Final Report

eDNA analysis of plant-pollinator relationships to improve Hass avocado production in south-west Western Australia

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eDNA analysis of plant-pollinator relationships to improve Hass avocado production in south-west Western Australia (PH19007)

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Public summary

Project PH19007, titled "The development and application of eDNA for the classification and management of plant-pollinator networks in an agro-ecosystem" set out to test a new molecular method, environmental DNA metabarcoding, for identifying potential pollinator, pest and predator insects, as well as the flowering plants upon which they rely. This work was undertaken in *Persea americana* 'Hass' avocado orchards in the south west of Western Australia (SWWA), which often experience sub-optimal pollination, despite prolific flowering. The contribution of managed (e.g. *Apis mellifera* – European Honeybee) and unmanaged (e.g. native flies, bees, moths, butterflies and wasps) pollinators to successful 'Hass' avocado fruit set remain an emerging area research, meaning that growers do not currently have access to management strategies which may enhance and maintain the presence of these insects within orchards. Project PH19007 aimed to test the usefulness of eDNA metabarcoding and add baseline research about which managed and unmanaged insects may provide pollination services for 'Hass' avocados in SWWA for growers in the region.

The work for project PH19007 was undertaken over three and a half years as part of a PhD thesis undertaken at Curtin University, Perth, Western Australia. All samples were collected from the Manjimup-Pemberton region in SWWA and processed and analysed at Curtin University. In 2020, 320 'Hass' avocado inflorescences were collected during peak flowering (October – November) from two orchards. Study A and B were undertaken using these samples. For study A, eDNA metabarcoding, using two common insect assays, was used to amplify insect DNA from these flowers. Detections from eDNA metabarcoding were then compared with two conventional methods often used in agriculture, pan traps and digital video recording (DVR) devices. While for study B, insects that were not previously available on the online reference sequence databases were barcoded and potential pollinators, pests and predator insects were classified from inflorescence samples using eDNA metabarcoding and DVR devices. In 2021, 120 pan trap samples were collected from six orchards adjacent to either pasture habitat or natural vegetation over four time points (before flowering, low flowering, moderate flowering and post-flowering). These pan trap samples were used in studies C and D. For study C, pan-trap water samples were amplified using two common plant assays and one insect assay to detect the DNA from both captured insects and the plants upon which they foraged. Lastly for study D, pan-trap water samples across the six sample orchards were amplified for only plant eDNA and analysed with environmental co-variates (time of sampling, adjacent habitat, avocado flowering, temperature and canopy cover).

The key outputs from this research were: i) the identification of potential pollinators for 'Hass' avocados in SWWA, ii) the collection and identification of insect species that were not previously available on the online reference databases, and iii) the trial of a new method for isolating pollen and insect DNA from pan traps. The outcomes from this research were: a). the identification of Syrphidae (hoverfly) species as the most common flower visitor for 'Hass' avocado trees (130 ± 15.5 visits per hour), followed by honeybees (10 ± 1.1 visits per hour) and Diptera (flies, Muscidae and Calliphoridae; 4 ± 1.1 visits per hour), b). sequencing of 29 insect specimens that were not previously available on the publicly available online DNA sequence repositories (e.g. GenBank), and c). the development of pan-trap water eDNA metabarcoding as a method to identify insect collected eDNA. This method was able to classify over 30 plant families, the three most common of which were all known to require insect to facilitate or enhance fruit set. One of the most important recommendations emerging from this project was that cross-validation remains necessary for eDNA-based surveys to help prevent biased detections. Overall, this research highlights that eDNA metabarcoding is a cost-effective and reliable method to detect insects in agricultural systems and the data generated can provide improved baseline knowledge to support avocado growers.

Keywords

Environmental DNA, metabarcoding, monitoring, insects, pollinators, pests and pathogens.

Introduction

Global insect biomass, abundance and diversity is under threat from a mixture of biotic and abiotic threats (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). Insects 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 insect 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), though, the practices associated with agriculture often harm insect 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 insect monitoring into agriculture have remained the exception, rather than the rule.

Surveys and monitoring of insects have largely been omitted from agricultural practices and farm management decisions (Martínez-Sastre, García, Miñarro, & Martín-López, 2020; Rader et al., 2016b). Insects 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, insects are often managed without adequate 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 insect predators remain largely unknown (Furlong, 2015; Martínez-Sastre et al., 2020). Furthermore, among the existing literature measuring insects 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., 2016b). Though, such studies remain necessary, 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 insects 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.

Insects 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 insect 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 insects 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. insects), 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 insect 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 insects in agriculture, one that is particularly well-suited to agroecosystems in Australia.

Australia is home to approximately 320,465 insect 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 insect 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 insects to facilitate cross pollination (DIPIRD, 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 insects 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 insect pollinations, predators, crop pests and pathogens, as well as the foraging resources upon which they rely.

The aims for this project were:

1) Use eDNA metabarcoding to compare floral-visitor networks across orchards with surrounding remnant native vegetation present or absent,
2) Establish a DNA reference database for potential pollinators of 'Hass' avocado in south west Western Australia, and
3) Develop and test a new method for isolating pollen and insect DNA from pan trap samples.

Methodology

The work reported for this project was undertaken in collaboration with PH16002: Managing flies for crop pollination. Bi-monthly meeting between the collaborators on both projects established sampling orchards in the Manjimup-Pemberton district, experimental setups that could capture the widest diversity of native pollinators, as well as fly species that this project would need to collect for the development of a custom reference database. The first stage of this project (Aim 1), we compared insect detections from eDNA metabarcoding 'Hass' avocado flowers with two conventional methods used to monitor insects in agriculture, digital video recording devices (DVRs) and pan traps. eDNA has previously been shown to reliably detect flower-visiting insects from flowers (see Thomsen & Sigsgaard, 2019), though this method had never been trailed in agriculture. For this study, inflorescences were collected from a single 'Hass' avocado orchard, Marron Brook Farm (34°18'52 S, 116°08'36 E), located in the avocado production region of Manjimup-Pemberton. 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 insects 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 'Fuerte' pollinisers. 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 1.1A). With our multi-method approach, we determined that eDNA metabarcoding, with two conventional insect assays, was able to identify a wide range of beneficial (e.g. pollinators) and antagonistic (e.g. crop pests) insects. Further, we found that this molecular method was most reliable when used in tandem with DVRs. Thus, in the second stage of this project, where we examined how insect communities in orchards vary both over time and space, we used eDNA metabarcoding and DVRs together.

For the second study of this project (Aims 1 and 2), 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. 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 while inflorescences were collected from both study orchards. 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). In the second stage of this project, we determined that many of the native insects that we observed on the DVRs were not present in the online databases used to inform the eDNA metabarcoding results. To deal with this problem, we captured insects not previously sequenced and sequenced them ourselves for the most taxonomically informative region currently available, Cytochrome Oxidase 1 (CO1) (Saccò et al., 2022). With these additional sequences available, we were able to improve the reliability of our eDNA detections.

