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IMPACT OF LANDSCAPE ON HONEY BEE POLLEN DIET, PESTICIDE EXPOSURE AND COGNITION

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Abstract

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Impact of Landscape on Honey bee Pollen Diet, Pesticide Exposure and Cognition

by

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The European honey, *Apis Melifera L.*, is the most commonly managed bee in the world, but despite an increase in the global stocks of honey bees, areas such as Europe (-26.5%) and North America (-49.5%) have experienced notable declines at the colony level. Although less data is available on the position of honey bees in the tropics, what is available suggests that there is a deficit of 210,00 honey bee hives compared to global trends. These declines are driven by a number of synergistic stressors, including land use change, pesticide exposure, as well as pathogen and parasite infection. This thesis investigates how in a tropical environment, Belize, the Africanised honey bee is impacted by the landscape, and in particular their subsequent diet and exposure to pesticides. Apiculture is one of the most widespread agricultural activities practised globally, with an estimated 94 million honey bee colonies present worldwide in 2020. Beekeeping offers substantial benefits to local people, particularly in rural developing communities, where it provides economic opportunities, through the production of honey, wax and bee bread. In addition, the impact of landscape on honey bee learning in the UK was also analysed, and for the first time directly compares visual learning across a gradient of landscapes, contributing to the growing knowledge of bee behaviour and cognition.

Analysis of the honey bee pollen diet across the Toledo and Cayo districts of Belize was carried out by identifying the pollen grains in bee bread samples. The most abundant and frequently occurring taxa were identified and community compositions studied. Tree species were found to be of particular importance, making up 80% of the 10 most relatively abundant species. This study contributes to the growing body of research highlighting the importance of trees in bee diets. No relationship was found between the most abundant pollen taxa and their nutritional make up based on crude protein levels, suggesting that the abundance of the pollen in the environment is more likely the driver behind its dominance in the bee bread.

The effects of landscape diversity on species richness, species diversity and community compositions of pollen found in bee bread was compared in the Toledo and Cayo districts. A negative relationship was found between landscape diversity and both pollen richness and diversity. This may be due to the complex relationship between each plant/pollen taxa and the landscape, as well as bee preference. A high abundance of preferred species within the landscape acts to lower the diversity and richness of pollen with the honey bees diet. This data suggests that the presence and dominance of preferred species in the landscape is more important than landscape diversity when it comes to determining honey bee diet.

The presence of pesticides in bee bread samples from honey bee hives located across a gradient off natural and agricultural landscapes was also investigated. Pesticides were not found in any of the samples, suggesting that exposure of honey bees to pesticides via their pollen diet does not represent a risk in Belize. The lack of pollen contamination of pesticides is likely due to the bees preference of tree pollen which does not receive pesticide treatment, and is unlikely to experience accidental treatment due to the main application methods in Belize, knapsack spraying.

Finally, the effects of landscape complexity and composition on honey bee cognition was studied directly in the field. Visual learning, a crucial component of bee behaviour used to find food resources, was compared across a gradient of landscapes with a range of complexity, whilst compositional changes were kept to a minimum. To do this a field adapted version of the proboscis extension response was utilised, and bees were taught to associate different coloured paper strips with positive and negative rewards. Results showed that as landscape edge density increased bee learning reduced, and while landscape diversity increased so did bee learning. This is important as to ensure colony survival, bees must learn foraging routes, find profitable flowers, develop spatial maps as well as recognise intruders.

Landscape has been shown to be an influential factor in both determining the pollen diet of bees as well as their learning ability, but in Belize did not contribute to pesticide exposure through their pollen diet, likely due to the pesticide application methods and bee preference for tree species. When placing bee hives in both Belize and the UK, it is therefore important to consider there placement in terms of landscape, to increase the likelihood of developing a strong and successful colony.

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Research Thesis: Declaration of Authorship

Print name: GEORGINA HOLLANDS

Title of thesis: Impact of Landscape on Honey bee Pollen Diet, Pesticide Exposure and Cognition

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signature: .... Date: 13/12/2022

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Abbreviations

Abbreviations	Term
AHB	Africanised Honey Bee
AIC	Akaike's Information Criterion
EHB	European Honey Bee
EMF	Electromagnetic Field
GLY	Glyphosate
HPG	Hypopharyngeal glands
HPLC-MS/MS	High Performance Liquid Chromatography with Tandem Mass Spectrometry
IICA	Inter-American Institute for Cooperation on Agriculture
LA	Latin America
MGL	Mayan Golden Landscape
PER	Proboscis Extension Response
SER	Sting Extension Reflex
2,4-D	2,4-Dichlorophenoxyacetic acid

Chapter 1: Introduction

1.1 Background

Natural habitats are under increasing pressure from worldwide land-use transitions driven by anthropogenic expansion and development, resulting in losses of native vegetation, fragmentation, and degradation of habitat, all of which have been implicated in the global decline of biodiversity (Winkler et al., 2021). Understanding the relationship between landscape and biodiversity is therefore critical in reversing such declines.

Bees play a key role in terms of pollination globally, this is particularly true in the tropics where insect assisted pollination is high (Bawa and Crisp, 1980), making plant reproductive success dependent on animal-plant interactions. It has been estimated that 98-99% of tropical plant species are animal pollinated, with the greatest contribution from bees (Bawa, 1990).

The European honey, *Apis Melifera L.*, is the most commonly managed bee in the world (vanEngelsdorp and Meixner, 2010), and not only contributes to pollination efforts but is also supports the livelihoods of farmers and local people, particularly in low and middle income countries though the production of honey, bee bread, and bees wax (Kumar Gupta et al., 2014; Schouten et al., 2020). Despite an increase in the global stocks of honey bees, areas such as Europe (-26.5%) and North America (-49.5%) have experienced notable declines at the colony level (FAOSTAT, 2009). Figures for Latin America are less clear, although data suggests a deficit of honey productivity of 460,000 tons for LA compared to global patterns (Requier et al., 2018). These decreases are thought to be due to an array of anthropogenic drivers including pesticides, introduced pathogens, climate change and land-use change (Havard et al., 2020). In addition, the interactions between these individual stressors are potentially resulting in stronger negative effects. For example, honey bee survival has been shown to be synergistically reduced by pesticide exposure and poor nutrition (Tosi et al., 2017). When honey bees were exposed to Thiamethoxam but did not experience nutrition stress survival was not significantly impaired, whilst synergistically experiencing Thiamethoxam exposure and nutritional stress reduced surival by 50% (Tosi et al., 2017).

Consequently, this thesis aims to contribute to the understanding of some of the factors that affect the health of honey bees across a gradient of landscapes, including the their nutrition/pollen diet, exposure to pesticides, and cognitive abilities. The following text will provide a comprehensive background as to the economic and social importance of honey bees, the threats bees face due to a land use change, including pollen diet and pesticide exposure, as well as the importance of cognition and potential effects of landscape.

1.1.1 A history to apiculture

Apiculture is one of the most widespread agricultural activities practised globally (Vural and Karaman, 2011), and as of 2020 there was an estimated 94 million managed honey bee colonies worldwide, an increase of almost 14 million since 2010 (Shahbandeh, 2020). Despite the overall increase of the honey bee, particularly in Asia and Africa (+130%) (FAOSTAT, 2009), not all areas have experienced these gains, with both Europe and North America seeing losses (-26.5%, -49.5% respectively). Whilst data regarding beekeeping and hive numbers are readily available for these areas, information regarding honey bee populations in Latin America is limited, although it is estimated that there is a deficit of 210,000 hives compared to the global pattern (Requier et al., 2018).

