diversity for orchard arthropods

Foraging resources include not only crop flowers, but also the co-flowering species which grow in cultivated (e.g. weed species) and unmanaged areas (e.g. native herbs, shrubs and trees) (Aviron et al., 2023). The presence of these co-flowering species can enhance the resilience of arthropod-plant networks when crops are not in flower and improve the delivery of pollination and predator services (Allasino et al., 2023; Aviron et al., 2023, but see Kleijn et al., 2015). In the present study, the number of plant genera detected by eDNA metabarcoding pan-trap water in orchards adjacent

to natural vegetation was over two times the number detected in orchards adjacent to pasture at the beginning of *P. americana* flowering. Further, during this sampling period we also observed that the number of shared arthropod-plant foraging genera between the two adjacent habitats fell by ca. 60%. Though some of our pan-trap water eDNA detections may represent false positives due to wind contamination (see section *5.5.3 Pan trap pollen – ambient or animal mediated*), our conservative marble trap thresholds minimised this issue and reduced the possibility of over-inflated foraging species diversity measures. Thus, our findings indicate that natural vegetation may provide more stable foraging resources in contrast to the disturbance-prone pasture habitats. Pasture habitats are strongly influenced by grazing from large herbivores, which can reduce reproductive plant biomass (i.e. flowering stems) and alter foraging diversity, as well as vegetation structure (Ferreira et al., 2020; Hickman & Hartnett, 2002; van Klink, van der Plas, van Noordwijk, Wallisdevries, & Olff, 2015). Our findings align with previous research showing that semi-natural habitats can support diverse arthropod foraging despite greater grazing intensities (Helden, Chipps, McCormack, & Pereira, 2020; Sjodin, Bengtsson, & Ekbom, 2008). In the context of SWWA, the retention of natural and semi-natural habitats may help stabilise arthropod community diversity in agricultural areas, although our eDNA metabarcoding results also indicated that even where natural vegetation is rarer, arthropod taxa may extend their flight ranges to visit these natural vegetation fragments.

Flight distances can vary significantly between foraging arthropod taxa, and certain bees species for instance may forage over kilometres, while some fly species may be restricted to foraging distances of less than a meter (Olesen & Warncke, 1989; Somanathan, Saryan, & Balamurali, 2019). Often however, the foraging distance that arthropods are willing to travel are proportional to the rewards on offer (Pope & Jha, 2018; Rutschmann, Kohl, & Steffan-Dewenter, 2023). In our study, orchard pan trap samples adjacent to pasture or native vegetation shared over 70% of the same plant genera, including numerous native taxa (e.g. *Acacia*, *Hibbertia*, *Hovea* and *Sowerbaea*). Indeed, for orchards adjacent to pasture, these findings suggest that some of the captured arthropods may forage beyond the orchard and pasture boundary (Albrecht et al., 2010; O'Donnell & Wright, 2021; Taki et al., 2010). Similar results were reported by Kohler, Verhulst, Van Klink and Kleijn (2008), where Hymenoptera and Diptera foraging remained consistent in agricultural fields up to 300 m away from adjacent natural vegetation. In our study system, natural vegetation and co-flowering weed species are likely critical for sustaining arthropod communities, as the main crop species for the region, *P. americana*, flowers for a relatively brief period of time (Ish-Am, 2005), meaning phenological gaps where little or no flowering resources are available are more likely (González-Varo & Vilà, 2017; Ramirez & Kallarackal, 2018). Morphological

analysis of orchard arthropod diversity determined, however, that the presence of adjacent natural vegetation does not benefit all arthropod taxa. These observations raise numerous questions for future research about the complex interactions between arthropod communities and unmanaged areas in agroecosystems.

6.5.4 Benchmarking pan trap eDNA metabarcoding

The present study has demonstrated that eDNA metabarcoding pan-trap water can detect arthropod foraging resources within agroecosystems. As eDNA-based surveys become more routine in agricultural monitoring (see Kestel et al., 2022), it is critical that new substrates are benchmarked against conventional approaches (e.g. Ryan, Bateman, Fernandes, van der Heyde, & Nevill, 2022; Newton et al., 2023). When we compared the taxa identified using eDNA metabarcoding against those from conventional floral surveys, we found that Asteraceae, Fabaceae and Poaceae were the most common plant families detected by both methods and that eDNA was able to detect 76% of the plant genera identified using conventional floral surveys. Despite this, eDNA metabarcoding failed to detect eight flowering plant genera, six of which likely rely upon animal pollination, found in the conventional floral surveys. These eight genera all had reference sequences available, although they were rare and occurred in 11% or fewer of the quadrats. Amongst conventional and eDNA-based surveys, taxonomic differences are not uncommon (Johnson, Fokar, Cox, & Barnes, 2021; Newton et al., 2023), though these differences emphasise the need to include complementary methods to account for these taxonomic gaps. The inclusion of additional complementary methods is also necessary because the presence of eDNA does not necessarily mean an ecological interaction occurred (i.e. flower visit without cross-pollination; see Thomsen & Sigsgaard, 2019). Thus, complementary methods (e.g. digital video recordings of crop flowers, as well as adjacent weed and native species) can provide additional ecological insights, not possible when eDNA used in isolation.

In the present study, marble trap reads were used as MSCTs for the corresponding pan traps when plant taxa were detected in both samples (method following Kestel et al., 2024). Using marble traps to set MSCTs allowed us to significantly reduce the contribution of airborne plant DNA to the final pan trap dataset, though we acknowledge that additional modifications may help further increase the accuracy and reliability of this filtering approach. The quantity of pollen captured by different passive air samplers may vary significantly depending on the medium used. Indeed, Goossens (2005) measured the deposition of airborne dust on water and marbles, finding that although both surfaces had comparable catch efficiencies, water samples accumulated a larger quantity of dust overall compared to the marble samples (water surface average flux of 182 g m^{-2}

s^{-1} vs. marble surface average flux of 113 g m^{-2} s^{-1}). In the context of this study, greater relative quantifications of plant DNA in the pan traps compared to the marble traps may have over-inflated the diversity measures by reducing our ability to 'correct' for non-target eDNA. Thus, we recommend that future studies include additional high volume active air samplers (see Emenyeonu et al., 2018) to produce higher MSCTs for the corresponding pan trap data. As well as quantifying the different capture rates of passive samplers, additional research remains necessary to examine how DNA is preserved in different trap types. The current understanding of pollen preservation remains limited, though initial studies have found that pollen grains from different species may show unique degradation rates, potentially biasing measures of arthropod community foraging towards particular species (Twiddle & Bunting 2010; Phuphumirat et al., 2015). Consequently, we recommend that future studies include pilot studies that measure the degradation rates of locally relevant pollen mixtures in different trap types over time. Such research would highlight if certain traps are biased towards particular species or if the hard outer exine of pollen grains (see Lalhmangaihi et al., 2014) preserves DNA quality irrespective of the capture method.

Pan trap eDNA metabarcoding is at a nascent stage and, like any emerging technology, requires further baseline research to establish detection thresholds, as well as any taxonomic biases (Kestel et al., 2022). Firstly, the development of new plant primers that help achieve genus- or species-level detections within certain families where the resolution of current markers is limited (e.g. Eucalyptus), would increase the accuracy and reliability of pan trap eDNA metabarcoding detections (Cheng et al., 2016). Secondly, it is possible that some of the plants identified from the pan traps may have occured as a result of 'secondary detections', whereby plant DNA present on one arthropod was exchanged with another on a common substrate (i.e. multiple arthropods visiting the same flower; see Thomsen & Sigsgaard, 2019). To date, no research has examined how frequent such 'secondary detections' are, however incorporating these measures inot eDNA surveys is necessary to account for potential false positives, as well as overinflated diversity measures (Gomez et al., 2023; Thomsen & Sigsgaard, 2019). Thirdly, arthropod size and morphological characteristics can significantly alter the quantity of pollen incidentally collected during flower visits (see Cook et al., 2020). For instance, larger and hairier arthropods may be expected to contribute more pollen in the pan trap sampels, which may skew the detections for foraging plant species (Silberbauer et al., 2004; Walton et al., 2020). Therefore, greater research is needed to identify the quantity of pollen collected by locally-relevant managed and unmanaged arthropod taxa and to relate these measures with arthropod morphological characteristics (i.e. size and hairiness; see Stavert et al., 2016), as well as the length of time that these species spend visiting flowers (see section *4.5.4 Smaller and more frequent flower-visiting arthropods have a greater*

probability of being detected). Detection limits and the suitability of pan traps to infer plant abundance also present critical areas for greater research.

The presence of rare species or species with few ecological interactions (e.g. flower visits) may be missed as these taxa tend to have fewer oppurtunties to deposit a sufficient quantity of DNA to be detected by eDNA-based surveys (Johnson, Fokar, Cox, & Barnes, 2021; Newton et al., 2023). Here, we identify that greater research is needed for pan trap eDNA metabarcoding to both estimate minimum detection densities for flowering plant taxa, and establish if DNA sequence abundance from pan-trap water is correlated with the quantity of foraging plant material collected by captured arthropods. To estimate minimum detection densities, researches could compare alternative arthropod-specific eDNA survey methods (e.g. pan-trap water, captured arthropods and homogenised bulk-arthropod samples) targeting the same arthropod taxa visiting flowering plants at varying densities. Lastly, eDNA read abundance, can, in some circumstances, be used to infer plant abundance (see Parducci et al., 2019), though these correlations are often confounded by taxonomic biases and PCR inhibition (Johnson et al., 2023). For pan traps, researchers could correlate conventional pollen counts from the pan-trap water (e.g. pollen grains counted using a haemocytometer) against eDNA read abundance generated by metabarcoding and PCR-free shotgun sequencing. Such research could be used to inform the most appropriate contexts for pan trap metabarcoding, while also accounting for any biases associated with this method.

6.5.5 eDNA metabarcoding for natural capital assessment

Within agroecosystems, the natural capital available for farmers to support arthropod diversity and ecosystem services is disappearing at ever increasing rates (Potts, Imperatriz-Fonseca, & Ngo, 2016; UNEP, 2007, 2010). At the same time, the uptake and inclusion of natural capital in individual farm-scale management decisions remains novel as harmonised approaches to valuation remain a work in progress (Marais et al., 2019; NCC, 2014). With the greater inclusion of eDNA metabarcoding to monitor biodiversity in agriculture (see Kestel et al., 2022), we envisage that eDNA could provide the foundation upon which a harmonised approach for detecting and valuing biodiversity may be built. Such work is already occurring in the United Kingdom, where the Natural Capital committee has adopted the recommendations to incorporate biodiversity "big data" into natural asset-based assessments (Castle, Hebert, Clare, Hogg, & Tremblay, 2021; United Kingdom Natural Capital Committee, 2021). For individual farmers, the ability to sample local areas of natural capital (e.g. soil, foraging plant resources, or arthropod predator diets) and generate summary metrics (see Dardonville et al., 2022), as well as valuations (see Marais et al., 2019) could help encourage farmers to maintain natural and semi-natural habitats within agroecosystems. Further, the inclusion of timely and reliable metrics/valuations could inform

farmers about which practices are harming overall biodiversity (e.g. generalised pesticide applications; see Leskey, Lee, Short, & Wright, 2012), as well as the ecosystem services which support crop yields. In conclusion, through using eDNA metabarcoding in combination with conventional methods, we were able to determine how the foraging preferences of arthropods, as well as the communities themselves fluctuated according to adjacent habitat types and crop flowering. By incorporating eDNA-based surveys into agriculture more broadly, this molecular method could help guide management decisions that maintain areas of natural capital and improve the delivery of ecosystem services.

6.6 References

A thorough effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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6.7 Supplementary materials

S6.7.1 Plant barcoding

In total, 22 sequences were generated for the custom reference database. For both *rbcL* and *trn*L we sequenced: *Hordeum glaucum*, *Bossiaea ornata*, *Hardenbergia comptoniana*, *Acacia pulchella*, *Patersonia umbrosa*, *Pimelea clavata*, *Tremeda stelligera*, *Agrostocrinum hirsutum*, *Tetrarrhera laevis*, *Leucopogon verticillatus*, *Banksia grandis*. For *trn*L, we sequenced: *Lysimachia arvensis*, *Trifolium repens*, *Stachys arvensis*, *Epilobpium tetragonum*, *Festuca rubra*, *Lotus subbiflorus*, *Rumex acetosella*, *Hovea elliptica*, and *Hibbertia cuneformis*. Finally, for *rbcL*, we sequenced: *Bossiaea aquifolium* and *B. linophylla*. All samples were taken through Sanger sequencing by the Australian Genome Research Facility.

Table S6.1 Taxonomic composition of the orchard and adjacent pasture or natural vegetation Diptera, Hymenoptera, Coleoptera, Lepidoptera and Other cohorts captured in pan traps and used in the GLMM analysis.

