THE UNIVERSITY OF HULL

# 3D structure of pollen provisions in *Osmia bicornis*: consistent patterns and their implications for future research.

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# AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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## General introduction

Diet plays a pivotal role in shaping the health and development of animals across diverse ecosystems (He et al, 2021., Souza et al, 2019., Ojala et al, 2005., Cahu et al, 2003., Kaspi et al, 2002). Animals are highly dependent on the chemical composition of their food, with micro- and macronutrients playing key roles in their dietary requirements (Friend, 1958., House, 1962., Simpson & Raubenheimer, 2012). Yet, the range of diet breadths available to animals is vast, ranging from those that are free to select their nourishment (as in many free-living animals) to others with more constrained diets (as in many sedentary animals).

In the broader animal context, dietary freedom manifests in the choices animals make when sourcing their nutrition. Some animals, free-living and adaptable, roam their environments indulging their appetite in a variety of dietary options. One great example, are humans (*Homo sapiens*), they are free to pick and choose what they eat and when they eat, specifically tailoring their diets to what they prefer (Naghii et al, 1996., Tur et al, 2005). Furthermore, free-living species can adapt and change their diet to accommodate to different requirements, such as pregnancy and illness (Krockenberger & Hume, 2007). In contrast, certain sedentary species or those living within their food sources, (like the wood-eating termites, Poissonnier et al, 2018), have their diets already dictated by their ecological niches.

Insects, with their immense diversity, demonstrate a variety of different ways to gain nutrients (Daly et al, 1978), some are free-living and adaptive (Polilov & Beutel, 2009., Jones et al, 2003., Davies et al, 2011), whilst others are not (Poissonnier et al, 2018., Mitchell, 1983., Hanks & Denno, 1998), yet both survive adequately. Alternatively, one major adaptation many insects have developed, is provisioning; a form of parental care behaviour whereby the adults provide food for their offspring until they reach full development (Clark et al, 2011., Field, 2005). Found predominantly in Hymenoptera (mainly bees and wasps, Bingham & Morley, 1897), the food sources often take the form of masticated or immobilised prey items (predatory wasps, Doutt, 1959) or combinations of nectar and pollen, only rarely do you see other forms of provisioning techniques such as carrion (decay) or glandular secretions (Field, 2005).

There are different types of provisioning; the first example comes from species who change their offsprings provisions according to the food available in their environment. Although organisms do regulate their nutritional intake towards an intake target, they can still have a distinct response towards environmental biodiversity. For example, when nutritional generalists such as the desert locust (*Schistocerca gregaria*) are given a largely diverse food source, they consume greater excess of the abundant nutrients (Raubenheimer & Simpson, 2003). Additionally, Donkersley found that Honey bee (*Apis mellifera*) hive provisions differ according to what is available in the landscape (Donkersley et al, 2017). Thus, showing that although provisioning is preplanned, it can still change depending external factors.

Often, offspring needs are not static; they can change over time, meaning their dietary requirements will shift as they age (Howard, 2020., Slansky, 1982). Therefore a second type of provisioning is known as progressive provisioning, where parents continually adapt their offspring's diet in response to evolving requirements (Suzuki, 2010., Gayubo, 2011., Wilson, 2008., Wong et al, 2013), ensuring a better match between nutritional resources and dietary requirements (Wilson et al, 2008). In contrast, others employ mass provisioning, where a fixed quantity of food is provided without regard to the offspring’s needs (Simpson & Raubenheimer, 2012., Smith et al, 2003., Wilson, 2008., Wong et al, 2013). While efficient in some cases, this may prevent the offspring from communicating its needs and therefore limiting the parents ability to maximise their offspring’s development (Wong et al, 2013., Warbrick-Smith et al 2009., Poissonnier et al, 2018). Nevertheless, if adults that perform mass provisioning are to optimise offspring diet, without the ability to dynamically adjust the food offspring receive, one option is to structure the food provision so that offspring encounter different nutrition at different stages of their growth. To my knowledge, this has never been demonstrated.

### *Study organism*

Red mason bees (*Osmia bicornis,* Apoidea: Megachilidae) are a cavity-nesting solitary bee species known to perform parental mass provisioning. They are native to the UK, and easily recognised by their dense ginger hair covering their abdomens (Split et al, 2022). Red mason bees are classed as pollen generalists (polylectic, Haider et al, 2014), and commonly considered to be a habitat generalist too; although they do display a preference for untidy nesting areas in the periphery of undisturbed fields, orchards and forests (Fliszkiewicz et al, 2015., Gruber et al, 2011), as well as man-made holes such as brickwork and "Bug hotels".

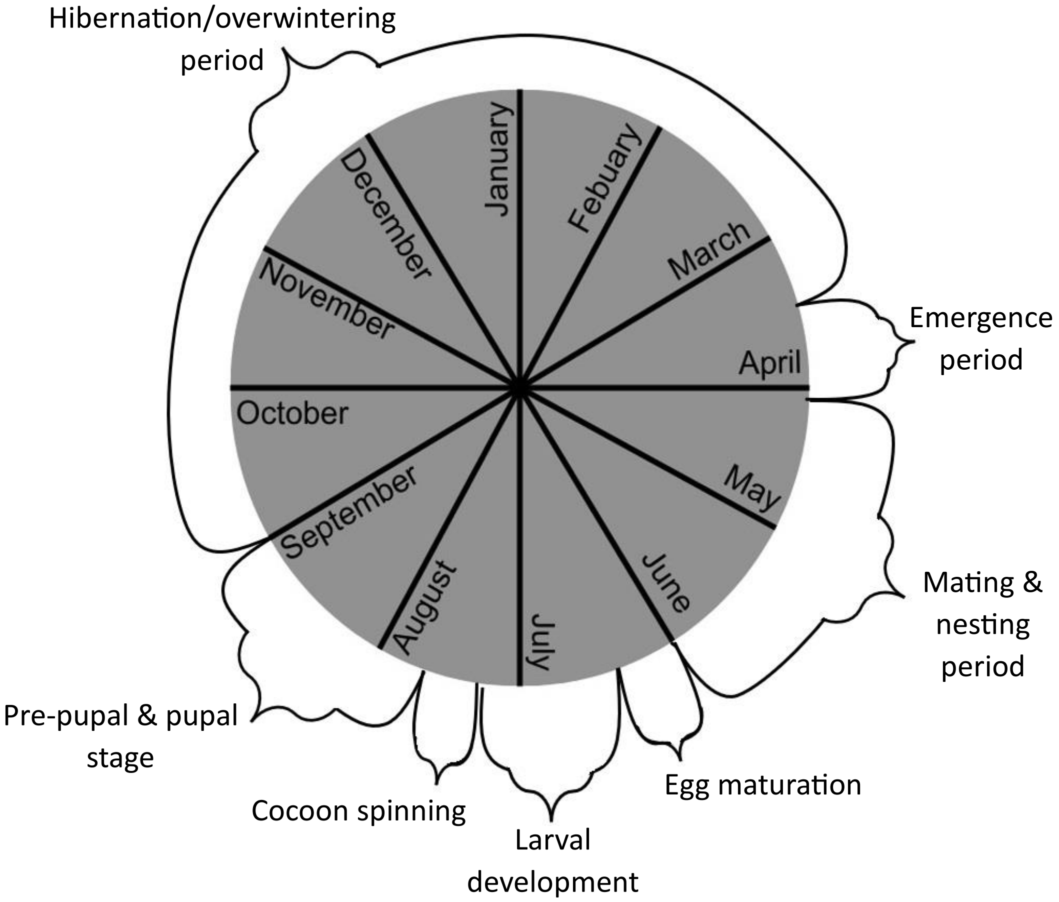

*Figure 1: A rough diagram demonstrating the annual lifecycle of Osmia bicornis. Information references: Raw, 1992., Krunić & Stanisavljević, 2006., Van der Steen & De Ruijter, 1991., Giejdasz & Wilkaniec, 2002 and Splitt et al, 2022.*

*O.bicornis* are univoltine (produce one brood in a season), with a lifespan of roughly 300 days for females and 260 for males (Splitt et al, 2022). As seen in figure 1, the eggs go through a short (~8 days) maturation before hatching and develop into larvae, during this time (~36 days female, ~32 days male, Giejdasz & Wilkaniec, 2002) they consume their pollen ball provisions and end by spinning themselves a cocoon (Krunic & Stanisavljevic, 2006). The fat reserves they consumed are then used to develop into a pupa and begin their overwintering period (hibernation-like state), which should last 120-170 days for optimal diapause (Van der Steen & De Ruijter, 1991). The female eggs (fertilised) are laid towards the back end of the nests as they take longer to emerge than males (unfertilised), there can be as little as 2 days or up to 2 weeks between the first male and last female emergence (Bosch, 2008., Van der Steen & De Ruijter, 1991). Also known as protogyny, their overwintering period can vary in length; 31.6 ± 2.41 days for males compared to 35.5 ± 1.99 for females (Giejdasz & Wilkaniec, 2002., Splitt et al, 2022) as it depends of temperature (around 15 degrees, Krunić & Stanisavljević, 2006). However, emergence usually always occurs in early spring (March/April). Now the emerged bees can mate and the female bees will begin their nesting activities, whilst the males die off (~30 days, Raw, 1992) and the cycle repeats with the next generation.

These bees exhibit specific nesting behaviours and foraging patterns (Split et al, 2022) that facilitate the observation and analysis of how they construct their nests and provisions for their offspring. This biological reproducibility allows researchers to examine the intricacies of provisioning strategies in a controlled manner. They are highly tractable for experiments; their relatively small size (Radmacher & Strohm, 2010) and low-maintenance life cycles make them amenable to laboratory and field studies, allowing scientists to manipulate various aspects of their foraging/provisioning behaviours. This tractability provides a valuable opportunity to design controlled and precise experiments.

### *Provisioning behaviour*

Female Osmia spend around 70 days (splitt et al, 2021., Raw, 1972) actively creating nests for their offspring. They create 10-12 individual cells in tube-like holes that are roughly 8-9mm in diameter and 150-200mm in length (Gruber et al, 2011). Constructing each cell takes time, as they are lining the cells with mud and laying one egg per cell, these eggs are then provisioned with a singular "pollen ball". A pollen ball is constructed of various pollen grains held together by small volumes of nectar (often 10mm in diameter) that are obtained by the female over an average of 6 – 7 hours, via ~ 17 - 30 foraging trips per ball (Seidelmann, 2006., Strohm et al, 2002., Tasei & Picart, 1973). However, this is dependent on female size and pollen mass needed for egg, i.e male or female (Seidelmann, 2006).

The mass of the pollen ball, how it is constructed and its nutritional content can be an effecting factor on offspring size, development time, fitness and mortality rates (Radmacher & Strohm, 2009., Filipiak & Filipiak, 2020., Austin & Gilbert, 2021., Seidelmann, 2006., Bosch & Vicens, 2006., Howard, 2020). This emphasizes the idea that these pollen balls are essential to the correct development of *Osmia* larvae and raises the question what the "correct" pollen ball would be. Recently, a study looking into the macronutrient content of said pollen balls, showed that *O.bicornis* larvae would always consume the same amount of carbohydrate regardless of whether they were given carbohydrate based pollen diet or protein based pollen diet (Austin & Gilbert, 2021). In other words, when provisioned a protein based diet; the larvae chose to consume more overall pollen mass (when compared to the carbohydrate groups) in order to maintain a high intake of carbohydrates. Implying the "rule of compromise" (a set of rules that an organism undertakes when optimal nutrition is not available. Raubenheimer & Simpson, 1999., Morimoto, 2023) for *Osmia* larvae is essentially a fixed carbohydrate target (Austin & Gilbert, 2021).

This shows that *O.bicornis* larvae, although dependent on adults for all food, play an active role in balancing their own nutrition to some extent. Nevertheless, the question remains – do females purposely provide a balanced diet for their offspring or are the larvae responsible for their own diet balancing? As stated previously, an offspring's dietary requirements can change over time, if females optimise larval performance via mass provisioning, then, we might expect the provisions to show a degree of structure to reflect these changing needs. My first research question (Chapter 1) was:

1) Are *Osmia* pollen provisions structured so as to provide larvae with different diets at different points in development?

To address this, in chapter one I will be collecting pollen balls that were provisioned in mason bee nests I set up throughout the Hull area. I will orientate the pollen balls so that "top" of the ball is where the larvae was situated and slice horizontally down the ball, creating "top to bottom" layers where I can take point samples. From these samples I will identify the pollen grains present and look for any patterns in the spatial placement and species richness across the layers. For the hypothesis to be supported, I would expect to see a repetitive pattern present across *all* pollen balls, irrespective of the samples location and time of construction (as pollen availability can change daily and seasonally, Norris-Hill & Emberlin, 1991., Wood et al, 2018). Additionally, I expect to find no patterns present from east to west or north to south and only from top to bottom or inner to outer, depending on how the provisions are constructed.

The pursuit of scientific knowledge often leads researchers down a path of discovery that balances the idea for precision with the practicalities of efficiency. This is especially prevalent in ecological studies, where understanding the smaller, finer details and intricacies of species interactions/behaviours can be paramount. If *O.bicornis* pollen balls are structured, then taking a singular pollen sample may not adequately capture the full pollen composition present within the pollen ball. Yet, researchers have often done this on the assumption that the pollen provisions are homogeneous (e.g. Gresty et al, 2018., Suzuki et al, 2023).

Although a precise and accurate method is necessary to study the complex provisioning behaviours of *O.bicornis*, with it, often comes a considerable demand on time, resources, and researcher effort. In addition to the invasive sampling method (rehousing and replacing food sources for the mason bee larvae), these can prevent future studies from taking place due to high commitment of time and resources. A simpler, more time-efficient and species-friendly approach which could potentially extract the same structural information would be beneficial. In an attempt to strike a balance between sufficient, detailed results and minimal researcher effort, my second research question (Chapter 2) was:

2) Can you reduce researcher effort by using a smaller, minimally invasive pollen sampling technique and still gain insights into the pollen provisions structure?

*Aims and objectives*

To summarise, the two chapters of this thesis will focus on the pollen provisions provided by female *Osmia bicornis*, and whether there is a top (relative to larvae) to bottom structure present. I aim to look into the three dimensional structure and composition of the pollen provisions, and identify any potential patterns or signs of heterogeneity. In addition to this, In chapter 2, I aim to re-evaluate the sampling methods and researcher effort required to obtain a minimal adequate understanding of the whole provision. I will apply different sampling techniques in an attempt to reach a minimum sampling protocol all while maintaining the integrity of the data collected.

# **Chapter one -** Pollen provision structure in *Osmia bicornis*: pollen arrangement changes with offspring development.

## Introduction

An animal's diet can affect an array of biological traits, both physiologically (Sansone et al, 2019., Pearson et al, 2009., De Wit et al, 2012) and behaviourally (Han & Dingemanse, 2015., Birch & Fisher, 1998). Nutrition is a key element in becoming a healthy adult, as previous studies have shown that an offspring's diet can affect their developmental outcome (He et al, 2021., Souza et al, 2019., Ojala et al, 2005., Cahu et al, 2003., Kaspi et al, 2002). Animals need multiple nutrients to maximize their fitness; if animals fail to ingest an optimal balance, their body's development and health can be compromised (Simpson & Raubenheimer, 2012). Optimal foraging theory is based on three assumptions; that the animal's foraging abilities increase the next generation's fitness, that the foraging abilities are somewhat hereditary and thirdly, that the relationship between foraging and fitness is known (Pyke, 1984).

Optimal foraging theory is especially relevant in species with high maternal investment in the form of provisioning, as the offspring often cannot acquire nutrition for themselves. The offspring rely entirely on the parents' ability to provide them with a sufficiently nutritious diet to maximise their fitness (Royle et al, 2012). However, optimal nutrition and consumption patterns can change throughout the offspring's development; becoming more or less dependent on different nutrients over time (Orłowski et al, 2015). Under this scenario it may be advantageous for females to provision strategically to provide specific nutrition targeted to the offspring's development stages.

One example of strategic progressive provisioning behaviours is in nesting birds. In general, birds will feed insects and small prey to their offspring regardless of adult diet, which is often different from that of offspring (Catry et al, 2016). However, some species are known to bring different types and proportions of food at different stages of their offspring's development (Mitrus et al, 2010., Radford, 2008). For example, Blue Tits (*Cyanistes caeruleus*) feed a high proportion of spiders early on in their offspring's development (García-Navas et al, 2013.) Presumably to target the maximum fitness as spiders contain higher concentrations of taurine and calcium, both essential for nestling's early development (Jacobsen & Smith, 1968., Haleswood, 1962). Furthermore, as they age, the food provided to nestlings changes, showing reduced numbers of spiders and a greater proportion of tortricid larvae (García-Navas et al, 2012). In another example; analysis of the Barred Warbler (*Sylvia nisoria*) nestlings' faeces showed different compositions of prey size, diversity and chitin content (necessary for body hardness and digestibility) depending on the age of the nestling. Thus, showing a strong correlation between parental provisioning and nestling age/body mass (Orłowski et al, 2015). Nestlings vary immensely in body mass between the ages of 2 to 9 days old, therefore their dietary requirements change greatly throughout this developmental period (Orłowski et al, 2015).

Parental care, including offspring provisioning, is particularly diverse in the insect world, making an appearance throughout 13 different orders and a minimum of 45 families (Tallamy & Brown, 1999). Many of these species show behaviours such as egg guarding (Modeer, 1674), and provisioning (Killewald et al, 2019). Provisioning can be progressive e.g. earwigs, stink bugs and some species of wasps (Suzuki, 2010., Tsukamoto & Tojo 1992., Kelstrup et al, 2018) or alternatively social insects like ants and honey bees display group provisioning, where they take one resource to feed the workers and produce another resource for the colony (Ydenberg & Schmid-Hempel, 1994). However, 90% of bee species are solitary, meaning the bees do not live in a colony, and have a strategy called mass provisioning, where larvae exclusively feed on a single food mass provisioned by their mother (Strohm et al, 2002) - in this case pollen, the most protein-rich part of a plant (Mattson, 1980). It would be interesting to know if there could still be strategic provisioning occurring within the species that execute mass provisions, for example; the individuals might layer their provisions to tend to specific life stages of their offspring. Alternatively, they could create different mass provisions for the same offspring and consume them at different developmental stages.