The origins of beekeeping date back 10,000 years ago where depictions have been found of humans collecting honey from wild bees. Sealed pots of honey have been found in graves of pharaohs such as Tutankhamun (Praduman et al., 2020). Honey was the only sweetener available to early African, Middle Eastern, and European civilizations, and the demand for honey led to the domestication of bees by the Ancient Egyptians, sometime before 2600 BC (van Engelsdorp & Meixner, 2010). The practise of beekeeping was passed to the ancient Greeks by 650 BC and later to the Romans by 150 BC who then spread the art of beekeeping throughout what would become medieval Europe. From here, it was the decedents that would eventually spread both the practise of beekeeping and management of bees across the world (van Engelsdorp & Meixner, 2010). In 1851 Lorenzo Lorraine Langstroth, also known as the father of beekeeping, discovered that honey bees maintained a constant distance of 8 mm between combes, placing propolis is spaces smaller than this, and on that the basis invented the Langstroth bee box (Praduman et al., 2020). Further discoveries, including the division of labour in honey bee colonies and rearing techniques, have led to the successful domestication of large honey bee populations globally (Praduman et al., 2020).

1.1.2 Honey bees in the neotropics/Latin America

Early European settlers initially introduced the Western European honey bee (*A. m. mellifera and A. m. iberiensis)* and later Eastern European races such as *A. m. ligustica* into the Americas (Whitfield et al., 2006), and therefore prior to 1956 the Americas predominantly consisted of honey bees of European decent. In 1956 queens of *A. m. scutellata* and *A.m. adansonii* were introduced into Brazil from east and southern Africa with the aims of breeding them with the European honey bee, as it was thought these hybrids would be better adapted to the warm climate and, therefore, achieve greater honey production. The African colonies were accidently released, however, and this led to rapid hybridisation with the European honey bee, creating the Africanised honey bee (AHB) (Rinderer and Hellmich, 1991). Whilst numerous efforts were made to stop the spread of the AHB, the populations expanded greatly and over 30 years colonised most of the Americas, displacing the majority of the European honey bees, except in particularly cool climates. This process led to the initial disruption of beekeeping in the Americans which until the release of the African bee had been strong (Caron, 2001; Guzman-Novoa, 1996; Hellmich, 1991).

There are a number of differences between the AHB and European honey bee which initially made them harder to manage. These included:

Aggressiveness – While European honey bees may attack an intruder with a few bees, usually between 10 and 20, an Africanised bee colony will attack with upwards of 100 bees. The Africanised colony will defend larger areas around their nests and will require a lower level stimulus to initiate an attack (O'Malley et al., 2009).

Reproduction and nest abandonment – Africanised honey bees swarm and abscond at greater frequencies than the European honey bee, making it more likely that beekeepers will lose their bees. Africanised bees reproduce at a quicker rate which means that hives become saturated quicker, and the bees are then more likely to abscond. European bees usually swarm one to three times a year whilst Africanised bees may swarm more than ten times (O'Malley et al., 2009).

Honey store – Africanised bees store proportionately less food (honey) than European honey bees, likely because they are native to an environment where food resources are available throughout the year. If too much honey is removed for sale, they are more likely to abscond (O'Malley et al., 2009).

Whilst both the European and Africanised bees forage for nectar and pollen, the frequency and resources put towards pollen foraging is much greater in the Africanised honey bee. Couvillon et al. (2015) found that Africanised honey bees allocated approximately 60% more foragers to pollen collection than European bees (Couvillon et al., 2010), whilst Schneider & Hall (1997) found that Africanised honey bees carried out 1.5 times the amount of pollen foraging (Schneider and Hall, 1997). This could be explained by the contrasting ecological landscapes, in which temperate bees need to store sufficient carbohydrates (honey) over the winter months in order to survive so they therefore need to prioritise nectar foraging. By comparison the AHB does not experience a prolonged winter season and therefore does not need to store as much honey. This means that more energy and time can be put into pollen collection needed for the developing brood (Couvillon et al., 2015).

After the introduction and spread of the AHB there was an expectation that this would lead to a collapse of beekeeping across South and Central America. This however did not occur, and not all countries however were impacted to the same extent. For example, while honey production decreased in Mexico in the first 10 years after the introduction of the Africanised honey bee, the reduction was not as great as it was in other Central American countries (Caron, 2001; Guzman-

Novoa, 1996; Hellmich, 1991). Countries which experienced large colony declines include Venezuela, where 10 years after AHB arrival honey production was almost 15,000 tons lower than it was prior to the AHB (Sader, 2020). By 2006, however, honey production exceeded 55,000 tons despite other factors negatively impacting beekeeping, such as the mite *Veroa destructor* (Chihu et al., 1992) and detrimental climatic events including hurricanes, showing a period of recovery (Borst, 2011). Honey production has since stayed consistent (Guzman-Novoa et al., 2020). The initial increase in production was likely due to beekeepers being able to adapt to new management practices suitable for the Africanised honey bee; however, levels did not return to the same highs as pre-Africanisation levels (Guzman-Novoa et al., 2020).

New management strategies included:

- Locating apiaries away from roads, animals and people. Africanised bees are able to pursue people up to 200 m and therefore there is a greater risk of colonies being disturbed by humans and animal activity and then pursuing an attack (Reátegui, 2020).
- Use of abundant smoke. Smoke has been consistently used to calm bee colonies by disrupting pheromone signals (Visscher et al., 1995); however, at least twice the traditional amount is needed to calm Africanised colonies down to a workable level (Reátegui, 2020).
- Thick gloves and bee suits are needed with multiple layers underneath to protect from bee stings (Reátegui, 2020). Beekeepers use white faced veils as Africanised bees typically attack dark colours (De Jong et al., 2007), whereas traditionally black face veils were used to reduce the suns glair.

Despite the introductions of Africanised bees, beekeeping is still of huge importance in the neotropics. Within Latin America (LA) seven countries are in the top 20 leading countries of the world honey market, revealing a critical role of LA in the global honey supply and economical importance of honey production with this region where they produce 14% of global honey production (Requier et al., 2018). Mexico for example, has one of the highest concentrations of managed honey bee colonies worldwide and a great diversity of climate, ecosystem and beekeeping regions (Quezada-Euán, 2007). As of 2010 it had 1.9 million beehives and 45,000 beekeepers (Frausto-Reyes et al., 2017). Mexico is the world's sixth largest honey producer and the third largest honey exporter, making beekeeping greatly important both socially and economically for the country (Programa Nacional para el Control de la Abeja Africana-PNCAA, 2010). It produces a wide variety of honey due to the diversity of climate and flora within the country, the geographical origin of which can be identified though pollen grains (Frausto-Reyes et al., 2017).

In Belize, there is currently a push towards beekeeping to supplement incomes and contribute to a growing economy. For example, in 2019 the Ministry of Agriculture launched a Honey Production Redevelopment Support Project to support farmers ("Ministry of Agriculture launches Honey

Production support project”, 2019). This occurred as the ministry identified beekeeping as a priority area for development due to its high potential for enterprise development and economic opportunities for rural communities. The aims were to increase rural livelihood options through beekeeping, particularly for women and youths, increase the national production and productivity of beekeeping and increase the quality of honey and honey products (“Ministry of Agriculture launches Honey Production support project”, 2019). This initiative was supported by the Inter-American Institute for Cooperation on Agriculture (IICA) (“IICA Supports Local Beekeepers in Belize”, 2019) which provided training including familiarization of honey standards, management practices and group organisation development as well as additional hives enabling apiaries from the Cayo Quality Honey Producers Cooperative to expand by 40%.