In the third stage of this project (Aim 3), we examined if eDNA metabarcoding could be used to enhance a conventional method, pan-traps, by detecting both insects and the pollen and plant material that they carry on their bodies. This research represented the first application of eDNA metabarcoding with pan traps in either natural or agricultural systems. We sampled three 'Hass' avocado orchards located in the Manjimup – Pemberton region of south west Western Australia 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. To survey insects and the pollen they collect on their bodies, in each orchard, five pan trap arrays (Figure 1.2A) were deployed at 10 m intervals 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). We determined that eDNA metabarcoding was able to consistently detect the plant resources upon which insects forage, however, insects were only detected in 17% of the pan trap samples. The lack of consistent insect detections likely occurred as a result of the insect exoskeletons preventing the exchange of DNA with the water substrate (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 herbivorous plant material from the gut contents of insects by preserving whole specimens, however, if future studies wish to detect a greater number of insect taxa from pan-trap water, then we would recommend suspending bulk insect samples in a lysis buffer as an initial step in DNA extraction. This non-destructive approach has previously generated comparable insect detections to homogenised bulk samples (see Kirse et al., 2022), while also retaining whole specimens for morphological identification and abundance data.

For the final stage of this project (Aims 1 and 3), six 'Hass' avocado orchards located in the Manjimup – Pemberton region of south west Western Australia 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 1.3A). 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 1.3B). To survey insects 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 1.3). 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 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, Melles, Vaz, Parker, & Burgess, 2018; Gervais et al., 2018). After eight hours, each pan trap was stirred using sterilised tweezers , 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 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. Insects 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).

Refer to the Appendices for detailed information on methodologies used in the project.

Photos/images/other audio-visual material

Figure 1.1 Three methods used to measure flower-visiting insects 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.

Figure. 1.2 (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.

Figure 1.3 *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).

Results and Discussion

Aim 1. Use eDNA metabarcoding to compare floral-visitor networks across orchards with surrounding remnant native vegetation present or absent.

To complete the first aim of this project, we tested if eDNA metabarcoding crop flowers could be used to detect a similar cohort of insects compared to those measured by Digital Video Recordings (DVRs) and pan traps. Here, we demonstrated that eDNA-based surveys can generate similar community diversity measures to these conventional methods, while also increasing the total number of insect taxa detected (Evans & Kitson, 2020; Thomsen & Sigsgaard, 2019) (Figure 1.4)(see Appendix 1for all Results and Discussion figures and tables). This project highlights that each survey method characterised a different aspect of the total insect community present within the orchard, likely a reflection of detection biases (e.g. DVRs may not consistently detect small insect taxa; see Johnson et al., 2023) (Table 1.1).

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. 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. While for the DVRs, 14,032 flower visits were observed across 96 hours of recordings. In total, 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. Overall, 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). For the pan traps, a total of 499 individual insects were collected, 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).

Additionally, the inclusion of DVRs helped to cross-validate the eDNA detections and reveal false negatives within the eDNA dataset. Some insect families (e.g. Calliphoridae and Pompilidae) were observed visiting sample flowers by the DVRs, but were completely absent from the eDNA dataset (Table 1.1), suggesting that eDNA metabarcoding may fail to detect some common flower-visiting insects (Gomez, Sørensen, Chua, & Sigsgaard, 2023; Thomsen & Sigsgaard, 2019). By comparing the detections from the two eDNA metabarcoding assays, we were 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 insect families (Muscidae, Culicidae and Latridiidae) not detected by the assay targeting Cytochrome Oxidase subunit 1 region (COI). In contrast, 14 insect families were unique to the assay targeting the COI region, while seven families were shared between both assays.

The methods used in the second stage of this research represent an applied use of eDNA metabarcoding to generate invertebrate diversity measures from flowers. Our previous work established that eDNA metabarcoding of flowers could generate comparable diversity measures to those generated by conventional survey methods (Figure 1.4). To determine how floral-visitor networks varied across orchards (Aim 1), we used eDNA-metabarcoding of flower samples to examine temporal (crop flowering intensity) and spatial (within trees and between orchards) variation for insect pollinators, pests and predator.

Here, eDNA metabarcoding detected a greater diversity and relative abundance of known pollinators, pests and predators in response to greater crop flowering, detections that were confirmed with the DVRs (Figure 1.5). AIC testing showed that flowering intensity and orchard location were significant co-variates for all insects (flowering; $p < 0.001$, orchard; $p = 0.04$), flies (Diptera spp.) (flowering; $p = 0.001$, orchard; $p < 0.001$) and bees and wasps (Hymenoptera spp.) (flowering; $p < 0.001$, orchard; $p < 0.001$). The use of eDNA also allowed for a relatively accessible measure of insect diversity in response to inflorescence location within the canopy, something not easily achievable with the DVRs (Figure 1.6). Inflorescence location in the canopy was found to be a significant explanatory co-variate for bees and wasps (Hymenoptera spp.) ($p = 0.02$). Using non-parametric testing, we determined that the probability of detecting bees and wasps were greater in the upper canopy (> 2m) compared to the lower canopy (< 2m) (upper; 0.36 ± 0.04 SE, lower; 0.23 ± 0.04 SE; $p = 0.05$) (Figure 2). Sample tree (1 – 8) was only found to be a significant explanatory co-variate for bees and

wasps Hymenoptera (p = 0.02).

At the spatial scale of separate orchards, eDNA detections confirmed that insect communities became more similar during increased crop flowering, suggesting that mass-flowering crops may attract similar insects taxa from the surrounding landscape (Willcox et al., 2019). A greater cumulative contribution of species was identified for the eDNA dataset compared to the DVRs. For the eDNA detections between two flowering intensities (low and peak flowering), 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). 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).

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). 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 insect 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). 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). 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).

This research also affirms some necessary considerations for eDNA metabarcoding, namely, the tendency to detect smaller and more frequently visiting insects, likely due to more opportunities for DNA deposition and subsequent detection (Johnson et al., 2023; Valentin et al., 2018). The majority (88%) of the insects detected by DVRs were 10 mm in length or smaller. The largest insect 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 insect ($p < 0.01$) and the number of visits ($p < 0.01$) were significant factors for explaining eDNA detections. Smaller insects were more likely to be detected (Odds ratio = 0.44), as were those that visited the flowers multiple times (Odds ratio = 1.64). Thus, the accuracy of eDNA metabarcoding 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.

Aim 2. Establish a DNA reference database for potential pollinators of 'Hass' avocado in south west Western Australia.