Order	Family	Species
Coleoptera	Buprestidae	Buprestidae sp.5
Coleoptera	Cerambycidae	Cerambycidae sp.20
Coleoptera	Cleridae	Cleridae sp.12
Coleoptera	Coccinellidae	Coccinellidae sp.15
Coleoptera	Ripiphoridae	Ripiphoridae sp.
Coleoptera	Scarabaeidae	Scarabaeidae sp.3
Coleoptera	Scarabaeidae	Scarabaeidae sp.4
Coleoptera	Scarabaeidae	Scarabaeidae sp.7
Coleoptera	Scarabaeidae	Scarabaeidae sp.8
Coleoptera	Scarabaeidae	Scarabaeidae sp.10
Coleoptera	Scarabaeidae	Scarabaeidae sp.14
Coleoptera	Scarabaeidae	Scarabaeidae sp.17
Coleoptera	Scarabaeidae	Scarabaeidae sp.19
Coleoptera	Silvanidae	Silvanidae sp.13
Coleoptera	Staphylinidae	Staphylinidae sp.27
Coleoptera	Staphylinidae	Staphylinidaesp.28
Coleoptera	Tenebrionidae	Tenebrionidaesp.1
Diptera	Acroceridae	Acroceridae sp.sp.16
Diptera	Anthomyiidae	Anthomyiidae sp.sp.27
Diptera	Athericidae	Athericidaesp.3
Diptera	Calliphoridae	Calliphoridae sp.sp.44
Diptera	Carnidae	Carnidae sp.sp.48
Diptera	Carnidae	Carnidae sp. sp.29
Diptera	Coelopidae	Coelopidae sp.sp.18
Diptera	Coelopidae	Coelopidae sp.sp.32
Diptera	Diptera family	Diptera sp.sp.67
Diptera	Diptera family	Diptera Unknown
Diptera	Dolichopodidae	Dolichopodidae sp.sp.36
Diptera	Dolichopodidae	Dolichopodidae sp.sp.50
Diptera	Empididae	Empididae sp.1
Diptera	Empididae	Empididae sp.4
Diptera	Empididae	Empididae sp.5
Diptera	Empididae	Empididae sp.9
Diptera	Ephydridae	Ephydridae sp.21
Diptera	Empididae	Empididae sp.62
Diptera	Syrphidae	*Eristalis tenax*
Diptera	Fanniidae	Fanniidae sp.7
Diptera	Teratomyzidae	Heleomyzidae Teratomyzidae
Diptera	Lauxaniidae	Lauxaniidae sp.13
Diptera	Lauxaniidae	Lauxaniidae sp.15
Diptera	Lauxaniidae	Lauxaniidae sp.52
Diptera	Lauxaniidae	Lauxaniidae sp.63
Diptera	Syrphidae	*Melangyna viridiceps*
Diptera	Micropezidae	Micropezidae sp.55

Order	Family	Species
Diptera	Milichiidae	Milichiidae sp.22
Diptera	Muscidae	*Musca domestica*
Diptera	Muscidae	Muscidae sp.8
Diptera	Muscidae	Muscidae sp.23
Diptera	Muscidae	Muscidae sp.28
Diptera	Muscidae	Muscidae sp.30
Diptera	Muscidae	Muscidae sp.34
Diptera	Muscidae	Muscidae sp.41
Diptera	Muscidae	Muscidae sp.45
Diptera	Muscidae	Muscidae sp.47
Diptera	Muscidae	Muscidae sp.66
Diptera	Muscidae	Muscidae sp.68
Diptera	Muscidae	Muscidae sp.69
Diptera	Muscidae	Muscidae sp.70
Diptera	Muscidae	Muscidae sp.71
Diptera	Muscidae	Muscidae sp.72
Diptera	Muscidae	Muscidae sp.73
Diptera	Muscidae	Muscidae sp.74
Diptera	Muscidae	Muscidae sp.75
Diptera	Muscidae	Muscidae sp.76
Diptera	Platystomatidae	Platystomatidae sp.17
Diptera	Rhagionidae	Rhagionidae (Atherimorpha)
Diptera	Rhagionidae	Rhagionidae sp.20
Diptera	Rhagionidae	Rhagionidae sp.40
Diptera	Sciaridae	Sciaridae sp.39
Diptera	Sciomyzidae	Sciomyzidae sp.19
Diptera	Syrphidae	*Simosyrphus grandicornis*
Diptera	Syrphidae	*Sphaerophoria macrogaster*
Diptera	Tachinidae	Tachinidae sp.2
Diptera	Tabanidae	Tabanidae sp.6
Diptera	Tabanidae	Tabanidae sp.12
Diptera	Tabanidae	Tabanidae sp.14
Diptera	Tabanidae	Tabanidae sp.33
Diptera	Tachinidae	Tachinidae sp.31
Diptera	Tachinidae	Tachinidae sp.37
Diptera	Tachinidae	Tachinidae sp.38
Diptera	Tachinidae	Tachinidae sp.43
Diptera	Tachinidae	Tachinidae sp.53
Diptera	Tachinidae	Tachinidae sp.56
Diptera	Tachinidae	Tachinidae sp.58
Diptera	Tachinidae	Tachinidae sp.65
Diptera	Tipulidae	Tipulidae sp.46
Diptera	Tipulidae	Tipulidae sp.51
Hymenoptera	Alysiinae	*Alysiinae* sp.
Hymenoptera	Apidae	*Amegilla chlorocyanea*
Hymenoptera	Bethylidae	Bethylidae sp.21
Hymenoptera	Bethylidae	Bethylidae sp.24
Hymenoptera	Bethylidae	Bethylidae sp.25
Hymenoptera	Bethylidae	Bethylidae sp.5

Order	Family	Species
Hymenoptera	Bibionidae	Bibionidae sp.26
Hymenoptera	Braconidae	Braconidae sp.3
Hymenoptera	Apidae	*Exoneurella* sp. Unnamed
Hymenoptera	Formicidae	Formicidae sp.2
Hymenoptera	Formicidae	Formicidae sp.10
Hymenoptera	Formicidae	Formicidae sp.11
Hymenoptera	Formicidae	Formicidae sp.22
Hymenoptera	Formicidae	Formicidae sp.26
Hymenoptera	Halictidae	*Homalictus dotatus*
Hymenoptera	Halictidae	*Homalictus urbanus*
Hymenoptera	Hymenoptera family	Hymenoptera sp.
Hymenoptera	Hymenoptera family	Hymenoptera sp.18
Hymenoptera	Ichneumonidae	Ichneumonidae sp.1
Hymenoptera	Ichneumonidae	Ichneumonidae sp.4
Hymenoptera	Ichneumonidae	Ichneumonidae sp.8
Hymenoptera	Ichneumonidae	Ichneumonidae sp.9
Hymenoptera	Ichneumonidae	Ichneumonidae sp.19
Hymenoptera	Halictidae	*Lasioglossum* (*Parasphecodes*)
Hymenoptera	Halictidae	*Lasioglossum castor*
Hymenoptera	Halictidae	*Lasioglossum erythrurum*
Hymenoptera	Halictidae	*Lasioglossum lanarium*
Hymenoptera	Halictidae	*Lasioglossum occidens*
Hymenoptera	Halictidae	*Lasioglossum* sp.
Hymenoptera	Halictidae	*Lassioglossum* (*Chilalictus*)
Hymenoptera	Colletidae	*Leioproctus* (*Minycolletes*)
Hymenoptera	Sphecidae	*Podalonia* sp.
Hymenoptera	Thynnidae	Thynnidae sp.6
Lepidoptera	Castniidae	Castniidae sp.
Lepidoptera	Ditrysia family	*Ditrysia* sp.
Lepidoptera	Gelechioidea	Gelechioidea sp.
Lepidoptera	Lepidoptera family	Lepidoptera sp.
Lepidoptera	lycaenidae	*Neolveia agricola occidens*
Lepidoptera	Pieridae	*Pieris rapae*
Other	Orthoptera family	Orthoptera sp.
Other	Acanthosomatidae	Acanthosomatidae sp.11
Other	Auchenorrhyncha family	Auchenorrhyncha (Hemiptera) sp.21
Other	Cicadellidae	Cicadellidae sp.2
Other	Auchenorrhyncha Family	Hemiptera (Auchenorrhyncha) sp.26
Other	Miridae	Miridae sp.6
Other	Miridae	Miridae sp.9
Other	Miridae	Miridae sp.18
Other	Miridae	Miridae sp.22
Other	Pentatomidae	Pentatomidae sp.16
Other	Arachnid family	Arachnid sp.
Other	Hemerobiidae	Hemerobiidae sp.
Other	Leptophlebiidae	Leptophlebiidae sp.25
Other	Zygoptera family	Zygoptera sp.

Table S6.2 Similarity percentage analysis (SIMPER) of species detected with pan traps in orchards adjacent to pasture during flowering and non-flowering phases for *Persea americana*.

Species	Average contribution	Cumulative contribution	Position
Tachinidae sp. 56	0.22	0.25	1
Muscidae sp. 47	0.22	0.49	2
Ditrysia sp.	0.02	0.73	11
Acanthosomatidae sp.11	0	1	58
Acroceridae sp. 16	0	1	59
Anthomyiidae sp. 27	0	1	60
Athericidae sp. 3	0	1	61
Bethylidae sp. 21	0	1	62
Bethylidae sp. 24	0	1	63
Bethylidae sp. 25	0	1	64
Braconidae sp. 3	0	1	65
Castniidae sp.	0	1	68
Cerambycidae sp. 20	0	1	69
Coccinellidae sp. 15	0	1	71
Coelopidae sp. 18	0	1	72
Diptera sp. 67	0	1	73
Empididae sp. 1	0	1	75
Empididae sp. 4	0	1	76
Ephydridae sp. 21	0	1	78
Empididae sp. 62	0	1	79

Table S6.3 Similarity percentage analysis (SIMPER) of species detected with pan traps in orchards adjacent to natural vegetation during the flowering and non-flowering phase for *Persea americana*.

Species	Average contribution	Cumulative contribution	Position
Formicidae sp. 26	0.13	0.14	1
Melangyna viridiceps	0.10	0.24	2
Buprestidae sp. 5	0.08	0.33	3
Lasioglossum sp.	0.06	0.40	4
Pieris rapae	0.04	0.56	7
Dolichopodidae sp. 36	0.04	0.60	8
Calliphoridae sp. 44	0.03	0.63	9
Lassioglossum (*Chilalictus*)	0.02	0.72	12
Tachinidae sp. 38	0.02	0.78	15
Ichneumonidae sp. 8	0.01	0.80	16
Homalictus urbanus	0.01	0.85	20
Muscidae sp. 45	0.01	0.88	24
Muscidae sp. 34	0.01	0.90	27
Acanthosomatidae sp. 11	0	1.00	55
Acroceridae sp. 16	0	1.00	56
Anthomyiidae sp. 27	0	1.00	58
Athericidae sp. 3	0	1.00	59
Bethylidae sp. 21	0	1.00	61
Bethylidae sp. 24	0	1.00	62
Bethylidae sp. 25	0	1.00	63

Table S6.4 Similarity percentage analysis (SIMPER) of species detected with pan traps in orchards adjacent to either pasture or natural vegetation during the flowering phase for *Persea americana*.

Species	Average contribution	Cumulative contribution	Position
Formicidae sp. 26	0.12	0.43	3
Buprestidae sp. 5	0.07	0.51	4
Podalonia sp.	0	0.98	40
Orthoptera sp.	0	0.98	41
Acanthosomatidae sp. 11	0	1.00	51
Acroceridae sp. 16	0	1.00	52
Anthomyiidae sp. 27	0	1.00	54
Athericidae sp. 3	0	1.00	55
Bethylidae sp. 21	0	1.00	57
Bethylidae sp. 24	0	1.00	58
Bethylidae sp. 25	0	1.00	59
Braconidae sp. 3	0	1.00	60
Castniidae sp.	0	1.00	63
Cerambycidae sp. 20	0	1.00	64
Coccinellidae sp. 15	0	1.00	65
Coelopidae sp. 18	0	1.00	66
Diptera sp. 67	0	1.00	67
Empididae sp. 1	0	1.00	70
Empididae sp. 4	0	1.00	71
Ephydridae sp. 21	0	1.00	74

Table S6.5 Significance of the GLMM co-variates (flowering period and orchard adjacent land use) for arthropods species richness captured in the pan trap arrays within orchards determined using Type II Wald χ^2 test. = Sampling Period and OALU = Orchard Adjacent Land Use.

Species cohort – fixed effect	Wald χ^2	DF	*p* – value
Coleoptera – SP	2.32	3	.51
Coleoptera – OALU	1.19	1	.27
Coleoptera – Interaction	0.04	3	1.00
Lepidoptera – SP	1.69	3	.64
Lepidoptera – OALU	0.00	1	.95
Lepidoptera – Interaction	1.37	1	.71
Other – SP	2.95	3	.40
Other – OALU	1.79	1	.18
Other – Interaction	1.87	3	.60

Table S6.6 Pairwise comparisons between flowering periods for species richness of; All, Diptera and Hymenoptera species cohorts. Arthropods were captured in pan trap arrays located within orchards. All pairwise comparisons were undertaken with a Bonferroni adjustment. OALU = Orchard Adjacent Land use and SP = Sampling Period. NV = natural vegetation and P = pasture. Diptera and Hymenoptera OALU is presented with NA as there was no significant interaction effect found between OALU and SP.