After talking to beekeepers in Belize during this study (accompanying materials 2/chapter 4), 9 out of 10 identified income as their primary reason for beekeeping. For some, selling honey was the main source of income and, therefore, extremely important to their livelihoods, whilst for others it was there to supplement their income from farming. A few mentioned that keeping bees also increased pollination and therefore productivity of their farms, specifically their cacao, plumb and coffee trees. A few others mentioned beekeeping for enjoyment although income was still their primary motivation. Interestingly 4 out of 10 beekeepers identified access to materials and equipment, and lack of knowledge as barriers to preventing more people from getting involved in beekeeping and stated that more training would be beneficial. Therefore, investment such as that from the IICA is likely to greatly encourage and allow more people to get involved in beekeeping in Belize. In the Trio community (Toledo district), apiculture has been identified as an important way to complement the sale of cacao beans as well as other traditional crops (Requena et al., 2020). Here, apiculture has also proved to be an important opportunity and source of income for women, allowing them to take a leading role in income-generation for the family, in a traditional Maya community. There is also the opportunity to diversify the products which are harvested, and include, pollen, wax and royal jelly (Requena et al., 2020). In the Yucatán peninsula in Mexico, 95% of its honey production goes to global markets, beekeeping acts as many locals’ only jobs and primary source of income (Güemes-Ricalde et al., 2006). Here, the commercial sector comprises 1,700 beekeepers of largely non-Mayan decent and a further sector of 16,000 loosely organised beekeepers of indigenous Mayan descent (Güemes-Ricalde et al., 2006). This is likely to be the direction beekeeping will be moving towards in Belize and shows how important it can become to local people for both social and economic reasons.

As honey bees carry great importance for people’s livelihoods in Belize it is important that the colonies remain healthy and strong in order to sustain a high level of honey production and income. Stressors such as poor nutrition in N American and European regions, have been shown to result in low honey and wax production, and well as decreased brood rearing and a decreased adult population, which will further reduce colony productivity in the long run (Hoover and Ovinge, 2018). This is true for other stressors such as land use, which has been shown to impact annual survival of commercial honey bees and their productivity, due to a reduction in resource availability (Smart et al., 2016). Stressors such as the Veroa destructor and pesticide application can also cause reductions in honey/income by causing colony collapse (Carreck et al., 2010; Goulson et al., 2015). These stressors also have the potential to be present in tropical countries such as Belize, effecting bee productivity and thus income from beekeeping. Therefore understanding these stressors in a tropical setting is essential.

1.1.3 Impact of Africanised bees on wild bees

The Neotropics are rich in bee fauna with 5000 recognised species, including 33 genera of Meliponini, the stingless bee, however estimations of total species richness are 3 fold greater (Freitas et al., 2009). Obtaining precise species richness figures for the Neotropics is difficult due to misidentifications and many poorly sampled and understudied regions, and thus the information on native bee fauna in Belize, and more widely Central America, is scarce. The apifauna of Mexico and Panama is probably the best studied of the Mesoamerican region (Freitas et al., 2009). The estimations from Mexico are of 8 families, 144 genera and 1800 species with the greatest number of species found in the genera: *Centris, Deltoptila, Exomalopis, Mesoxaca, Peponapis* and *Protoxea* (Ayala, 2006). Limited data could be found in studies investigating bee diversity in northern coastal Belize, with a total of 43 species of bees identified from the tribes, Apini, Ceratinini, Epeolini, Eucerini, Euglossini, Exomalopsini, Meliponini, Osirini and Tapinotasoidini (Schüepp et al., 2012). The greatest number of species were from the tribe Meloponini and the Genus *Triona,* sometimes called stingless honey bees. The second most commonly occurring was *Euglossa* from the tribe Euglossini, also known as the orchid bee (Schüepp et al., 2012).

The interaction of the non-native honey bee with wild bee species is important to consider as competition may occur between these species if there is an overlap of floral and nesting resources (Paini, 2004). It has been reported that competition from managed honey bees may intensify the already present threat to wild bees by depleting common food resources and this impact is likely to be stronger in simplified landscapes where flower-rich habitats have been lost (Thomson, 2016).

Roubik (2009) studied the impact of the AHB on native bees in French Guiana, Panama and Yucatan, Mexico over a 10 - 17 year period. It was found that the AHB did not exert any negative effect on either solitary or highly social wild bees, with relatively steady population dynamics occurring. It was noted, however, that the native bees shifted their foraging time and/or floral species in order to reduce competition with the AHB (Roubik, 2009). Similarly, Roubik and Villanueva-GutiÉrrez (2009) found the presence of the AHB lead to a floral shift by native bees. They found that two major plant families, Anacardiaceae and Eurphorbaceae, were lost due to competing honey bees but were compensated for by greater use of Fabaceae, Rubiaceae and Sapotaceae (Roubik and Villanueva-GutiÉrrez, 2009). This may have long lasting importance as not all plant families offer the same levels of crude protein in their pollen, and thus overall nutrition may be impacted (Forcone et al., 2011). Wilms et al. (1996) suggest that whilst the AHB is the main competitor for many wild bee species in the neotropics, its impact on the native species is buffered by mass-flowering trees that are the most important food plants for the indigenous highly eusocial bees. Similar ideas are suggested by Gilberto et al (2012) who found that the AHB induced significant changes in the structure of native pollination networks by monopolising many interactions. The AHB has a number of advantages which help it to out-compete native stingless bees: the ability to communicate the distance and direction of a food source from the nest; an often larger forager size; and a larger colony size, which provides Africanised bee colonies with a competitive ability superior to that of stingless bees in terms seeking rich, compact resources (Roubik, 1980).

This suggests that whilst the AHB may play an important role in the livelihoods of local people and offer economic opportunities for Belize, there is potential impact on native bee species, likely by altering the floral resources they utilise, although there seems to be limited effect on overall population numbers. When interviewing beekeepers (chapter 4 of this study) in Belize 7 out of 10 stated they had seen a decline in native stingless bees; however, only 2 suggested this was due to the presence of the Africanised honey bee, the others all stated that deforestation is the main cause. It is worth recognising that when trying to preserve honey bee health in Belize to improve and sustain honey productivity and colony strength, that they are an invasive species, which have potential negative effects on native fauna (Elbgami et al., 2014; Paini and Roberts, 2005; Walther-Hellwig et al., 2007). No data is available about the number of unmanaged honey bee colonies in Belize; however anecdotally the majority of beekeepers initially obtained their colonies through the capture of feral honey bees suggesting that honey bees in Belize survive well unmanaged, and may therefore pose a threat to other bee species outside of the managed systems. The survival of unmanaged honey bees in Latin America is further supported by their rapid spread from the point of their release in Brazil to almost all of Latin America in a few decades (Calfee et al., 2020).

1.1.4 Economic, social and environmental background to Belize

Belize is the second smallest country in Central America, with a land area of 22,963 km^2 (Kongsager and Corbera, 2015). Belize is considered to be a small, upper middle-income developing country (“Belize”, 2017), with a GDP of $2.07 Bn, ranking it at 168 in the world out of 211 countries, with a population of 404,914 people (“GDP Ranked by Country”, 2019). According to the 2002 Belize poverty report 25.2% of households in Belize and 33.0% of individuals were considered poor on the basis of their expenditure on food and non-food items, whilst 9.6% of households and 13.4% of individuals were considered extremely poor, meaning they could not meet basic food requirements (“Report 495 Poverty Assessment Report - Belize”, 2002). Unicef identified that 42% of the country were affected by poverty in 2019 (Unicef, 2019). Cayo and Toledo were identified as the poorest districts in the county where 19.7% and 47.2%, respectively, were suffering from extreme poverty, and over half the rural population in these districts were considered poor. The Maya were the most heavily represented in groups of poverty relative to their percentage make up in the population ("Report 495 Poverty Assessment Report - Belize", 2002). The majority of people considered poor depended on the Agriculture and Fishing sector in the Corazol, Orange Walk, Stann Creek and Toledo districts and to a lesser extent in the Cayo district. Those in the Belize district depended on a number of sectors including construction and retail ("Report 495 Poverty Assessment Report - Belize", 2002). This highlights how additional sources of income such as beekeeping may act to improve the livelihoods of local people.