To improve the reliability of our eDNA-based surveys, it was necessary to sequence any orchard insect species that were not available on the online reference databases for the CO1 (Aim 2). In total, 29 insects captured in pan traps were identified that were not available on the online reference databases. These species were primarily flies belonging to Muscidae family. Previous research has shown that these species visit 'Hass' avocado flowers and may facilitate cross-pollination (Kestel et al., 2023; Cook et al., 2020).

Aim 3. Develop and test a new method for isolating pollen and insect DNA from pan trap samples.

In the third stage of this research, we attempted to use a conventional survey tool (pan traps) in a novel way, by metabarcoding pan trap water to detect both captured insects and the pollen and plant material upon which they foraged (Aim 3). Insects were not consistently detected, despite specimens being present in all of the pan trap samples (Figure 1.7). Further, when insects were detected using eDNA, there was little overlap with the morphological identifications (Figure 1.7). Only 17% of pan-trap water samples, all extracted with the Blood and Tissue extraction kit, showed successful amplification for insect DNA, while no amplification was achieved for samples extracted with the Plant Pro kit. The amplification success of insect eDNA was low despite all of the pan traps showing the presence of insect taxa during the morphological identifications. In total, 13 insect 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. Morphological identifications of the pan trap samples yielded 17 insect families, although here the most common taxa belonged to Apidae (15% of detections), Coelopidae (20% of detections), and Tenebrionidae (13% of detections). We note that further methodological refinements (e.g. suspending captured insects in lysis buffer; see Kirse, Bourlat, Langen, Zapke, & Zizka, 2023) are needed to increase the reliability of eDNA metabarcoding pan trap water to detect insects.

For plant detections, 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 (Table 1.2). 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 1 and Fig. 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 S3 in Appendix S1). 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. 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 1m2 quadrat. Predictably, species composition differed significantly between the eDNA and floral survey methods ($R = 0.365$, $p < 0.001$). Although neither method alone appeared to capture the total plant diversity present within the orchards. 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).

To examine the influence of adjacent native remnant vegetation on plant-pollinator networks and the diversity of pollen upon which orchard insects forage (Aims 1 and 3) we wanted to assess whether the presence of adjacent natural vegetation enhanced orchard insect diversity and abundance and provided more diverse insect-plant foraging resources compared to orchards adjacent to pasture. We 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 insects, which were preserved for morphological identification. The nectar, pollen and plant tissue carried on insect bodies was targeted by eDNA metabarcoding the pan-trap water. Within orchards, insect diversity and abundance was not significantly enhanced by the presence of natural vegetation (Figure 1.8).

Overall, 2,078 insect 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. With a PERMANOVA analysis, we found that for insect communities captured in orchards, both adjacent habitat type ($DF = 1$, $p < .01$) and sampling period ($DF = 3$, $p < .01$) were significant. While for insect 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. The alpha diversity of insects captured in orchard pan trap samples showed no correlations with flowering resource availability within orchards or adjacent habitat. The alpha diversity of insects (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 insect 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 insect 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).

However, pan traps within the adjacent natural vegetation captured significantly higher levels of insect diversity and abundance, compared to pan traps in pasture habitats (Figure 1.8). These patterns of insect 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, insect diversity increased with greater maximum daily temperatures, although not with greater crop flowering (Steen, 2017; Wilson & Jamieson, 2019). Alpha diversity for insects 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 insect diversity (canopy cover; Spearman's rho = 0.38, p < .01, maximum daily temperature; Spearman's rho = 0.41, p < .01). These patterns likely reflect the increase in foraging opportunities with less stochastic weather conditions (Prendergast et al., 2020).

By eDNA metabarcoding pan-trap water, we were able to show that orchards adjacent to natural vegetation, during low *P. americana* flowering, contained a greater insect-plant foraging diversity compared to those collected adjacent to pasture (Figure 1.9). 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.

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 1.8). Plant foraging genera composition recorded by eDNA showed some distinct partitioning for both adjacent habitat type and sampling period (Figure 1.8, 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 1.8; 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 1.8; 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). 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). 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). These findings indicate that natural vegetation habitats may enhance foraging resources for insects in agroecosystems at certain time periods (e.g. when adjacent pasture is actively grazed), and that certain insect 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 insects in agroecosystems, are often overlooked during monitoring (see Rader et al., 2016). By combining pan traps with eDNA metabarcoding, we were able to demonstrate that this novel approach can provide a scalable method capable of evaluating how natural capital enhances insect foraging diversity in agroecosystems.

Outputs

Table 1.3. Output summary

Output	Description	Detail
Pemberton Regional Forum presentation, June 2021	Presentations on successful applications of eDNA metabarcoding to detect beneficial and antagonistic insects from 'Hass' avocado flowers.	Presentations were done at the Pemberton regional forum. This is the first of two planned workshops over the project lifetime.
Media release, 2022	Article on the use of eDNA metabarcoding for food security	The media release was taken up by South West NRM
Talking Avocados article, March 2022	Article on the comparison of eDNA metabarcoding with DVRs and pan traps.	This is the first of the articles planned over the project lifetime.
Conference presentation, 2022	Interstate conference presentation at the Ecological Society of Western Australia in Wollongong on comparison of eDNA with conventional survey methods.	Presentation was done in Wollongong to an audience of 30 domestic and international scientists.
Conference presentation, 2023	Interstate conference presentation at the Southern eDNA society conference in Hobart on the comparison of eDNA with conventional survey methods	Presentation was done in Hobart to an audience of over 300 domestic and international scientists.
Media release, 2023	Article on the comparison of eDNA metabarcoding with conventional survey methods.	The media release was taken up by Curtin University.
Grower presentation, May 2023	Presentation on the findings from the project, focusing on results of the method comparison, novel application of pan traps and testing of plant foraging diversity between orchard	Presentations were done at the Manjimup regional forum. This is the second of two planned workshops over the project lifetime.

	adjacent land uses.	
Establish a DNA reference database of potential pollinators of Hass avocados in south-west Western Australia, 2023	Collect and identify insect specimens collected from orchards in the Manjimup-Pemberton region and barcode any species not currently available on the online databases.	Insect specimens were identified by three entomologists, Terry Huston from the WA Museum, David Knowles from Spineless Wonders and Chris Swinstead from Curtin University. In total, 29 insect specimens were identified that were missing from the online databases. These specimens were barcoded and the sequences generated will be uploaded to GenBank (NCBI) to complement future efforts to monitor native insects in orchards. The link will be made available on the project page on Hort Innovation website: https://www.horticulture.com.au/growers/help-your-business-grow/research-reports-publications-fact-sheets-and-more/ph19007/
Completion of research thesis, December 2023	Printable 370 page document with descriptions of the applications of eDNA metabarcoding for avocado orchards in the Manjimup-Pemberton region.	Attached as Appendix