Species cohort	OALU	SP comparison	Ratio	SE	*DF*	*p* – value
All	P					
		A – B	1.17	0.37	1	1.00
		B – C	0.28	0.07	1	< .01
		C – D	1.02	0.18	1	1.00
	NV					
		A – B	0.43	0.12	1	.05
		B – C	0.85	0.17	1	1.00
		C – D	0.81	0.15	1	1.00
Diptera	NA					
		A – B	1.27	0.36	1	1.00
		B – C	0.39	0.10	1	< .01
		C – D	0.84	0.15	1	1.00
Hymenoptera	NA					
		A – B	0.42	0.23	1	.64
		B – C	0.38	0.14	1	.04
		C – D	0.74	0.17	1	1.00

Table S6.7 Significance of GLMM co-variates sampling period and orchard adjacent land use, as well as the interaction effect between the two, for orchard arthropod abundance determined using Type II Wald χ^2 test. SP = Sampling Period and OALU = Orchard Adjacent Land Use.

Species cohort – co-variate	Wald χ^2	DF	*p* – value
Coleoptera – SP	3.74	4	.44
Coleoptera – OALU	1.36	2	.51
Coleoptera – Interaction	0.37	3	.95
Hymenoptera – SP	30.01	3	< .01
Hymenoptera – OALU	0.00	1	.98
Hymenoptera – Interaction	7.40	3	.06
Lepidoptera – SP	2.26	3	.52
Lepidoptera – OALU	0.26	1	.61
Lepidoptera – Interaction	2.87	3	.52
Other – SP	2.95	3	.40
Other – OALU	1.79	1	.18
Other – Interaction	1.87	3	.60

Table S6.8 Pairwise comparisons between sampling periods for abundance of All, Diptera and Hymenoptera species cohorts. Arthropods were captured in pan trap arrays located within orchards. All pairwise comparisons were undertaken with a Bonferroni adjustment. OALU = Orchard Adjacent Land use and SP = Sampling Period. NV = natural vegetation and P = pasture. Hymenoptera OALU is presented with NA as there was no significant interaction effect found between OALU and SP.

Species cohort	OALU	SP comparison	Ratio	SE	*DF*	*p* – value
All	P					
		A – B	1.11	0.40	1	1.00
		B – C	0.10	0.03	1	< .01
		C – D	0.78	0.17	1	1.00
	NV					
		A – B	0.36	0.11	1	.03
		B – C	0.93	0.24	1	1.00
		C – D	0.69	0.17	1	1.00
Diptera	P					
		A – B	1.93	0.94	1	1.00
		B – C	0.04	0.02	1	< .01
		C – D	0.77	0.18	1	1.00
	NV					
		A – B	0.93	0.35	1	1.00
		B – C	0.67	0.23	1	1.00
		C – D	0.51	0.15	1	.69
Hymenoptera	NA					
		A – B	0.29	0.15	1	.11
		B – C	0.53	0.18	1	.36
		C – D	0.57	0.15	1	.19
		A – C	0.15	0.07	1	< .01
		A – D	0.09	0.04	1	< .01

Table S6.9 Significance of GLMM co-variates sampling period and adjacent habitat type, as well as the interaction effect between the two for arthropod species richness determined using the Type II Wald χ^2 test. Arthropods were captured in pan trap arrays located within adjacent pasture or natural vegetation. SP = Sampling Period and AHT = Adjacent Habitat Type.

Species cohort – co-variate	Wald χ^2	DF	*p*–value
Coleoptera – SP	0.46	3	.93
Coleoptera – AHT	0.07	1	.79
Coleoptera – Interaction	3.34	3	.34
Diptera – SP	9.65	3	.02
Diptera – AHT	15.17	1	< .01
Diptera – Interaction	6.57	3	.09
Hymenoptera – SP	10.02	3	.02
Hymenoptera – AHT	1.59	1	.21
Hymenoptera – Interaction	9.03	3	.03
Lepidoptera – SP	0.29	4	.99
Lepidoptera – AHT	0.21	2	.90
Lepidoptera – Interaction	0.12	3	.99
Other – SP	4.14	4	.39
Other – AHT	3.31	2	.19
Other – Interaction	1.77	3	.62

Table S6.10 Pairwise comparisons for species richness of All, Diptera and Hymenoptera cohorts. Arthropods were captured in pan trap arrays located in adjacent pasture or natural vegetation. All pairwise comparisons were undertaken with Bonferroni adjustments. AHT = adjacent habitat type, NV = natural vegetation and P = pasture. Hymenoptera is presented with NA under contrast as there was no significant effect for adjacent habitat type.

Species cohort	AHT Contrast	Sampling period	Ratio	SE	DF	*p* – value
All	NV – P					
		All	2.18	0.29	1	< .01
All	NV – P					
		A	3.21	1.00	1	< .01
		B	4.00	1.10	1	< .01
		C	1.28	0.24	1	1.00
		D	1.40	0.30	1	1.00
Diptera	NV – P					
		All	1.91	0.59	1	.03
	P					
		A – C	0.12	0.08	1	.02
Hymenoptera	P					
		A – B	0.37	0.25	1	1.00
		B – C	0.33	0.13	1	.19
		C – D	1.16	0.52	1	1.00
		A – C	0.12	0.08	1	.02
	NV					
		A – B	0.83	0.29	1	1.00
		B – C	0.95	0.31	1	1.00
		C – D	0.76	0.23	1	1.00

Table S6.11 Significance of GLMM co-variates sampling period and adjacent habitat type, as well as the interaction effect between the two for arthropod abundance determined using the Type II Wald χ^2 test. Arthropods were captured in pan trap arrays located within adjacent pasture or natural vegetation. SP = Sampling Period and AHT = Adjacent Habitat Type.

Species cohort – co-variate	Wald χ^2	DF	*p* – value
Coleoptera – SP	2.24	3	.52
Coleoptera – AHT	0.30	1	.58
Coleoptera – Interaction	4.10	3	.25
Diptera – SP	29.58	3	< .01
Diptera – AHT	1.02	1	.31
Diptera – Interaction	7.76	3	.05
Hymenoptera – SP	10.17	3	.02
Hymenoptera – AHT	1.34	1	.25
Hymenoptera – Interaction	16.96	3	< .01
Lepidoptera – SP	0.29	4	.99
Lepidoptera – AHT	0.21	2	.90
Lepidoptera – Interaction	0.12	3	.99
Other – SP	8.33	4	.08
Other – AHT	3.31	2	.19
Other – Interaction	1.77	3	.62

Table S6.12 Pairwise comparisons between adjacent habitat type and sampling periods for arthropod abundance of All, Hymenoptera and Diptera. Arthropods were captured in pan trap arrays located in adjacent pasture or natural vegetation. All pairwise comparisons were undertaken with a Bonferroni adjustment. SP = Sampling Period and AHT = Adjacent Habitat Type, NV = natural vegetation and P = pasture.

Species cohort	AHT	SP comparison	Ratio	SE	*DF*	*p* – value
All	NV-P					
		All	1.93	0.50	1	.01
All	P					
		A – B	0.46	0.17	1	1.00
		B – C	0.23	0.07	1	< .01
		C – D	1.47	0.42	1	1.00
All	NV					
		A – B	0.59	0.17	1	1.00
		B – C	0.94	0.26	1	1.00
		C – D	1.47	0.41	1	1.00
Diptera	P					
		A – B	0.45	0.21	1	1.00
		B – C	0.23	0.09	1	.01
		C – D	1.60	0.60	1	1.00
Diptera	NV					
		A – B	0.72	0.29	1	1.00
		B – C	0.56	0.22	1	1.00
		C – D	1.60	0.62	1	1.00
Hymenoptera	P					
		A – B	0.49	0.31	1	1.00
		B – C	0.23	0.10	1	.03
		C – D	1.88	0.68	1	1.00
Hymenoptera	NV					
		A – B	0.48	0.15	1	.53
		B – C	1.64	0.50	1	1.00
		C – D	0.97	0.33	1	1.00

Table S6.13 Conventional floral surveys for orchards (O), adjacent pasture (P) and adjacent natural vegetation (NV). Surveys were undertaken following Fisher, Gonthier, Ennis, and Perfecto (2017). Average proportions are calculated from four repeated measures of flower cover using $1m^2$ quadrats.

Sampling period	Site	Site (O/P/NV)	Species	Average proportion	SE
A	BA	P	*Trifolium subterraneum*	0.40	0.08
			Romulea rosea	< 0.01	0
			Arctotheca calenolula	0.01	0.01
A	BA	O	*Lysimachia arvensis*	<0.01	0
			Arctotheca calenolula	0.03	0.02
			Raphanus raphanistrum	< 0.01	0
			Trifolium repens	< 0.01	0
A	SD	O	*Lysimachia arvensis*	0.01	0.01
			Trifolium repens	0.01	0.01
A	SD	NV	*Bossiaea aquifolium*	0.31	0.10
			Hovea elliptica	0.15	0.08
			Bossiaea ornata	0.01	0.01
A	BA	O	No flowering	-	-
A	BA	P	*Lysimachia arvensis*	0.01	0.01
			Arctotheca calenolula	0.02	0.01
			Poa annua	0.01	0.01
			Trifolium subterraneum	0.04	0.02
A	DC	O	*Sonchus oleraceus*	0.04	0.02
			Trifolium subterraneum	0.01	0
			Cerastium glomeratum	0.03	0.02
			Poa annua	0.01	0
			Stachys arvensis	0.01	0.01
A	DC	NV	*Hibbertia cuneformis*	0.03	0.02
			Clematis pubescens	0.01	0
			Hovea elliptica	0.01	0.01
			Hardenbergia comptoniana	0.01	0.01
			Trymalium lediforium	0.15	0.13
A	MB	O	*Trifolium subterraneum*	0.15	0.10
			Arctotheca calenolula	0.03	0.02
			Raphanus raphanistrum	0.12	0.08
A	MB	P	*Arctotheca calenolula*	0.01	0
			Trifolium subterraneum	0.03	0.01
A	PB	O	*Arctotheca calenolula*	< 0.01	0
			Raphanus raphanistrum	0.01	0.01
			Brassica tournefortii	0.01	0
			Capsella bursa-pastoris	0.01	0
A	PB	NV	*Clematis pubescens*	0.03	0.02
			Hardenbergia comptoniana	0.08	0.06
			Hibbertia cuneformis	< 0.01	0
B	PB	O	*Trifolium subterraneum*	< 0.01	0
			Orobanche minor	0.01	0

Sampling period	Site	Site (O/P/NV)	Species	Average proportion	SE
B	PB	O	*Arctotheca calenolula*	0.14	0.04
			Trifolium repens	0.17	0.06
B	PB	NV	*Hibbertia cuneformis*	0.01	0
			Hardenbergia comptoniana	0.01	0
			Tremandra stelligera	0.01	0
			Clematis pubescens	0.01	0
B	BD	O	*Bromus catharticus*	0.06	0.02
B	BD	P	*Cerastium glomeratum*	< 0.01	0
			Geranium molle	< 0.01	0
			Trifolium subterraneum	0.02	0.01
			Arctotheca calenolula	< 0.01	0
B	BA	O	*Raphanus raphanistrum*	0.01	0.01
B	BA	P	*Arctotheca calenolula*	0.03	0.01
			Trifolium subterraneum	0.01	0.01
			Erodium moschatum	< 0.01	0
B	SA	O	*Poa annua*	0.01	0
			Trifolium repens	0.01	0
B	SA	NV	*Hovea elliptica*	0.06	0.05
			Bossiaea ornata	0.03	0.02
			Hardenbergia comptoniana	0.04	0.03
			Tremandra stelligera	0.01	0.01
B	MB	O	*Arctotheca calenolula*	0.04	0.02
			Trifolium subterraneum	< 0.01	0
			Hordeum glaucum	< 0.01	0
B	MB	P	*Raphanus raphanistrum*	0.03	0.02
			Arctotheca calenolula	0.01	0
			Trifolium subterraneum	0.06	0.01
			Taraxacum officinale	< 0.01	0
B	DC	O	*Arctotheca calenolula*	0.01	0
			Trifolium subterraneum	0.02	0.01
			Poa annua	0.04	0.02
			Hordeum glaucum	0.01	0
B	DC	NV	*Hibbertia cuneformis*	0.02	0.01
			Tremandra stelligera	0.01	0
			Hovea elliptica	0.01	0.01
C	SD	O	*Lysimachia arvensis*	0.01	0
			Lolium perenne	0.03	0.02
			Cerastium glomeratum	0.01	0
			Trifolium repens	< 0.01	0
C	SD	NV	No flowering	-	-
C	BD	O	*Lolium perenne*	0.23	0.09
			Epilobium tetragonum	< 0.01	0
C	BD	P	*Lolium perenne*	0.11	0.03
			Trifolium subterraneum	< 0.01	0
			Geranium molle	0.01	0.01
			Cerastium glomeratum	<0.01	0
C	PB	O	*Arctotheca calenolula*	0.06	0.02
			Trifolium repens	0.01	0.01