*Osmia bicornis* (Red Mason Bees, Apoidea: Megachilidae) are cavity nesting solitary bees, common in England, Wales, and parts of Scotland. The species are known to nest in small tube-like holes such as wall cavities, and are frequent visitors to "bug hotels" and other man-made nesting sites; usually constructed of hollow bamboo canes. They are easily recognised by the dense ginger hair covering their abdomens (Splitt et al, 2022) with the females being larger than the males in body size (Radmacher & Strohm, 2010). Due to the decline of honey bees, solitary bees have become much more important in pollination (O'Toole, 2000), they perform up to 90% of pollination, especially prevalent in crop plants such as orchards (Lee et al, 2010., Gruber et al, 2011). There are different factors that can influence the development of *Osmia* larvae, such as nesting temperatures (Radmacher & Strohm, 2010., Kierat et al, 2017) and moisture (Pitts-Singer, 2004). However, the main influence comes from the female's provisioning techniques, with provisioning-related factors such as parasitic activity (Seidelmann, 2006., Ostap-Chec et al, 2021), provisioning quantity (Radmacher & Strohm, 2009) as well as pollen community (Lawson et al, 2020), all affecting the outcome of the larvae's development. Although the females' individual abilities play a major role in provisioning techniques, there are external factors that can also effect the construction of the provisions. When it comes to pollen selectivity; external factors such as temperature can have an effect on the female's pollen choices (Russel & McFrederick, 2022) and weather in general can also affect the amount of pollen produced by the local plants (Corden & Millington, 1999., Emberlin, 1994., Corbet, 1990). Similarly, diurnal pollen variation can create different pollen availabilities throughout the day (Norris-Hill & Emberlin, 1991) and depending on when the females are foraging, it could lead to provisions with different pollen communities and qualities (Donkersley et al, 2017., Baum et al, 2011., van Doorn & Van Meeteren, 2003).

Referring back to the optimal foraging theory (Pyke, 1984); specifically the third assumption (the relationship between foraging/fitness), the relationship between foraging and fitness in *Osmia* is to do with the quality and quantity of pollen provisioned. Smaller masses of pollen provisions can cause a decrease in adult bee size and create issues later in adult life (Roulston & Cane, 2002). Smaller male bees are less likely to fend off larger males during mating and smaller females will have smaller scopa (pollen collection utensils) and therefore struggle to collect enough pollen within a reasonable timeframe (Bosch, 2008., Bosch & Vicens, 2006). Similarly, different pollen species have different nutritional composition; which can determine the outcome of the larvae's development (Bukovinszky et al, 2017., Austin & Gilbert, 2021., Howard, 2020., Lawson et al., Filipiak & Filipiak, 2020). For example, there are correlations between the nutritional quality of the pollen provisions and the bacterial microbes in the bee's gut; fatty-acid provisioning has a strong effect on pollen-derived bacteria development (Leonhardt et al, 2022). Furthermore, *O.bicornis* that are nutrient deficient have different rates of mortality and cocoon growth (Filipiak & Filipiak, 2020). For instance, there is an 80% mortality rate in Na (sodium) deficient *Osmia* females and only 7% in those supplemented with Zn+ (zinc). Similarly, the cocoon growth was prevented in the bees that were K-deficient, Na-deficient and Na+ supplemented, resulting in a lower number of bees developing to adulthood (Filipiak & Filipiak, 2020).

Therefore it makes evolutionary sense for provisioning females to provide a special diet for their offspring, in order to avoid bad health effects. This hypothesis is known as 'oviposition preference-offspring performance' or the 'mother-knows-best' hypothesis (Jaenike, 1987., Mayhew, 2001) and it is thought to have emerged from selection processes favouring parents who maximise their offspring's fitness via specialised diets (McAulay & Forrest, 2021). However, recent data has shown *O.bicornis* larval requirements change over time; as the larvae age, their nutritional requirements focus on carbohydrate-based pollen (Howard, Austin & Gilbert, in prep., Howard, 2020). This results in larvae with higher protein-biased provisions consuming more overall provision mass than those with carbohydrate-biased pollen, in order to maintain a high intake of carbohydrates. While species such as honey bees that provision progressively are able to change the composition of provisions dynamically over time, species with mass provisioning, such as red mason bees, are unable to do so. One way they might ensure offspring receive targeted nutrition appropriate to their developmental stage is by structuring the provision, so that larvae are likely to encounter different nutrition at different stages as they age and eat through the pollen ball.

There is plenty of previous literature focusing on the composition of solitary bee pollen provisions; however, these studies make the assumption that pollen provisions are homogenous, and we lack an understanding of the structure within the pollen balls (Bedinger, 1992., Moore et al, 1991., Austin & Gilbert, 2021). I address these issues with my hypothesis: Are *Osmia* pollen provisions structured so as to provide larvae with different diets at different points in development?

Red mason bees are classed as polylectic (pollen generalists, Haider et al, 2014), which could be taken to imply they have little preference over pollen types, suggesting that their pollen provisions would be relatively unstructured due to the unplanned foraging. Furthermore, Mason bees are commonly considered to be a habitat generalist, although they do display preferences for periphery of undisturbed fields, orchards and road verges (Fliszkiewicz et al, 2015., Gruber et al, 2011). This “unplanned” nature translates into their provisioning techniques too: unlike honeybees or bumblebees, mason bees do not have pollen baskets, known as corbiculae, to fill and carry pollen grains (Michener, 1999). Instead, they dive into pollen resources and coat themselves in pollen grains, using their thick dry fur on their underbellies (“scopae”) to store the pollen (Zesiger et al, 2021). In summary, the nesting patterns, *Osmia* scopae and provisioning methods imply a lack of precision when compared to other species, hence, the prediction for this investigation is that there will be little or no structure within the pollen provisions.

In this chapter, I extract and examine the pollen balls provided by female *Osmia*, to identify if there are any spatial or structural patterns in pollen grain placement that could imply the females are attempting to target feed their offspring at different developmental stages. By slicing horizontally down through the pollen provisions, with the “top” being where the larvae was situated, I take multiple point samples and identify the pollen species present, providing insight into the pollen ball’s top to bottom structure. In contrast, I would expect not to see any such structure when slicing the pollen ball in directions orthogonal to larval development, e.g. east-to-west, north-to-south and inner-to-outer relative to the larva (see schematic in fig 7). If the pollen provisions are not homogeneous and my hypothesis is supported, I would expect to see a repetitive and consistent difference in the placement of pollen species across multiple provisions. The potential patterns could be comprised of different quantities of pollen species (species community), different fluctuations in proportions of the same set of species, or different numbers of species (species richness). I combine this with vegetation surveys of the nearby landscape, providing information on pollen availability, and thus, indirectly, pollen selectivity and preferences in female *O.bicornis*.

## Method

In April and May of 2021 I set up four experimental mason bee houses in local Hull areas. Houses were made from plywood and coated in odourless sealant for protection against the weather. Each bee house consisted of a wooden spike, topped with a small block with two solid sides, a hinged door on the back and an open front with a slanted roof, see figure 2 for reference. The inside is a nesting area which is separated by 3 horizontal shelves (figure 3), with the bottom three spaces filled with 13-16 polystyrene tubes (the inside tubing is 8-9mm in diameter) with a removable, transparent slip-lid, see figure 4.

*Figure 2: An image of one of the Mason Bee houses.*

The top layer of the block was seeded with a mix of 20 male and 20 female *Osmia bicornis* cocoons, roughly sexed by cocoon size (females are typically larger, Radmacher & Strohm, 2010), ordered from the Mason Bee Company (https://www.masonbees.co.uk). Bees can see in the ultra-violet (UV) colour spectrum (Peitsch et al, 1992), making bright coloured flowers attractive to them (Kevan et al, 2001). Therefore, to make the houses more attractive to the female bees and entice them to nest, I painted the outside of the houses with UV spray paint. The houses were placed in locations with low foot-fall with high volumes of vegetation and nearby mud for nesting. See figure 5 for site locations via a satellite map and table 1 for their map co-ordinates and what3word addresses.

*Figure 3: Close up image of the bee house nesting area.*

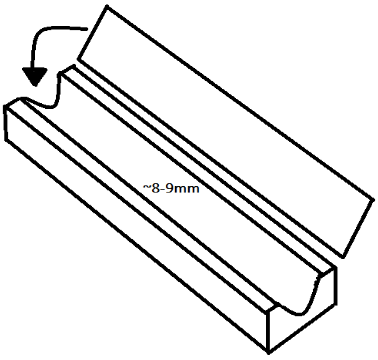

*Figure 4: A sketch diagram of the polystyrene tubes, with a transparent lid.*

*Figure 5: A map of hull showing the bee house locations. Point 1 is Thwaite Gardens, point 2 is Westfield Campus and point 3 is Salmon Grove.*

| Site Location | Map Co-ordinates | What3Words Address |
|---|---|---|
| **Thwaite Gardens (1)** | 53.780092, -0.402854 | https://w3w.co/called.cities.sunk |
| **Westfield Campus (2)** | 53.772734, -0.374557 | https://w3w.co/regime.final.lame |
| **Salmon Grove (3)** | 53.769991, -0.366319 | https://w3w.co/miles.funded.giant |

*Table 1: A table showing the bee house locations and their co-ordinates and What3words locations.*

After the seeded *O.bicornis* emerged and started nesting, the tubes became capped and I collected the pollen provisions. I removed the slip lids (figure 4) and carefully took the pollen balls from the bee larvae and placed them into a separate container. Making note of its original orientation with respect to the larva, as well as the location within the nest (front or the back), to provide an estimate of larval sex as males tend to be at the front (Splitt et al, 2022., Giejdasz & Wilkaniec, 2002). The “larval crater/indent” is what I used to describe the hole in the pollen provisions left behind by the squirming larvae. This is how I oriented the balls, this indent would be the top of my sample, in slice 1 (figure 7). Larvae were provided with alternative pollen on which to develop for separate research.

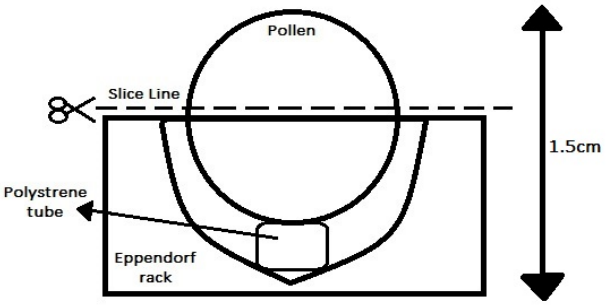

*Figure 6: A diagram showing how the Eppendorf rack and polystyrene tube was used to stabilise the pollen provisions during slicing.*

Sampling the internal structure of a pollen ball without destroying their structure was a novel challenge without literature precedent. I tried a number of methods for slicing the pollen ball, which are detailed in the supplementary material, but the final method was as follows. I hand sliced the pollen provisions with a scalpel or razor blade, whilst using a small Eppendorf rack to stabilise the pollen provision (see figure 6 for reference). I used very small polystyrene tubes, cut to specific lengths inside the Eppendorf rack, as a slicing guide and placed each pollen ball on top. Thus, making sure all slices were taken at the same height and location in each sample, creating the reproducibility necessary for a study of this nature. I created 5 equally spaced slices (2mm for each slice, see figure 7) in each provision, cut vertically with the larval indent at the uppermost point.

The slices are classed as fifths, 1/5 being the first slice and 5/5 being the last. On each slice, I took 9 point samples using a cocktail stick dampened with deionised water. I coded the samples from one to nine - with one to five evenly spaced across the centre and sample three being the innermost point. Samples six to nine run vertically, also through the centre point, forming a cross on each section (see figure 7). Nine point samples from each of the five slices gave 45 samples from every whole pollen provision.

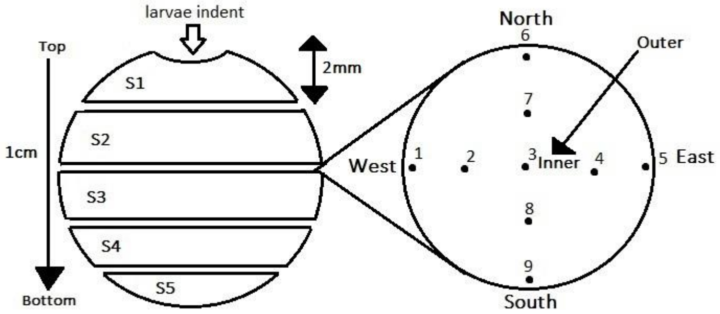

*Figure 7: Pollen provision slice diagram. showing the full pollen provision with slices 1 - 5 labelled on the left. On the right is the labelled point samples 1 - 9 on an individual slice. With the variables top to bottom, North to South, East to West and inner to outer.*

I placed each point sample onto a microscope slide, using silicone oil and a coverslip to preserve it before placing it under a light microscope. I counted 100 individual pollen grains per slide, starting each slide at the top right, moving down, across and back up the slide until I had reached 100 grains. This provided me with 4,500 individual pollen grains per whole pollen provision to identify. My final sample included 5 whole pollen provisions from each bee nesting location (Westfield campus, Thwaite gardens and Salmon Grove nature area, see figure 5 and table 1), leading to identification of 67,500 individual pollen grains.

To ensure a consistency in recording of the pollen species, I took multiple reference photos for each new pollen grain I encountered, which I used to begin the identification process. I managed to identify each pollen into types using a combination of size, shape, apertures (where pollen grain walls thin/soften Božič & Šiber, 2020) and surface morphological features (Moore et al, 1991). Types were then assigned taxonomic identifications, for example (figure 8); *Quercus* (oak) is a tricolpate (contains three slits) pollen grain, whereas *Ranunculus* (buttercup) is a pantoporate (pores scattered over the surface). These descriptions plus, image comparisons were from a variety of standard dichotomous keys (Wiethold, 2009., Moore et al, 1991), as well as consultations with an expert palynologist and the reference collections held at the University of Hull. The taxonomy of pollen grains was adjusted in accordance with "Stace", a system developed by Clive A. Stace; a British botanist. This alignment was achieved using a reference list tailored for the UK, specifically prepared by K. D. Bennett.

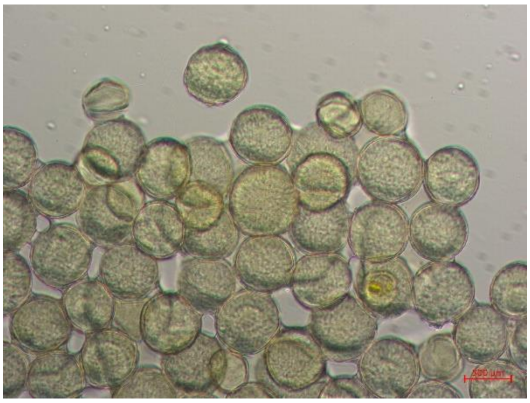

*Figure 8: An image of multiple Quercus (small lined grains) and Ranunculus (large spherical grains) pollen under a light microscope.*

All data analysis was conducted in R (v4.2.0; R Core Team, 2022) and using the vegan package (Ksanen et al, 2022), I created 4 variables to describe sample position within the pollen ball structure, see figure 7 for point references:

- Position relative to ball top (larval indent): five values from 1 (top slice) to 5 (bottom slice).
- Position relative to centre of pollen ball: three values of 'outer' (points 1, 6, 5, 9), 'inner' (points 7, 4, 2, 8), and 'centre' (point 3).
- Position relative to East-West axis: three values of 'East' (points 6 & 7), 'EW central' (points 1, 2, 3, 4, 5) and 'West' (points 8 & 9).
- Position relative to North-South axis: three values of 'North' (points 1 & 2), 'NS central' (points 6, 7, 3, 8, 9) and 'South' (points 4 & 5).

If the pollen provisions are layered, then I would expect to see a strong pattern from the top to bottom variable and potentially the inner to outer; but no patterns should be present in the north to south and east to west. Additionally, if there is a stronger significance seen in the inner to outer variable, then it could demonstrate that the pollen balls are constructed by progressively adding outer layers to the central core. Therefore, we might still see a similar statistical significance, however the pollen could be structure in a different way.

*Estimating pollen availability*

During the emergence period (June - July 2021), I completed a vegetation survey for each location. I followed five, 20 meter transects starting from the bee house extending into the surrounding vegetation (see figure 9) whilst making a note of each plant species present that was directly under the transect line. By using an app called Seek (iNaturalist. Available at: https://www. inaturalist.org) and the book "Wildflowers of Britain and Ireland" (Blamey et al, 2003), I noted the abundance of each species, counting the number of inflorescences visible under the transect line.

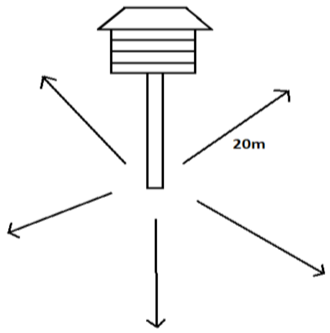

*Figure 9: vegetation survey diagram, bee house in centre with 5, 20m transects*

*Statistical methods*

I first characterised differences in the pollen communities among the pollen balls. As my data were distribution free, I needed an ADONIS permutation-based statistical test. This determines whether the pollen communities in different provisions are significantly different from one another. I fit a model with the pollen community as the response variable and pollen ball ID as a predictor.

Then, to test my hypothesis that provisions would show internal top-to-bottom spatial structure, I performed further ADONIS and ANOSIM (a similar non-parametric technique based on ranks) tests using different variations of slicing the pollen provisions as predictors (respectively, top to bottom, inner to outer, east to west and north to south), restricting permutations to those within each pollen ball, rather than among all the pollen balls. To visualise community differences, I used non-metric multidimensional scaling (NMDS) to reduce the dataset size, by grouping any correlated variables together into a combined axis.

Furthermore, I checked for a difference in species diversity (quantity of different species present in each sample) using both Simpson ($D = \Sigma n_i(n_i\text{-}1) / N(N\text{-}1)$, where N = the total number of organisms and $N_i$ = the number of organisms that belong to species i) and Shannon diversity indices ($H = -\Sigma p_i * ln(p_i)$, where In = the natural log and $P_i$ = the proportion of the entire community made up of species i). A diversity index is a quantitative measure reflecting the number of different species and how the individuals from each species are distributed, therefore allowing me to see if there are structured differences within the provisions in the evenness of distribution of different pollen species.

Looking at the individual pollen species present within the provisions, I used multiple T-tests to see if there was a difference in their abundance at the different layers in the pollen. I then used these results to calculate the percentage differences of specific pollen species present within the provisions.

Finally I tested whether any structural differences I identified with the methods described above were best described as continuous (i.e. changing gradually throughout the provision), or discrete (i.e. showing a distinct breakpoint). Using the R packages vegan and lme4, I fitted 5 models (ADONIS for community differences, mixed models to look at metrics such as species richness) using each of the variables in table 2 as predictors. In each case I compared the 5 models using AIC (Akaike information criterion), to see which described the data best. I regarded a difference of 2 AIC points between two models as evidence in favour of the model with the lower AIC (Burnham & Anderson, 2000).

| Variable | Predictor Variable | Random effect |
|---|---|---|
| Pollen community or metric (species richness or diversity) | All slices (i.e. the original continuous data) | Pollen ID |
| Pollen community or metric (species richness or diversity) | Slices 1 vs slice 4-5 | Pollen ID |
| Pollen community or metric (species richness or diversity) | Slices 1-2 vs slices 3-5 | Pollen ID |

| | | |
|---|---|---|
| Pollen community or metric (species richness or diversity) | Slices 1-3 vs slices 4-5 | Pollen ID |
| Pollen community or metric (species richness or diversity) | Slices 1-4 vs slice 5 | Pollen ID |

*Table 2: A table describing the models used to find the break point in composition difference within the pollen balls. Showing random effect, predictor and response variables for each model.*

## Results

Looking at the initial ADONIS test performed across all of the pollen provision samples (using pollen ball ID as a predictor) revealed that communities showed a significant difference among the provisions (P = 0.001, Df = 12, SumOfSqs = 77.027, R2 = 0.647 and F = 87.222). Thus, meaning it would be necessary to account for pollen ball ID in subsequent data analysis, as each pollen ball had a different composition to the next.