As stated previously the agricultural sector is incredibly important in Belize to sustain livelihoods, particularly in rural regions of the country. It is estimated that there are approximately 10,000 farm holdings of which 75% are small scale farms of 25 acres or less (Holder, n.d.). Many of these farms are mixed, containing both livestock and crops both for subsistence and income generation. Two farming systems are most dominant in Belize: the Milpa or "slash and burn" system which is practised by traditional Mayans; and the Amish small farming system. Both systems are mixed farming and practice low external input agriculture (Holder, n.d.).

Belize is the only country remaining in Central America where there is still over 60% forest cover; however, in recent years deforestation has been increasing, and if the current trend continues by 2045 forest cover will have fallen below 50% (Latha et al., 2016). Land tenure in Belize can be divided into four broad categories: national land (owned by the government); forest reserves (administered by the government); private land and Mayan Reservations (Kongsager and Corbera, 2015). As of 2016 the total national territory that was under some form of protection was 26.22%, of which 9.3% was strictly preserved for conservation purposes (Chicas et al., 2016). The majority of deforestation has occurred outside protected areas, through the conversion of forest to agricultural land and plantations (Kongsager and Corbera, 2015). This has resulted in fragmentation, isolated forest patches, and has led to degradation processes such as soil erosion, and thus has the potential to affect biotic communities in these environments (Vernet, 2016). The current conversion of forest to agriculture across Belize makes it a prime location to study the effects of land use change on bee health as it has created clear gradients between these environments. Additionally, whist over 60% of forest cover is still present in Belize, it is important to identify the value and necessity of these resources to biotic communities, such as for the present bee population.

Agriculture in Belize is focused to the North, along the Belize River Valley in central Belize, as well as towards the South (Fig. 1.1). In the North, agriculture is largely dominated by sugarcane which is exported to the United States and the European Union (Alford et al., 2019). Citrus crops (oranges and grapefruit) and bananas are produced in the central areas, with bananas also produced in the South. Rice is cultivated on large mechanized farms along the Belize River Valley, whilst corn, roots, and vegetables tend to be produced on smaller plots (Alford et al., 2019). Lowland broad-leaved forest dominates the forest habitat occurring in the North and South of the country, whilst submontane broad-leaved forests are located mainly in the Central to South of the country. Savannah habitat runs along the East coast and across Northern regions.

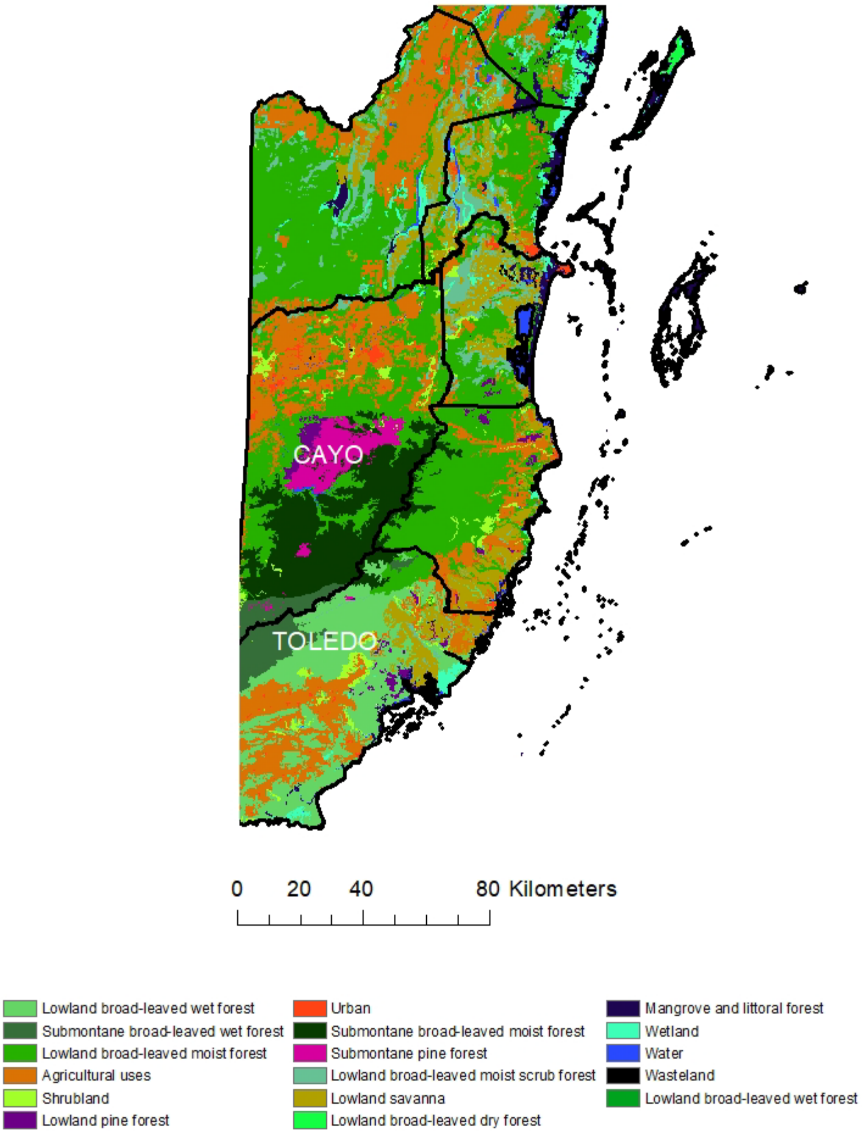


Figure 1.1 Land use in Belize 2017. Ecosystems are based on the 2017 land use map provided by Meerman and Clabaugh (2017).

Whilst the northern districts of Belize are already undergoing, or have undergone, a move to more intensive agriculture, the Maya Golden Landscape (MGL), located in the Toledo district in southern Belize is still mostly forested and has retained a greater amount of mature forest cover in comparison to other areas of Belize (Voight et al., 2019). Historically, the livelihoods of the Rural Maya in Toledo were based on long term fallow subsistence agriculture, with supplementary income from work as labourers and from non-timber forest products (Chicas et al., 2016). In recent years, the MGL has become increasingly threatened by unsustainable land use practices, which have reduced the land's capacity to provide life-supporting ecosystem services. This region of Belize has been traditionally farmed through slash and burn agriculture in which farmers would cultivate a plot until it decreased in productivity when it would be left for 10 to 15 years for the natural vegetation, nutrients and fertility to be restored. More recently, however, this fallow period has been reduced to two or three years due to a shortage of land created by a growing population, and consequently, the soil has not regained its fertility to the same extent as it would have over 15 years. Thus, more land is needed to produce the same crop yield and shorter fallow seasons continue. This feedback loop results in increased deforestation and conversion of land from forest to agriculture (Voight et al., 2019). Other causes of deforestation in the south of Belize include the intensive harvest of rosewood between 2010 and 2012 for international exportation, as well as the construction of the Jalacte highway that connects Belize to Guatemala as it passes through several Maya villages (Chicas et al., 2016). This highway is thought to have increased access to previously inaccessible areas and thus created conditions conductive to deforestation (Chicas et al., 2016).

1.2 Honey bee stressors and their effects

Significant losses of both wild and managed bee species have been reported across the world (Benaets et al., 2017). No one single factor or stressor has been identified to explain these losses, but is instead thought to be multifactorial (Goulson et al., 2015). The factors identified include pesticide exposure (Potts, Biesmeijer, et al., 2010), increased parasitic infection (Cameron et al., 2011), climate change (Ploquin et al., 2013), habitat loss (Santos-Leal, 2006) and decreased floral diversity (Arien et al., 2015). Honey bee colony losses are likely the result of the interaction and synergistic effects of these stressors. These stressors result in both lethal and non-lethal effects; however both mechanisms lead to overall losses in bee populations. Non-lethal effects can include behavioural changes (Colin et al., 2019), impaired cognitive functions (Shepherd et al., 2018), as well as physiological and molecular alterations (Havard et al., 2020; Zanni et al., 2017). So far, many of these stressors have been reported and studied in temperate regions; however there is comparatively little research as to whether the same stressors are relevant in the tropics. This is important as the most relevant stressors are likely to vary between regions due to varying anthropogenic and environmental factors.