Outcomes

Table 1.4. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Intermediate outcomes			
Increased grower knowledge and confidence in eDNA-based surveys	Outcome 3: Alternate pollination options developed for increased productivity Strategy 3.2: Develop and enable novel technologies to support pollination. KPI: Number of growers indicating an increase in knowledge of how and why eDNA analyses of pollinators should be implemented	By increasing grower knowledge of and confidence in using molecular techniques practices for arthropod surveys they will be more likely to implement them.	At workshop 1 and 2, attendees reported that the workshop was relevant to their avocado growing enterprise.
Increased researcher knowledge of eDNA metabarcoding relevant to management including; taxonomy, within orchard distribution and seasonal community variations	Outcome 2: Crop pollination requirements are understood and integrated into best practice Strategy 2.3: Integrate pollination into crop management systems and best practice across all horticultural production systems Outcome 3: Alternate pollination options developed for increased productivity Strategy 3.3: Develop alternative pollinator options KPI: Number of growers indicating an increase in knowledge of how and why eDNA analyses of pollinators should be implemented	Improving precision and capability of research staff will lead to more diverse and relevant areas of interest being included in the project. Giving a greater probability of relevant outputs and successful outcomes.	Connections developed between Australian researchers and those in NSW that also study native insects in avocado orchards. Also connections developed with fly and bee specialists in the field of taxonomy to identify species which are not currently available on the online reference databases used in project PH19007. Extensive literature reviews in the area of eDNA monitoring and sampling, processing, assay choice, substrate choice and future directions conducted. Project team made themselves available growers for assistance with eDNA metabarcoding queries.
Increased researcher knowledge of effectiveness of native insects as flower visitors for avocado trees, management strategies; encourage persistence and diversity.			

Improved connectivity between growers, local and interstate researchers facilitates efficient and targeted research and extension		Improving connectivity between growers, local and interstate researchers will lead to more relevant outputs being delivered and ensure the Australian industry is exposed to the most up-to-date international knowledge and experience.	
End of project outcome			
Increased uptake of alternative pollinator management practices by orchardists in areas of southwest WA.	Outcome 3: Alternate pollination options developed for increased productivity Strategy 3.4: Extension of best practice use of alternative pollinator options in an integrated approach to pollination. KPI: Practice change by growers	Increased uptake of biodiversity 'friendly' practices for native insect management is desirable to reduce unnecessary pesticides application and increase the resilience of pollinator networks in agricultural ecosystems. The ultimate aim of eDNA-based surveys is to reduce the loss of beneficial insect species in orchards and ensure greater opportunity for successful pollination and higher quality fruit.	33% of growers taking part in the research studies for project PH19007 reported altering their pollinator management strategies as a direct result of being involved in the project and obtaining genetic data concerning the diversity of insects present within their orchards.

Monitoring and evaluation

The project evolved over time, which is typical for a PhD thesis.
The following changes were made to the original project description:
-METHODOLOGY-Aim 2 "Determine the factors that affect DNA deposition and persistence" deleted. Replaced by new Aim 2 "Australian insect species are poorly represented in DNA sequences databases but are essential for robust eDNA based surveys. We will establish a DNA reference database of potential pollinators of Hass avocados in south-west Western Australia" and Aim 3 "Incorporation of novel substrates is critical for the continued refinement of eDNA based insect surveys. We will develop and test a new method for isolating pollen and insect DNA from pan trap samples"
-OUTPUTS-Objective c "Identify the factors that affect DNA deposition and persistence on flowers" deleted. Replaced by new objective c "Establish a DNA reference database of potential pollinators of Hass avocados in south-west Western Australia" and objective d "Develop and test a new method for isolating pollen and insect DNA from pan trap samples".

The key evaluation questions, from the project M&E plan are in table 1.5 below along with project performance indicators for each KEQ and continuous improvement opportunities when relevant.

Table 1.5. Milestone details

Key Evaluation Question	Project performance	Continuous improvement opportunities
Q1. To what extent has the project achieved its expected outcomes?		
a. Is there an eDNA metabarcoding information package available for orchardists to learn about eDNA and to manage native insects in their orchards?	Yes, the package is complete and is available on the South West NRM's website.	
b. To what extent has the project improved knowledge and confidence of growers to adopt eDNA-based surveys and sustainable management strategies to encourage native pollinators?	In the final feedback from growers taking part in this research: 33% of respondents reported altering their pollinator management strategies as a direct result of being involved in the project and obtaining genetic data concerning the diversity of insects present within their orchards.	
Q2. How relevant was the project to the needs of intended beneficiaries?		
a. To what extent has the project met the needs of industry levy payers?	Through feedback from growers over the life of the project additional activities and outputs were completed. Namely, a custom database of insects that were not previously available on the online database, case study into alternative ways to measure the plant foraging resources upon which orchard insects rely, a literature review of applications of eDNA in agriculture. Growers were also given the	

	opportunity to comment on areas of further research beyond this project as part of the final forum These are integrated into the recommendations section of this report.	
Q3. How well have intended beneficiaries been engaged in the project? Q4. To what extent were engagement processes appropriate to the target audience/s of the project		
a. How many participants attended workshops?	Between the two different grower workshops, over 50 growers attended the two growers presentations. Three grower entities made direct contact with the project team to discuss ways to enhance native insects in orchards. There was also an opportunity to present at the eDNA Society of Western Australia and the Ecological Society of Australia in 2022 and 2023, where there were 30 and 300 attendees, respectively. These presentations were focused on comparing the insect detections generated from eDNA with conventional monitoring methods. Further, more broadly, information the project, eDNA metabarcoding in avocado orchards, it's application and use was included on both occasions. These events further extended the reach of the project, it's activities and outcomes, to the WA avocado industry.	
b. Have regular project updates been provided through linkage with the industry communication project and other extension channels?	The industry was kept regularly updated of project activities and given relevant seasonal information through 2 workshops over the life of the project, 1 article in the industry magazine Talking Avocados. Presentations coordinated by the industry communication project at the WA regional forum in 2021 and Grower presentation in May 2023. Growers that hosted monitoring sites received regular emails.	
c. How accessible (including timing and location) were workshops and extension material to southwest WA avocado growers?	All workshops were held in the Manjimup/Pemberton area, the focus region for native insect detections. All except one workshop was held in autumn or winter, avoiding the busier spring and summer months for avocado crops.	