The ADONIS tests looking into the internal structure of the pollen balls (with pollen ball ID as a predictor), revealed no significant difference in the composition from north to south or east to west. However pollen communities differed significantly between outer vs inner (DF = 1, SumOfSqs = 0.170, R2 = 0.00202, F = 1.363, Pr(>F) = 0.013) and top to bottom (Df = 1, SumOfSqs = 0.211, R2 = 0.00251, F = 1.6965, Pr(>F) = 0.007). Repeating this analysis using ANOSIM, revealed no significance across inner to outer, but still showed a strong significance with top to bottom (R=0.004, Sig=0.001).

Looking closer at differences in community metrics between the top and bottom layers, there was no difference found within the species diversity via the Simpson (mixed model, Min = 0, 1st Qu.= 0.175, Median = 0.939, Mean = 0.394, 3rd Qu.= 0.606, Max. = 0.797) or Shannon diversity index (mixed model, Min = 0.00, 1st Qu.= 0.41, Median = 0.78, Mean = 0.79, 3rd Qu.= 1.17, Max. = 1.79). Yet, species richness showed a difference from top to bottom (mixed model, dropping "slice" from the model, X2 = 12.361, DF = 3, p=0.004. Min = 1.00, 1st Qu.= 4.00, Median = 5.00, Mean = 4.76, 3rd Qu.= 6.00, Max. = 11.00).

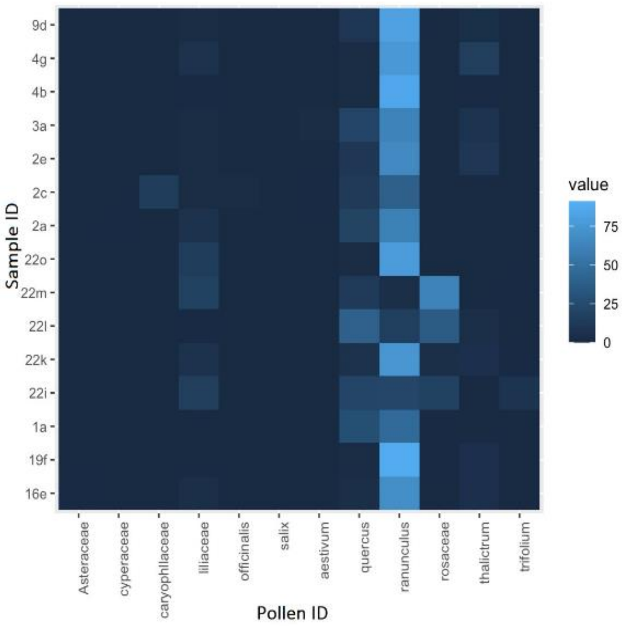

*Figure 10: A heatmap showing the concentrations of the different pollen species present in the pollen provisions.*

I investigated the differences in pollen community and species richness from top to bottom of the provision further, specifically to see whether the community was composed of discrete layers or continuous change. To do this, I investigated potential break point(s) in the community by creating 5 new binary variables, each splitting the pollen provision at a different point, see table 2. For community differences (with ADONIS models) none of the breakpoint models, nor the continuous model, was better than any other by more than 2 points (table 3) indicating all models were equivalent. I took this to mean that there was no particular evidence to support a break point in the pollen species community, and that the top-bottom difference in the community was best described as continuous throughout the whole pollen provision equally.

In contrast, for species richness (with mixed models) there was a difference among the models. The lowest AIC value was produced with the model for slice 1 vs slices 2-5 (difference to next best model = 5.9, see table 4), providing evidence for a break point in species richness with the difference occurring between slices 1 and slices 2-5.

| Predictor variable | AIC value |
|---|---|
| Slice 1 vs slices 2-5 | 2173.7 |
| Slices 1-2 vs slices 3-5 | 2179.6 |
| Slices 1-3 vs slices 4-5 | 2180.8 |
| Slices 1-4 vs slices 5 | 2182.6 |
| Continuous model | 2179.7 |

*Table 4: A table showing the AIC values for species richness for the 5 different mixed models.*

| Predictor variable | AIC value |
|---|---|
| Slice 1 vs slices 2-5 | 2993.56 |
| Slices 1-2 vs slices 3-5 | 2993.18 |
| Slices 1-3 vs slices 4-5 | 2993.59 |
| Slices 1-4 vs slices 5 | 2993.25 |
| Continuous model | 2993.05 |

*Table 3: A table showing the AIC values for species community for the 5 different ADONIS models.*

As shown in figures 10, 11 and 12, there was a very strong presence of *Ranunculus* pollen, occurring throughout the whole pollen provisions, with pollen from *Acer, Asteraceae, Betula, Cyperaceae, Hemlock, Liliaceae, Potentilla, Rosaceae, Thalictrum, Trifolium* and *Quercus* abundant in smaller proportions. Referring to table 5, there is very little overlap between the species noted in the vegetation survey and figure 12 . The only species seen in the samples that appear in the vegetation survey are *Ranunculus* (buttercup), *Rosaceae* (rose), *Acer* (oak), *Betula* (birch) and *Trifolium* (clover).

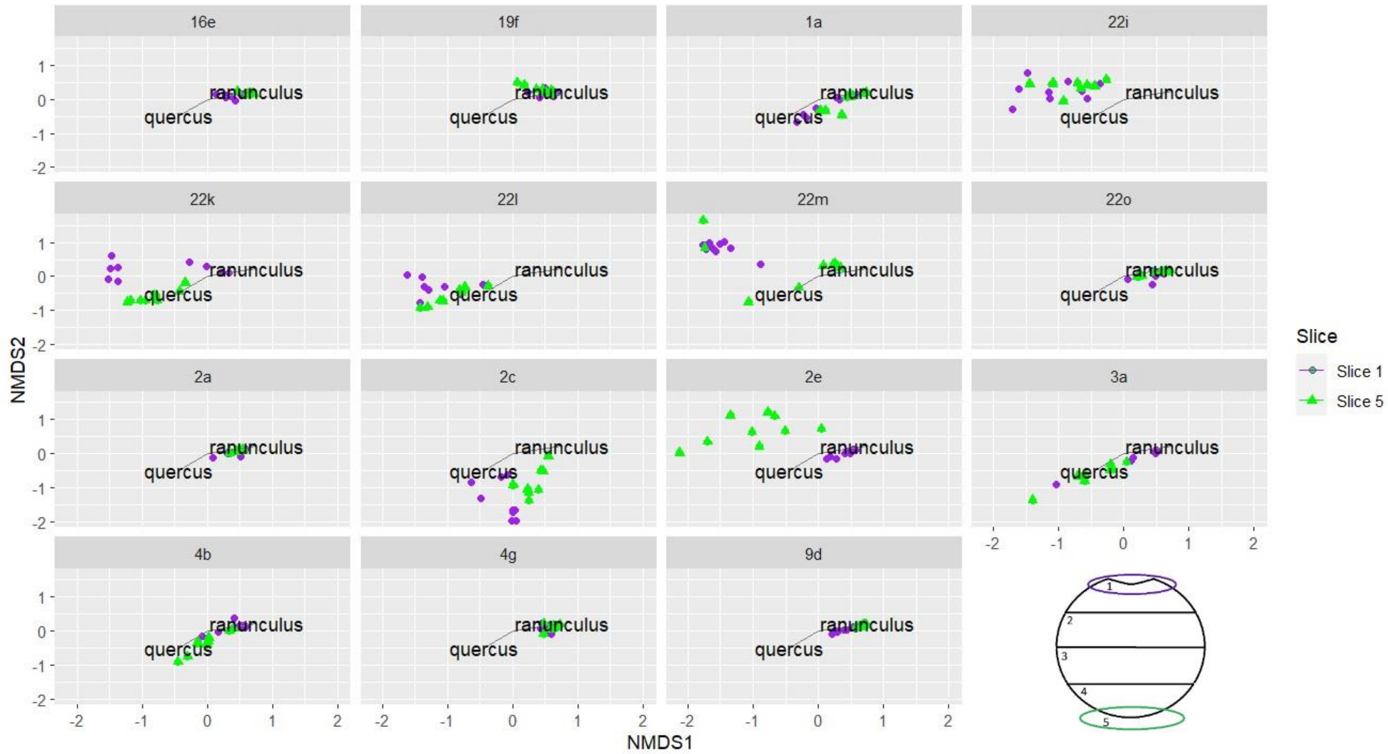

*Figure 11: An NMDS plot, showing the samples from slices 1 (Green triangles) and 5 (purple circles) from all pollen provisions. Loadings for the two most abundant pollen taxa, Quercus and Ranunculus, are indicated.*

I created some linear models to compare the amounts of each pollen species between each slice. There was no difference in the proportions of *Ranunculus*, *Liliaceae*, *Asteraceae*, *Cyperaceae*, *Hemlock*, *Rosaceae*, *Thalictrum* and *Trifolium*. However there was a significant difference in the amounts of *Quercus* (t = -2.084, df = 673, p = 0.038), *Betula* (t = 2.197, df = 673, p-value = 0.028), *Acer* (t = -3.953, df = 673, p-value = 8.543e-05), and *Potentilla* (t = -2.167, df = 673, p-value = 0.031). Looking closer at the species that show a significant difference, the majority show an increase in the mean abundance as you move towards the bottom of the pollen ball (from slice 1 towards slice 5, using T-Tests). *Quercus* (23 %), *Acer* (205 %) and *Potentilla* (113 %) all show an increase in their mean abundance of pollen grains as you go down the slices (1-5). In contrast, the pollen species *Betula* shows a 71 % decrease in percentage abundance from top to bottom. Referring to figure 11, you can see how the 23% increase in *Quercus* is demonstrated, with plots from slice 5 bearing towards *Quercus* when compared to slice 1 plots.

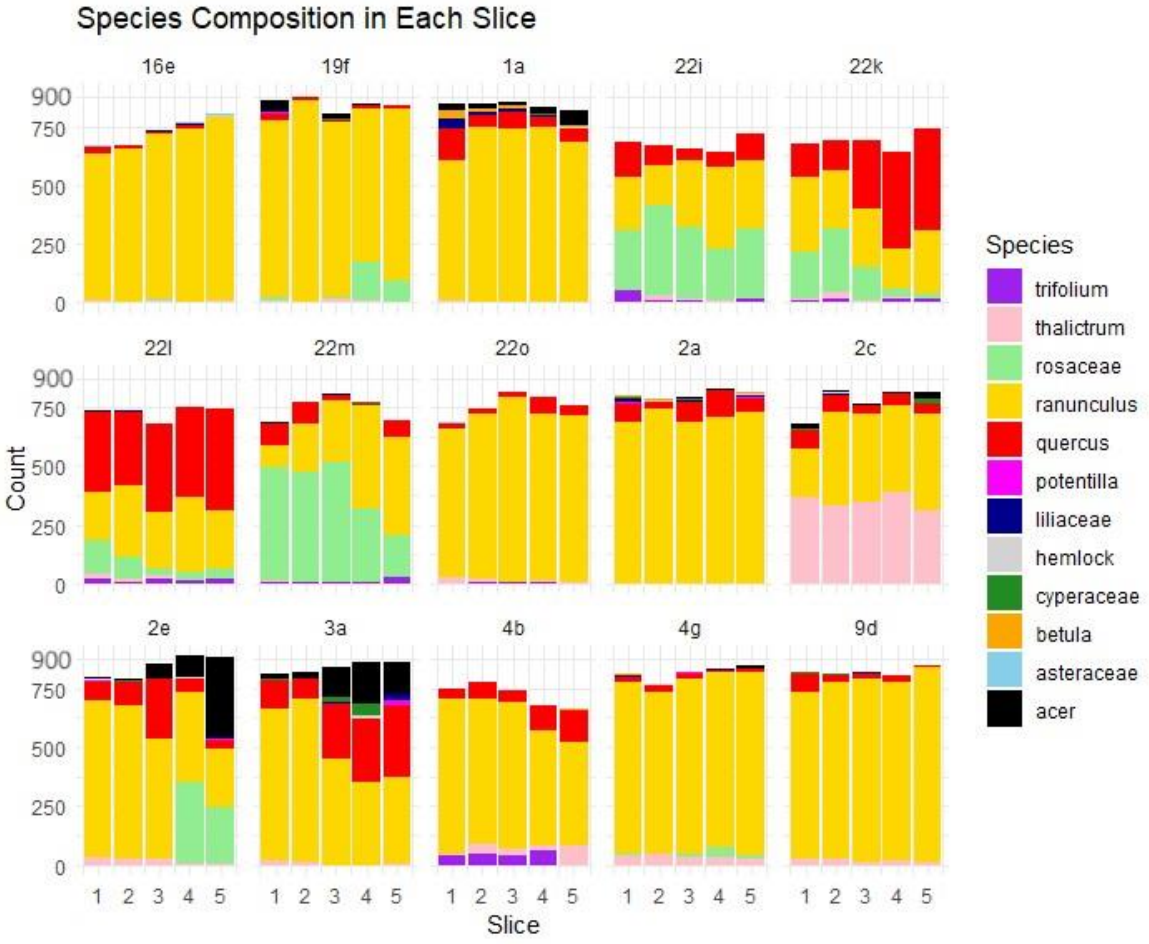

*Figure 12: A stacked bar plot showing the count of each species present in slices 1 to 5 for all 15 pollen provisions.*

| **Plant Species** | **Latin Name** | **Species Quantity** | | |
|---|---|---|---|---|
| | | **Westfield Campus** | **Thwaite Gardens** | **Salmon Grove** |
| Armenia blackberry | *Rubus armeniacus* | | 255 | 37 |
| Bell flowers | *Campanula* | | 100 | |
| Birdsfoot trefoil | *Lotus corniculatus* | 20 | | |
| Blue erygo | *Eryngium planum* | | 40 | |
| Broadleaf docks | *Rumex obtusifolius* | 4 | 12 | 2 |
| Bull thistle | *Cirsium vulgare* | 1053 | 8 | 30 |
| Burnet rose | *Rosa pimpinellifolia* | | 100 | |
| Catchweed bedstraw | *Galium aparine* | 240 | 90 | 420 |
| Common buttercup* | *Ranunculus* | 159 | 136 | |
| Common daisy | *Bellis perennis* | 9 | 80 | 100 |
| Common dandilion | *Taraxacum officinale* | | 4 | |
| Common hawthorn | *Crataegus monogyna* | | | bush |
| Common snowberries | *Symphoricarpos albus* | 53 | | |
| Common sorrel | *Rumex acetosa* | 9 | 51 | 100 |
| Common vetch | *Vicia sativa* | 13 | | |
| Common yarrow | *Achillea millefolium* | 134 | 2 | |
| Creeping buttercup* | *Ranunculus repens* | | | 60 |
| Cutleaf teasal | *Dipsacus laciniatus* | 2 | | |
| Druce cranes bill | *Geranium endressii* | | 510 | |
| European ash tree | *Fraxinus excelsior* | | 1 | |
| European beech tree | *Fagus sylvatica* | | 1 | |
| European black elderberry tree | *Sambucus nigra* | 1 | | |

| | | | | |
|---|---|---|---|---|
| European holly | *Ilex aquifolium* | | | 1 |
| False bindweed | *Calystegia* | | 9 | |
| Feverfew | *Tanacetum parthenium* | | 5 | |
| Firethorns | *Pyracantha* | | 250 | |
| Fishpole bamboo | *Phyllostachys aurea* | | 1 | |
| Forget-me-nots | *Myosotis* | | 64 | |
| Goutweed | *Aegopodium podagraria* | | 12 | |
| Great willowherb | *Chamaenerion angustifolium* | 10 | | |
| Ground ivy | *Glechoma hederacea* | 500 | | |
| Heart podded hoary cress | *Lepidium draba* | 5 | | |
| Hedge bindweed | *Calystegia sepium* | 10 | | |
| Herb robbert | *Geranium robertianum* | | 182 | 70 |
| Hogweed | *Heracleum mantegazzianum* | 38 | | 570 |
| Japanese pagoda tree | *Styphnolobium japonicum* | | 1 | |
| Kingsspear | *Asphodeline lutea* | | 4 | |
| Large bindweed | *Convolvulaceae* | 4 | | |
| Lavender | *Lavandula* | | 110 | |
| Marsh cranes-bill | *Geranium palustre* | | | 30 |
| Meadow buttercup* | *Ranunculus acris* | 73 | | |
| Mono maple* | *Acer pictum mono* | | | 1 |
| Mountain pine | *Pinus mugo* | | 1 | |

| | | | | |
|---|---|---|---|---|
| Natal grass | *Melinis repens* | | | 560 |
| Oxeye daisy | *Leucanthemum vulgare* | 256 | | |
| Palmgrass | *Setaria palmifolia* | | 7 | |
| Prairie onion | *Allium stellatum* | | 19 | |
| Purple foxglove | *Digitalis purpurea* | | 3 | |
| Ragwort | *Senecio jacobaea* | 4 | | |
| Red campion | *Silene dioica* | | | 100 |
| Red clover* | *Trifolium pratense* | 11 | | |
| Ribwort plantain | *Plantago lanceolata* | 278 | | |
| Rush skeletonweed | *Chondrilla juncea* | | 27 | |
| Scots pine tree | *Pinus sylvestris* | 1 | | |
| Shrubby rock roses* | *Helianthemum* | | 100 | |
| Silver birch tree* | *Betula pendula* | 1 | | |
| Stinging nettles | *Urtica dioica* | 600 | 150 | 690 |
| Sycamore maple tree* | *Acer pseudoplatanus* | 2 | | 2 |
| Tutsan | *Hypericum androsaemum* | | 160 | |
| Wall lettuce | *Lactuca muralis* | | | 60 |
| Welsh poppy | *Meconopsis cambrica* | | 7 | |
| White clover* | *Trifolium repens* | 1 | 35 | 20 |
| White dead nettle | *Lamium album* | | | 3 |
| Wild cherry tree | *Prunus avium* | 1 | | |
| Wild garlic | *Allium ursinum* | | 20 | |
| Wood avens | *Geum urbanum* | | 20 | 40 |
| Yellow archangel | *Lamium galeobdolon* | | 50 | |

| | | | | |
|---|---|---|---|---|
| Yorkshire fog | *Holcus lanatus* | 8 | | |

*Table 5: A table showing the observed plant species, their Latin name and their quantities (numbers of inflorescences) at each bee house location. The species highlighted green (*) are also present in the samples.*

## Discussion

The data shows there is a significant heterogeneity within the pollen balls. The pollen provisions have a continuous significant difference in their species community from top to bottom within the provision, in addition to a difference in species richness, which is more prevalent between slices 1 and slices 2-5. The visualisations seen in figure 12 demonstrate a small but significant difference in the proportions of the different pollen species per slice.