Sampling period	Site	Site (O/P/NV)	Species	Average proportion	SE
C	PB	O	*Raphanus raphanistrum*	< 0.01	0
			Poa annua	0.01	0
			Lolium perenne	0.01	0
C	PB	NV	*Hibbertia cuneformis*	<.0.01	0
C	BA	O	*Avena fatula*	0.04	0.03
			Lolium perenne	0.04	0.03
			Raphanus raphanistrum	0.03	0.02
C	BA	P	*Bromus catharticus*	0.09	0.06
			Lolium perenne	<0.01	0
C	MB	O	*Bromus catharticus*	0.03	0.01
			Raphanus raphanistrum	0.01	0.01
C	MB	P	*Taraxacum officinale*	0.02	0.01
			Bromus catharticus	0.05	0.03
C	DC	O	No flowering	-	-
C	DC	NV	*Hibbertia cuneformis*	0.02	0.01
			Tremandra stelligera	0.01	0
D	SD	O	*Trifolium repens*	< 0.01	0
			Cerastium glomeratum	< 0.01	0
			Raphanus raphanistrum	< 0.01	0
			Rumex acetosella	< 0.01	0
			Holcus lanatus	0.01	0.01
			Taraxacum officinale	< 0.01	0
			Lotus subbiflorus	< 0.01	0
D	SD	NV	*Agrostocrinum hirsutum*	< 0.01	0
			Tetrarrhena laevis	< 0.01	0
D	BD	O	*Rumex acetosella*	0.01	0
			Lysimachia arvensis	0.02	0.01
			Epilobium tetragonum	0.01	0
			Lolium perenne	0.01	0
D	BD	P	*Lolium perenne*	0.05	0.02
D	BA	O	No flowering	-	-
D	BA	P	*Bromus catharticus*	< 0.01	0
D	PB	O	*Raphanus raphanistrum*	< 0.01	0
			Capsella bursa-pastoris	< 0.01	0
			Trifolium tomentosum	< 0.01	0
			Trifolium repens	0.03	0.02
			Arctotheca calenolula	< 0.01	0
			Lolium perenne	0.01	0.01
D	PB	NV	*Hibbertia cuneformis*	0.01	0
			Banksia grandis	0.01	0.01
D	DC	O	*Lolium perenne*	0.02	0
			Stachys arvensis	0.01	0.01
			Cerastium glomeratum	< 0.01	0
D	DC	NV	*Tremandra stelligera*	< 0.01	0
			Hibbertia cuneformis	< 0.01	0
D	MB	O	*Lolium perenne*	0.01	0
			Raphanus raphanistrum	0.03	0.03
			Sonchus oleraceus	< 0.01	0
D	MB	P	*Taraxacum officinale*	< 0.01	0

Chapter 7. General Discussion

7.1 Summary of findings

Surveys that use environmental DNA (eDNA) metabarcoding in agroecosystems, either in isolation or as a complementary method, remain a developing field, meaning there exists significant potential for the application of this molecular tool. In this thesis, I explored technical and methodological considerations when undertaking eDNA-based surveys within agroecosystems. Thesis findings are synthesised in Figure 1.1. In addition, I also discuss key knowledge gaps, as well as the limitations identified during the process of this research, which, once addressed, will help increase the capability and reliability of eDNA-based monitoring. Overall, the goal of this thesis is to develop and assess the utility of eDNA metabarcoding for surveying arthropods and the foraging resources upon which they rely. The main findings are summarised below.

eDNA is an emerging survey tool, which is currently underutilised in agriculture

The published global literature review in Chapter 2 examined the diverse applications of eDNA-based surveys in agriculture, while also highlighting that eDNA remains a nascent method in these systems. This chapter identified the majority of eDNA-based surveys in agriculture targeted either arthropods or microorganisms, largely from soil and plant substrates (Figure 1.1). Current applications of this molecular technology have highlighted the benefits for detecting a wide range of ecologically significant taxa (e.g. antagonistic crop pests and beneficial pollinators), without the need for significant time commitments (for field-based observations) or extensive taxonomic expertise. However, this chapter also highlights that greater integration of eDNA for agricultural monitoring relies upon the following considerations: a) the local factors that influence DNA deposition, dispersal and persistence, b) whether a 'needle and haystack' approach is required to target specific species or broader biological communities, c) an *a priori* understanding of which locally relevant species may be affected by biased amplification, d) which unknown species are missing from online databases, such as GenBank and the Barcode of Life Data System (Meiklejohn, Damaso, & Robertson, 2019), and e) integration of cross-validation techniques to generate abundance data and capture detections missed by eDNA surveys.

eDNA metabarcoding characterised different assemblages of arthropod diversity compared to conventional survey methods

Chapter 3 tested if the eDNA metabarcoding of crop flowers could be used to detect a similar cohort of arthropods compared to those measured by Digital Video Recordings (DVRs) and pan traps (Figure 1.1). I demonstrated that eDNA-based surveys can generate similar community diversity measures to these conventional methods, while also increasing the total number of

arthropod taxa detected. This research also highlights that each survey method characterised a different aspect of the total arthropod community present within the orchard, which is likely a reflection of detection biases (e.g. DVRs may not consistently detect small arthropod taxa; see Johnson et al., 2023). Additionally, the inclusion of DVRs helped to cross-validate the eDNA detections and reveal false negatives within the eDNA dataset. Some arthropod families (e.g. Calliphoridae and Pompilidae) were observed visiting sample flowers by the DVRs, but were completely absent from the eDNA dataset, suggesting that eDNA metabarcoding may fail to detect some common flower-visiting arthropods (Gomez, Sørensen, Chua, & Sigsgaard, 2023; Thomsen & Sigsgaard, 2019). By comparing the detections from the two eDNA metabarcoding assays, I was able to determine that the assay targeting the 16S ribosomal RNA subunit gene had a limited number of reference sequences and was only able to find three additional arthropod families not detected by the assay targeting Cytochrome Oxidase subunit 1 region (COI). In contrast, 14 arthropod families were unique to the assay targeting the COI region, while seven families were shared between both assays.

Beneficial and antagonistic arthropods vary both spatially and temporally

Chapter 2 identified that eDNA metabarcoding can be used to detect both beneficial and antagonistic arthropod taxa, while Chapter 3 established that eDNA metabarcoding of flowers could generate comparable diversity measures to those generated by conventional survey methods (Figure 1.1). Subsequent to this research, Chapter 4 explored the use of eDNA-metabarcoding of flower samples to examine temporal (crop flowering intensity) and spatial (within trees and between orchards) variation for arthropod pollinators, pests and predator taxa. In congruence with DVR sampling, eDNA metabarcoding detected a greater diversity and relative abundance of known pollinators, pests and predators during greater crop flowering. The use of eDNA also allowed for a relatively accessible measure of arthropod diversity in response to inflorescence location within the canopy, something not easily achievable with the DVRs. At the spatial scale of separate orchards, eDNA detections confirmed that arthropod communities became more similar during increased crop flowering, suggesting that mass-flowering crops may attract similar arthropods taxa from the surrounding landscape (Willcox et al., 2019). This chapter affirms some necessary considerations for eDNA metabarcoding arthropods in agroecosystems, namely, the tendency to detect smaller and more frequently visiting arthropods, which is likely due to more opportunities for DNA deposition and subsequent detection (Johnson et al., 2023; Valentin, Fonseca, Nielsen, Leskey, & Lockwood, 2018). Thus, the accuracy of eDNA sampling can be increased with the inclusion of multiple methods to account for the inherent biases of this molecular technique (Newton, Bateman, Heydenrych, Mousavi-Derazmahalleh, & Nevill, 2023).

The inclusion of eDNA metabarcoding, in conjunction with other complementary methods, can enable rapid and accurate assessments which could help inform agricultural management practices by providing timely feedback on biodiversity.

Pan-trap water is a suitable substrate to capture plant, but not arthropod, eDNA

In Chapter 2, I identified that unmanaged arthropod taxa (e.g. native bees, flies, wasps and moths) are generally omitted from agricultural surveys, which is largely a result of *a priori* information availability and that conventional surveys normally used for detecting them are difficult to apply at the scale of agriculture (Rader et al., 2016) (Figure 1.1). For Chapters 3 and 4, flower samples were collected and tested using invertebrate assays (Figure 1.1). However, these surveys did not provide information about what other plant resources may encourage the persistence of these unmanaged arthropods within orchards. In Chapter 5, used a conventional survey tool (pan traps) in a novel way, by metabarcoding pan trap water to detect captured arthropods and the pollen and plant material upon which they foraged (Figure 1.1). eDNA metabarcoding of pan-trap water enabled the consistent detection of plant taxa across all of the pan trap arrays and the three most commonly detected plant families are all known to require animals to facilitate or enhance fruit set. In contrast to the plant detections, arthropods were not consistently detected, despite specimens being present in all of the pan trap samples. Further, when arthropods were detected using eDNA, there was little overlap with the morphological identifications. I highlight that further methodological refinements (e.g. suspending captured arthropods in lysis buffer; see Kirse, Bourlat, Langen, Zapke, & Zizka, 2023) are needed to increase the reliability of eDNA metabarcoding pan trap water to detect arthropods.

Adjacent natural vegetation alters arthropod community composition and enhances foraging resources in orchards

In Chapter 5, eDNA metabarcoding was used to assess the foraging resources supporting arthropods in agroecosystems across three orchards in SWWA (Figure 1.1). For Chapter 6, I aimed to identify whether the presence of adjacent natural vegetation enhanced orchard arthropod diversity and abundance and provided more diverse arthropod-plant foraging resources compared to orchards adjacent to pasture. I sampled across six orchards, adjacent to either to pasture or natural vegetation, at four time points corresponding to different crop flowering intensities. Pan traps were used to capture arthropods, which were preserved for morphological identification. The nectar, pollen and plant tissue carried on arthropod bodies was targeted by eDNA metabarcoding the pan-trap water. I found that within orchards, arthropod diversity and abundance was not significantly enhanced by the presence of natural vegetation. However, pan traps within the adjacent natural vegetation captured significantly higher levels of arthropod diversity and

abundance, compared to pan traps in pasture habitats. These patterns of arthropod diversity and abundance likely reflect greater habitat complexity and resource availability of natural vegetation habitats (Chaplin-Kramer, O'Rourke, Blitzer, & Kremen, 2011). In both orchards and adjacent habitats, arthropod diversity increased with greater maximum daily temperatures, although not with greater crop flowering (Steen, 2017; Wilson & Jamieson, 2019). These patterns likely reflect the increase in foraging opportunities with more favourable weather conditions (i.e. low wind and warmer temperatures; see Prendergast, Menz, Dixon, & Bateman, 2020). By eDNA metabarcoding pan-trap water, I was able to show that orchards adjacent to natural vegetation, during low *P. americana* flowering, contained a greater arthropod-plant foraging diversity compared to those collected adjacent to pasture. These findings indicate that natural vegetation habitats may enhance foraging resources for arthropods in agroecosystems at certain time periods (e.g. when adjacent pasture is actively grazed), and that certain arthropod taxa may extend their foraging ranges to utilise the rich foraging rewards afforded from these areas of natural capital (Albrecht et al., 2010; O'Donnell & Wright, 2021). The foraging resources provided by areas of natural capital, which support managed and unmanaged arthropods in agroecosystems, are often overlooked during monitoring (see Rader et al., 2016). By combining pan traps with eDNA metabarcoding, I demonstrate that this novel approach can provide a scalable method capable of evaluating how natural capital enhances arthropod foraging diversity in agroecosystems.

Monitoring arthropods and their foraging preferences can be challenging

In this thesis, I used eDNA metabarcoding to survey for arthropod diversity, as well as the diversity of the plants upon which they rely. Chapters 4 and 5 illustrate how eDNA-based surveys do not always capture the taxa of interest, and can be highly variable depending on the time of sampling and the characteristics of the organisms being targeted (Kudoh, Minamoto, & Yamamoto, 2020; Thomsen & Sigsgaard, 2019). To accurately detect taxa using eDNA metabarcoding, surveys must be tailored towards the question and system of interest. These topics are discussed in the next section.

7.2 Technical and methodological considerations for the application of eDNA metabarcoding to monitoring arthropods and associated foraging resources in agroecosystems

The purpose of this thesis was to assess the efficacy of eDNA-based surveys and ultimately improve monitoring in agroecosystems. This technique was successfully applied for both arthropod and plant taxa (see previous section) and across multiple orchards. Accordingly, this thesis examines many of the factors that need to be taken into consideration when designing and implementing an eDNA-based surveys. This section encapsulates all of the learnings from these studies, the limitations to this technology, as well as the future research necessary to increase the reliability and accuracy of eDNA metabarcoding for agricultural monitoring. I use these considerations to develop a framework for eDNA-based survey design in agriculture (Figure 7.1).