Recommendations

The purpose of this project was to assess the efficacy of eDNA-based surveys and ultimately improve monitoring in agroecosystems. This technique was successfully applied for both insect and plant taxa (see previous section) and across multiple orchards, below are the recommendations obtained from this research:

Grower practice

- It is recommended that growers wishing to use eDNA contact local organisations who can provide a consultation on the cost to collect, process and analyse the data generated from eDNA surveys. These organisations which specialise in the eDNA workflow include:

  1. eDNA Frontiers (http://www.ednafrontiers.com),

  2. EnviroDNA (https://www.envirodna.com/about/company),

  3. University of Canberra (https://www.canberra.edu.au/research/institutes/iae/our-research/environmental-dna), and

  4. Wilderlab (https://www.wilderlab.co.nz/).

- The findings from our applications of eDNA metabarcoding of 'Hass' flowers indicate that far from relying on only honeybees, avocado trees are visited by an array of native species (e.g. hoverflies, blowflies and moths). To increase the diversity and persistence of native insects in orchards, growers should:

  1. Growers should provide habitat for native pollinators by planting bare patches of earth with native species and providing nesting areas with commonly available materials (Prendergast et al., 2020).

  2. Growers should avoid the use of pesticides close to crop flowering.

  3. All orchards should be regularly monitored for emerging pests (i.e. six spotted mite).

- Based on the pan-trap water eDNA metabarcoding results, the following on-farm management practices are recommended to all avocado growers to support diverse foraging in and around orchards.

  1. Retain remnant areas of native vegetation in and around orchards.

  2. If possible, refrain from removing weed species in the understorey and inter-row of avocado orchards until avocado flowering has commenced.

Monitoring and sampling

It is recommended that all growers wishing to collect eDNA samples (i.e. crop flowers, soil or leaf material) themselves take into account the following considerations.

- If unmanaged/native insects are of interest, complementary methods (e.g. pan traps) should be included to capture whole specimens for morphological identification and barcoding.
- 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, Lekang, Jonassen, Thompson, & Troedsson, 2017; Mauvisseau et al., 2019).
- 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.
- 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 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.
- 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).

Extension

A major extension recommendation from this project is that resources be put towards improving the monitoring of beneficial and antagonistic insects within orchards through activities such as eDNA metabarcoding and morphological identification, workshops, one-on-one insect community assessments and adjustments and general media on the benefits and how-to of collecting eDNA samples. This recommendation comes largely from the potential pollinators studies and the high occurrence of unknown insect communities within orchards and about eDNA technology. This would not only be beneficial for improving insect management practices but also management of all pollinators, pests, predators and pathogens that emerge within orchards.

Research

There are several areas of future research that the project recommends to increase understanding of the insect communities in orchards/eDNA metabarcoding surveys and improve sustainable management of these beneficial and antagonistic insects. These research areas could be addressed individually or as part a larger project.

In the area of eDNA surveys there are three specific research topics that the project recommends pursuing in order the improve the use of eDNA metabarcoding for orchard management.

• Conduct multi-year eDNA surveys in tandem with conventional insect capture (e.g. pan traps) to identify shifts in insect communities and the plant resources they utilize.

• Assess the use of in-field eDNA sequencing technologies (i.e. portable PCR machines with species-specific assays) to target beneficial (i.e. native bees) and antagonistic (i.e. mites) insects from informative substrates (i.e. crop flowers). This will provide 'same day' results which can then be used to inform management strategies for orchards (i.e. increase hive density).

• Identify and sequence additional insects that are absent on the online reference databases. This will improve the accuracy and reliability of eDNA metabarcoding specific to these crop ecosystems.

Refereed scientific publications

Journal articles

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.

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 insects in food production systems? *Molecular Ecology Resources*, 23(7), 1473-1755. doi:10.1111/1755-0998.13814.

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

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 insect-plant interactions, *Environmental DNA.* (In review).

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

Acknowledgements

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Appendix 1: Figures and Tables

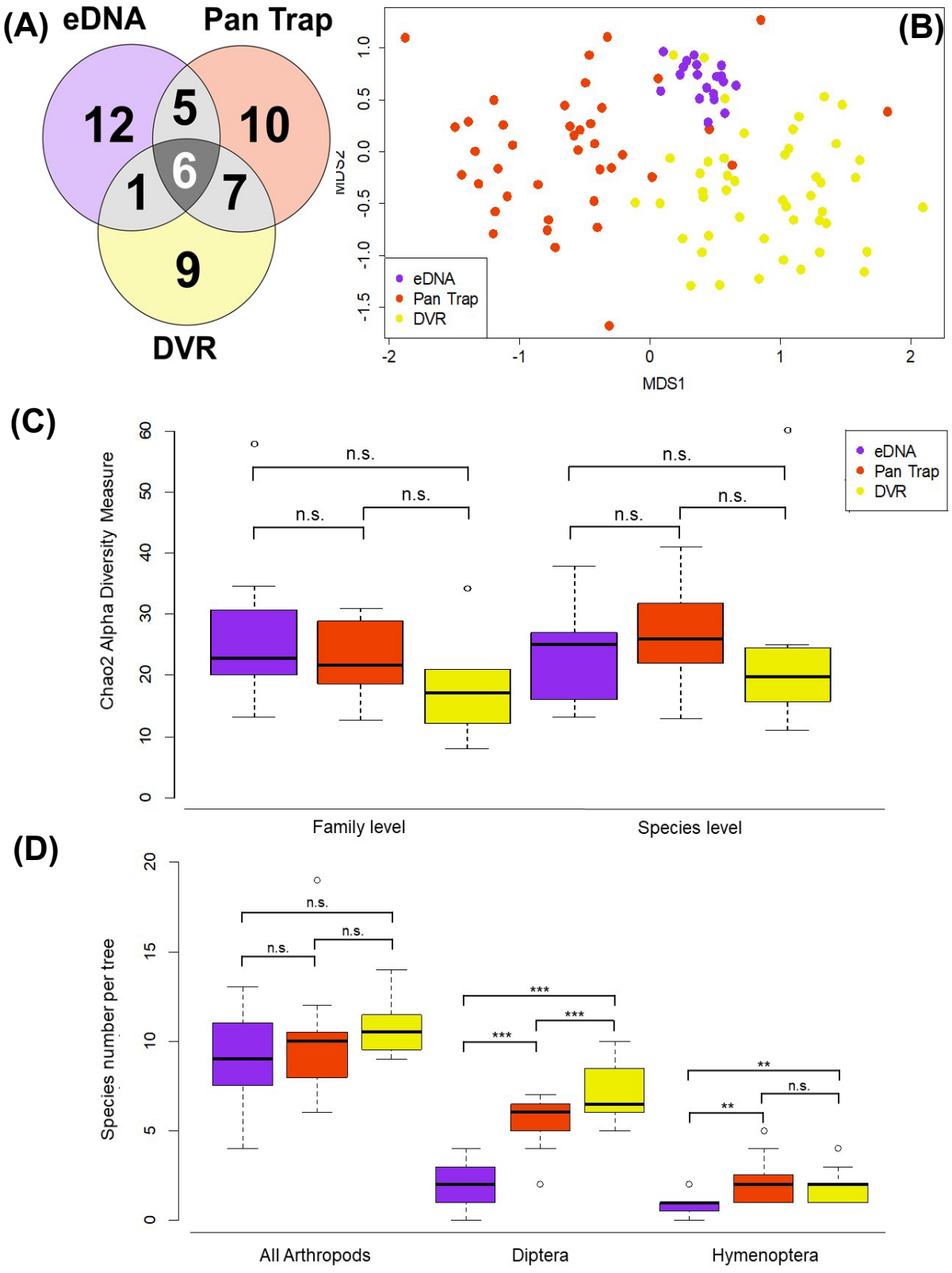

Figure 1.4 (A) Number of families identified for each survey method; eDNA (*N* = 24), DVR (*N* = 23) and Pan Trap (*N* = 28).