Due to the top vs bottom (effectively “distance from egg”) being the only variable to show significant community differences, and not the north vs south (left to right), east vs west or inner vs outer, it implies that there may be a specific layered structure to the pollen community present in *O.bicornis* provisions. If none of the variables showed significance, it would suggest that there is no structure to the provisions relative to the larva. Additionally, if all variables showed significantly different compositions, it would suggest there is little structure and the pollen provisions are assembled randomly with many different pollen species, implying a lack of specific organisation with respect to the larva.

### *Environment (pollen community) is an important determinant of pollen provisioning?*

Previous research has shown that there could be a preference in *O.bicornis* pollen selection, with bees selecting trees: especially *Quercus* (oak), *Rosaceae* (rose), *Salix* (willow) in addition to the herbaceous plant *Ranunculus* (buttercup) as the basis of their pollen provisions (Raw, 1974., Radmacher & Strohm, 2010., Sedivy et al, 2011., Hansted et al, 2014., Coudrain et al, 2016., Splitt et al, 2021). Looking at the pollen I sampled from the nests, a strong preference is evident for *Ranunculus*, see figures 10, 11 and 12, compared to its availability in the environment as indicated in the vegetation surveys. This selective behaviour was spread throughout the whole provision. Based on the results from the linear models, there is no difference in the proportions of *Ranunculus* throughout the slices of the provision, whereas there is a statistically significant difference in the amounts of *Quercus* (+23 %), *Betula* (-71 %), *Acer* (+205 %) and *Potentilla* (+113%) as you move down the slices (1-5). Additionally, there was a difference in the species richness, showing that, on average, the number of species present per slice is around 4-5 in the top slices but 3-4 in the bottom slices. However, the analysis did not find a significant difference in Simpson Diversity Index between the layers of the pollen provision. The mean value of 0.394 suggests that, on average, there is a moderate diversity of species running throughout the layers. Similarly, the Shannon Diversity Index did not find a significant difference, the mean value of 0.79 indicates a moderate to high level of diversity with a relatively even distribution between the slices.

To summarise, there is a significant difference in species richness among the layers, however, the distribution and evenness of species within each layer do not differ significantly. The community composition might be changing in terms of the number of species present, but the overall diversity and evenness within the layers remain relatively constant. These are important factors to investigate as they may provide insight into selectivity by female *Osmia* for larvae at each developmental stage, although we cannot rule out diurnal changes in pollen availability (see below). For example; if female *Osmia* differentially focus on gathering one particular pollen species for development at one specific larval stage, then we could expect to see a lower species richness and diversity in the corresponding layer of the pollen ball.

One possible alternative explanation for the observed patterns is that this top to bottom layered structure could be due to pollen availability changing across the multiple trips female *O.bicornis* make to create one pollen ball (Seidelmann, 2006). The difference between the top to bottom community could be due to the females visiting different pollen resources during their multiple foraging trips. However, the pollen balls were not initiated simultaneously, and yet the communities within the provisions were significantly and predictably different from top to bottom across all 15 sampled provisions. This shows that the top-to-bottom differences within pollen balls are not random, instead showing a potentially predictable pattern. I suggest this could be due to the female *O.bicornis* purposely selecting different floral resources at different points during construction of the provision, in order to target their offspring's dietary requirements. Although an attractive possibility, there are other alternative explanations for such results. One of which could be the local pollen availability (amount of pollen present on an inflorescence at any one time). Pollen availability can change seasonally, which was deemed unlikely as an explanation for my findings because female *Osmia* create a single pollen provision within a few hours (Seidelmann, 2006), so provision structure is unlikely to be affected by seasonal differences. However, some plants also experience diurnal variation, whereby the pollen availability changes throughout the day (Norris-Hill & Emberlin, 1991). To expand further, one study looking into honey bee (*Apis Mellifera, L.*) pollen collection showed that the quantities of certain collected pollen species changed depending if it was and early or a late foraging trip (Baum et al, 2011., van Doorn & Van Meeteren, 2003). Although factors such as resource availability and individual bee preference exist, there are chances that the change in resource collection is due to floral patterns of pollen availability (Baum et al, 2011).

Different species of flora exhibit different opening and closing signals; light intensity (diurnal vs nocturnal), humidity and also temperature. One example of diurnal pollen is seen in the *Liliaceae* family, they tend to open in the early mornings and close in the evenings (van Doorn & Van Meeteren, 2003), which corresponds to *Osmia* foraging times (Seidelmann, 2006). Similarly, Oak trees (*Quercus*), a popular species seen in almost every sample I took, can have different rates of daily pollen production; with the peak production being recorded at 15:00 hours (Corden & Millington, 1999). Furthermore, external factors such as weather (drought, heavy rainfall, heatwaves and wind) prior to and during pollination times can have an effect on the amount of pollen produced by the plant (Corden & Millington, 1999., Emberlin, 1994., Corbet, 1990). Plus, weather can affect the plants daily opening/closing patterns (Corbet, 1990), which would restrict the *Osmia's* abilities to collect that pollen species. This could offer an alternative explanation for the divide I found within the pollen provision's species richness (slices 1 vs slices 2-5). Which could imply that the difference might not be due to the female *O.bicornis* selecting different pollen grains, but instead due to diurnal patterns of pollen availability.

Prior to the commencement of pollen analysis, I completed a vegetation survey (table 5), looking back, a more effective method would have been a pollen availability survey. Although the vegetation survey provides details on the local plant species, it does not accurately detail the amounts of available pollen in the area. Additionally, I believe the survey to be too small, as there are very few noted species that appear in the pollen samples. For example, the main source of pollen grain collected is *Ranunculus*, which has very little notation in the survey (a combination of *R.Acris, R.Ranunculus* and *R.Repens*). For future studies I would recommend a quantitative estimation of production for the main floral types attractive to bees, using automated counting methods for pollen-per-inflorescence. To do this, I would find the average number of inflorescences per plant per species (for example; *Ranunculus*); by counting the number of inflorescences present on 20 different plants, then divide by 20 to get your average. From here you can randomly select 5 different plants of the same species and take one inflorescence per plant to quantify the total pollen content using a Coulter Counter following the method of Rush et al (1995). Once the total number of plants have been quantified, using a vegetation survey similar to the one I completed (or another method such as community mapping), you can scale up your pollen content results and gain an estimation of total pollen availability per species. Repeat this for each plant species that could potentially be attractive for the *Osmia*.

Due to mason bees being a small bodied species, it was previously thought that they would prefer to forage over very small distances, although, more recent studies have shown that they will travel up to 900m for the correct provisioning species (Zurbuchen et al, 2010). In general, foraging distance increases with the female's body size (Greenleaf et al, 2007), making a full detailed and accurate pollen availability/vegetation survey quite a considerable task without a large team, hence why an estimation is a much better fit for independent researchers. Alternatively, a more time efficient method would be comparing passively dispersed pollen in the air, via aerial trapping (aeropalynology, Biesboer, 1977). Although it is not 100% accurate, this has standardised methods and provides an indication of which plants are flowering/releasing pollen for the area around the trap (Biesboer, 1977., Roger, 1993., Stephen, 2014). Although this focuses more on wind pollinated plants, so you may need to take extra steps to ensure that bee pollinated plants are accounted for.

Additional to pollen availability, there is another environmental change that could potentially influence pollen selectivity: temperature (Russel & McFrederick, 2022). As temperatures change, the nectar-inhabiting micro-organisms can alter nectar chemistry, changing sugar concentrations as well as releasing volatile organic compounds (Russel & McFrederick, 2022., Vannette & Fukami, 2016., Rering et al, 2018). This can cause the pollen species to be "less attractive" and therefore effect a bees preference for that species (Schaeffer et al, 2017). Russel and McFrederick (2022) found that temperature increased the abundance of bacteria in the pollen, significantly altering the bees liking for said pollen species. Although red mason bees are not pollen specialists, changes in temperature during foraging seasons could potentially alter their pollen selectivity. As temperatures tend to fluctuate in UK springtime, this could be a cause behind the difference in species richness within the pollen provisions.

Furthermore, temperature can affect the growth rate of overwintering species such as mason bees, its known as the “Temperature-size rule” (Mayr, 1956). Originally discovered by Bergmann in 1847, it was proposed that species developed larger bodies in response to higher latitudes, which was shortly rephrased as colder temperatures. Thus, leading to thorough investigations on rearing temperatures and body size (Walters & Hassall, 2006., Ashton, 2002., Ashton, 2000., Gilbert & Raworth, 1996), it was noted that 80% of ectotherms conformed to this rule (Ray, 1960., Atkinson, 1994). The idea that higher temperatures lead to faster development times and therefore smaller adults (Begon, 1983., Gibert & De Jong, 2001., Gilbert & Raworth, 1996) could shift nutritional requirements of larvae; smaller bodies and higher metabolic rates would put on body tissue faster. If female Osmia can predict a warmer growth season for their larvae, that may alter their pollen selectivity. Which could affect how the females structure their pollen provisions as different quantities of pollen may be selected, as well as different species all together. It may even result in one species of pollen being incredibly dominant over the rest, if that is best for a warmer growth season.

### *Top to bottom is an important determinant of pollen structures?*

These results show a complexity of foraging implications; there may be a much finer control of the pollen grain placement than previously assumed. This would be consistent with the idea that there may be some benefit to the offspring which is achieved by the structure and composition of the provisions the female bees are creating. Our current understanding is that larval diet *can* affect bee health and development (Austin & Gilbert, 2021., Tainsh et al, 2021., Filipiak, 2019., Roulston & Cane, 2002., Sedivy et al, 2011., Filipiak et al, 2022., Filipiak & Filipiak, 2020., Lan et al, 2021). As we know, a lack of key nutrients can cause fluctuations in a bees’ development time and cocoon growth, causing some to emerge underdeveloped and more likely to die, increasing their mortality rates up to 80% (Filipiak & Filipiak, 2020). Similarly, it can cause a decrease in adult size (Roulston & Cane, 2002) and smaller bodied *Osmia* can have a decreased fitness; males are less able to fend off others during mating and females can struggle with large or lengthy foraging trips (Bosch, 2008., Bosch & Vicens, 2006., Greenleaf et al, 2007). Hence why a correctly provisioned meal is vital in *Osmia* offspring survival and fitness, but, can the placement of these nutrients effect larvae in similar ways?

Austin and Gilbert (2021) as well as Howard (2020) found that carbohydrate based pollen is key to offspring survival (mediates growth and survival to pupation) and is therefore prioritised by the *Osmia* larvae. Howard states that "compensatory feeding behaviour shifted across development, becoming more apparent in the final growth periods. Where larvae on the low-carb diets tolerated more protein ingestion in order to converge at a similar level of carbohydrate ingestion." (Howard, 2020). Thus, Implying the need for carbohydrate intake is much greater during the larvae's late developmental stages. Therefore, we could assume that when structuring a pollen ball for the larvae's development and fitness, the pollen species higher in carbohydrates would be beneficial at the bottom (slices 4 and 5) of the provision.

Comparing this assumption to my investigation, some of the pollen species showed a significant difference in their proportions throughout the provision. For example; *Acer*, *Quercus* and *Potentilla* all showed an increase in abundance (205 %, 23 % and 113 % respectively) as you move down through the provision. Which means the larvae will consume more of those 3 pollen species during their later developmental stages. If female *Osmia* are attempting to maximise their offspring fitness via targeted nutrition, then we could predict that *Acer*, *Potentilla* and *Quercus* might be higher in carbohydrates than *Betula* (which decreased in frequency by 71%). While beyond the scope of the current study, the macronutrient content of these pollen species should now be analysed to further identify any patterns or beneficial traits they may contain, as macronutrient content varies massively among different pollen species (Vaudo et al, 2020., Vaudo et al, 2016 (1)., Vaudo et al, 2016 (2)., Austin & Gilbert, 2021., Doskey & Ugoagwu, 1992).

Additionally the micronutrient content could play a role in pollen selection, as it varies within different pollen species too, and also within the landscape that the plants grow in (soil content). One study looked at the volumes of different metals and found a difference in the levels of Sodium, Potassium, Calcium and Magnesium (Harmanescu et al, 2007) present within the pollen collected by honey bees. This could be a key influencing factor as metals like sodium and potassium are extremely important in the day to day function of an animals body. Potassium for example, is involved in protein synthesis as well as maintaining cell membrane potential (Nomura et al, 2019), both key elements of survival.

## *Conclusion*

To conclude, the results did not meet the expectations predicted for this investigation based on the apparently "messy" nature of typical *Osmia* pollen collection. Instead, they show a clear heterogeneity within the pollen ball, indicating that there *is* an internal structure to the provisions. The community difference present within the top to bottom paired with the difference in species richness between slices 1 and slices 2 – 5 suggests a predictably layered structure to the provision, which implies a timeline through the pollen ball. Although assumptions can be made about the female *Osmia* purposefully creating this structure, I cannot prove the females are differently selective at different points throughout construction of their provisions because of the possible alternative explanations; temperature related selectivity and diurnal pollen variation.

Suggesting future studies, I would recommend replicating this study, with the bee houses located in monitored areas with different pollen availabilities. It would be interesting to see if the female bees would still create similar provision structures when given the option of different pollen species such as *Rosaceae. Rosaceae* pollen is usually fruits including apples, strawberries, pears etc, seen in orchard farms and is known to be a very efficient diet source for *Osmia* (Gruber et al, 2011., Gardner & Ascher, 2006., Horth & Campbell, 2018). This could begin an interesting future for artificial bee-breeding set ups for agricultural use and potentially opens the studies of bees to more researchers interested in palynology.

Investigating the internal structure of the provisions sheds light on the red mason bees unexpectedly complex foraging and provisioning behaviours. Understanding how these provisions are organized and what factors influence their construction can help us comprehend the factors affecting bee reproductive success. *Osmia bicornis* are essential pollinators and like many other bee species, are facing challenges due to habitat loss, pesticide use, and climate change (Drossart & Gerard, 2020., Lima et al, 2022., Kearns et al, 1998). These findings can provide information for the conservation and management of these bees, by promoting the use of mason bees in economically important land such as orchards (Batra, 1995., Klein et al, 2007, Klein et al, 2011., Winfree et al, 2007). With knowing their pollen preferences and nesting/provisioning behaviours, we can help implement targeted measures to protect and enhance both wild and artificial populations of *Osmia bicornis*.

## **Chapter two -** Exploring a minimum sampling protocol to understand *Osmia bicornis* pollen provision structure.

### Introduction

Researching animals can be challenging, and invasive sampling methods may skew or bias data, leading to inaccurate conclusions (Balcombe, 2009., Zemanova, 2020., Reeve et al, 2014). Animal behavioural studies exemplify this issue, as invasive observation or monitoring techniques can disrupt natural behaviour patterns, resulting in altered responses and potentially compromising the accuracy and reliability of research findings (Carlo, 2005., Beauchamp, 2009., Carlo et al, 2003). Alternative methods such as camera trapping, covert recording devices, and animal tagging are less invasive and do not interfere with the natural behavioural patterns of species (Qiao et al, 2020., Nassar et al, 2018., Ternman et al, 2012., Francisco et al, 2020., Stewart et al, 2005). There is an increasing emphasis on employing less invasive sampling methods while still achieving necessary results, adhering to the minimum sampling protocol required (Jermalowicz-Jones, 2018., Bager, 2012., Nedreaas, 2009., Le, 1984). This approach focuses on the three Rs: reduction, refinement, and replacement (UK Health & Security Industry, 2023). The goals are to reduce the number of animals used in research, refine experimental techniques to minimize harm, and, where possible, replace animals with non-animal models.

Chapter one found a clear, significant difference within *O.bicornis* pollen provisions, indicating they are not homogeneous. Although the method provided detailed results, the sampling was time consuming and invasive on the *O.bicornis* larvae. To replicate this method, researchers would be required to find alternative nesting spaces and food for the removed larvae, which could disrupt the larvae's natural development (Steffan-Dewenter & Westphal, 2008., Cane et al, 2007., Mandeville & Novak, 2015). To minimise the interference with the mason bee larvae, many researchers (e.g. Gresty et al, 2018., Suzuki et al, 2023) have instead opted to collect residual pollen samples after the larvae have eaten their share of the pollen and started to spin their cocoons. In theory, this creates a much less invasive sampling method, leaving no affected larvae in the aftermath. However, if the pollen ball is structured, as demonstrated in chapter 1, the residual pollen may not be representative of the entire pollen ball, and sampling residual pollen may not sufficiently capture the pollen community within the provision.

The results from chapter one indicate that the provisions show a continuous difference (in species community) from the top to the bottom of the provision, as well as a break point in species richness between slice 1 vs slices 2-5. In practice, one sample from the top of the provision (slice 1) and one from the bottom (slice 5) may be sufficient data to capture the essential structure of the pollen ball, reducing the need for more detailed sampling. To reduce time and effort needed to sample pollen balls adequately, researchers could take only these two samples, creating a much more efficient method that opens palynology and solitary bee studies to more researchers by saving time, resources and money.

This chapter will first look into a more efficient, minimally invasive sampling method, testing whether it is possible to simplify 45 samples per pollen provision down to 2 (per provision) while preserving the same significant community differences. Thus, providing adequate insight into the three dimensional structure of pollen provisions in *Osmia bicornis*. Secondly, I test whether a similar insight can be gained by allowing the larvae to eat and spin a cocoon prior to sampling, thus again testing a much less invasive method of pollen sampling.

For data collection, I used identical methods to chapter one, excluding the timeframe. Instead of sampling the provisions as soon as the nests were constructed, I waited two weeks, allowing the larvae to consume what they needed from the provisions and start spinning cocoons prior to entering their nests. For the sampling, I again used a similar method to chapter one, but simplifying the sampling technique down to only two per pollen provision, which required no slicing of the provisions. Given this method, I expected to see a mix of results. With the findings from Chapter 1 indicating the provisions have a continuous change from top to bottom, in addition to the break point (in species richness) between slice 1 and slices 2-5, I believe this minimum sampling protocol could capture similar results. In contrast, allowing a prolonged waiting period that enables the larvae to consume significant parts of the provision, in addition to free movement in the nest (potentially homogenising the provision), could pose a challenge, as the original layered structure may become disrupted.

Method

As the bee houses were already in place from the previous year, during the months of January-March 2022, I replaced any damaged or used nesting tubes and seeded the houses with new *Osmia bicornis* cocoons (from The Mason Bee Company). Each house was seeded with 40 cocoons; 20 males and 20 females, roughly sexed by size as females are larger than males (Radmacher & Strohm, 2010). The breeding season began in April of 2022 and sampling started soon after, until early July. Due to reasons outside of my control only one research site was successful in a second season of breeding. Therefore, my entire pollen samples for 2022 came from Thwaite Gardens.