It is important also to recognise that colony losses are not only due to stressors within the environment, but also socio-economic reasons such as a reduction in the number of beekeepers. A good example of this was seen with the introduction of the Africanised honey bees across Latin America, which resulted in a reduction in beekeeping due to their aggressive nature, and subsequently a reduction in honey bee colonies as described in section 1.1.3.

The following section aims to explore the literature on these stressors, particularly the effects of pesticides and floral resources, as well as identifying the potential effects they have on both lethality, behaviour and developments changes. This literature will be reviewed in the context of Belize and the tropics, where the pollen and pesticide experiments were carried out for this thesis.

1.2.1 Impact of deforestation and fragmentation on bees

The tropics are home to a range of faunal and floral diversity and encompass much of the worlds biodiversity as well as agricultural products such as coffee, cacao, and rice (Jha and Vandermeer, 2010). Much of the tropics, however, exist as a mosaic of forest and agricultural patches which are becoming increasingly isolated as deforestation and agricultural intensification proceeds (Norris, 2016). Species in these landscapes vary in their susceptibility to these processes, with those that are specialised, more limited in dispersal and have small ranges, being more greatly affected, with the potential of population decline and local extinctions (Norris, 2016).

Deforestation and fragmentation have been shown to dramatically reduce the abundance and richness of wild bee species. For example in Yucatan, Mexico, a significantly higher number of stingless bee nests were found in undisturbed areas compared to those where agriculture and ranching had resulted in mass deforestation (Santos-Leal, 2006). Declines of both solitary and social bees have also been noted in Tucuman, Argentina, due to deforestation in order for the expansion of crop fields for soybean (Zak et al., 2004). In Ceará, Brazil, *Melipona quinquefasciata*, a ground-nesting stingless bee, was classified as endangered due to agricultural expansion and firewood gathering, resulting in deforestation of the landscape, reducing nesting opportunities as well as reducing floral host plants (Lima-Verde and Freitas, 2005).

Fragmentation can cause changes in community composition. The conditions of a small fragmented area are likely to be more suitable for generalist bee species, due to their ability to make use of a variety of resources, as well as species with a high dispersal capacity, which are able to move between forest patches (Steffan-Dewenter, 2003). Social bees may be better adapted for intensely managed agricultural areas as sociality may be able to buffer the negative impacts of fragmentation (Steffan-Dewenter et al., 2002). In contrast, however, many studies have found more severe impacts of deforestation and agriculture on social bees (Klein et al., 2003). One study carried out in Indonesia found that the diversity of social bees decreased with increased distance to forest, whereas the diversity of solitary bees increased with light intensity and flowering plants (Klein et al., 2003). The higher densities of social bees near the forest was suspected to be due to a greater number of nesting sites for stingless and honey bees. Solitary bees however preferred less shaded and humid environments, where there are greater opportunities for ground-nesting species (Klein et al., 2003).

Alternatively, studies from temperate regions have found that social bees are less impacted by forest fragmentation than solitary bees (Steffan-Dewenter et al., 2002) and this highlights why it is so important that studies on deforestation and land use change are carried out not just in temperate regions, as the effects on species in different environments may not be the same. It is possible that temperature and humidity as well the plant and animal taxa present, interact with deforestation and agricultural intensification differently in tropical environments compared to temperate regions.

1.2.1.1 The impact of deforestation and fragmentation in the tropics

The impact of deforestation and fragmentation on honey bees in the tropics is limited with much of the literature focusing on impacts on wild bee species. Brown et al. (2016) investigated the presence and absence of the AHB across landscape gradients in Rondonia, Brazil. Data were collected from 187 sites across Rononia, these sites were either within forests or outside of forests. It was found that the AHB preferred to forage outside rather than inside the forest, although they were present in both landscapes (Brown et al., 2016). In addition, they were most prevalent in recently deforested areas, which the study suggested was due to reduced competition with stingless bee species as well as an abundance of nesting sites present at ground level. Alternatively a study carried out in Indonesia found that social bees including the honey bee decreased with land use intensity (Klein et al., 2003).

Although not focusing only on honey bees, Brosi et al (2007) studied the effect of distance to forest, tree management and floral resources on both social and solitary bee communities in pastures in Costa Rica over a two-year period and compared the effects between the social and solitary species. Distance to forest was found to impact both solitary and social bee communities. At the forest edges, around 50% of the sampled bees were native social stingless bees and honey bees only made up 5% of the community. With greater distances from forests the proportion of stingless bees dropped to 20%, whilst the number of honey bees increased to 45%. This, therefore, suggests that honey bee survival and growth rate is better in areas of low forest and higher amounts of agriculture area (Brosi et al., 2008). This is supported by another study in Costa Rica that showed that wild bee richness, visitation rate, and pollen deposition rate were all significantly higher at coffee plantations when surrounded by forest fragments closer than 100m. The abundances of honey bees, however, did not decline with distance from forest fragements, suggesting that honey bees are less effected by a reduction in forest availability than other bee species (Ricketts, 2004). These studies contrast with those by Basu et al., 2016 and Klein et al., 2003 who found that in the tropics social bees, such as the honey bee, are found in greater abundances in forest environments as opposed to open, highlighting inconsistencies within the literature.

It is worth considering that as honey bees are primarily a managed species, and so there abundances in the environment will also be determined by the location of the beekeepers and thus the positioning of their hives. This relationship between the forest and the percentage of honey bees in the community may therefore actually be to do with where the beekeepers are placing the hives, and less to do with the suitability of the environment for the bees. This is an important distinction to make between honey bees and wild be species; honey bees will be impacted by human choice, such as the location of the hives, whilst wild bee species will be impacted only by the environment. This difference is highlighted particularly by nesting resources, which will be important to wild bee species but not to honey bees.

Deforestation and habitat fragmentation have been shown to effect the community compositions of bees, both in the tropics and temperate regions (Basu et al., 2016; Carrié et al., 2017; Klein et al., 2003; Santos-Leal, 2006; Zak et al., 2004), due to changing nesting habitats as well as floral resources and pesticide presence (Brosi et al., 2008; Dormann et al., 2007). Whether either solitary or social bees are negatively or positively impacted by this deforestation appears to be influenced by local factors as well as the geographic location, temperate or tropical due to the variation of effects noted by the literature. Whilst the literature on wild bees in the tropics is useful due to the limited amount available on the honey bee specifically it is important to consider that managed bee species may be effected differently to wild bee species due to the presence a nesting sites created by artificial bee boxes placed by beekeepers.

The papers discussed above have all focused on the effects seen on bees through changes in abundance and species richness and have connected these to changes in the landscape. The fitness of bees could, however, be impacted without noticeable changes in abundance or species richness, for example, foraging frequency, reproductive rates or honey production may decrease, as well as possible negative effects on bee cognition. Furthermore, this may impact the success of beekeeping, with reduced honey yields and an increased chance of abscondment, and thus lower income. It is important to analyse the effects of a changing landscape in order to assess which components of land use change are effecting bee species. Examples include a change in floral resources, nesting sites, pesticides presence and there interactions, as well as investigating the wider picture of land use change on bee fitness. The following sections will provide more detail on the effects of land use change in terms of diet and pesticide presence on bee species both in tropical and temperate systems.

1.2.2 Nutritional stress and the importance of diet

1.2.2.1 A background to honey bee nutrition

Nutritional stress caused by habitat loss has been identified as one of the factors leading to honey bee colony collapse, and is thought to interact synergistically with other stressors impacting the immunocompetence of individuals, their death rates, and colony development (Naug, 2009).