(B) Non-metric multidimensional scaling ordination (Stress value = 0.1098) showing the relationship between insect 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 insect 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 insect species collected per tree and both major flower-visiting insect 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 1.1 Taxonomic identifications of the 49 insect 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)

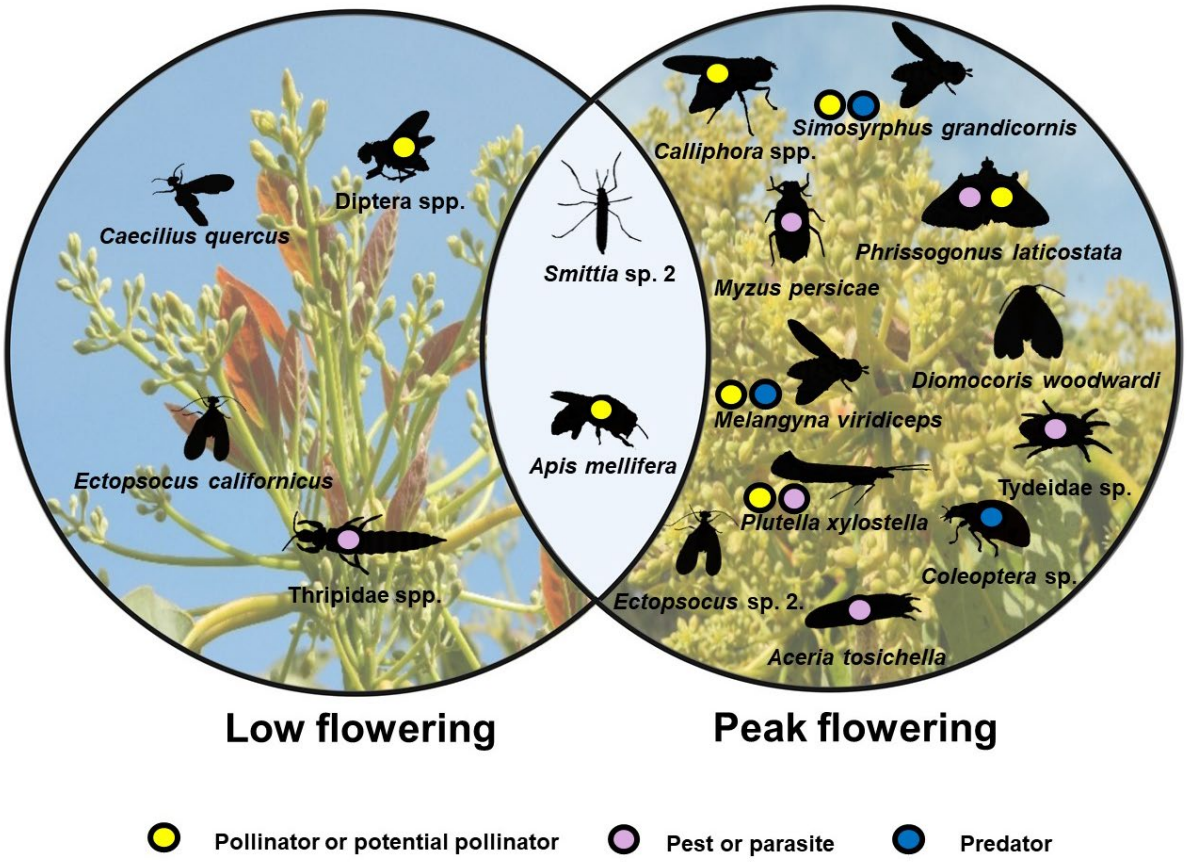

Figure 1.5 The key species driving dissimilarity identified in the eDNA and DVR SIMPER analyses at both sample orchards at low and high *P. americana* flowering. Where known, species have been colour-coded as either pollinator, pest or insect predators.

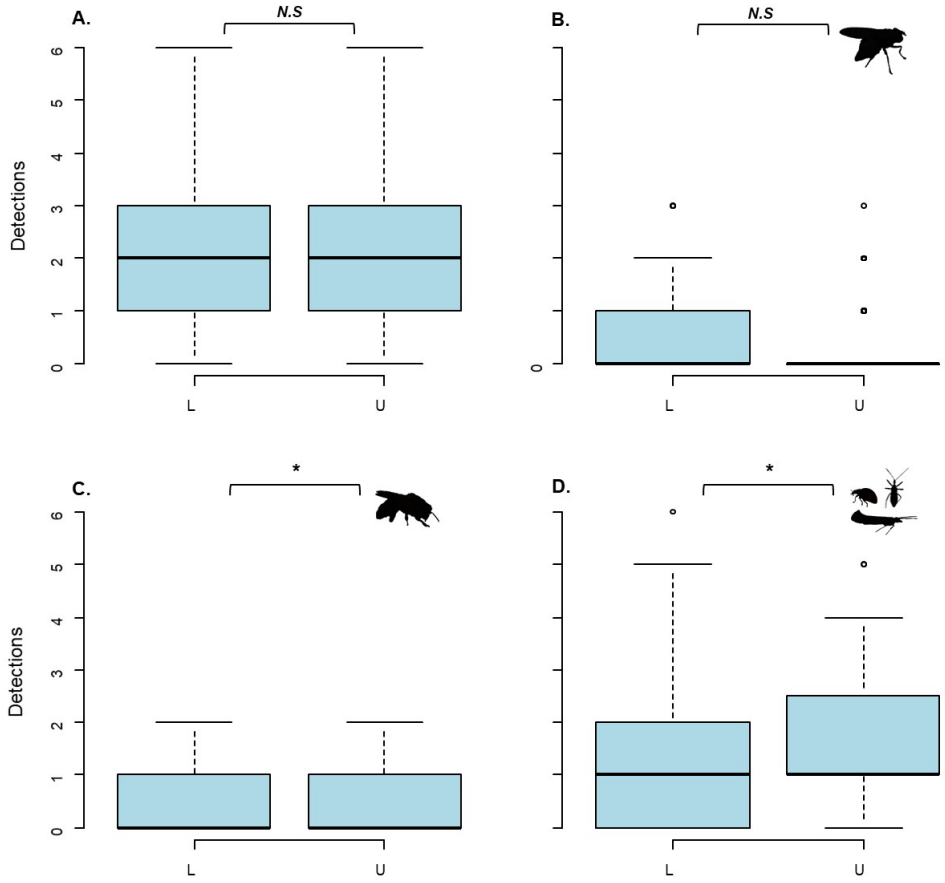

Figure 1.6. eDNA-detected diversity changes significantly temporally, spatially and within canopies. Vertical stratification for insect functional groups (A) all insects, (B) Diptera, (C) Hymenoptera, and (D) 'Ancillary' detected using eDNA metabarcoding. N.S = $p > .05$ and * = $p \leq .05$.