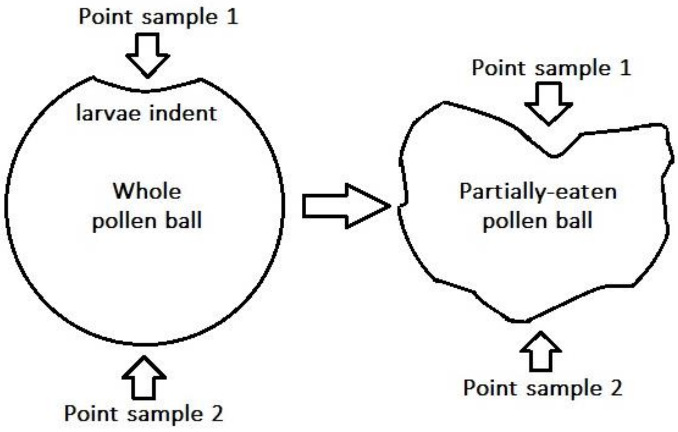

*Figure 13: A diagram demonstrating the structural difference between a whole, uneaten pollen provision and a half-eaten/damaged provision.*

After collection, I randomly allocated the capped tubes into two groups. Group one, consisting of 8 whole pollen provisions, were sampled in an identical method to chapter 1: Sliced 5 times, with slice 1 being the uppermost point and slice 5 being the lowest and furthest from the larvae (figure 7). On each slice, I took 9 point samples and coded them from one to nine - with one to five evenly spaced across the centre and sample three being the innermost point. Samples six to nine run vertically, also through the centre point, forming a cross on each section (figure 7). Nine point samples from each of the five slices gave 45 samples from every whole pollen provision, this helps to test the original investigation for validity and reproducibility (which included taking a secondary vegetation survey of thwaite gardens, see table 6).

The sampling of group two was delayed by two weeks in order to achieve half-eaten residual pollen, which resulted in a total of 20 provisions. I used a similar method to chapter 1 for the sampling: with the only changes being the removal of the slicing and instead of 9 point samples on each slice, I would take 1 point sample from the top of the pollen ball and a second from the bottom (see figure 13). This allowed me to investigate whether a much less invasive data collection method and smaller sample size of half-eaten residual pollen provisions can provide enough information to sufficiently describe the whole composition and structure of the complete pollen ball.

Additionally to the *Osmia bicornis* nests, an unknown Megachilid (presumed to be a species of *Osmia*) nested in the 2022 bee houses. Although the species was unknown (identification is pending adult emergence), I decided to investigate and sample these pollen provisions with the same method and analysis as group two, labelling it group three.

For group one, the results were analysed using the same statistical tests as chapter 1: first, I performed an ADONIS permutation-based statistical test looking into the differences between pollen communities throughout the samples, using pollen ball ID as a predictor. Then I performed ADONIS tests looking at the four variables (detailed in chapter one): north to south, east to west, top to bottom and inner to outer, using pollen ball ID as a random effect. Similarly, I used ADONIS tests to look for differences in the pollen communities and species richness, using different break-points between slices as predictors (table 2), as an attempt to find the best break point between the slices where the pollen community differed. In addition to this, I used a one-way ANOVA test, with continuous slices (1-5) as a predictor variable, to investigate differences in the proportions of individual pollen species present throughout the slices.

Repeating the same tests for sample groups two and three, I used an ADONIS permutation-based statistical test, with pollen ball ID as the predictor, to see if the top and bottom samples can replicate the same significant differences as the whole-sampled provisions in chapter one. Furthermore, I investigated the abundance of pollen species present (species richness) within the pollen provisions, using an ADONIS test with slice (1 or 5) as the predictor.

In order to create a direct top to bottom comparison between the groups, I repeated analysis on group one, whilst using only 2 samples, from slices 1 and 5 (pollen ball ID as random effect, looking for a difference within the pollen community and slice as the predictor, looking for differences in pollen species proportions). I also used a one-way ANOVA to look at the differences between individual pollen species and their abundance between the samples. Additionally, I used only the samples from slices 1 and 5 in group one and compared them to group 3 (unknown *Osmia* species), using Multivariate analysis of variance (MANOVA) with group (1 or 3) as a predictor, to see if the different species (*Osmia bicornis* vs unknown) could explain potential differences in the pollen communities. As well as an independent T-test to look for differences between the proportions of pollen species present in each group.

## Results

### *Group one*

The initial ADONIS test for group one, (with pollen ball ID as predictor), showed significant differences among the pollen communities across provisions (Df = 4, SumOfSqs = 0.633, R2 = 0.278, F = 3.368, P = 0.001). Looking into the variables, further ADONIS tests revealed there were no significant differences found among inner to outer, north to south or east to west in the samples, but, as in Chapter 1, a strong significant difference in the community was revealed from top to bottom (Df = 4, SumOfSqs = 0.594, R2 = 0.268, F = 3.328, P = 0.001).

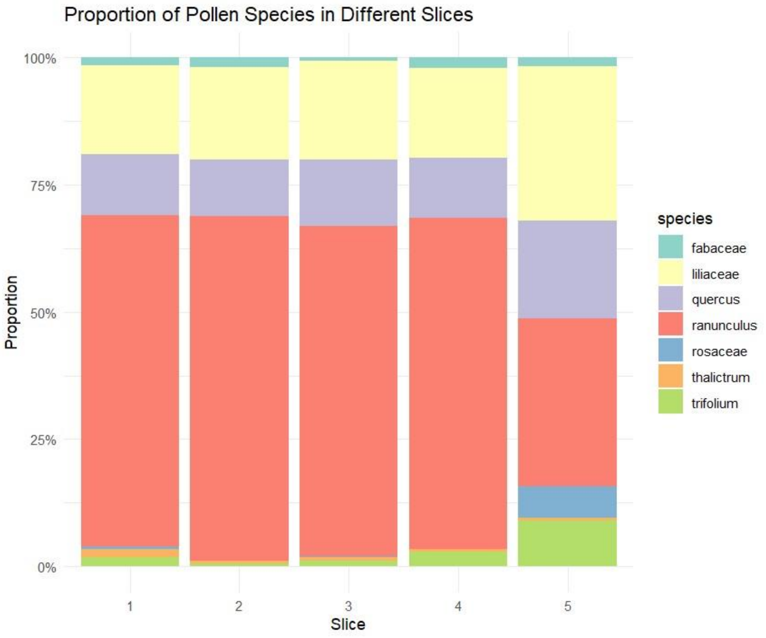

*Figure 14: A stacked bar plot showing the proportions of each species in each slice for group 1.*

Using the individual slices as predictor variables, further ADONIS tests revealed a high significance in the breakpoint between slices 1-3 and 4-5 (Df = 4, SumOfSqs = 0.633, R2 = 0.278 , F = 3.368 and Pr(>F) = 0.001), as well as slices 1-4 versus slice 5 (Df = 1, SumOfSqs = 0.513, R2 = 0.225, F = 11.056 and P = 0.001). Both comparisons are statistically significant, as both breakpoint scenarios show clear differences in pollen communities, however considering the F-value and R-squared value, the second comparison (Slices 1-4 vs. 5) appears to have a stronger effect and a better fit to the data (see figure 14).

The predominant species present was *Ranunculus*, which is the same as the previous year's samples seen in chapter one. Similarly, it is also present in the vegetation survey (table 6) detailed at the end of this section. Using a one-way ANOVA, with slice (1-5) as the predictor variable, I tested for a difference in proportions of each pollen species. This revealed a significant difference in the proportions of *Ranunculus* (Df = 1, sum sq = 2486, F = 16.84, Pr(>F) = 0.002), *Quercus* (Df = 1, sum sq = 84, F = 4.884, Pr(>F) = 0.033), *Trifolium* (Df = 1, sum sq = 125, F = 5.736, Pr(>F) = 0.022), *Rosaceae* (Df = 1, sum sq = 54.45, F = 16.75, Pr(>F) = 0.002) and *Liliaceae* (Df = 1, sum sq = 231.2, F = 6.462, Pr(>F) = 0.015) throughout the slices. *Thalictrum* and *Fabaceae* (other non-*Trifolium Fabaceae*) showed no differences across the slices. Looking closer at the species with significant differences, *Quercus* (24 %), *Trifolium* (391 %), *Rosaceae* (1250 %) and *Liliaceae* (25 %) all showed a percentage increase, as you move down through the pollen provision. However *Ranunculus* showed a 27 % decrease from slice 1 to slice 5.

### *Group two*

Group two (residual pollen with reduced sample size) showed no significant difference in pollen communities among the different pollen ball provisions with the initial ADONIS test using pollen ball ID as a predictor (df = 1, SumOfSqs = 0.054, R2 = 0.005, F = 0.193 and P = 0.491). The majority of the samples in group two were made up of *Asteraceae* and *Ranunculus* pollen, however, there are no significant differences in the abundance of any pollen species between sample 1 and 2 (figure 15).

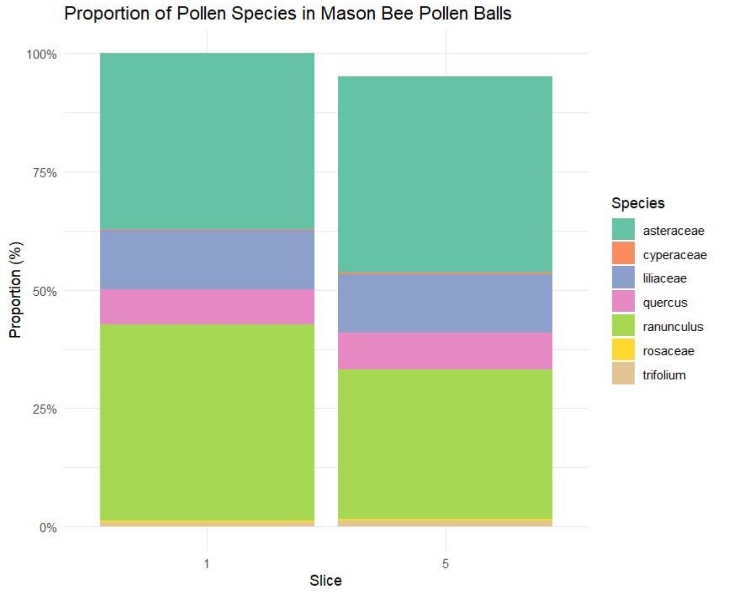

*Figure 15: A stacked bar plot showing the proportion of each pollen species in both Samples in the pollen balls for group 2.*

### *Group 1 (slices 1 and 5 only)*

Using samples from only slice 1 and slice 5 from group one (whole, uneaten pollen provisions), I ran an ADONIS test using pollen ball ID as predictor, to allow a direct comparison between group one and group two. Whereas for Group 2 I had identified no differences in pollen communities between slices 1 and 5, for Group 1 the results indicated that there was a significant difference in the pollen community composition between slices 1 and 5 (R2 = 0.241, f = 12.087 and p = 0.002). The R-squared value suggests that 24.13% of the variation in the community composition can be explained by the difference between the two slices. The one way ANOVA (with slice 1 or 5 as predictor) also revealed significant differences in the abundance of *Ranunculus* (Df = 1, sum sq = 24, F = 16.900, p-value = 0.007), *Quercus* (p-value = 0.021), *Rosaceae* (Df = 1, sum sq = 287, F = 6.441, p-value = 0.022) and *Liliaceae* (Df = 1, sum sq = 86, F = 8.109, p-value = 0.022) between slices 1 and 5. However, there was no difference in the abundance of *Trifolium*, *Thalictrum* and (non-*Trifolium) Fabaceae*.

Group three

The pollen provisions of the unknown species of *Osmia* revealed no significance among the pollen communities with an ADONIS test, using pollen ball ID as a predictor (df = 1, SumOfSqs = 0.054, R2 = 0.005, F = 0.184, P = 0.331). Additionally, to check for differences in species abundance, I used another ADONIS test with slice (1 and 5) as the predictor, which revealed no significant differences between the two samples (figure 16). Showing there is no significant patterns in the unknown osmia provisions when only sampleing once at the top (slice 1) and once at the bottom (slice 5).

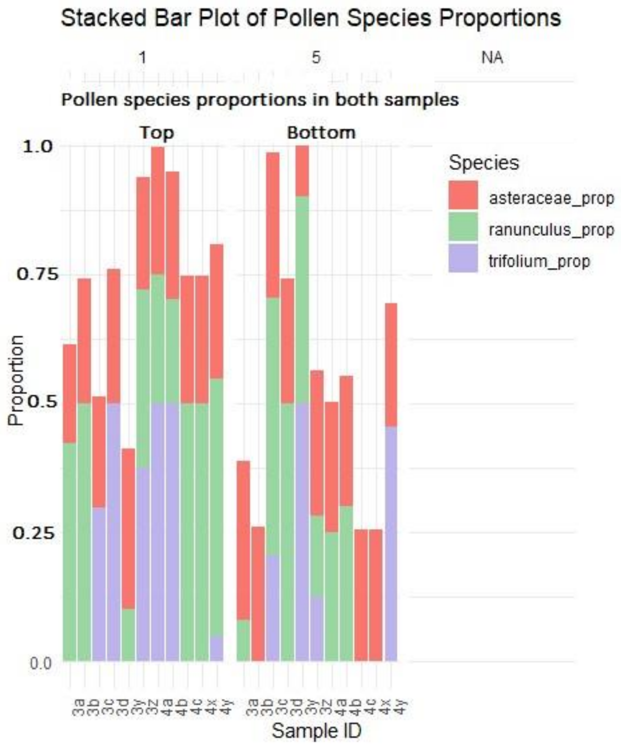

*Figure 16: A stacked bar plot showing the proportions of each species present in slices 1 and 5 for group 3.*

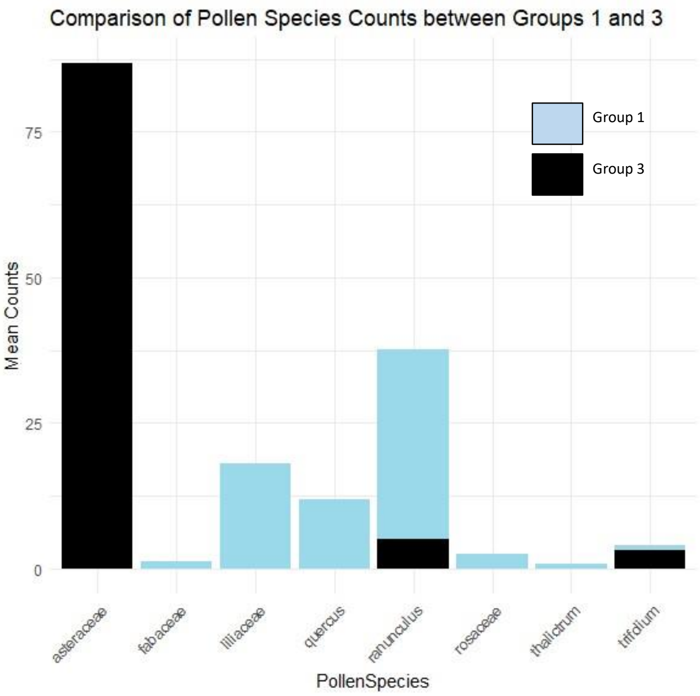

*Figure 17: A stacked bar plot showing the proportions of each pollen species present in both group 1 (blue) and group 3 (black).*

Comparing group 3 and group 1 (using only samples from slice 1 and 5), we can visualise a huge difference in pollen species present (see figure 17). Although the difference is quite drastic, I proceeded to run an independent T-test to look for statistical differences in pollen species abundance between the two groups. *Ranunculus* (t = 7.414, df = 1, p = 7.344e-07), *Quercus* (t = 1.180, df = 1, p = 5.426e-09), *Rosaceae* (t = 3.420, df = 15, p = 0.003), *Liliaceae* (t = 2.089, df = 15, p = 1.378e-07), non-*Trifolium Fabaceae* (t = 3.371, df = 15, p = 0.004), and *Asteraceae* (t = -2.698, df = 2,p = 8.77e-18) all showed significant differences in their proportions between the groups. While *Trifolium* (t = 0.499, df = 24, p = 0.622) and *Thalictrum* (t = 9.251, df = 1, p =0.055) did not. Additonally, I performed a MANOVA test, using group (1 or 3) as a predictor, which revealed significant results (Df = 1, F = 162.010, P = 2.2e-16), indicating that the different species (*Osmia bicornis* vs unknown) is highly significant in explaining the variation in pollen communities.

| **Plant Species** | **Latin Name** | **Quantity** |
|---|---|---|
| Bull thistle | *Cirsium vulgare* | 113 |
| Bush vetch | *Vicia sepium* | 3 |
| Catchweed bedstraw | *Galium aparine* | 49 |
| Common buttercup | *Ranunculus acris* | 53 |
| Common hawthorn | *Crataegus monogyna* | 12 |

| | | |
|---|---|---|
| Common hazel tree | *Corylus avellana* | 4 |
| Common ivy | *Hedera helix* | 109 |
| Common st-johns-wort | *Hypericum perforatum* | 51 |
| Corn spurrey | *Spergula vulgaris* | 1 |
| Creeping buttercup | *Ranunculus repens* | 98 |
| European ash tree | *Fraxinus excelsior* | 1 |
| European bramble complex | *Rubus fruticosus* | 7 |
| Feild horsetail | *Equisetum arvense* | 7 |
| Hedge woundwort | *Stachys sylvatica* | 9 |
| Herb-robert | *Geranium robertianum* | 24 |
| Kikuyu grass | *Cenchrus clandestinus* | 2 |
| Large-leaved avens | *Geum macrophyllum* | 8 |
| Little-robin | *Erithacus rubecula* | 12 |
| Marsh cranes-bill | *Geranium palustre* | 31 |
| Nodding spurge | *Euphorbia nutans* | 1 |
| Ragwort | *Senecio jacobaea* | 19 |
| Ribwort plantain | *Plantago lanceolata* | 36 |
| Sycamore maple tree | *Acer pseudoplatanus* | 2 |
| White clover | *Trifolium repens* | 53 |
| White dead nettle | *Lamium album* | 25 |
| Wood avens | *Geum urbanum* | 23 |
| Yorkshire fog | *Holcus lanatus* | 67 |

*Table 6: A table that shows the plant species, their Latin name and the quantity found at thwaite gardens in 2022. The highlighted (green) species are also identified in the samples.*

## Discussion

The results of the previous chapter indicated that *O.bicornis* pollen balls are not homogeneous, with the top to bottom axis accounting for the most variation in the pollen community. In the present chapter, the focus shifted onto the minimum sampling protocol necessary to reduce researcher effort (Tista & Fiedler, 2011., Shimano et al, 2018., De Almeida Papa et al, 2020., Nascimbene et al, 2010) and sampling invasiveness (UK Health & security industry, 2023). I investigated whether a simplified technique consisting of only two samples; one from the top and one from the bottom of each pollen ball, would yield similar insights into the pollen ball structure as the more detailed sampling seen in chapter 1. To develop the method further, I waited two weeks after the nests were built, before taking the pollen samples, allowing the larvae to eat and cocoon themselves prior to disturbance.