Honey bees forage on flowering plants as their primary source of nutrition (Donkersley et al., 2017), from which they acquire amino acids, vitamins, proteins and carbohydrates from the available pollen and nectar (Morgano et al., 2012). Bees will, therefore, experience nutritional stress when their choice of host plants is limited or only sub-optimal plants are available (Vaudo et al., 2015). It is important to consider that while there may be flowering plants in the surrounding landscapes not all plants provide the same quantities and qualities of these nutrients. Malnutrition of pollen in bees has been shown to lead to smaller hypopharyngeal glands (HPGs) (a source of queen and worker jelly) (DeGrandi-Hoffman et al., 2010), as well as increased susceptibility to deformed wing virus (DeGrandi-Hoffman et al., 2010), parasitism, particularly by the microsporidium *Nosema ceranae* (Belzunces et al., 2013), and vulnerability to pesticides (Wahl and Ulm, 1983), in addition to suffering a compromised immune system (Alaux et al., 2010) and a shorter life span (Wang et al., 2014).

Pollen is an essential source of amino acids and protein. For honey bees 10 amino acids have been identified as being essential and include arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, and valine (De Groot, 1953). It has been recommended that crude protein levels are kept above 25% in order to satisfy the requirement of a colony for brood rearing, and those under 20% cannot meet the requirements needed (Somerville and Nicol, 2006). Forcone et al. (2011) suggested that between 23% and 30% is the optimum crude protein content for bees to successfully rear brood.

The nutritional requirements of bees vary with life stage. Larvae, for example, depend highly on protein availability from pollen, and begin consuming pollen just a few hours after emerging, and continue to rely heavily on this diet during their first two weeks to sustain normal growth and development (Li et al., 2012). Adult honey bees require greater amounts of carbohydrates and less protein (Donkersley et al., 2014). For the honey bee, bumblebee, and sweat bee, protein concentration in pollen is positively correlated with larval development and adult reproduction, and abundances and reproductive output often correlate with floral abundances (Crone, 2013). Other essential materials gained from pollen include lipids and linoleic acid which are required for egg production, wax production and a higher reproductive output (Avni et al., 2014). For a colony to be successful, adult bees must collect sufficient protein stores to allow for the next generation to be healthy and of sufficient size.

1.2.2.2 The importance of amino acids, crude protein and lipid availability

Different amino acids are required for different roles. Lysine, for example, plays a very important role in honey bee physiology, where fortification of pollen substitute with DL lysine and methionine has been shown to significantly raise the size of the fat body of the bee (Ghosh et al., 2020). In addition, the highest counts of haemocytes in the haemolymph were recorded for bees which received pollen substitute supplemented with L isomers of methionine, lysine, histidine and threonine. Arginine supplements have been shown to enhance the immune response. Non-essential amino acids are also important. Glutamic acid and proline are often found in high concentrations, and proline is used as fuel for flight in insects including honey bees and also helps in increasing cold hardiness (Ghosh et al., 2020).

Amino acids, crude protein levels and lipid availability has been shown to effect bee fitness through numerous mechanisms. For example, HPG development in honey bees is influenced by the quantity and quality of protein ingested, and its size is related to their total protein content (Brouwers, 1982). The HPG is of importance as it is responsible for the for royal jelly production, this can in turn impact growth of larvae and future queens (Omar et al., 2017). For the most optimal development of the HPG, newly emerged bees must be fed on protein food (Crailsheim, 1990). In support of this Pernal & Currie (2000) found that development of the HPG was strongly correlated with the amount of protein workers consumed from pollen diets, after newly emerged honey bees were fed either a sucrose based diet, or one of 6 pollen diets ranging in protein content.

Protein rich diets have been found to result in high expression levels of vitellogenin and transferrin (Bitondi and Simões, 1996; Di Pasquale et al., 2013). This is to be expected as both vitellogenin and transferrin are expressed in the fat bodies, and pollen is known to promote their development (Alaux et al., 2010). It is important that vitellogenin levels remain high as this protein is recognized as one of the most important regulators of immunity and longevity of honey bees (Amdam et al., 2004). There have, however, been cases where vitellogenin levels have been greater in lower protein diets compared to higher protein diets, and this is thought to be due to levels of other nutritional factors such as lipid concentration, which can cause an increase in vitellogenin (Di Pasquale et al., 2013). Another study found that when honey bees were fed a high protein content diet there was an increase in vitellogenin in the haemolymph of the bees (Basualdo et al., 2013). Moreover, hexamerin (Hex 70 a) bands on polyacrylamide gels were very dense, suggesting accumulation of hexamerins were associated with diet. This is also supported by Martins et al. (2008) who found that transcription of HEX 70 was associated with the diet in young workers. While the function of the protein HEX 70 is unknown it is thought to be an additional source of amino acids (Basualdo et al., 2013).

A greater head and thorax weight has also been seen in bees fed supplementary amino acids in comparison to controls (Hendriksma et al., 2019). Here it was suggested that higher head and thorax weights may reflect increased hypopharyngeal glandular development in nurse bees and greater flight muscle mass in foragers bees. In terms of colony health, it was found that a 17% increase in nurse bee head size resulted in 100% more capped brood cells (Hendriksma et al., 2019).

When honey bees were fed a variety of pollen diets containing different concentrations of crude protein, at 15%, 20%, 25%, 30%, and 35%, egg hatch, pupation success and pupal weight were affected (Li et al., 2012). It was found that a diet of 30% crude protein was of sufficient quality to allow for maximum body weight, while a protein content of 35% exerted the greatest effect on increasing the percentage of hatch and percentage of pupation (Li et al., 2012).

A protein based diet has also been considered to be important in venom production, where it was found that bees fed with only sugar solution produced only 25% of the venom produced on a normal diet (Lauter and Vrla, 1939). The effects of different levels of crude protein found in pollen on venom production, however, have not been compared, but could potentially be used as a means to evaluate the nutrient value of a given pollen diet (Omar et al., 2017).

Learning and memory in honey bees has also been shown to be impacted in *Nosema* infected bees not fed on a pollen diet. These bees have been shown to have a reduced ability to learn to associate a positive stimulus (sugar solution) with an odour by extending their proboscis, a method known as the proboscis extension response (PER) (Gage et al., 2020) (more details of honey bee cognition and PER can be found in chapter 1.3). Another study examining learning and memory in honey bee workers found no long term effects of pollen stress. Three treatments were tested, including pollen rich, pollen poor or an intermediate level. PER was used to test if bees learnt the association between an odour and a reward (Mattila and Smith, 2008). It was found that colony level nutritional status had no effect on worker bee learning or memory, likely because colonies used brood-rearing capacity to buffer nutrient stress at the individual level.

Protein and amino acid content are not the only elements of pollen which contribute to honey bee health as lipids also play a key role, however they tend to be discussed less in the literature. Honey bees obtain lipids largely from pollen (Brodschneider and Crailsheim, 2010) and are important not only as a source of energy, but also contribute to the synthesis of fat reserves, glycogen, and the membrane structure of cells (Manning, 2001). Lipid components such as fatty acids and sterols have also been shown to be important in honey bee development and reproduction. They are primarily metabolized during the brood stage of development and are an important precursor for further biosynthesis (Cantrill et al., 1981). The fatty acid content of pollen has been shown to range between 0.8 and 18.9% depending on the plant species (Brodschneider and Crailsheim, 2010).

Oleic and palmitic fatty acids have been shown to dominate the body fats of both adult worker bees (60%), larvae (40%) and queen pupae (58%) (Robinson and Nation, 1970). The importance of lipids to honey bee diet was demonstrated by Herbert et al. (1980) where bees were fed a pollen substitute with either a 0%, 2%, 4%, 6% or 8% lipid concentration. Honey bees fed on a 0% lipid diet reared significantly less brood than those fed with a lipid presence. However, no difference was found between 2%, 4%, 6% and 8% levels. In a later study, eucalypts pollen which contains low concentrations of lipids, were enhanced with fatty acids, and it was found that bees fed the enhanced pollen experienced increased longevity compared to control bees (Manning et al., 2007). These effects were greater when pollen diets were enhanced with oleic acids as opposed to linoleic acids; however only up to a 6% enrichment. When pollens were enriched with more than 6% oleic acids mortality rates increased (Manning et al., 2007). In addition, Manning (2006) found that when the fatty acids, oleic and linoleic, were added to pollen at concentrations greater than 2%, the longevity of bees decreased and the queen failed to lay eggs. Taken together these results suggest that while lipids are a very important component of the honey bee diet; too high concentrations are detrimental to the health of the individuals and thus colony.