7.2.1 Research questions

The nature of the research question posed will influence how an eDNA-based survey will be designed and implemented (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). For instance, researchers need to determine if a general survey (detecting the widest possible diversity of taxa) or targeted approach (detecting only certain species or taxonomic groups, e.g. flower-visitors) to capture certain taxonomic groups is necessary. Both questions diverge in terms of sampling methods. General surveys often require a greater number of substrates with multiple metabarcoding assays to target a diverse array of taxa (e.g. targeting both arthropod and fungal taxa in vineyards; see Rasmussen et al., 2021). Whilst targeted surveys may only sample one substrate, generally with a single metabarcoding and/or species-specific assay (e.g. crop wash water used for species-specific barcoding of the spotted lanternfly, *Lycorma delicatula*; see Valentin et al., 2020). The question posed must also acknowledge the ecological process that researchers wish to understand better.

eDNA-based detections for arthropod pollinators remains a largely unexplored area of research (Evans & Kitson, 2020). One of the principal reasons for this is that eDNA metabarcoding alone cannot be used to discern which taxa pollinated a flower (King, Ballantyne, & Willmer, 2013; Newton et al., 2023). Instead, eDNA-based surveys are only able to capture flower visits, which are detected either from plant-centric (e.g. flower tissue; see Thomsen & Sigsgaard, 2019), or animal-centric sampling (e.g. pollen from the bodies of arthropods; see Pornon, Andalo, Burrus, & Escaravage, 2017) (Evans & Kitson, 2020). This thesis has shown that arthropods detected from flower surfaces can be representative of a wide variety of ecological niches (e.g. pests and parasites) and are, therefore, not only representative of potential pollinators (Chapters 3 and 4). To

identify pollination by a particular taxa, bagging experiments are required to align a particular flower-visit with the successful fertilisation and fruit development for the visited flower (Bishop & Nakagawa, 2021). Thus, when designing a research question around detecting animals that interact with crop flowers, researchers must determine if the resolution of 'flower visitor' is sufficient, or if additional bagging experiments are necessary to classify the pollination services offered by particular taxa.

eDNA-based surveys hold significant potential to allow for the rapid identification of arthropods and plants over entire agroecosystems (Galimberti et al., 2014; Gomez et al., 2023). However, such surveys are not without their challenges, especially for accurately monitoring unmanaged flower visiting species. 'Dark taxa', otherwise known as 'false negatives' may arise within a dataset when a particular species has not previously been sequenced (Ficetola et al., 2015; Saccò et al., 2022). Even though the DNA from these species may be present in the sample, the absence of a matching reference sequence will lead to this taxa being erroneously classified as undetected (Ficetola et al., 2015; Saccò et al., 2022). False negatives are especially problematic for locally restricted and endemic species, such as unmanaged flower-visitors, which may not have been previously sequenced (Hebert et al., 2016). This thesis has demonstrated that if unmanaged arthropod taxa are of interest, complementary methods (e.g. pan traps; Chapters 3, 5 and 6) should be included to capture whole specimens for morphological identification and barcoding.

7.2.2 The type of agricultural system being surveyed

The question of 'how best to represent a particular agroecosystem using eDNA-based surveys?' will vary depending on the system under study. The size of areas used for farming can vary substantially, for instance, farms in emerging economies typically occupy an area of ca. 1.6 ha, while in more developed economies, the average area used is ca. 51 ha (Adamopoulos & Restuccia, 2014). Thus, the number of samples required to detect individual species or capture community diversity will reflect: a) the size of the farm, b) the level of habitat heterogeneity within the agroecosystem (e.g. monoculture vs. mixed-planting) , and c) the number of microhabitats of interest (e.g. measuring vertical stratification within crop trees; see Frimpong, Gemmill-Herren, Gordon, & Kwapong, 2011) (Erickson, Merkes, & Mize, 2019). Further, as this thesis has shown, the accuracy of eDNA-based surveys increases with a greater number of field and laboratory replicates, although at increased costs (Ficetola et al., 2015; Lanzén, Lekang, Jonassen, Thompson, & Troedsson, 2017). Therefore, if the agroecosystem under study is large and requires a greater number of samples than is economically viable, or if survey budgets are limited, pooling samples and increasing the number of technical replicates in the laboratory is recommended to provide

greater survey accuracy and reliability (Lanzén et al., 2017; Mauvisseau et al., 2019). As well as accurately representing the habitat characteristics of a particular agroecosystem, researchers may also need to include measures from adjacent habitats.

Surveys of habitats adjacent to agricultural systems (e.g. pasture lands, natural vegetation, etc.) are rarely incorporated into agricultural monitoring programs (see Chaperon et al., 2022), meaning, the value of these areas for maintaining and increasing beneficial ecosystem services (e.g. pollination) are largely unexplored (Cole, Brocklehurst, Robertson, Harrison, & McCracken, 2017; Garibaldi et al., 2014). This thesis demonstrates that the presence of adjacent natural vegetation can increase arthropod diversity and abundance, as well as the diversity of foraging resources within agroecosystems (Chapter 6). The inclusion of these measures in future agricultural surveys could help detect reservoirs of diversity within these typically monoculture systems, thereby adding tangible value to these otherwise overlooked natural assets (Kleijn et al., 2015; Morandin & Winston, 2006). Depending on the target substrate, researchers may wish to sample flowers (to measure arthropod diversity) or arthropods (to examine pollen loads) in the adjacent habitat(s) (e.g. Gervais, Chagnon, & Fournier, 2018).

Crop flowering provides a temporal constraint for eDNA-based surveys (Gomez et al., 2023). These flowering windows are unique to each crop species and may change between sampling years. For instance, in this thesis, *P. americana* bloomed for one month in 2020 (Chapter 4), while in 2021, blooming occurred sporadically over two months (Chapter 6). Seasonal variation of crop flowering can also lead to fluctuations in arthropod diversity and abundance (Senapathi et al., 2021). For example in this thesis, Syrphidae spp. were highly abundant in 2020, although in 2021 they were detected in much lower numbers (Chapters 4 and 6). Thus, crop flowering windows are a necessary consideration when designing eDNA-based surveys and can be adequately accounted for by incorporating measurements from multiple years (see Mathieu et al., 2020) to account for inter-annual variability.

7.2.3 Target substrate

The choice of target substrate is critical for detecting specific species or entire communities of interest (Evans & Kitson, 2020). In this thesis, I targeted both DNA left on flowers by visiting arthropods, as well as the pollen collected on the bodies of arthropods. By sampling the DNA deposited on flowers (Chapters 3 and 4), I was able to detect potential pollinators, as well as crop pests and predators. Whilst sampling the pollen carried on the bodies of captured arthropods (Chapters 5 and 6), helped me determine which plant species provided foraging resources for the

captured community. However, one limitation of this thesis is that it did not combine eDNA metabarcoding with bagging experiments to determine which flower-visiting arthropods facilitated pollination of *P. americana*. Instead, I relied on previous research and bagging experiments performed by other researchers (Cook et al., 2020; Dymond et al., 2021; Sagwe, Peters, Dubois, Steffan-Dewenter, & Lattorff, 2022). To date, no studies have combined eDNA-barcoding or metabarcoding with bagging experiments, although this approach may prove useful for detecting plant-animal interactions that are often brief and difficult to observe. Such an approach would require swabbing of the flower (see Harper et al., 2023), rather than collecting whole flowers, to determine if the flower visit results in fertilisation and subsequent fruit development. As well as highlighting the potential of additional substrates, this thesis has also demonstrated that pilot studies are necessary to examine if a chosen substrate can provide reliable detections for the taxa of interest. For instance, consistent arthropod detections were found from flower eDNA samples (Chapter 3), but not from pan-trap water samples (Chapter 5), likely due to the exoskeletons of arthropods preventing the exchange of DNA with the water substrate (Zizka, Leese, Peinert, & Geiger, 2018). Thus, before eDNA monitoring can be used in a particular agroecosystem, substrate calibration with pilot studies remains a critical initial step to optimise sampling for reliable eDNA detections.

7.2.4 Survey design

As a general rule, the detections from any one monitoring method will always be biased for or against certain taxa (Newton et al., 2023; Prendergast et al., 2020; Spafford & Lortie, 2013). This thesis has demonstrated that eDNA metabarcoding of flowers is biased towards detecting smaller and more frequently visiting arthropods compared to DVRs (Chapters 3 and 4). For example, with eDNA, we were able to consistently detect Syrphidae spp., the most common flower-visiting species observed by DVRs, however, we did not detect larger Calliphoridae sp., which had far fewer flower visits (Chapter 4). Likely, lower flower visits resulted in fewer opportunities for DNA deposition and subsequent detection (Johnson et al., 2023). These findings highlight that cross-validation remains necessary for eDNA-based surveys to help prevent biased detections (Kelly et al., 2017). In the present thesis, complementary methods were included in each data chapter to determine if arthropod (Chapters 3 and 4) and plant taxa (Chapters 5 and 6) were missed by eDNA metabarcoding. Additionally, the inclusion of complementary methods may enable measures of abundance, which is often difficult to infer from eDNA data (Johnson et al., 2023), as well as detections of dark taxa not available in the online databases.

Arthropod and plant detections may fluctuate in response to spatial (e.g. microhabitat, farm or landscape) and temporal (e.g. diurnal, seasonal or annual) variation (Campbell, Melles, Vaz, Parker, & Burgess, 2018; Smart et al., 2017). When incorporating eDNA-based surveys into agricultural monitoring, there may be a need to include additional replicates and sampling points to capture spatial and/or temporal fluctuation for the taxa of interest (Chapters 4 and 6). The inclusion of these additional samples can, for example, help detect shifts in arthropod diversity and abundance across farms in response to flowering patches (Fisher, Gonthier, Ennis, & Perfecto, 2017; Hemberger & Gratton, 2018). In this thesis, the inclusion of samples in orchards at low and peak flowering helped to identify that arthropod diversity and abundance increased in response to peak flowering (Chapter 4). However, these measures were only collected over one season and may not reflect inter-annual variation (Bosch, Martín González, Rodrigo, & Navarro, 2009; Senapathi et al., 2021; Willcox et al., 2019). Thus, future monitoring efforts using eDNA metabarcoding may wish to survey over multiple seasons or years to determine how long taxa persist within the agroecosystem under study.

One of the strengths of eDNA metabarcoding is the ability to tailor this molecular method to suit almost any sampling approach (Ruppert, Kline, & Rahman, 2019; Taberlet et al., 2012). Through combining conventional passive approaches (e.g. vane traps and pitfall traps) or active approaches (e.g. sweep netting and vacuuming) with eDNA technology, previous studies have been able to specifically target a community of interest (e.g. vane-traps to capture arthropods when crops are not in flower; see Rasmussen et al., 2021), while also benefiting from the significant taxonomic resolution afforded by this molecular technology (e.g. Macgregor et al., 2019; Pornon et al., 2017; Rasmussen et al., 2021). Despite this extant research, there remains a paucity of multi-method approaches for monitoring in agriculture. In Chapter 5, a novel multi-method research design was undertaken which combined pan traps and dust collectors with eDNA metabarcoding to target both arthropod and plant communities in orchards. For eDNA monitoring in agroecosystems, there exists numerous opportunities to link conventional survey methods and mechanised equipment with eDNA sampling (Chapter 2). By tailoring these ‘typical’ approaches in agriculture, researchers may be able to generate timely and accurate taxonomic detections over broader areas and at a higher frequency.

7.2.5 Sampling considerations

Prior to undertaking field work, it was deemed necessary to consider: the available time, budget constraints, experimental design and locally relevant factors affecting DNA degradation (Takahashi et al., 2023). Two of the limitations pertaining to this research were the time available

to collect all of the given samples and the budget to accommodate this sampling effort. First, the time available depends on the agricultural system under study (see section *7.2.2 The type of agricultural system being surveyed*), where the duration of crop flowering may be the most significant factor limiting time in the field. Such limitations can make sampling difficult, especially when trying to capture numerous ecological interactions (Gomez et al., 2023; Johnson et al., 2023). If time is a limiting factor, then a multi-method approach may prove useful to maximise the amount of taxonomic and ecologically information obtainable over shorter sampling periods (Valdivia-Carrillo, Rocha-Olivares, Reyes-Bonilla, Domínguez-Contreras, & Munguia-Vega, 2021). In Chapter 3, by combining eDNA metabarcoding of flowers with DVRs and pan traps, both flower-visits and general arthropod diversity measures were captured in a single orchard over two sampling days. Second, the available budget for monitoring will influence the number of samples able to be processed. With smaller budgets, pooling of eDNA samples can help to reduce overall expenditure, without omitting potentially ecologically informative samples (Brunner, 2020). In Chapters 5 and 6, pan traps were pooled (one blue, one white and one yellow bowl combined per pan trap array) and this reduced the number of samples for extraction from 135 to 45. However, it is important to note that when samples are pooled, there is a corresponding reduction in spatial resolution (Brunner, 2020). Thus, by pooling samples across multiple sites, researchers will not be able to determine where particular taxa were detected, meaning that site specific management actions (e.g. hive placement or pesticide application) may be more difficult to implement.