Table 1.2. Plant taxa (*N* = 64) detected using eDNA collected from pan traps (after putative false positives were removed) and floral surveys at three *P. 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

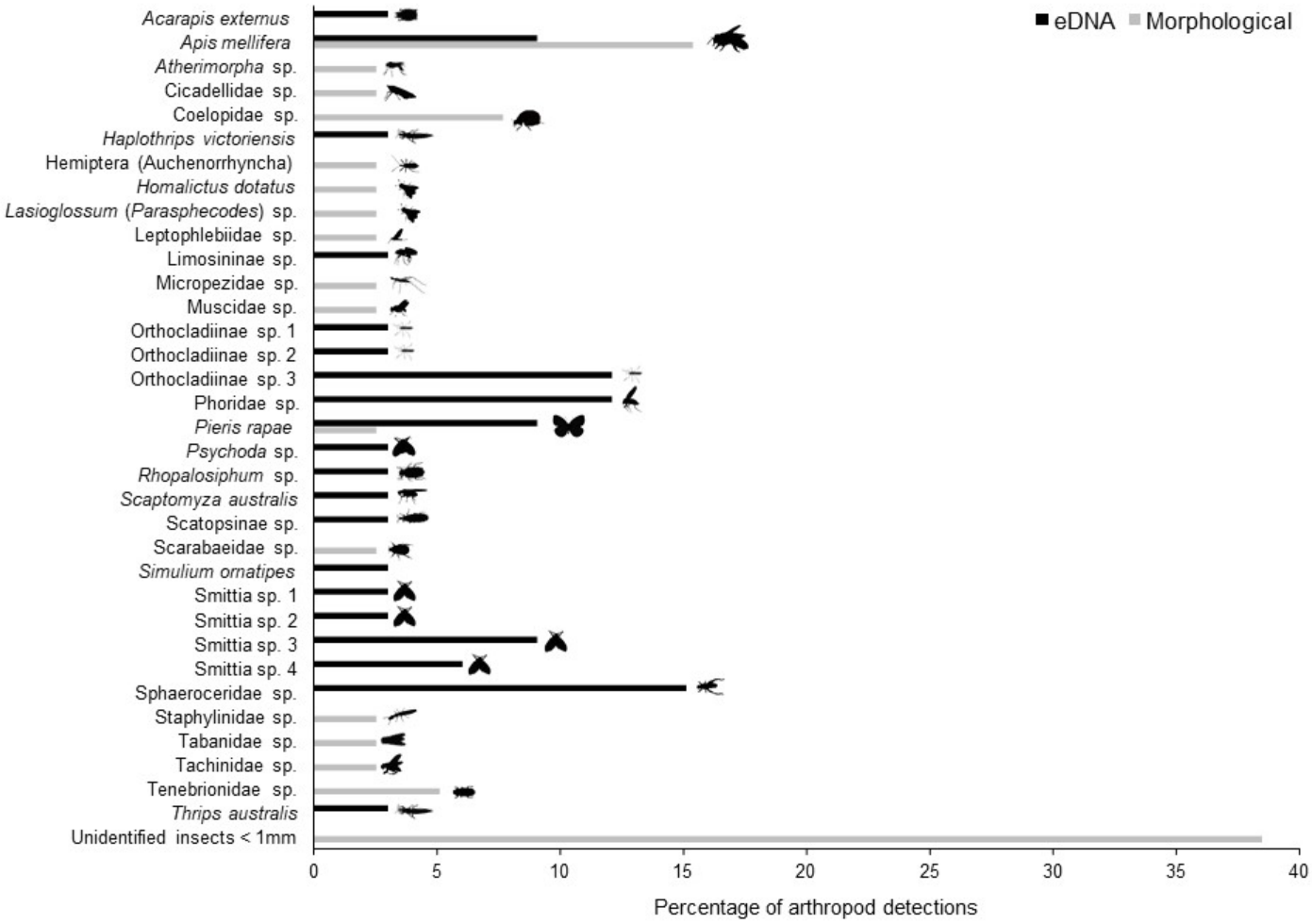

Figure 1.7. Percentage of taxa detected for insects classified by eDNA metabarcoding of pan-trap water (*N* = 20 taxa total) and morphological identification (*N* = 17 taxa total). For the morphological identifications, insects that were less than 1mm in size were not identified, due to the focal orchard species, *Persea americana*, typically requiring pollinators with larger body sizes (Ish-Am, 2005).