Arien et al. (2018) showed that the lipid omega 3 (alpha-linolenic acid) was an important component in the diet of bees. The effect of the absolute amount of omega-3 in the diet of honey bees as well as the omega-6:3 ratio was compared in terms of associative learning. The concentration and ratios between these two fatty acids is known to vary between pollen species (Manning, 2001). The PER was used to test a bees' ability to learn to associate an odour with a sugar regard. Results showed that at the greatest omega-6:3 ratios, honey bee olfactory learning was greatly reduced regardless of the absolute amount of omega-3 in the diet, whilst when the omega - 6:3 ratios were at their lowest honey bee performance was significantly better. It was concluded that learning is best when the diet is sufficiently rich in fatty acids, as long as the omega-6:3 ratio is not high (Arien et al., 2018).

Lipids are also thought to be important when it comes to pollen choice (Ruedenauer et al., 2021; Vaudo et al., 2020). Studies have shown that chemicals attractive to honey bees reside in the lipid fraction of the surface of pollen grains, also known as the pollenkitt (an oily sticky substance that coats the pollen grains) (Dobson 1988). Dobson. (1988) investigated the chemistry of the pollenkitt to understand its role in producing olfactory cues to pollinators. It was found that pollenkitt lipid compositions vary from species to species and that they almost all belong to neutral classes (low polarity). Patterns in these neural lipid compositions were suggested to provide pollen with species specific odours. Dobson's. (1988) study, however made no direct comparison between the composition of the pollenkitt and the pollen's attractiveness to bees.

1.2.2.3 Amino acids in pollen families

Amino acids have been shown to be largely conserved within plant families. A study by Weiner et al. (2010) found that closely related species only differed slightly in their proportions of amino acids suggesting that the profiles are a highly conserved trait, whilst differences across families were more significant. They also found that whilst most plant species contained a full spectrum of essential amino acids, some were available in small quantities (Weiner et al., 2010). One study compared amino acids profiles between 8 interrelated families of angiosperms, the Acanthaceae, Bignoniaceae, Boraginaceae, Convolvulaceae, Labiatae, Scrophulariaceae, Solanaceae and Verbenaceae (Mondal et al., 2009). A considerable amount of homology was found between species belong to the same family, although homology was more pronounced at the genus level. A study focusing solely on the Asteraceae family found a degree of homology between 9 members of the family, whereby amino-n-butyric acid, aspartic acid and proline were present in almost all the taxa, and Tyrosine in all but 3. Proline was abundantly present in all the investigated samples constituting between 0.085 and 0.420 mol/mg dry weight. Some variation was seen in the presence of leucine, histidine, arginine and trace levels of glycine (Mondal et al., 1998). An earlier study did not detect any qualitative differences in the free amino acids extracted from pollen of 11 members of Asteraceae (Shellard and Jolliffe, 1968). Somerville & Nicol (2006) support the idea that pollens collected from species of the same genus demonstrate similar amino acid and protein profiles, and suggested that isoleucine is the most frequent limiting amino acid, next to valine and methionine (Somerville and Nicol, 2006). Fabaceae is an example of a family with relatively high protein content and contains all essential amino acids (Haider et al., 2014).

Asteraceae have consistently been shown to be a poor pollen type due to its low protein quality (Nicolson and Human, 2013). One study by Frias et al. (2016) used eight different pollen diet mixes consisting of three to five familial pollen types (8.4% - 18.1% protein) to feed to worker bees. They found that mixtures containing largely Asteraceae pollen led to increased bee mortality. Moreover, the mean survival rate was positively related to the mean amount of protein consumed per bee and negatively related to the amount of Asteraceae in the mixture (Frias et al., 2016). This was thought to be due to two reasons: firstly protein levels of Asteraceae are usually low compared to other familial pollen types (Roulston et al., 2000); secondly, Asteraceae (sunflower and dandelion) have also been shown to lack essential amino acids (Loper and Cohen, 1987; Nicolson and Human, 2013), as well leading to low rates of digestion in honey bees (Peng et al., 1985; Roulston and Cane, 2000).

1.2.2.4 Crude proteins in plant families

Crude protein levels have been shown to vary substantially across different families, ranging from 2.5% to 61% (Roulston et al., 2000). Three hundred and seventy seven plant species from 96 families were compared by Roulston et al. (2000), who found that pollen protein concentrations were highly conserved within plant genera and families. Roulston et al. (2000) also found that crude protein variation within families did not necessary correspond with floral variation. The Solanaceae family, for example, have uniformly protein rich pollen but flowers which ranged from small bee pollinated flowers to much larger bat pollinated flowers. Species shown to have particularly low levels of crude protein are *Zea* (maize, Poaceae) and *Helianthus* (sunflower, Asteraceae), whilst other crop species such as *Senna* (Fabaceae) and *Solanum* from the Solanaceae family contain much higher levels (Pamminger et al., 2019).

Both amino acids and crude protein content are used throughout the literature as indicators of diet nutrition, however, there appears to be some discrepancy to which indicator is best. Nicolson (2011) argues that amino acid composition determines the amount of pollen required by bees, more than the protein content. This is supported by Weiner et al. (2009) who argue that crude protein content may not adequately reflect the availability and composition of amino acids, as two diets containing the same protein content may differ due to an imbalance of essential amino acids (Weiner et al., 2010). Whilst most pollen contain the majority of essential amino acids particularly leucine, lysine and arginine, pollen species sometimes lack phenylalanine, tryptophan, highlighting the need for amino acid levels to be quantified (Roulston and Cane, 2000)

The protein content of pollen varies widely and is generally accepted as a measure of its nutritional value for bees (Pernal and Currie, 2000; Pirk et al., 2010; Roulston and Cane, 2000; Somerville and Nicol, 2006). One argument for using crude protein over amino acid levels is that amino acid profiles between plants are highly conserved and thus few qualitative differences are seen between plant species (Keller et al., 2005; Vanderplanck et al., 2017). On the other hand protein concentrations in pollen has been shown to vary considerably between species of different families with values ranging from 2.3% to 61.7%. This is supported by Vanderplanck et al. (2014) who found that the proportions of free and bound protein were variable amongst different diets, but the proportion of essential amino acids was highly conserved. Leonhardt & Blüthgen (2012) similarly found that while protein levels and amino acid compositions from different plant taxa varied considerably, they were qualitatively similar with regards to amino acids essential for honey bees. Radmacher & Strohm (2010) found pollen protein concentration to be one of the most important factors in influencing larval growth and body size in bees, and noted that it is highly conserved within families (referencing back to Roulston et al. 2000). They stated that determination of pollen to plant family is sufficient to investigate the effects of pollen diversity on larval performance. Pernal and Currie

(2000), likewise, suggested that crude protein content could be used as a general guideline for evaluating pollen quality.

To summarise for the most part crude proteins appear to be more important than amino acid levels as there is greater variance between families, whereas the majority of pollens contain sufficient levels of essential amino acids for bees. Where essential amino acids are missing or are in low concentrations, however, this becomes more important than total crude protein level. A pollen with high crude protein levels but missing an essential amino acid will be a lower quality pollen than one with lower crude protein levels but contains all essential amino acids.