The samples collected as part of group one (whole pollen provisions, with detailed sampling method) from *O.bicornis* nest in 2022 were very similar to the results from chapter one. They confirmed a continuous change, in both pollen community and species richness, throughout the provision (from top to bottom) and additionally confirmed that there is a structure to the *O.bicornis* pollen provisions when sampled adequately. Similar to chapter one, among the pollen species that showed a significant difference in proportion, the majority showed an increase as you move down through the pollen provision (*Quercus* = 25 %, *Trifolium* = 391 %, *Rosaceae* = 1250 %, *Liliaceae* = 25 % and *Ranunculus* = -27 %).

The analysis of group two (the simplified sampling technique, using residual pollen provisions) did not reveal a significant difference in the pollen communities between the top and bottom of the pollen provisions. This discrepancy suggests that the structural variation within the pollen provisions, as observed in chapter one and group one, might not be adequately captured by the simplified sampling approach. This implies that researchers cannot sample half-eaten residual pollen balls (e.g. Gresty et al, 2018., Suzuki et al, 2023) and capture a full insight into the three dimensional structure and species composition of the pollen provision. Something which is necessary to thoroughly capture pollen selection by female *O.bicornis*.

These results were not unexpected, and they did meet the expectation of non-significance, there are several factors which could contribute to the disparity in results between the two sampling techniques. Firstly, As the bee larvae eat through the pollen provision, they may disrupt the layers sampled in chapter one (slices 1-5), leaving a smaller pollen community with little structure left to analyse. Furthermore, the remaining pollen, that uneaten by the larvae, will not necessarily remain in their original location. By moving through the pollen provision the larvae can shift the pollen grains, homogenising it and disrupting the original structure created by the female *O.bicornis*. Hence why it was expected that such a corrupted structure would show a lack of top-bottom difference. Additionally, the reduced number of samples taken in group two (45 samples per pollen ball reduce to 2 samples) might have led to a decrease in statistical power, making it more challenging to detect significant differences (Prajapati et al, 2010., Hong & Park, 2012., Akobeng, 2016). Whichever factor(s) caused the inaccurate representation, I have proved that this simplified technique is not a suitable replacement for the original method noted in chapter one.

Looking at the vegetation survey (table 6), the only species present in both the survey and the pollen provisions (from group one and two) are *Trifolium* and *Ranunculus*. While, the majority of both groups are made up of *Ranunculus* (buttercup), a common found pollen preference in Osmia (Bednarska et al, 2022., Radmacher & Strohm, 2010., Persson et al, 2018., Haider et al, 2014., Sedivy & Dorn, 2014., Splitt et al, 2022), there are still differences between the pollen composition of the two groups (figures 14 and 15). Both groups show different proportions of *Ranunculus, Quercus, Trifolium, Rosaceae* and *Liliaceae* present, however only group one has *Thalictrum* and non-*Trifolium Fabaceae* present. Additionally, in group two there is a high abundance of *Asteraceae* present as well as smaller quantities of *Cyperaceae*, which are not seen in group one.

The results from group one confirm the findings from chapter 1, in that the significant difference observed between the slices, have important implications for understanding the provisioning behaviours of female *Osmia*. The structural variations within the pollen provisions suggest that different layers of the provision may contain varying nutrient compositions or spatial arrangements, which could have implications for the development and fitness of the *O.bicornis* larvae – detailed in the discussion of chapter one (Howard, 2020., Austin & Gilbert, 2021., Tainish et al, 2021., Filipiak, 2019., Roulston & Cane, 2002., Sedivy et al, 2011). By identifying these structural differences, we can shed light on the sophisticated strategies employed by female *O.bicornis* to provision their offspring.

It is crucial to acknowledge the limitations of the simplified sampling method seen in group two: sampling residual pollen is insufficient to capture the pollen ball structure. However, looking at the direct comparison from group one, sampling only once at the top and once at the bottom of a whole, uneaten pollen provision *can* provide an adequate representation of the structure of the provision. Although this simplified technique resulted in the same pollen species present, there were still small disparities seen amongst the proportions of species. The full sampling method (slices 1-5) in group one showed a significant difference between the proportions of *Ranunculus*, *Quercus*, *Trifolium*, *Rosaceae* and *Liliaceae* throughout the slices, while *Thalictrum* and non-*Trifolium Fabaceae* showed no difference. Yet, looking at the samples only from slices 1 and 5 shows a significant difference in the proportions of *Ranunculus*, *Quercus*, *Rosaceae* and *Liliaceae;* the difference being a lack of significance in *Trifolium*. This implies that a simplified sampling technique, applied to a whole pollen provision would be a sufficient method to analyse pollen composition and basic structure, as it is mostly accurate. For example, when group one was analysed fully, the R2 value was 0.278, yet, when analysed with only slices 1 and 5, the R2 fell to 0.241. Although the difference is not huge, it shows by simplifying the sampling method you are losing 3.7% of explanatory power. This highlights the importance of comprehensive sampling, and further emphasizes the need for more research into the minimum sampling protocol necessary for a comprehensive understanding of pollen provisions. Whether this loss of accuracy can be seen as a reasonable compromise will depend on the needs and resources of future researchers.

Future studies could consider exploring alternative sampling methods that strike a balance between efficiency and the necessary structural information. There is still potential for a minimum sampling protocol to be discovered; I would encourage researchers to try similar methods with different sampling quantities, I would suggest exploring the layered sampling method with reduced sample size, I.E. one sample from each slice. Additionally, examining a larger sample size would allow enhanced statistical power and could potentially uncover more nuanced differences between the top and bottom of the provisions.

To conclude, the simplified sampling method in chapter 2, both in group one (samples from slices 1 and 5 only) and group two did not exactly replicate the findings of the more comprehensive sampling protocol in Chapter one. The nonsignificant results from group two serve as a reminder that female *O.bicornis* have complex provisioning behaviours and further investigation into the minimum sampling protocol is necessary to understand this species. However, this does not discount the findings in chapter one, which are reinforced by the repeated significance of group one (slices 1-5) in chapter two. The confirmed patterns and structural differences observed between slices 1-3 and 4-5 still provide valuable insights into provisioning behaviours of female *Osmia*.

## General discussion

Analysis in Chapter 1 of this thesis revealed a strong bias towards *Ranunculus* pollen species across all sampled *O.bicornis* provisions. Nevertheless, there was a small but significant and clearly discernible structure within the pollen provisions of *Osmia bicornis*. A distinct community difference was evident from the top (near the larva) to bottom of the pollen provision. Also, typically one fewer pollen species was found in slice 1, next to the larva, than in the remaining layers. This implies that pollen provisions are not homogeneous, when sampled using adequate methods. This chapter shows that female *O.bicornis* do create an internal structure during their provision assembly, however I do not have enough evidence to confirm that this is due to the females targeting the differential developmental stages of their offspring. As detailed in the discussion of chapter one, there are alternative explanations such as diurnal pollen variation and weather related factors that could affect the outcome of the pollen provisions structure.

Analyses in chapter 2 showed that the findings from chapter 1 are a confirmed pattern, as group one (whole, uneaten pollen provisions) resulted in very similar significant differences. The similarities in the significance between the two chapters, showed that there is a continuous difference, in both species community and richness, throughout the pollen provision as you move from top to bottom. This confirmed that the pollen provisions are heterogeneous. It also showed that the method used to sample the provisions is reproducible and reliable and will yield similar results irrespective of sample location and time.

In addition to this, looking at the percentage change of species abundance as you move down through the pollen provision (slice 1 to 5), there are similarities in chapter 1 and chapter 2's data. The percentage of *Quercus* in both the 2021 and 2022 pollen provisions increased by roughly a quarter from slice 1 to slice 5 (23% in 2021 and 25% in 2022). Although I cannot prove this pattern is due to the female *Osmia* purposely selecting more *Quercus* for the later stages of the offspring development, I can confirm that there is a repeated structure present within the provisions.

Additionally, the findings from group two (residual pollen with reduced sample size), which showed no significance, highlight the importance of future research into the minimum sampling protocol. Although group two failed to capture the subtle variations within the provisions, when using only two samples from group one (slice 1 and 5) there was very similar significance found when compared to the fully sampled group one (R2 value of 0.278 and 0.241, respectively). The only major change in results was in the breakdown of individual pollen species and their percentage proportions, as *Trifolium* was observed as no longer having a significant change in abundance. This accentuates the pivotal role of meticulous sampling techniques, which in this case, are needed to capture subtle variations. This demonstrates that simple sampling techniques, such as taking one point sample from old, used pollen is not an adequate representation of the whole pollen provision.

It is important to note the consistency of my findings across chapters in the face of the variability of plant species surrounding the bee houses in Chapter 1 and Chapter 2 (conducted in 2021 and 2022, respectively). Looking solely at Thwaite Gardens; the 2021 survey showed 38 different plant species, with *Geranium endressii* being the most abundance. In contrast, the survey in 2022 identified 27 species, with *Ranunculus* (*R.repens* and *R.acris*) being most prevalent. Despite the variability in available pollen sources, the outcomes from group one in Chapter 2 consistently align with those of Chapter 1, confirming that the identified structural pattern within the pollen provisions are not due to localized pollen resources and represents a reliable and repeated structure.

Together, these chapters highlight the importance for further research into pollen provision structure and what effects different structures could have on *Osmia bicornis* larva. As we know, nesting conditions, pollen species and micro-macronutrient content plays a huge role in the development of *O.bicornis* larvae (Howard, 2020., Austin & Gilbert, 2021., Tainsh et al, 2021., Filipiak, 2019., Roulston & Cane, 2002., Sedivy et al, 2011., Lawson et al, 2020., Seidelmann, 2006., Ostap-Chec et al, 2021., Radmacher & Strohm, 2009., Radmacher & Strohm, 2010., Kierat et al, 2017., Pitts-Singer, 2004., Doskey & Ugoagwu, 1992), it would be interesting to see similar studies, investigating into the effects of different structures on the larvae. As well as future studies looking further into the minimum sampling protocol, as the results from chapter 2 (group 1, using only slice 1 and 5 samples) indicate that it is possible for the sample size to be reduced and still gain a full comprehensive understanding of the whole provision structure and composition.

The provisions provided by female bees serve as an essential and vital source of nutrition for the larvae, without it, the larvae would not develop. Therefore, understanding the provisioning behaviour of bees is crucial for comprehending their species populations ((2)Engelke et al, 2016, (1)Engelke et al, 2016). Investigating the structural variations within these pollen provisions has contributed to the understanding of how bees ensure the optimal development and survival of their offspring, enhancing our knowledge of the mechanisms underlying successful reproduction and population dynamics in these important pollinators, as well as practical implications for conservation efforts, as it helps inform the management and conservation of *Osmia* bees.

Overall, these findings contribute to the existing body of literature on bee provisioning behaviour and pave the way for further research aimed at unravelling the relationships between female provisioning techniques and resource availability, provisioning strategies and reproductive success in *Osmia bicornis*.

## Appendix

Methods development:

Due to the pollen provisions being fragile and the necessity for reproducibility, the pollen slicing method needed to be precise and efficient. The first method I attempted was to embed the whole pollen provisions in paraffin wax, a technique used to stabilise soft tissue samples for microscopy (An et al, 2003). Paraffin wax has very neutral properties making it a great holding agent for delicate samples, such as crumbly pollen provisions.

Pollen grains are known to withstand extreme conditions due to their exine structure composed of one of the most inert biological polymers: sporopollenin (Wiermann & Gubatz, 1992). An organic biopolymer of extremely high stability ornamented in a dense sculpture provides ultimate protection for the male gametes hidden within the pollen grain (Domínguez et al, 1999). It is this structure that meant the pollen grains would be able to withstand the relatively high temperatures (68˙C) of the molten wax submission.

Once fully submerged for 30 minutes the sample was then solidified and placed into the microtome for slicing. The slices created by the microtome are 0.2 microns thick, which after depressing in a water bath (50˙C), is thin enough to be placed onto a microscope slide. Thus, allowing insight inside the pollen provision, where the individual pollen grains can be identified under a light microscope. Repeating this throughout the pollen provision, creating multiple slices provides inside knowledge on how the pollen grains are arranged whilst still maintaining the circular structure, eventually leading to the rough 3D structure of the whole pollen provision.

Upon slicing on the microtome, the pollen provisions still crumbled in the centre destroying the 3D structure. I experimented with different wax submergence times between 0.5 to 4 hours. Each treatment still produced the same effect of a crumbly centre, yet, above 5 hrs (5 – 12hrs) caused the whole provision structure to fall apart before the wax had a chance to solidify.

A paper by Nicolson and Thornburg states that plants susceptible to water stress I.E. those located in dryer environments, produce less nectar than plants with access to increased volumes of water (Nicolson & Thornburg, 2007). Implying that nectar contains water, however the percentage of water and level of nectar dilution does vary between plant species (Potts et al, 2004). Pollen provisions are made from pollen grains held together by small volumes of nectar (Poderoso et al, 2013). Knowing Paraffin wax is hydrophobic, a reason for the failed method could be due to the small volume of water-based nectar in the provisions, preventing the wax from fully infiltrating the centre. To combat this, I attempted to "clean" the pollen balls with a series of chemical baths used in tissue sample cleaning prior to embedding in paraffin wax. The treatment went as follows:

1) PBS – 15 mins
2) 70% ethanol – 15 mins
3) 90% ethanol – 15 mins
4) 100 % ethanol – 15 min
5) 100% ethanol – 15 mins
6) 100% ethanol – 30 mins
7) 100% ethanol – 45 mins
8) Histoclear – 20 mins
9) Histoclear – 20 mins
10) Histoclear – overnight

After the first chemical treatment the pollen provisions began to moult pollen grains and eventually caused the structure to collapsed creating a disorganised pile of grains, making the samples unsuitable for this study. Although the individual grains were unaffected, the pollen balls must maintain their shape for a comparison of the three dimensional structure.

The next trial method I tied was an attempt to replicate an epidermal peel: using a substance or object to obtain an imprint or copy of the surface structure in question (Wu et al, 2009., Eisele et al, 2016). After slicing the pollen ball by hand with a razor blade, I would use Sellotape to try and remove a neat top layer from each slice. The first attempt at this created a very densely populated peel, making individual grains hard to identify. Many attempts to reduce the grain density did not work, dissolving the tape with acetone and creating a peel of the peel all fell unsuccessfully. The final method, as stated in chapter one in the methods section, was the only reproduceable and therefore successful method discovered for slicing *Osmia bicornis* pollen provisions.

References

- Akobeng, A.K., 2016. Understanding type I and type II errors, statistical power and sample size. *Acta Paediatrica*, *105*(6), pp.605-609.
- An, Y.H., Moreira, P.L., Kang, Q.K. and Gruber, H.E., 2003. Principles of embedding and common protocols. In *Handbook of histology methods for bone and cartilage* (pp. 185-197). Humana Press, Totowa, NJ.
- Anderson, D.R. and Burnham, K.P., 2002. Avoiding pitfalls when using information-theoretic methods. *The Journal of wildlife management*, pp.912-918.
- Ashton, K.G., 2002. Patterns of within-species body size variation of birds: strong evidence for Bergmann's rule. Global Ecology and Biogeography, 11(6), pp.505-523.
- Ashton, K.G., Tracy, M.C. and Queiroz, A.D., 2000. Is Bergmann's rule valid for mammals?. The American Naturalist, 156(4), pp.390-415.
- Atkinson, D., 1994. Temperature and organism size-a biological law for ectotherms?. Adv. Ecol. Res., 25, pp.1-58.
- Austin, A.J. and Gilbert, J.D., 2021. Solitary bee larvae prioritize carbohydrate over protein in parentally provided pollen. *Functional Ecology*, *35*(5), pp.1069-1080.
- Bager, A. ed., 2012. *Ecologia de Estradas: tendências e pesquisas*. Editora UFLA.
- Balcombe, J. P. (2009). Animal research: Invasive or non-invasive? ALTEX - Alternatives to Animal Experimentation, 26(4), 201-205.
- Batra, S.W.T., 1995. Bees and pollination in our changing environment. *Apidologie*, *26*(5), pp.361-370.
- Baum, K.A., Rubink, W.L., Coulson, R.N. and Bryant Jr, V.M., 2011. Diurnal patterns of pollen collection by feral honey bee colonies in southern Texas, USA. Palynology, 35(1), pp.85-93.
- Beauchamp, G. (2009). How prey detect predators: A review and reinvestigation of approaches. Animal Behaviour, 77(3), 621-629
- Bednarska, A.J., Mikołajczyk, Ł., Ziółkowska, E., Kocjan, K., Wnęk, A., Mokkapati, J.S., Teper, D., Kaczyński, P., Łozowicka, B., Śliwińska, R. and Laskowski, R., 2022. Effects of agricultural landscape structure, insecticide residues, and pollen diversity on the life-history traits of the red mason bee Osmia bicornis. *Science of The Total Environment*, *809*, p.151142.
- Begon, M., 1983. Grasshopper populations and weather: the effects of insolation on Chorthippus brunneus. Ecological Entomology, 8(4), pp.361-370.

- Biesboer, D., 1977. Aeropalynology. The American Biology Teacher, pp.88-92.
- Bingham, C.T. and Morley, C., 1897. Hymenoptera (Vol. 1). Taylor & Francis.

- Birch, L.L. and Fisher, J.O., 1998. Development of eating behaviors among children and adolescents. *Pediatrics*, *101*(Supplement_2), pp.539-549.
- Blamey, M., Fitter, S.R. and Fitter, A., 2003. *Wild Flowers of Britain & Ireland*. A. & C. Black.
- Bosch, J. and Vicens, N., 2006. Relationship between body size, provisioning rate, longevity and reproductive success in females of the solitary bee Osmia cornuta. Behavioral Ecology and Sociobiology, 60, pp.26-33.
- Bosch, J., 2008. Production of undersized offspring in a solitary bee. Animal Behaviour, 75(3), pp.809-816.
- Božič, A. and Šiber, A., 2020. Mechanical design of apertures and the infolding of pollen grain. Proceedings of the National Academy of Sciences, 117(43), pp.26600-26607.
- Bukovinszky, T., Rikken, I., Evers, S., Wäckers, F.L., Biesmeijer, J.C., Prins, H.H. and Kleijn, D., 2017. Effects of pollen species composition on the foraging behaviour and offspring performance of the mason bee Osmia bicornis (L.). *Basic and Applied Ecology*, *18*, pp.21-30.
- Cahu, C., Infante, J.Z. and Takeuchi, T., 2003. Nutritional components affecting skeletal development in fish larvae. *Aquaculture*, *227*(1-4), pp.245-258.
- Cane, J.H., Griswold, T. and Parker, F.D., 2007. Substrates and materials used for nesting by North American Osmia bees (Hymenoptera: Apiformes: Megachilidae). *Annals of the Entomological Society of America*, *100*(3), pp.350-358.
- Caro, T., (2005). *Antipredator defenses in birds and mammals*. University of Chicago Press (p-28).
- Caro, T., Graham, C. M., & Stoner, C. J. (2003). Sampling locality affects estimates of bird density from point
- Catry, I., Catry, T., Alho, M., Franco, A.M.A. and Moreira, F., 2016. Sexual and parent–offspring dietary segregation in a colonial raptor as revealed by stable isotopes. *Journal of Zoology*, *299*(1), pp.58-67.
- Clark, K.E., Hartley, S.E. and Johnson, S.N., 2011. Does mother know best? The preference–performance hypothesis and parent–offspring conflict in aboveground–belowground herbivore life cycles. *Ecological Entomology*, *36*(2), pp.117-124.
- Corbet, S.A., 1990. Pollination and the weather. Israel Journal of Botany, 39(1-2), pp.13-30.
- Corden, J. and Millington, W., 1999. A study of Quercus pollen in the Derby area, UK. Aerobiologia, 15, pp.29-37.
- Coudrain, V., Rittiner, S., Herzog, F., Tinner, W. and Entling, M.H., 2016. Landscape distribution of food and nesting sites affect larval diet and nest size, but not abundance of Osmia bicornis. *Insect Science*, *23*(5), pp.746-753.