Applications of eDNA to monitor arthropod and plant diversity required an understanding of between-sample variation to generate accurate and reliable results. This thesis used either five (Chapters 5 and 6) or ten (Chapters 3 and 4) in-field replicates to minimise the risk of false negatives and under-representative samples (Macher et al., 2021; West et al., 2020). The inclusion of three or more replicates will differ according to the diversity and variability within the chosen substrate. For instance, due to the stochastic nature of arthropod detections from flowers (see Newton et al., 2023; Thomsen & Sigsgaard, 2019), a larger number of replicates was deemed necessary in Chapters 3 and 4 to detect a wider range of arthropod taxa. In general, three replicates is the minimum number required and additional replicates should be added according to species accumulation curves, ideally established with pilot studies in the agricultural system of interest (Chapters 2 and 3). However, additional replicates also add additional cost, while the diversity of some taxa (e.g. arthropods) may be difficult to adequately capture even with a large number of samples (Chapter 4). Under such circumstances, it may be necessary to pool samples into one composite homogenised sample (e.g. Kamoroff & Goldberg, 2018), which can then be

subsampled, though this may come at the cost of reduced statistical power and ability to detect change in the chosen study system.

False positives generated from contamination are persistent issues for eDNA-based surveys, largely due to the ubiquitous nature of DNA in almost any environment (Clare et al., 2021; Johnson, Fokar, Cox, & Barnes, 2021). Negative controls were included in all of the chapters in this thesis to reduce the generation of false positives and over-inflation of diversity measures (Ficetola et al., 2015). For example, in Chapters 5 and 6, I included marble traps as negative controls to capture airborne pollen and plant material (see Johnson, Cox, & Barnes, 2019). The detections from these controls were then used as minimum sequence copy thresholds for those same species in the pan traps (see section *7.2.7 Laboratory processing and bioinformatics*). It is important to note however, that using marble traps in this way assumes that DNA in pan traps and marbles traps is similar, though to date this assumption has not yet been validated. Thus, we recommend that future studies use multiple approaches to generate minimum sequence copy thresholds (see section *5.5.3 Pan trap pollen – ambient or animal-mediated?*) to reduce the biases associated with any one method. As well as including informative negative controls in the field, it was also critical to incorporate these measures of contamination throughout the eDNA workflow, from DNA extraction through to PCR (West et al., 2020). This was especially true of plant eDNA, which is often prevalent in the air (Jantunen & Saarinen, 2011; Johnson et al., 2021). As eDNA-based surveys are gradually incorporated into agricultural monitoring, reporting of common contaminants is essential for replicability, establishing thresholds, above which, a species should be counted as detected (see Drake et al., 2022) and identifying where additional negative controls may be included to help standardise applications of this molecular technology.

Locally relevant factors affecting the persistence of DNA (e.g. UV, temperature and microbial presence) are an important consideration for eDNA-surveys in agriculture. In Chapter 3, I found that only 17% of pan trap samples contained a sufficient quantity of arthropod eDNA to be detected, despite morphological identifications confirming the presence of arthropod taxa in all of the pan trap samples. Here, the increase in temperature of pan-trap water in the field may have accelerated the degradation of arthropod eDNA, potentially contributing to the lower detection rate for these taxa (Strickler, Fremier, & Goldberg, 2015). To account for degradation, pilot studies are necessary to measure the persistence of DNA on the target substrate. For instance, Valentin, Kyle, Allen, Welbourne & Lockwood (2021) showed that simulated rainfall can remove between 75 – 100% of arthropod DNA from leaf surfaces. Such prior information is critical for accurately interpreting community assemblages detected with eDNA. A limitation of this thesis is that I did

not undertake these baseline studies for either arthropod DNA collected from flowers (Chapters 3 and 4) or plant and arthropod DNA collected from pan traps (Chapter 5). Instead, I relied on shorter sampling windows to minimise DNA degradation (e.g. Gomez et al., 2023; Newton et al., 2023; Thomsen & Sigsgaard, 2019). Future studies should, however, use *in vitro* (lab-based) pilot studies to establish how long DNA remains detectable and what sized fragments amplify successfully (e.g. Kudoh et al., 2020; Macher et al., 2016). With this information, eDNA-based surveys can account for potentially challenging sampling environments (e.g. tropical climates; see Sirois & Buckley, 2019) with more frequent sampling, or by using assays that target shorter (and more degraded) fragments of DNA (Goldberg, Strickler, & Fremier, 2018).

7.2.6 Sample preservation

eDNA preservation can have a significant impact on the successful detection of taxa from environmental samples (Chung et al., 2017; Haile et al., 2009). Through a combination of microbial and enzyme activity, as well as chemical reactions (e.g. oxidative), DNA fragments degrade, even after they have been collected (Lindahl, 1993; Takahashi et al., 2023). Thus, the means by which a sample is preserved is often critical to slowing this degradation and maintaining the diversity captured within the sample. Three options are generally available for preserving samples; i) fridge/ice, ii) freezing, and iii) preservation buffer (Takahashi et al., 2023). Immediately freezing samples is often regarded as the best approach for preventing DNA degradation, although there are numerous practical limitations for achieving this in the field (Bowers et al., 2021). In this thesis, flower and pan-trap samples were kept on ice to maintain low temperatures until the samples could be moved to a freezer. This approach minimised the potential for DNA degradation which can result from sample warming or inconsistent temperatures (see Chung et al., 2017) and allowed for the collection of large quantities of bulky samples. However, preservation buffers (e.g. ATL buffer or cetyl trimethyl ammonium bromide (CTAB)) may present a viable alternative to freezing samples, without the need to keep refreshing ice (Harper et al., 2023; Johnson et al., 2023). For flower samples, the use of preservation buffers will depend on the size and morphology of the crop flower being collected, where large mass flowering species (e.g. *P. americana*) may require sub-sampling or storage on ice.

7.2.7 Laboratory processing and bioinformatics

One of the most critical decisions after a sample has been collected is what assay to use (Alberdi, Aizpurua, Gilbert, & Bohmann, 2018). Whether an assay is appropriate for a given agricultural substrate will depend on; i) the community or taxa of interest, ii) the DNA reference database for the chosen barcode region, iii) the amplicon length, and iv) primer biases (Alberdi et al., 2018; Esling, Lejzerowicz, & Pawlowski, 2015). Here, previously tested and validated assays were used,

rather than developing any new assays to target the novel substrates of flowers and pan trap water (Clarke, Soubrier, Weyrich, & Cooper, 2014; Poinar et al., 1998; Taberlet et al., 2007; Taberlet, Gielly, Pautou, & Bouvet, 1991; Vamos, Elbrecht, & Leese, 2017). In regards to arthropod detections, the fwhF2/R2n primers were the most reliable due to the extensive database for the cytochrome c oxidase subunit I (COI) gene (Marquina, Andersson, & Ronquist, 2019; Mauvisseau et al., 2019). While for plants, the combination of both rbcLh2aF/R, targeting Ribulose-1,5-bisphosphate carboxylase-oxygenase (rbcL) region and trnL_c/h, targeting the *trnL* (UAA) intron region (trnL) benefitted from the extensive reference database associated with rbcL and the taxonomic depth afforded by trnL (Chase et al., 2005; Cheng et al., 2016). The use of two or more assays is generally recommended to help counteract the biases associated with any one assay, although at increased cost (Aizpurua et al., 2018; Bell et al., 2019). Though in general, further development of shorter arthropod assays (to account for DNA degradation and low deposition) and assays that can detect plant taxa at the species-level (Cheng et al., 2016) are needed to accommodate reliable and accurate detections from novel substrates in agriculture (Chapters 3 and 5). Once chosen, the primers chosen for a given survey will require *in silico* (computer-based) testing to verify taxonomic biases, *in vitro* (laboratory-based) assessment to confirm that the assay chosen works for the designated substrate and *in situ* (field-based) experimentation to prove that the assay can amplify target DNA in the field (Alberdi et al., 2018; Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005; Marquina et al., 2019).

The choice of using either barcoding or metabarcoding assays will depend on the research question posed (see section *7.2.1 Research questions*). For the purposes of this thesis, broader taxonomic groups of arthropods (Chapters 3, 4 and 5) and plants (Chapters 5 and 6) were targeted, rather than specific species. This decision meant that taxonomic assignments at the genus or family level were common. With these community measures, I was able to contrast different agricultural management practices (e.g. weed cover and adjacent habitat) on arthropod diversity (Chapters 3 and 6). However, if the purpose of the survey is to consistently detect specific species (e.g. crops pests), then barcoding assays are recommended, due to their high specificity and high sensitivity (Ashfaq, Hebert, & Naaum, 2016; DAWE, 2021; Valentin, Maslo, Lockwood, Pote, & Fonseca, 2016). While the choice of assay type can determine which taxa are successfully amplified from an eDNA sample, appropriate thresholds in the bioinformatics pipelines are critical for reliable detections.

Thresholds for filtering sequences, forming Zero-radius Operational Taxonomic Units (ZOTUs) and assigning taxonomic identifications can transform data in unexpected ways. Specifically,

‘over-conservative’ thresholds may introduce false negatives by removing ‘real’ species detections and thus under-representing measures of taxonomic diversity (Piggott, 2016). While, ‘under-conservative’ thresholds can introduce false positives by retaining ‘artefacts’, which may be introduced at any stage of the metabarcoding workflow (Alberdi et al., 2018). For Chapter 3, a ‘relaxed’ threshold was chosen to determine if arthropod eDNA could consistently be detected from crop flowers. While in Chapter 5, a ‘conservative’ threshold was chosen to evaluate pan-trap water as a medium for capturing plant species diversity. However, designating a particular threshold as ‘relaxed’ or ‘conservative’ for detecting species is a somewhat ambiguous area (Alberdi et al., 2018; Drake et al., 2022). Drake et al. (2022) defines the best practice for establishing detection thresholds as the utilisation of both reads generated from negative controls for particular OTUs and the inclusion of minimum sequence copy thresholds. In addition to this, appropriate thresholds will depend on the unique mutation and hybridisation rates of particular taxa (e.g. plant taxa may require higher thresholds compared to arthropods; see Smith & Keeling, 2015), as well as the detectability of a given organism for the selected substrate (e.g. Newton et al., 2023). For instance, in Chapter 5, due to the low detectability of arthropods from pan-trap water, only singletons were removed, compared to the more conservative threshold of 10 reads for plant ZOTUs. For agricultural surveys more broadly, eDNA-based methods will require multiple negative controls (field and laboratory), as well as PCR replicates sequenced separately (e.g. Gomez et al., 2023), to effectively calibrate detection thresholds and avoid the generation of false negatives.

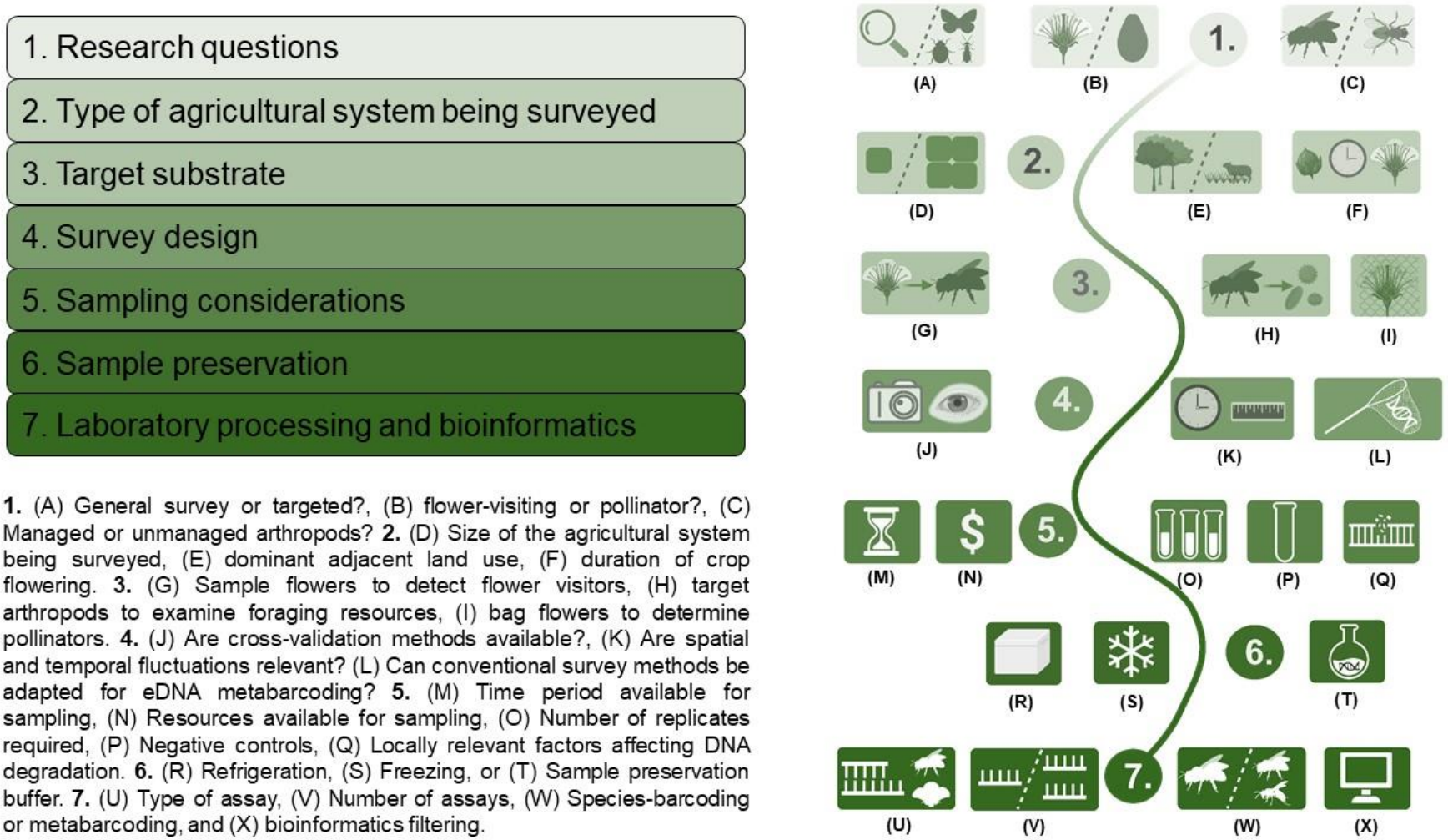

Figure 7.1 Critical considerations for eDNA-based surveys in agriculture for detecting flower-visiting arthropods, as well as the plants they forage upon and pollinate. Graphic created using Biorender.