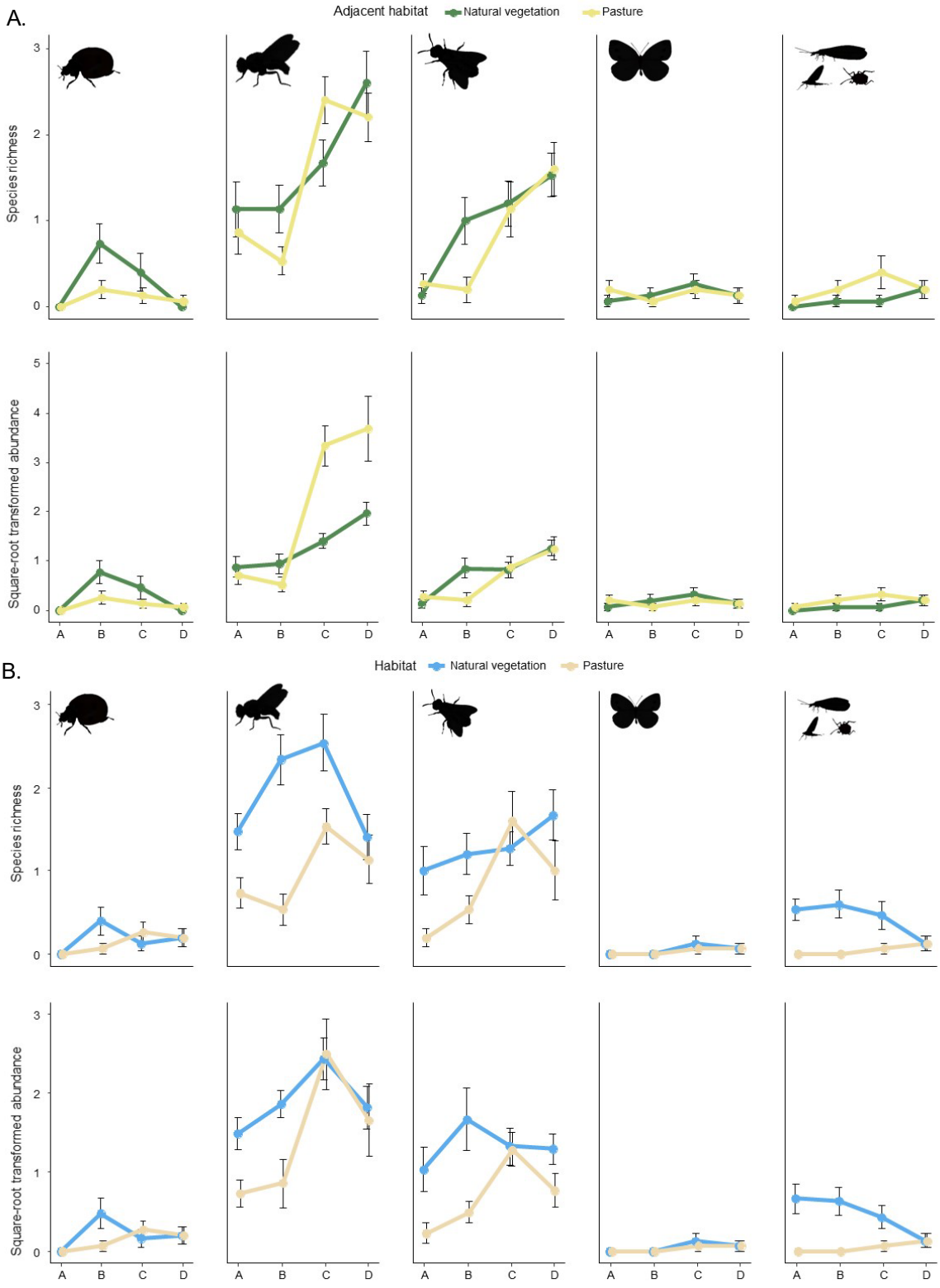

Figure 1.8. A. Orchard measures of species richness and square-root transformed abundance data, with standard error bars, for the five dominant orders collected in six orchards adjacent to either natural vegetation or pasture in the Manjimup-Pemberton region of SWWA. B. Adjacent habitat measures of species 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.

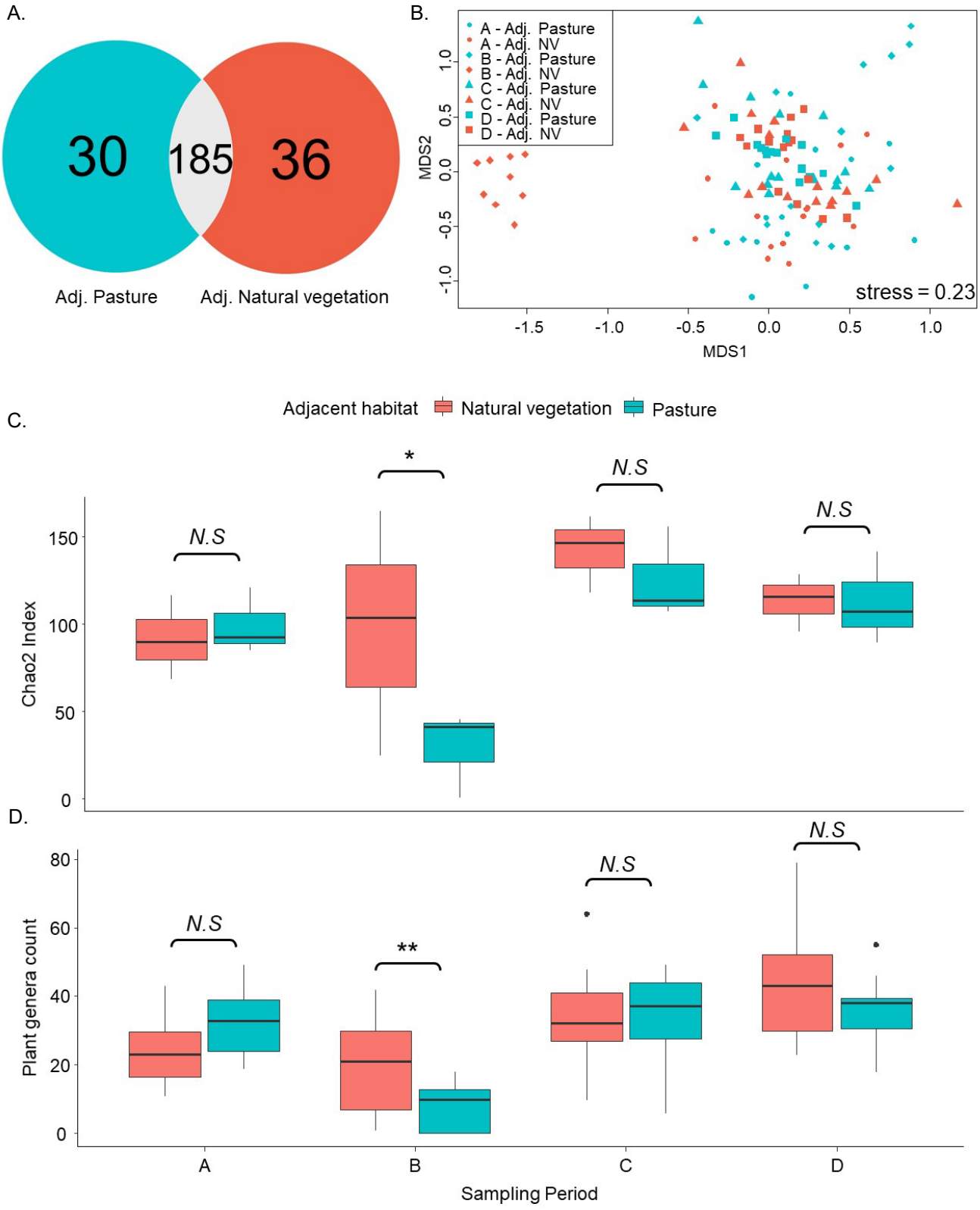

Figure 1.9. A. Number of plant genera identified in orchards adjacent to pasture and natural vegetation; Adj. Pasture (*N* = 215) and Adj. Natural vegetation (*N* = 221). B. Non-metric multidimensional scaling ordination based on a Jaccard dissimilarity matrix (Stress value = 0.23), showing the relationship 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 ***= $p \leq .001$.

Appendix 2: Published literature review

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

Review

Appendix 3. Published data chapter

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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

[illegible] *Persea americana* [illegible]

KEYWORDS
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Intellectual property

No project IP or commercialisation to report