- Daly, H.V., Doyen, J.T. and Ehrlich, P.R., 1978. *Introduction to insect biology and diversity*. McGraw-Hill Book Company.
- Davies, K.F., Melbourne, B.A., McClenahan, J.L. and Tuff, T., 2011. Statistical models for monitoring and predicting effects of climate change and invasion on the free-living insects and a spider from sub-Antarctic Heard Island. *Polar Biology*, *34*, pp.119-125.
- de Almeida Papa, D., de Almeida, D.R.A., Silva, C.A., Figueiredo, E.O., Stark, S.C., Valbuena, R., Rodriguez, L.C.E. and d'Oliveira, M.V.N., 2020. Evaluating tropical forest classification and field sampling stratification from lidar to reduce effort and enable landscape monitoring. *Forest Ecology and Management*, *457*, p.117634.
- de Sá-Otero, M.D.P., Armesto-Baztan, S. and Díaz-Losada, E., 2009. Analysis of protein content in pollen loads produced in north-west Spain. *Grana*, *48*(4), pp.290-296.
- De Wit, N.J., Afman, L.A., Mensink, M. and Müller, M., 2012. Phenotyping the effect of diet on non-alcoholic fatty liver disease. *Journal of hepatology*, *57*(6), pp.1370-1373.
- Domínguez, E., Mercado, J.A., Quesada, M.A. and Heredia, A., 1999. Pollen sporopollenin: degradation and structural elucidation. *Sexual Plant Reproduction*, *12*(3), pp.171-178.
- Donkersley, P., Rhodes, G., Pickup, R.W., Jones, K.C., Power, E.F., Wright, G.A. and Wilson, K., 2017. Nutritional composition of honey bee food stores vary with floral composition. Oecologia, 185, pp.749-761.
- Doskey, P.V. and Ugoagwu, B.J., 1992. Macronutrient chemistry of pollen. *Communications in soil science and plant analysis*, *23*(1-2), pp.15-23.
- Douglas Bates, Martin Maechler, Ben Bolker, Steve Walker (2015), Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48.doi:10.18637/jss.v067.i01.
- Doutt, R.L., 1959. The biology of parasitic Hymenoptera. Annual review of entomology, 4(1), pp.161-182.
- Drossart, M. and Gérard, M., 2020. Beyond the decline of wild bees: Optimizing conservation measures and bringing together the actors. *Insects*, *11*(9), p.649.
- Eilers, E.J., Kremen, C., Smith Greenleaf, S., Garber, A.K. and Klein, A.M., 2011. Contribution of pollinator-mediated crops to nutrients in the human food supply. *PLoS one*, *6*(6), p.e21363.
- Eisele, J.F., Fäßler, F., Bürgel, P.F. and Chaban, C., 2016. A rapid and simple method for microscopy-based stomata analyses. *PLoS One*, *11*(10), p.e0164576.
- Emberlin, J., 1994. The effects of patterns in climate and pollen abundance on allergy. Allergy, 49, pp.15-20.

- Engelke, U., Hutson, H., Nguyen, H. and de Souza, P., 2016, May. Melissar: Towards augmented visual analytics of honey bee behaviour. In *Proceedings of the 2016 CHI Conference Extended Abstracts on Human Factors in Computing Systems* (pp. 2057-2063).
- Engelke, U., Marendy, P., Susanto, F., Williams, R., Mahbub, S., Nguyen, H. and De Souza, P., 2016, December. A visual analytics framework to study honey bee behaviour. In *2016 IEEE 18th International Conference on High Performance Computing and Communications; IEEE 14th International Conference on Smart City; IEEE 2nd International Conference on Data Science and Systems (HPCC/SmartCity/DSS)* (pp. 1504-1511). IEEE.
- Field, J., 2005. The evolution of progressive provisioning. Behavioral Ecology, 16(4), pp.770-778.
- Filipiak, M., 2019. Key pollen host plants provide balanced diets for wild bee larvae: A lesson for planting flower strips and hedgerows. *Journal of Applied Ecology*, *56*(6), pp.1410-1418.
- Filipiak, Z.M. and Filipiak, M., 2020. The scarcity of specific nutrients in wild bee larval food negatively influences certain life history traits. *Biology*, *9*(12), p.462.
- Filipiak, Z.M., Denisow, B., Stawiarz, E. and Filipiak, M., 2022. Unravelling the dependence of a wild bee on floral diversity and composition using a feeding experiment. *Science of The Total Environment*, *820*, p.153326.
- Fliszkiewicz, M., KuśnierczaK, A.N.N.A. and Szymaś, B., 2015. Reproduction of the red mason solitary bee Osmia rufa (syn. Osmia bicornis)(Hymenoptera: Megachilidae) in various habitats. *EJE*, *112*(1), pp.100-105.
- Francisco, F.A., Nührenberg, P. and Jordan, A., 2020. High-resolution, non-invasive animal tracking and reconstruction of local environment in aquatic ecosystems. *Movement ecology*, *8*(1), pp.1-12.
- Friend, W.G., 1958. Nutritional requirements of phytophagous insects. *Annual review of entomology*, *3*(1), pp.57-74.
- García-Navas, V., Ferrer, E.S. and Sanz, J.J., 2012. Prey selectivity and parental feeding rates of Blue Tits Cyanistes caeruleus in relation to nestling age. *Bird Study*, *59*(2), pp.236-242.
- García-Navas, V., Ferrer, E.S. and Sanz, J.J., 2013. Prey choice, provisioning behaviour, and effects of early nutrition on nestling phenotype of titmice. *Ecoscience*, *20*(1), pp.9-18.
- Gardner, K.E. and Ascher, J.S., 2006. Notes on the native bee pollinators in New York apple orchards. *Journal of the New York Entomological Society*, *114*(1), pp.86-91.
- Gayubo, S.F., 2011. Are solitary progressive-provisioning wasps optimal foragers? A study with the digger wasp Bembix merceti (Hymenoptera: Crabronidae). *Behaviour*, *148*(2), pp.191-214.
- Gibert, P. and De Jong, G., 2001. Temperature dependence of development rate and adult size in Drosophila species: biophysical parameters. Journal of Evolutionary Biology, 14(2), pp.267-276.

- Giejdasz, K. and Wilkaniec, Z., 2002. Individual development of the red mason bee (Osmia rufa L., Megachilidae) under natural and laboratory conditions. J. Apic. Sci, 46, pp.51-57.
- Gilbert, N. and Raworth, D.A., 1996. Insects and temperature—a general theory. The Canadian Entomologist, 128(1), pp.1-13.
- Google (2023), Hull [Google maps]. Retrieved from: https://www.google.co.uk/maps/place/Hull/@53.7663425,-0.4143863,12z/data=!3m1!4b1!4m6!3m5!1s0x47d6208f7c0160ad:0x6d447e9d82f130ff!8m2!3d53.7676236!4d-0.3274198!16zL20vMGZtMl8?entry=ttu.
- Greenleaf, S.S., Williams, N.M., Winfree, R. and Kremen, C., 2007. Bee foraging ranges and their relationship to body size. *Oecologia*, *153*, pp.589-596.
- Gresty, C.E., Clare, E., Devey, D.S., Cowan, R.S., Csiba, L., Malakasi, P., Lewis, O.T. and Willis, K.J., 2018. Flower preferences and pollen transport networks for cavity-nesting solitary bees: Implications for the design of agri-environment schemes. *Ecology and Evolution*, *8*(15), pp.7574-7587.
- Gruber, B., Eckel, K., Everaars, J. and Dormann, C.F., 2011. On managing the red mason bee (Osmia bicornis) in apple orchards. *Apidologie*, *42*, pp.564-576.
- Haider, M., Dorn, S., Sedivy, C. and Müller, A., 2014. Phylogeny and floral hosts of a predominantly pollen generalist group of mason bees (Megachilidae: Osmiini). *Biological Journal of the Linnean Society*, *111*(1), pp.78-91.
- Han, C.S. and Dingemanse, N.J., 2015. Effect of diet on the structure of animal personality. *Frontiers in Zoology*, *12*(1), pp.1-9.
- Hanks, L.M. and Denno, R.F., 1998. Dispersal and adaptive deme formation in sedentary coccoid insects. *Genetic structure and local adaptation in natural insect populations: effects of ecology, life history, and behavior*, pp.239-262.
- Hansted, L., Grout, B.W., Toldam-Andersen, T.B. and Eilenberg, J., 2014. An assessment of Osmia rufa (syn. bicornis) as a pollinator of the sour cherry (Prunus cerasus) cv. Stevnsbaer in eastern Denmark. *Journal of Apicultural Research*, *53*(1), pp.177-182.
- Harmanescu, M., Popovici, D. and Gergen, I., 2007. Mineral micronutrients composition of bee's pollen. *J. Agroaliment. Processes Technol*, *13*(1), pp.175-182.
- Haslewood, G.A.S. 1962. Bile salts: structure, distribution and possible biological significance as a species character. *Biochem, vol. 3.*, pp.205-229.
- He, L.M., Wang, T.L., Chen, Y.C., Ge, S.S., Wyckhuys, K.A. and Wu, K.M., 2021. Larval diet affects development and reproduction of East Asian strain of the fall armyworm, Spodoptera frugiperda. *Journal of Integrative Agriculture*, *20*(3), pp.736-744.

- Hong, E.P. and Park, J.W., 2012. Sample size and statistical power calculation in genetic association studies. *Genomics & informatics*, *10*(2), p.117.
- Horth, L. and Campbell, L.A., 2018. Supplementing small farms with native mason bees increases strawberry size and growth rate. *Journal of applied ecology*, *55*(2), pp.591-599.
- House, H.L., 1962. Insect nutrition. *Annual Review of Biochemistry*, *31*(1), pp.653-672.
- Howard, C. (2020). *Moving targets: Nutritional geometry of development in solitary bee, Osmia bicornis*. Thesis. University Of Hull. Available at: https://docs.google.com/document/d/1pelJR3QusZwHTWg-mVr8T1_cuRaG8_FAdAXFLCSzI0/edit?pli=1
- iNaturalist. Available from https://www.inaturalist.org.
- Jacobsen, J.G. and Smith, L.H., 1968. Biochemistry and physiology of taurine and taurine derivatives. *Physiological reviews*, *48*(2), pp.424-511.
- Jaenike, J., 1978. On optimal oviposition behavior in phytophagous insects. *Theoretical population biology*, *14*(3), pp.350-356.
- Jermalowicz-Jones, J.L., Barron Lake Management Plan Study Cass County, Michigan.
- Jones, A.G., Chown, S.L., Webb, T.J. and Gaston, K.J., 2003. The free-living pterygote insects of Gough Island, South Atlantic Ocean. *Systematics and Biodiversity*, *1*(2), pp.213-273.
- Kaspi, R., Mossinson, S., Drezner, T., Kamensky, B. and Yuval, B., 2002. Effects of larval diet on development rates and reproductive maturation of male and female Mediterranean fruit flies. *Physiological Entomology*, *27*(1), pp.29-38.
- Kearns, C.A., Inouye, D.W. and Waser, N.M., 1998. Endangered mutualisms: the conservation of plant-pollinator interactions. *Annual review of ecology and systematics*, *29*(1), pp.83-112.
- Kelstrup, H.C., Hartfelder, K., Lopes, T.F. and Wossler, T.C., 2018. The behavior and reproductive physiology of a solitary progressive provisioning vespid wasp: evidence for a solitary-cycle origin of reproductive castes. The American Naturalist, 191(2), pp.E27-E39.
- Kevan, P.G., Chittka, L. and Dyer, A.G., 2001. Limits to the salience of ultraviolet: lessons from colour vision in bees and birds. Journal of Experimental Biology, 204(14), pp.2571-2580.
- Kierat, J., Szentgyörgyi, H., Czarnoleski, M. and Woyciechowski, M., 2017. The thermal environment of the nest affects body and cell size in the solitary red mason bee (Osmia bicornis L.). *Journal of Thermal Biology*, *68*, pp.39-44.
- Killewald, M.F., Rowe, L.M., Graham, K.K., Wood, T.J. and Isaacs, R., 2019. Use of nest and pollen resources by leafcutter bees, genus Megachile (Hymenoptera: Megachilidae) in central Michigan. *The Great Lakes Entomologist*, *52*(1), p.8.

- Klein, A.M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C. and Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences*, *274*(1608), pp.303-313.
- Kremen, C., Williams, N.M., Aizen, M.A., Gemmill-Herren, B., LeBuhn, G., Minckley, R., Packer, L., Potts, S.G., Roulston, T.A., Steffan-Dewenter, I. and Vázquez, D.P., 2007. Pollination and other ecosystem services produced by mobile organisms: a conceptual framework for the effects of land-use change. *Ecology letters*, *10*(4), pp.299-314.
- Krockenberger, A.K. and Hume, I.D., 2007. A flexible digestive strategy accommodates the nutritional demands of reproduction in a free-living folivore, the Koala (Phascolarctos cinereus). *Functional Ecology*, *21*(4), pp.748-756.
- Krunic, M.D. and Stanisavljevic, L.Ž., 2006. Population management in the mason bee species Osmia cornuta and O rufa for orchard pollination in Serbia (Hymenoptera: Megachilidae). *Entomologia generalis*, pp.27-38.
- Krunić, M.D. and Stanisavljević, L.Ž., 2006. Supercooling points and diapause termination in overwintering adults of orchard bees Osmia cornuta and O. rufa (Hymenoptera: Megachilidae). Bulletin of entomological research, 96(3), pp.323-326.
- Ksanen, J,. Simpson, G,. Blanchet, F,. Kindt, R,. Legendre, P,. Minchin, P,. O'Hara, R,. Solymos, P,. Stevens, M,. Szoecs, E,. Wagner, H,. Barbour, M,. Bedward, M,. Bolker, B,. Borcard, D,. Carvalho, G,. Chirico, M,. Deaceres, M,. Durand, S,. Evangelista, H,. FitzJohn, R,. Friendly, M,. Furneaux, B,. Hannigan, G,. Hil, l, M,. Lahti, L,. McGlinn, D,. Ouellette, M,. Ribeiro, Cunha, E,. Smith, T,. Stier, A,. Ter Braak, C,. Weedon, J (2022). _vegan: Community Ecology Package_. R package version 2.6-2, https://CRAN.R-project.org/package=vegan>.
- Lan, J., Ding, G., Ma, W., Jiang, Y. and Huang, J., 2021. Pollen source affects development and behavioral preferences in honey bees. *Insects*, *12*(2), p.130.
- Lawson, S., Kennedy, K. and Rehan, S., 2020. Pollen composition significantly impacts the development and survival of the native small carpenter bee, Ceratina calcarata. *Ecological Entomology*, 46(2), pp.232-239.
- Le, N.A., Ginsberg, H.N. and Brown, W.V., 1984. Protocol for efficient plasma sampling for low density lipoprotein turnover studies. *Journal of Lipid Research*, *25*(12), pp.1387-1391.
- Lee, K.Y., Yoon, H.J., Park, I.G., Kwon, C.R. and Lee, S.C., 2010. Survey on the current status of mason bees in apple orchard of Korea. *Korean Journal of Apiculture*.
- Leonhardt, S.D., Peters, B. and Keller, A., 2022. Do amino and fatty acid profiles of pollen provisions correlate with bacterial microbiomes in the mason bee Osmia bicornis?. *Philosophical Transactions of the Royal Society B*, *377*(1853), p.20210171.

- Lima, M.A.P., Cutler, G.C., Mazzeo, G. and Hrncir, M., 2022. The decline of wild bees: Causes and consequences. *Frontiers in Ecology and Evolution*, *10*, p.1027169.
- MacIvor, J.S., Cabral, J.M. and Packer, L., 2014. Pollen specialization by solitary bees in an urban landscape. *Urban Ecosystems*, *17*, pp.139-147.
- Maddocks, R. and Paulus, H.F., 1987. Quantitative Aspekte der Brut-biologie von Osmia rufa L. und Osmia cornuta Latr.(Hymenoptera, Megachilidae): Eine vergleichende Untersuchung zu Mechanismen der Konkurrenzminderunt zweier nahverwandter Bienenarten. *Zoologische Jahrbücher. Abteilung für Systematik, Ökologie und Geographie der Tiere*, *114*(1), pp.15-44.
- Mandeville, E. G., & Novak, S. J. (2015). Nesting success and behaviors of a managed solitary ground-nesting bee, Megachile rotundata (Hymenoptera: Megachilidae). Journal of Insect Science, 15(1), 1-10.
- Mattson Jr, W.J., 1980. Herbivory in relation to plant nitrogen content. *Annual review of ecology and systematics*, *11*(1), pp.119-161.
- Mayhew, P.J., 2001. Herbivore host choice and optimal bad motherhood. *Trends in ecology & evolution*, *16*(4), pp.165-167.
- Mayr, E., 1956. Geographical character gradients and climatic adaptation. Evolution, 10(1), pp.105-108.
- Michener, C.D., 1999. The corbiculae of bees. *Apidologie*, *30*(1), pp.67-74.
- Mitchell, R.O.D.G.E.R., 1983. Effects of host-plant variability on the fitness of sedentary herbivorous insects. *Variable plants and herbivores in natural and managed systems*, pp.343-370.
- Mitrus, C., Mitrus, J. and Sikora, M., 2010. Changes in nestling diet composition of the red-breasted flycatcher Ficedula parva in relation to chick age and parental sex. *Animal Biology*, *60*(3), pp.319-328.
- Modeer, A. 1764. Einige Merkwu¨rdigkeiten bei dem Insecte Cimex ovatus pallide griseus, abdominis lateribus albo nigrogre variis, alis albis basiscutelli nigricante. Vetenskaps Academiens Handlingar, 25, 41–57.
- Moore, P.D., Webb, J.A. and Collison, M.E., 1991. Pollen analysis. Blackwell scientific publications.
- Morimoto, J., 2023. Nutrigonometry IV: Thales' theorem to measure the rules of dietary compromise in animals. Scientific Reports, 13(1), p.7466.
- Naghii, M.R., Wall, P.M. and Samman, S., 1996. The boron content of selected foods and the estimation of its daily intake among free-living subjects. *Journal of the American College of Nutrition*, *15*(6), pp.614-619.