7.2.8 Using emerging technologies

Environmental DNA metabarcoding has the potential to revolutionise biodiversity surveys for agroecosystems by providing timely feedback for management practices, as well as assisting in the detection of economically significant species (Furlong, 2015; Gomez et al., 2023; Valentin et al., 2016). However, eDNA, like any other survey method, has shortcomings that must be recognised. Firstly, current monitoring efforts in agroecosystems using conventional techniques and taxonomic expertise (e.g. passive sampling, floral surveys, etc.) are able to capture species abundance, which may be an important measure for certain ecological groups (e.g. emerging parasites, like *Varroa destructor*; see Utzeri et al., 2019). Unfortunately, despite a wealth of research investigating the quantitative relationship between DNA deposition and the abundance of different organisms (e.g. Fonseca, 2018; Jo, Takao, & Minamoto, 2021; Kelly et al., 2017), the consensus remains that eDNA is unreliable for assessing abundance (Bell et al., 2019; Elbrecht & Leese, 2015). Thus, where abundance measures are critical, complementary methods remain necessary (see section *7.2.4 Survey design*). Secondly, the field of eDNA is rapidly changing as new taxonomically-informative substrates emerge (Aizpurua et al., 2018; Clare et al., 2021; Gregorič et al., 2022), a wealth of assays are developed (Cheng et al., 2016; Marquina et al., 2019), the mechanisms which affect the deposition and persistence of DNA in the environment are uncovered (Liu, Clarke, Baker, Jordan, & Burridge, 2020; Valentin et al., 2021) and new bioinformatics tools are tested (Mousavi-Derazmahalleh et al., 2021; Pafilis, Zafeiropoulos Viet, Quoc, Vasileiadou, & Potirakis, 2020). In the face of such significant progress, establishing standardised surveys and monitoring programs that use consistent methods over time may prove challenging. Thus, a single 'best practice' model for implementing eDNA-based surveys in agriculture may be an evolving concept, with the implication that surveys change through time, making backward compatibility with previous efforts difficult. These challenges are not unique to eDNA, after all, multi-decade arthropod surveys with imperfect survey methods are still critical for evaluating long-term diversity trends (e.g. Winter et al., 2015). Further, one advantage of eDNA-based surveys is that the archived DNA samples can be re-sequenced and re-analysed as new assays, sequencing technologies and bioinformatics pipelines emerge to maintain some continuity with evolving methods.

7.2.9 Reporting guidelines

As eDNA-based surveys are integrated into agricultural monitoring, the need for standardised reporting which encapsulates critical details (e.g. substrate choice, primers, DNA extraction procedure, contamination, bioinformatics pipelines and minimum sequence copy thresholds) is necessary for accuracy and the continued development of this molecular tool. For instance, by

targeting different terrestrial substrates, studies may develop completely contrasting views of the same ecosystem (Ryan, Bateman, Fernandes, van der Heyde, & Nevill, 2022; van der Heyde et al., 2020). Where, for example, soil samples may underestimate measures of plant and animal diversity (Newton et al., 2022; van der Heyde et al., 2020), samples collected from scat or rollers in the same area may provide a much closer estimate of the 'true' diversity (Newton et al., 2022; Ryan et al., 2022). This point is one of many that should be included in reporting from eDNA-based surveys in agriculture (Figure 7.2). The goal of reporting should be to convey the most important findings to the stakeholder, whilst also capturing technical details (e.g. contamination and false negatives) that may have influenced these detections (Dickie et al., 2018; Goldberg et al., 2016). Additionally, there is also a need to make this information publicly available, thereby allowing for auditing and the creation of a repository of trialled substrates, assays and DNA extraction methods. After all, the scientific literature omits many studies that have been unsuccessful (see Takahashi et al., 2023), though a 'failed' amplification is still informative and may prevent future researchers from repeating the same mistake.

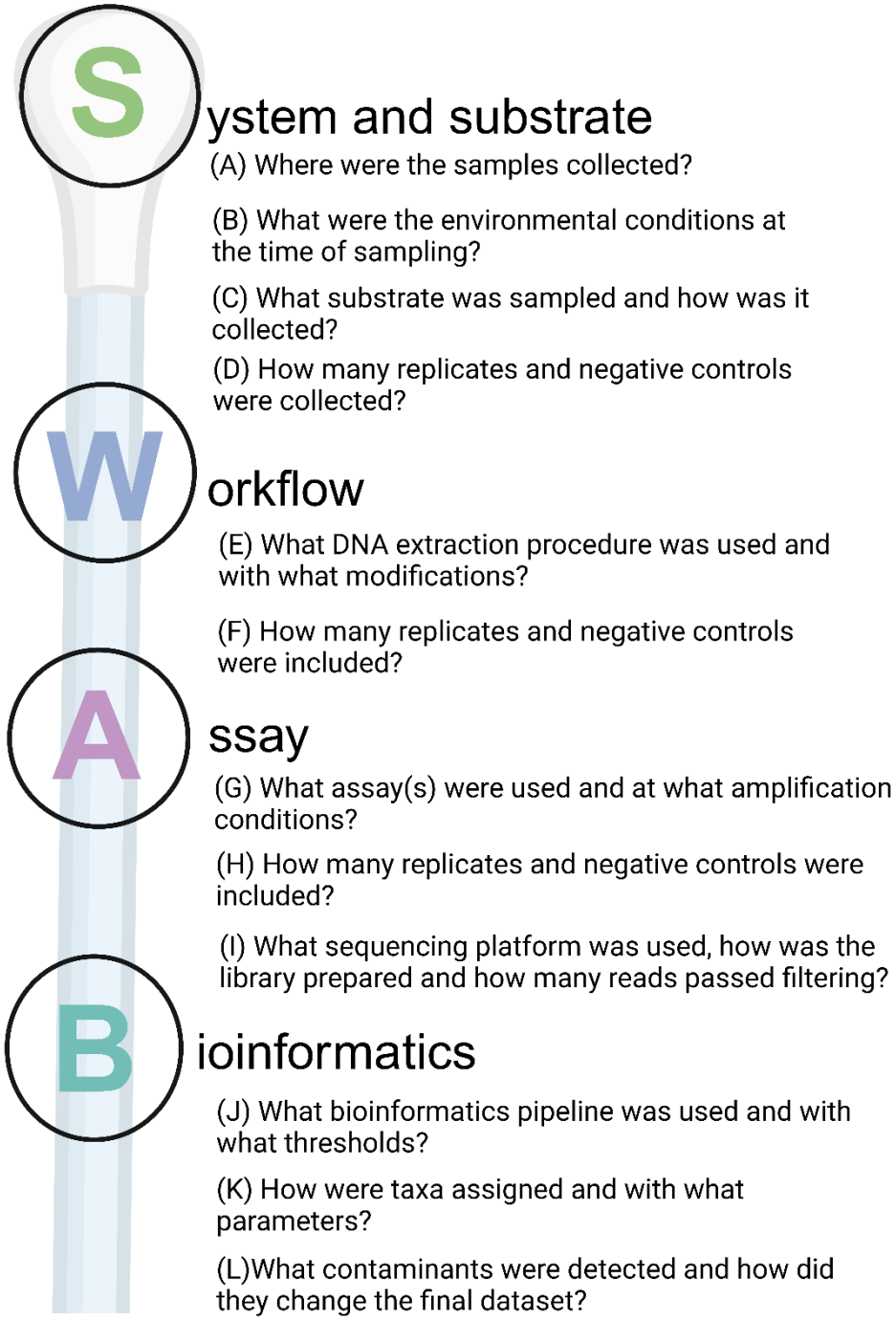

Figure 7.2 The SWAB acronym to highlight critical information to be included in eDNA reporting in agriculture. Topics and questions were adapted from Goldberg et al. (2016). Graphic created using Biorender.

7.3 Significance of the thesis

Globally, the diversity and abundance of terrestrial arthropods is threatened by a mixture of biotic (e.g. climate change, and introduced species) and anthropogenic (e.g. light pollution and generalised use of pesticides) stressors (Potts, Imperatriz-Fonseca, & Ngo, 2016; Wagner, Grames, Forister, Berenbaum, & Stopak, 2021). This problem is not confined to just diversity, however, as the ecosystem services arthropods mediate (e.g. pollination and nutrient recycling) are also at risk (Potts et al., 2016; Wagner, 2020). The implication for agroecosystems is that the reduced delivery of these ecosystem services, in conjunction with the emergence of antagonistic taxa (e.g. crop pests and parasites), may add a premium to food security (Basualdo et al., 2022; FAO, 2020). The research presented in this thesis evaluates eDNA metabarcoding as a potential survey tool to assess the diversity of flower-visitors, pests and predator taxa, as well as foraging resources upon which they rely in agroecosystems. This was the first study to analyse flower and pan trap substrates in agricultural systems, and the findings herein echo those from natural systems (Bell et al., 2019; Thomsen & Sigsgaard, 2019), confirming that eDNA can detect a broad array of ecologically significant taxa critical to the productivity of these managed landscapes.

Agricultural studies using eDNA-based surveys to detect specific-species, as well as entire biological communities, from diverse substrates ranging from water to air are rapidly emerging (Gomez et al., 2023; Tordoni et al., 2021; Valentin et al., 2020). At this juncture, the agricultural applications that will benefit from this approach and how this technology will change monitoring for agroecosystems more broadly are difficult to comprehend, although it is evident that eDNA is not without its limitations (e.g. false negatives, false positives, detection biases, etc.; see Section *7.2 Technical and methodological considerations for the application of eDNA metabarcoding to monitoring arthropods and associated foraging resources in agroecosystems*) and my research has highlighted that some substrates are better suited to detecting particular taxa than others. The implications of these findings are that eDNA-based techniques will require on-going pilot studies, cross-validation methods, consideration of locally relevant factors and data generated to be made publicly available as this technology is increasingly implemented. The continued evolution of eDNA will make it challenging to standardise, potentially reducing the backwards compatibility of future surveys. Despite these limitations, this thesis illustrates the taxonomic breadth and depth afforded by this molecular method to rapidly detect ecologically significant taxa which are critical to the health of agricultural systems.

7.4 Looking forward

As this research was completed, new discoveries, techniques and statistical analyses have continued to emerge. The following section discusses potential future directions that will allow for greater implementation of eDNA-based surveys in agriculture.

7.4.1 Air eDNA

Airborne molecules of DNA isolated and characterised for taxonomic identification are collectively known as 'air eDNA' (Clare et al., 2021). Despite the methods for characterising DNA from air existing since 2001 (see Williams, Ward, & McCartney, 2001), this technique has only recently been expanded to detect vertebrates and invertebrates (Clare et al., 2021; Gregorič et al., 2022), as well as airborne plant material and pollen (Johnson et al., 2021, 2019; Longhi et al., 2009). The implications for isolating and characterising biodiversity from air are manyfold, but one of the most significant is for assessing possible contamination. To date, when detections are generated from a substrate (e.g. soil, flowers, water), there has been an underlying assumption that the organism detected has been in direct contact with that substrate (Johnson et al., 2023; Valentin et al., 2020; van der Heyde et al., 2020). However, the discovery that DNA molecules from a wide variety of taxa may be airborne, raises urgent questions about this assumption. Specifically, how common are successful detections from 'incidental' airborne eDNA, compared to direct interactions between an organism and the chosen substrate? Currently, there is a need for more baseline studies to determine how common these incidental detections are, in order to verify the reliability of terrestrial eDNA-based surveys (Clare et al., 2021). In the meantime, pilot studies remain essential to determine the detection limits of air eDNA for the chosen system and substrate, thereby allowing for the implementation of appropriate minimum sequence copy thresholds (see Drake et al., 2022) to help account for this potential source of contamination.

7.4.2 Metagenomics and shotgun sequencing

The application of PCR-free shotgun sequencing to sequence genome fragments from environmental samples is well-established for microbes, but remains novel for macroorganisms (Linard, Arribas, Andújar, Crampton-Platt, & Vogler, 2016; Ruppert et al., 2019). Metagenomics-based techniques allow for the detection of multiple taxa, while also generating measures of functional diversity (e.g. classification of bacteria that support crop rhizobiome health by producing volatile organic compounds; see Ciancio et al., 2018) and allowing for the construction of metagenome-assembled genomes (MAGs) for dark taxa (Liu et al., 2021). Metagenomics also benefits from the lack of biased-amplification which is often attributed to eDNA surveys which rely upon PCR (Kelly et al., 2017; Serite et al., 2023). However, far from being a 'silver bullet'

for diversity assessments, metagenomics-based methods are often limited by a lack of reference sequences (e.g. Serite et al., 2023) and generally rely upon more costly long-read sequencing (ca. 30,000 – 50,000 bp) to overcome the issues associated with challenging genomic regions (e.g. long repeats and homologous regions) (Vuong, Wise, Whiteley, & Kaur, 2022). Thus, metagenomics surveys that rely upon shotgun sequencing do hold potential for future biodiversity assessments, although further methodological refinements remain necessary before this technology can be widely implemented.

7.4.3 Field-based DNA sequencing and detections

Agricultural surveys often require rapid identifications of the target organism of interest in order to plan appropriate management actions (e.g. replacing crops with disease-resistant variety) (Boykin et al., 2019; Rich et al., 2023). Thus, the development of portable amplification and sequencing platforms to provide same-day detections for crop pests and pathogens is an area of research that has gained significant interest (Boykin et al., 2019; Skinner, Murdoch, Loeza-Quintana, Crookes, & Hanner, 2020). Currently, eDNA sample processing often relies upon lengthy laboratory times that can extend from weeks to months depending on the number of samples (Thomas et al., 2020), as well as the expense of skilled lab technicians for processing and sequencing (Sanches & Schreier, 2020). Portable PCR and sequencing machines (e.g. Oxford MinION) presents a cheaper and more time-efficient alternative, capable of detecting species in as little as 60 minutes and as much as one day (Boykin et al., 2019; Rich et al., 2023; Thomas et al., 2019). Until recently, the biggest limitation to these technologies was the accuracy with which they could sequence samples (e.g. 10 – 15% error rate; see Lu, Giordano, & Ning, 2016), however, with recent technological and computational advances, these error rates have been reduced to as low 0.5 – 5% (Brown, Dreolini, Wilson, Balasundaram, & Holt, 2023). Nevertheless, these in-field approaches are limited by the need for *a priori* knowledge about the organism of interest, especially for portable PCR-based methods which require species-specific primers (e.g. Nguyen et al., 2018). Thus, for general surveys to capture known and unknown taxa, lab-based eDNA metabarcoding remains the most reliable and robust method, although with the continued development of online databases (e.g. Briski, Ghabooli, Bailey, & MacIsaac, 2016; Kjærandsen, 2022), this will undoubtedly change in the future.

7.5 Thesis conclusion

"*In other studies you go as far as others have gone before you, and there is nothing more to know; but in a scientific pursuit there is continual food for discovery and wonder*."

– Mary Shelly

The application of eDNA metabarcoding for agroecosystems is, at present, a balance between methodological refinement and technical limitations. Agricultural monitoring will be transformed by eDNA-based surveys, though to realise this goal, this method must be seen like any other, with strengths and weaknesses, as well as an understanding that it will not work equally well for all taxa in all ecological contexts. Agricultural monitoring urgently needs tools capable of providing reliable and timely feedback on biodiversity measures, as well as the detection of ecologically significant species. This thesis aimed to answer the overarching question "How do environmental DNA (eDNA) surveys contribute to monitoring arthropods and the plant resources they rely upon in agroecosystems". To this end, I have shown that eDNA metabarcoding is a viable tool for surveys in agroecosystems. First, the review of the current literature for eDNA in agriculture shows that this molecular method is currently underutilised, although there are numerous opportunities where it may be beneficial. Second, eDNA-based surveys were found to generate comparable diversity measures to those obtained from conventional methods and increase the overall number of arthropod taxa detected, though each method detected largely unique species, with little overlap (i.e. eDNA did not detect some flower-visitor families observed by DVRs). Third, eDNA and DVRs used in combination, showed that the diversity and abundance of beneficial and antagonistic arthropods varied significantly within tree canopies and between orchards, with communities gradually becoming more similar with greater crop flowering. Fourth, the use of a novel substrate (pan-trap water) was useful for detecting the plant foraging resources that help support arthropod communities in agroecosystems, however further methodological refinements are necessary to detect arthropods from this substrate. Fifth, the wide-scale surveys across multiple orchards revealed that adjacent natural vegetation habitats are important areas for arthropod biodiversity and abundance, which provide greater foraging resources than adjacent pasture lands. Together, these chapters demonstrate that eDNA metabarcoding can help detect and classify arthropod-plant interactions to allow for high resolution monitoring in agroecosystems. Sustainable agricultural practices require rapid and accurate measurements of biodiversity, as well as ecologically significant taxa, like pollinators, pests and predators. With further development and more baseline studies, this molecular method will be a strong complement to current agricultural monitoring efforts, which ultimately can be used to support greater crop yields and improved food security.

7.6 References

A thorough effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Appendices

Appendix I. Copyright statements

Chapter 2: Published in the journal *Science of the Total Environment*. As an author, permission is automatically granted to reproduce this copyright material to include the article in my thesis.

To Whom It May Concern, I, Joshua Kestel, contributed to the conceptualisation of the study, collected and processed the samples, analysed the data, and wrote and edited the resulting manuscript:

Kestel, J.K., Field, D. L., Bateman, P. W., White, N. E., Allentoft, M. E., Hopkins, A. J. M., … Nevill, P. (2022). Applications of environmental DNA (eDNA) in agricultural systems: Current uses, limitations and future prospects. *Science of the Total Environment*, 847, 157556. doi: 10.1016/j.scitotenv.2022.157556.

15/11/2023

X JK

Joshua Kestel

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David Field

X P.W. Bateman

Bill Bateman

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Morten Allentoft

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Kestel, J.K., Bateman, P.W., Field, D.L., White, N.E., Lines, R., & Nevill, P. eDNA metabarcoding of avocado flowers: 'Hass' it got potential to survey arthropods in food production systems? *Molecular Ecology Resources*, 23(7), 1473-1755. doi:10.1111/1755-0998.13814.

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Kestel, J.K., Bateman, P.W., Field, D.L., White, N.E., & Nevill, P. Spatio-temporal variation in arthropod-plant interactions identified using eDNA and digital video recordings. *Agriculture, Ecosystems & Environment,* (In review).

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Chapter 5: Published in the journal *Environmental DNA*. As an author, permission is automatically granted to reproduce this copyright material to include the article in my thesis.

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Kestel, J.K., Field, D.L., Bateman, P.W., White, N.E., Bell, K.L., & Nevill, P. Environmental DNA metabarcoding of pan-trap water to monitor arthropod-plant interactions, *Environmental DNA.* (2024).

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Kestel, J.K., Bateman, P.W., Field, D.L., White, N.E., & Nevill, P. Come Together: Adjacent natural vegetation affects arthropod community composition and alters arthropod-plant foraging diversity in 'Hass' avocado orchards, *Agriculture, Ecosystems & Environment,* (In preparation).

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Appendix II. Publications

Science of the Total Environment 847 (2022) 157556

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

Applications of environmental DNA (eDNA) in agricultural systems: Current uses, limitations and future prospects

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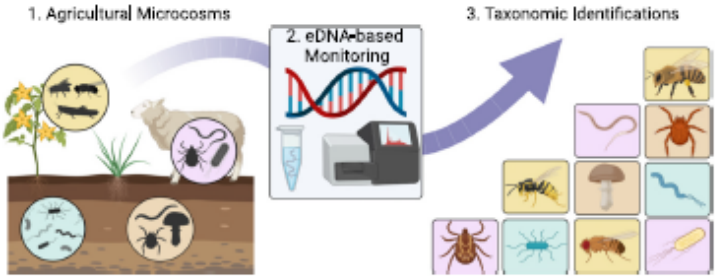
HIGHLIGHTS

- eDNA is a powerful but underused monitoring tool for agricultural systems.
- eDNA surveys can provide classifications for specific organisms and entire assemblages in substrates ranging from soil to air.
- Monitoring with eDNA can help detect and classify ecologically beneficial and harmful organisms in food production systems.
- In silico, in vitro, and in vivo approaches help overcome limitations and caveats associated with eDNA analysis.
- When combined with traditional techniques, eDNA-based surveys can help improve monitoring for agricultural systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Global food production, food supply chains and food security are increasingly stressed by human population growth and loss of arable land, becoming more vulnerable to anthropogenic and environmental perturbations. Numerous mutualistic and antagonistic species are interconnected with the cultivation of crops and livestock and these can be challenging to identify on the large scales of food production systems. Accurate identifications to capture this diversity and rapid scalable monitoring are necessary to identify emerging threats (i.e. pests and pathogens), inform on ecosystem health (i.e. soil and pollinator diversity), and provide evidence for new management practices (i.e. fertiliser and pesticide applications). Increasingly, environmental DNA (eDNA) is providing rapid and accurate classifications for specific organisms and entire species assemblages in substrates ranging from soil to air. Here, we aim to discuss how eDNA is being used for monitoring of agricultural ecosystems, what current limitations exist, and how these could be managed to expand applications into the future. In a systematic review we identify that eDNA-based monitoring in food production systems accounts for only 4 % of all eDNA studies. We found that the majority of these eDNA studies target soil and plant substrates (60 %), predominantly to identify microbes and insects (60 %) and are biased towards Europe (42 %). While eDNA-based monitoring studies are uncommon in many of the world's food production systems, the trend is most pronounced in emerging economies often where food security is most at risk. We suggest that the biggest limitations to eDNA for agriculture are false negatives resulting from DNA degradation and assay

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RESOURCE ARTICLE

MOLECULAR ECOLOGY RESOURCES WILEY

eDNA metabarcoding of avocado flowers: 'Hass' it got potential to survey arthropods in food production systems?

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Abstract

In the face of global biodiversity declines, surveys of beneficial and antagonistic arthropod diversity as well as the ecological services that they provide are increasingly important in both natural and agro-ecosystems. Conventional survey methods used to monitor these communities often require extensive taxonomic expertise and are time-intensive, potentially limiting their application in industries such as agriculture, where arthropods often play a critical role in productivity (e.g. pollinators, pests and predators). Environmental DNA (eDNA) metabarcoding of a novel substrate, crop flowers, may offer an accurate and high throughput alternative to aid in the detection of these managed and unmanaged taxa. Here, we compared the arthropod communities detected with eDNA metabarcoding of flowers, from an agricultural species (*Persea americana*—'Hass' avocado), with two conventional survey techniques: digital video recording (DVR) devices and pan traps. In total, 80 eDNA flower samples, 96 h of DVRs and 48 pan trap samples were collected. Across the three methods, 49 arthropod families were identified, of which 12 were unique to the eDNA dataset. Environmental DNA metabarcoding from flowers revealed potential arthropod pollinators, as well as plant pests and parasites. Alpha diversity levels did not differ across the three survey methods although taxonomic composition varied significantly, with only 12% of arthropod families found to be common across all three methods. eDNA metabarcoding of flowers has the potential to revolutionize the way arthropod communities are monitored in natural and agro-ecosystems, potentially detecting the response of pollinators and pests to climate change, diseases, habitat loss and other disturbances.

KEYWORDS
agroecosystem, arthropod, environmental DNA, flower-visitor and pollinator, metabarcoding

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Environmental DNA

WILEY

METHOD

Environmental DNA metabarcoding of pan trap water to monitor arthropod-plant interactions

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Abstract

Globally, the diversity of arthropods and the plants upon which they rely are under increasing pressure due to a combination of biotic and abiotic anthropogenic stressors. Unfortunately, conventional survey methods used to monitor ecosystems are often challenging to conduct on large scales. Pan traps are a commonly used pollinator survey method and environmental DNA (eDNA) metabarcoding of pan trap water may offer a high-throughput alternative to aid in the detection of both arthropods and the plant resources they rely on. Here, we examined if eDNA metabarcoding can be used to identify arthropods and plant species from pan trap water, and investigated the effect of different DNA extraction methods. We then compared plant species identified by metabarcoding with observation-based floral surveys and also assessed the contribution of airborne plant DNA (plant DNA not carried by arthropods) using marble traps to reduce putative false positives in the pan trap dataset. Arthropod eDNA was only detected in 17% of pan trap samples and there was minimal overlap between the eDNA results and morphological identifications. In contrast, for plants, we detected 64 taxa, of which 53 were unique to the eDNA dataset, and no differences were identified between the two extraction kits. We were able to significantly reduce the contribution of airborne plant DNA to the final dataset using marble traps. This study demonstrates that eDNA metabarcoding of pan trap water can detect plant resources used by arthropods and highlights the potential for eDNA metabarcoding to be applied to investigations of arthropod-plant interactions.

KEYWORDS
agroecosystems, environmental DNA, marble traps and minimum sequence copy thresholds, pan traps, pollen metabarcoding