- Nascimbene, J., Marini, L., Bacaro, G. and Nimis, P., 2010. Effect of reduction in sampling effort for monitoring epiphytic lichen diversity in forests. *Community Ecology*, *11*(2), pp.250-256.
- Nassar, J.M., Khan, S.M., Velling, S.J., Diaz-Gaxiola, A., Shaikh, S.F., Geraldi, N.R., Torres Sevilla, G.A., Duarte, C.M. and Hussain, M.M., 2018. Compliant lightweight non-invasive standalone "Marine Skin" tagging system. *npj Flexible Electronics*, *2*(1), p.13.
- Nedreaas, K.H., Stransky, C., Jardim, E. and Vigneau, J., 2009. Quality assurance framework–the concept of quality assurance applied to fisheries data and its operationalisation under the ICES scope. ICES.
- Neuwirth E (2022). _RColorBrewer: ColorBrewer Palettes_. R package version 1.1-3,<https://CRAN.R-project.org/package=RColorBrewer>.
- Nicolson, S.W. and Thornburg, R.W., 2007. Nectar chemistry. In *Nectaries and nectar* (pp. 215-264). Springer, Dordrecht.
- Nomura, N., Shoda, W. and Uchida, S., 2019. Clinical importance of potassium intake and molecular mechanism of potassium regulation. *Clinical and experimental nephrology*, *23*, pp.1175-1180.
- Norris-Hill, J. and Emberlin, J., 1991. Diurnal variation of pollen concentration in the air of north-central London. Grana, 30(1), pp.229-234
- Ojala, K., Julkunen-Tiitto, R., Lindström, L. and Mappes, J., 2005. Diet affects the immune defence and life-history traits of an Arctiid moth Parasemia plantaginis. *Evolutionary Ecology Research*, *7*(8), pp.1153-1170.
- Orłowski, G., Wuczyński, A. and Karg, J., 2015. Effect of brood age on nestling diet and prey composition in a hedgerow specialist bird, the barred warbler Sylvia nisoria. *PLoS One*, *10*(6), p.e0131100.
- Ostap-Chec, M., Kierat, J., Kuszewska, K. and Woyciechowski, M., 2021. Red mason bee (Osmia bicornis) thermal preferences for nest sites and their effects on offspring survival. *Apidologie*, *52*(3), pp.707-719.
- O'Toole, C., 2000. *The red mason bee: taking the sting out of bee-keeping: a practical guide to managing Osmia rufa as a pollinator in gardens, allotments, and orchards*. Osmia Publications.
- Pearson, J.R., Gill, C.I. and Rowland, I.R., 2009. Diet, fecal water, and colon cancer–development of a biomarker. *Nutrition reviews*, *67*(9), pp.509-526.
- Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F. and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. J. Comp. Physiol. A 170, 23–40.

- Persson, A.S., Mazier, F. and Smith, H.G., 2018. When beggars are choosers—How nesting of a solitary bee is affected by temporal dynamics of pollen plants in the landscape. *Ecology and Evolution*, *8*(11), pp.5777-5791.
- Pitts-Singer, T.L., 2004. Examination of 'pollen balls' in nests of the alfalfa leafcutting bee, Megachile rotundata. *Journal of apicultural research*, *43*(2), pp.40-46.
- Poderoso, J.C.M., Correia-Oliveira, M.E., Vieira, J.M., Ribeiro, G.T., Ribeiro, R.C. and Zanuncio, J.C., 2013. Lasioderma serricorne (Coleoptera: Anobiidae): first record in dehydrated bee pollen in Sergipe State, Brazil. *Florida Entomologist*, *96*(2), pp.682
- Poissonnier, L.A., Arganda, S., Simpson, S.J., Dussutour, A. and Buhl, J., 2018. Nutrition in extreme food specialists: An illustration using termites. *Functional ecology*, *32*(11), pp.2531-2541.
- Polilov, A.A. and Beutel, R.G., 2009. Miniaturisation effects in larvae and adults of Mikado sp.(Coleoptera: Ptiliidae), one of the smallest free-living insects. *Arthropod Structure & Development*, *38*(3), pp.247-270.
- Potts, S.G., Vulliamy, B., Roberts, S., O'Toole, C., Dafni, A., Ne'eman, G. and Willmer, P.G., 2004. Nectar resource diversity organises flower-visitor community structure. *Entomologia experimentalis et applicata*, *113*(2), pp.103-107.
- Prajapati, B., Dunne, M. and Armstrong, R., 2010. Sample size estimation and statistical power analyses. *Optometry today*, *16*(7), pp.10-18.
- Pyke, G.H., 1984. Optimal foraging theory: a critical review. Annual review of ecology and systematics, 15(1), pp.523-575.
- Qiao, K., Nickerson, A., MacDonald, S. and Ghafar-Zadeh, E., 2020, August. A Non-Invasive Wireless Respiratory Monitoring System for Animals' Behavioural Studies. In *2020 IEEE Canadian Conference on Electrical and Computer Engineering (CCECE)* (pp. 1-4). IEEE.
- R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
- Radford, A.N., 2008. Age-related changes in nestling diet of the cooperatively breeding green woodhoopoe. *Ethology*, *114*(9), pp.907-915.
- Radmacher, S. and Strohm, E., 2010. Factors affecting offspring body size in the solitary bee Osmia bicornis (Hymenoptera, Megachilidae). *Apidologie*, *41*(2), pp.169-177.

- Radmacher, S. and Strohm, E., 2010. Factors affecting offspring body size in the solitary bee Osmia bicornis (Hymenoptera, Megachilidae). *Apidologie*, *41*(2), pp.169-177.

- Raubenheimer, D. and Simpson, S.J., 1999. Integrating nutrition: a geometrical approach. In Proceedings of the 10th International Symposium on Insect-Plant Relationships (pp. 67-82). Springer Netherlands.

- Raubenheimer, D. and Simpson, S.J., 2003. Nutrient balancing in grasshoppers: behavioural and physiological correlates of dietary breadth. Journal of Experimental Biology, 206(10), pp.1669-1681.
- RAW, A., 1972. The biology of the solitary bee Osmia rufa (L.)(Megachilidae). Transactions of the Royal Entomological Society of London, 124(3), pp.213-229.
- Raw, A., 1974. Pollen preferences of three Osmia species (Hymenoptera). *Oikos*, pp.54-60.
- Ray, C., 1960. The application of Bergmann's and Allen's rules to the poikilotherms. Journal of morphology, 106(1), pp.85-108.
- Reeve, N., Molony, S. E., Clark, C. C., Corke, P. I., Hardaker, B. J., & Dwyer, C. M. (2014). Review: The implications of non-invasive sampling for wildlife biologists. Wildlife Research, 41(7), 527-541
- Rering, C.C., Beck, J.J., Hall, G.W., McCartney, M.M. and Vannette, R.L., 2018. Nectar-inhabiting microorganisms influence nectar volatile composition and attractiveness to a generalist pollinator. New Phytologist, 220(3), pp.750-759.
- Rogers, C.A., 1993. Application of aeropalynological principles in palaeoecology. Review of Palaeobotany and Palynology, 79(1-2), pp.133-140.
- Roulston, T.A.H. and Cane, J.H., 2002. The effect of pollen protein concentration on body size in the sweat bee Lasioglossum zephyrum (Hymenoptera: Apiformes). *Evolutionary Ecology*, *16*, pp.49-65.

- Roulston, T.A.H. and Cane, J.H., 2002. The effect of pollen protein concentration on body size in the sweat bee Lasioglossum zephyrum (Hymenoptera: Apiformes). *Evolutionary Ecology*, *16*, pp.49-65.

- Roulston, T.A.H., Cane, J.H. and Buchmann, S.L., 2000. What governs protein content of pollen: pollinator preferences, pollen–pistil interactions, or phylogeny?. *Ecological monographs*, *70*(4), pp.617-643.
- Royle, N.J., Smiseth, P.T. and Kölliker, M. eds., 2012. *The evolution of parental care*. Oxford University Press.
- Rush, S., Conner, J.K. and Jennetten, P., 1995. The effects of natural variation in pollinator visitation on rates of pollen removal in wild radish, Raphanus raphanistrum (Brassicaceae). American Journal of Botany, 82(12), pp.1522-1526.
- Russell, K.A. and McFrederick, Q.S., 2022. Elevated temperature may affect nectar microbes, nectar sugars, and bumble bee foraging preference. Microbial Ecology, 84(2), pp.473-482.

- Sansone, V., Applefield, R.C., De Luca, P., Pecoraro, V., Gianola, S., Pascale, W. and Pascale, V., 2019. Does a high-fat diet affect the development and progression of osteoarthritis in mice? A systematic review. *Bone & Joint Research*, *8*(12), pp.582-592.
- Schaeffer, R.N., Mei, Y.Z., Andicoechea, J., Manson, J.S. and Irwin, R.E., 2017. Consequences of a nectar yeast for pollinator preference and performance. Functional Ecology, 31(3), pp.613-621.
- Sedivy, C. and Dorn, S., 2014. Towards a sustainable management of bees of the subgenus Osmia (Megachilidae; Osmia) as fruit tree pollinators. *Apidologie*, *45*, pp.88-105.
- Sedivy, C., Müller, A. and Dorn, S., 2011. Closely related pollen generalist bees differ in their ability to develop on the same pollen diet: evidence for physiological adaptations to digest pollen. *Functional Ecology*, *25*(3), pp.718-725.
- Seidelmann, K., 2006. Open-cell parasitism shapes maternal investment patterns in the Red Mason bee Osmia rufa. *Behavioral Ecology*, *17*(5), pp.839-848.
- Shimano, Y., Cardoso, M. and Juen, L., 2018. Ecological studies of mayflies (Insecta, Ephemeroptera): Can sampling effort be reduced without losing essential taxonomic and ecological information?. *Acta Amazonica*, *48*, pp.137-145.
- Simpson, S.J. and Raubenheimer, D., 2012. The nature of nutrition: a unifying framework. *Australian journal of zoology*, *59*(6), pp.350-368.
- Simpson, Stephen J, and David Raubenheimer. 2001. "The Geometric Analysis of Nutrient-Allelochemical Interactions: A Case Study Using Locusts." *Ecology* 82 (2): 422–39
- Simpson, Stephen J. and Raubenheimer, David. The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity, Princeton: Princeton University Press, 2012.
- Slansky, F., 1982. Insect nutrition: an adaptationist's perspective. *The Florida Entomologist*, *65*(1), pp.45-71.
- Smith, A.R., Wcislo, W.T. and O'donnell, S., 2003. Assured fitness returns favor sociality in a mass-provisioning sweat bee, Megalopta genalis (Hymenoptera: Halictidae). *Behavioral Ecology and Sociobiology*, *54*, pp.14-21.
- Souza, R.S., Virginio, F., Riback, T.I.S., Suesdek, L., Barufi, J.B. and Genta, F.A., 2019. Microorganism-based larval diets affect mosquito development, size and nutritional reserves in the yellow fever mosquito Aedes aegypti (Diptera: Culicidae). *Frontiers in physiology*, *10*, p.152.
- Splitt, A., Schulz, M. and Skorka, P., 2022. Current state of knowledge on the biology and breeding of the solitary bee–Osmia bicornis. *Journal of Apicultural Research*, *61*(2), pp.163-179.
- Splitt, A., Skórka, P., Strachecka, A., Borański, M. and Teper, D., 2021. Keep trees for bees: Pollen collection by Osmia bicornis along the urbanization gradient. *Urban Forestry & Urban Greening*, *64*, p.127250.

- Steffan-Dewenter, I. and Westphal, C., 2008. The interplay of pollinator diversity, pollination services and landscape change. *Journal of Applied Ecology*, pp.737-741.
- Stephen, A., 2014. Pollen: A microscopic wonder of plant kingdom. International Journal of Advanced Research in Biological Sciences, 1(9), pp.45-62.
- Stewart, M., Webster, J.R., Schaefer, A.L., Cook, N.J. and Scott, S.L., 2005. Infrared thermography as a non-invasive tool to study animal welfare. *Animal Welfare*, *14*(4), pp.319-325.
- Strohm, E., Daniels, H., Warmers, C. and Stoll, C., 2002. Nest provisioning and a possible cost of reproduction in the megachilid bee Osmia rufa studied by a new observation method. *Ethology Ecology & Evolution*, *14*(3), pp.255-268.
- Suzuki, S., 2010. Progressive provisioning by the females of the earwig, Anisolabis maritima, increases the survival rate of the young. Journal of Insect Science, 10(1), p.184.
- Suzuki, T., Ikegami, M., Goka, K. and Sakamoto, Y., 2023. Insecticide residues, associated with apple orchard treatments, in the mason bee, Osmia cornifrons, and their nests. *Environmental Toxicology and Chemistry*.
- Tainsh, F., Woodmansey, S.R., Austin, A.J., Bagnall, T.E. and Gilbert, J.D., 2021. Sporopollenin as a dilution agent in artificial diets for solitary bees. *Apidologie*, *52*, pp.101-112.
- Tallamy, D.W. and Brown, W.P.,1999. Semelparity and the evolution of maternal care in insects. *Animal Behaviour*, *57*(3), pp.727-730.
- Tasei, J.N. and Picart, M., 1973. Le comportement de nidification chez Osmia (Osmia) cornuta Latr. et Osmia (Osmia) rufa L.(Hymenoptera Megachilidae). Apidologie, 4(3), pp.195-225.
- Ternman, E., Hänninen, L., Pastell, M., Agenäs, S. and Nielsen, P.P., 2012. Sleep in dairy cows recorded with a non-invasive EEG technique. *Applied Animal Behaviour Science*, *140*(1-2), pp.25-32.
- Tista, M. and Fiedler, K., 2011. How to evaluate and reduce sampling effort for ants. *Journal of Insect Conservation*, *15*, pp.547-559.
- Tsukamoto, L. and Tojo, S., 1992. A report of progressive provisioning in a stink bug, Parastrachia japonensis (Hemiptera: Cydnidae). Journal of Ethology, 10(1), pp.21-29
- Tur, J.A., Colomer, M., Monino, M., Bonnin, T., Llompart, I. and Pons, A., 2005. Dietary intake and nutritional risk among free-living elderly people in Palma de Mallorca. *Journal of Nutrition Health and Aging*, *9*(6), p.390.
- UK Health & security industry, (2023), *Corporate report: UKHSA research involving animals.* Accesses via: https://www.gov.uk/government/publications/uk-health-security-agency-ukhsa-research-involving-animals/ukhsa-research-involving-animals

- Van der Steen, J. and De Ruijter, A., 1991. The management of Osmia rufa L. for pollination of seed crops in greenhouses. In Proceedings of Experimental and Applied Entomology (Vol. 2, No. 1, pp. 137-141).
- van Doorn, W.G. and Van Meeteren, U., 2003. Flower opening and closure: a review. Journal of experimental botany, 54(389), pp.1801-1812.
- Vannette, R.L. and Fukami, T., 2016. Nectar microbes can reduce secondary metabolites in nectar and alter effects on nectar consumption by pollinators. Ecology, 97(6), pp.1410-1419.
- Vaudo, A.D., Patch, H.M., Mortensen, D.A., Tooker, J.F. and Grozinger, C.M., 2016. Macronutrient ratios in pollen shape bumble bee (Bombus impatiens) foraging strategies and floral preferences. *Proceedings of the National Academy of Sciences*, *113*(28), pp.E4035-E4042. (1)
- Vaudo, A.D., Stabler, D., Patch, H.M., Tooker, J.F., Grozinger, C.M. and Wright, G.A., 2016. Bumble bees regulate their intake of essential protein and lipid pollen macronutrients. *Journal of Experimental Biology*, *219*(24), pp.3962-3970. (2)
- Vaudo, A.D., Tooker, J.F., Patch, H.M., Biddinger, D.J., Coccia, M., Crone, M.K., Fiely, M., Francis, J.S., Hines, H.M., Hodges, M. and Jackson, S.W., 2020. Pollen protein: lipid macronutrient ratios may guide broad patterns of bee species floral preferences. *Insects*, *11*(2), p.132.
- Walters, R.J. and Hassall, M., 2006. The temperature-size rule in ectotherms: may a general explanation exist after all?. The American Naturalist, 167(4), pp.510-523.
- Warbrick-Smith, J., Raubenheimer, D., Simpson, S.J. and Behmer, S.T., 2009. Three hundred and fifty generations of extreme food specialisation: testing predictions of nutritional ecology. *Entomologia experimentalis et applicata*, *132*(1), pp.65-75.
- Wiermann, R. and Gubatz, S., 1992. Pollen wall and sporopollenin. In *International Review of Cytology* (Vol. 140, pp. 35-72). Academic Press.
- Wiethold, J., 2009. Hans-Jürgen Beug, Guide to pollen determination for Central Europe and neighboring areas. Germania: Announcement of the Roman-Germanic Commission of the German Archaeological Institute, pp.699-702.
- Wilson, E.O., 2008. One giant leap: how insects achieved altruism and colonial life. *BioScience*, *58*(1), pp.17-25.
- Wong, J.W., Meunier, J. and Kölliker, M., 2013. The evolution of parental care in insects: the roles of ecology, life history and the social environment. *Ecological Entomology*, *38*(2), pp.123-137.
- Wood, T.J., Kaplan, I. and Szendrei, Z., 2018. Wild bee pollen diets reveal patterns of seasonal foraging resources for honey bees. Frontiers in Ecology and Evolution, 6, p.210
- Wu FH, Shen SC, Lee LY, Lee SH, Chan MT, Lin CS. Tape-Arabidopsis Sandwich—a simpler Arabidopsis protoplast isolation method. Plant Methods. 2009; 5: 16. pmid:19930690

- Ydenberg, R. and Schmid-Hempel, P., 1994. Modelling social insect foraging. *Trends in ecology & evolution*, *9*(12), pp.491-493.
  1) Zemanova, M.A., 2020. Towards more compassionate wildlife research through the 3Rs principles: moving from invasive to non-invasive methods. *Wildlife Biology*, *2020*(1), pp.1-17.

Zesiger, C., Cohen, E., Jarvis, H., Spears, L.R. and Ramirez, R., 2021. Beginner's Guide to Common Native Bees.

Zurbuchen, A., Landert, L., Klaiber, J., Müller, A., Hein, S. and Dorn, S., 2010. Maximum foraging ranges in solitary bees: only few individuals have the capability to cover long foraging distances. *Biological Conservation*, *143*(3), pp.669-676.