1.2.2.5 Seasonal differences in pollen quality and bee fitness

One way in which the effects of pollen quality on bee fitness and development have been studied, is by comparing pollen mixes across different seasons. For example, one study found that spring and autumn pollens were similar in total protein and lipid concentrations, however, spring pollen had a higher concentration of essential amino and fatty acids, supporting HPG growth and brood production (DeGrandi-Hoffman et al., 2018). These diets were then fed to spring bees either infected or not infected with *Nosema*. They found that the HPG of both infected and non-infected bees was larger after being fed the spring diet. Spring bees differentially regulated more than 200 genes when fed in- *versus* out-of-season pollen. This increased to around 400 genes after they were infected with *Nosema*, showing that infection-induced gene expression varies with pollen type, and that pollen containing a higher concentration of essential amino acids allows for a stronger reaction to infection. HPG size in autumn bees was not effected by pollen type. Taken together this suggests that the physiological responses to seasonal pollens differs between bees reared in the spring and those in the autumn, and that spring bees gene expression is sensitive to pollen type especially when infected with *Nosema* (DeGrandi-Hoffman et al., 2018). Seasonal differences of crude protein content in pollen grains have also been shown by Liolios et al. (2015). They found that pollen collected from plants blooming in the spring had higher protein content (20%-24.7%) than those from summer (15.5%-19.9%) and autumn blooms (19.3%-23.1%) (Liolios et al., 2015). This further develops the idea that different mixes of pollen taxa can result in different nutritional values. Radev. (2018) found similar results where pollen blooming in the spring had a higher protein content (21.1%-27.8%) than those from summer (13.9%-23.5%) and autumn (15.1%-25.1%). Radev. (2018) suggested that this variation of crude protein content with season is the reason for the presence of strong brood and population growth during spring, and a reduction after. The study itself, however, did not directly investigate how these seasonal fluctuations in pollen level affects development.

1.2.2.6 Mono - and poly-floral diets

Not all studies have focused on the crude protein or essential amino acids contents of pollen diets, and instead a comparison was made between monofloral and polyfloral diets (Alaux et al., 2010; Di Pasquale et al., 2013). The assumption here is that polyfloral diets are likely to contain a greater range of amino acids, lipids and protein contents than a monofloral one. This is particularly true if the pollen type relied on in a monofloral diet is lacking in any essential amino acids or have a particularly low crude protein content (Alaux et al., 2010).

Alaux et al. (2010) exposed honey bees to a monofloral pollen diet, representative of what they might find in agriculturally intensive areas, whilst the second group of honey bees was exposed to a polyfloral pollen diet. The authors found that diet diversity increased the levels of baseline immuno-competence, particularly by increasing glucose oxidase levels, a proxy of social immunity. This was true even when the monofloral pollen diet contained high protein levels (Alaux et al., 2010), suggesting that the overall health of a hive can be improved by a varied diet. This has been supported by other studies where it has been shown that nurse bees fed on a polyfloral diet lived longer when infected by *Nosema caranae* than bees fed on a monofloral diet (Belzunces et al., 2013).

In Belize it is expected that more diverse landscapes will provide a more diverse pollen diet. It is unlikely that the honey bees in Belize will experience the extremity of a pure monofloral diet in any location as the areas of natural vegetation have a high floral diversity and the majority of the agricultural areas are of mixed farming practices at small or medium scale. However a mixed landscape will have floral contribution from the agricultural, the disturbed natural habitat and forest the plant communities. Hence a higher landscape diversity is expected to lead to a higher pollen diet diversity.

1.2.2.7 Honey bee pollen preferences

The studies discussed so far all point towards a benefit of a high protein pollen diet; however honey bee preferences for this diet has yet to be clearly demonstrated (Vaudo et al., 2015). Studies carried out in Western Australia for example, suggest bees do not have a preference for high protein or amino acid content and instead are more attracted to local plants in high abundances (Chemaly, 2002). Pernal & Currie (2001) argued that bees will increase the gross amount of pollen returned to the colony when protein levels are low, rather than specialising in collecting pollen with higher protein contents (Pernal and Currie, 2001). Corby-Harris et al. (2018) found that protein levels effect the development of the HPG, and that nurse honey bees were be unable to differentiate between pollen of high protein and that of low protein (Corby-Harris et al., 2018). Similar results were observed by Basualdo et al. (2013) who found that bees fed on a low protein content pollen did not increase pollen consumption to match those fed a high protein pollen diet. This is important as it suggests that the plant species immediately surrounding the hives are very important to the bee pollen diet, as bees are unlikely to discriminate or search for pollen with a higher protein content.

Alternatively, Requier et al. (2015) found that in an area of over 75% arable land in France, pollen was primarily collected from a wide diversity of herbaceous and woody plants in semi-natural habitats or from weeds, even when crop abundance was high. Results showed that rapeseed pollen was avoided due to a preference for higher nutritional value found in wild species (Requier et al., 2015), highlighting the importance of wildflowers in bee foraging. Additionally, there is some evidence that honey bees may identify pollens based on the amino acid content (Cook et al., 2003). For example, in a choice between oilseed rape pollen and field bean pollen, after previous exposure, bees would choose oilseed rape, however before exposure there was no preference (Cook et al., 2003). After the amino acid quantities were measured for each pollen species, oilseed rape was found to contain a greater amount of the most essential amino acids, valine, leucine, and isoleucine. This suggests that bees are able to somehow detect amino acid levels in pollen, and then make a choice to consume the one with more favourable amino acids.

Evidence has also been found to suggest that bumblebees will collect pollen that is high in essential amino acids and sterol content. Somme et al. (2015) compared the floral resource choice of four different bumble bee species occurring in Belgium. They found that the plant species offering the pollen of the highest chemical value (amino acid and phytosterol content) were the most dominant in the diet of the four sampled bee species (Somme et al., 2015). As some of the plants were also highly dominant in the environment, it is also possible that they were chosen for reasons of abundance, in a future study controlling the abundance of plants in the landscape would help to account for this.

Previous studies have suggested that bees will forage greater distances in order to achieve a more diverse pollen diet. Danner et al. (2014), for example, found that whilst there was a sufficient supply of maize close to hives, bees undertook longer foraging distances to collect diverse pollen types, despite this using more energy, showing a desire for a mixed diet (Danner et al., 2014). When a flowering field was available for foraging there was an extremely low proportion of maize foragers despite its close proximity to the hives, suggesting a preference for wildflowers. Similar results have been seen in additional studies foraging bees flew further in simple landscapes than in complex ones and that this was more pronounced when resources were scarce (Steffan-Dewenter and Kuhn, 2003).

1.2.2.8 Honey bee diet and landscape

The floral resources within a landscape are heterogeneous in space and time, and thus the resources bees have access to will vary over the year (Ogilvie and Forrest, 2017). A complex landscape is much more likely to ensure a continuous source of pollen and nectar than a simple one, where there may be huge quantities in some seasons and low levels in others. Complex landscapes enhance resource diversity available to its inhabitants (Donkersley et al., 2017) and thus the proteins and amino acids available.

Landscape scale studies have been carried out to investigate how honey bee diet is impacted by the surrounding environment, and some have then analysed how this translates to bee fitness. Donkersley et al. (2014) demonstrated that honey bee nutrition is influenced by the surrounding landscape composition by collecting beebread from hives across North West England between April and September. They found that landscape composition was important in determining nutritional composition, where protein content in the pollen was negatively correlated with the amounts of arable and horticultural land around the beehives. Their study used data from the Countryside Survey 2007 land cover map as a proxy for the composition of floral resources, however, it would have been more beneficial if the floral diversity in the surrounding landscapes has been identified in the field. Additionally, the pollen collected was not identified, and thus it cannot be confirmed where the bees were foraging. Kämper et al. (2016) however, when studying the buff-tailed bumble bee *Bombus terrestris* found that the pollen collected varied little in relation to the surrounding landscape. Early colony growth, however, was positively correlated with the amount of woody pollen and protein content but not pollen diversity (Kämper et al., 2016). Williams et al. (2012) investigated how the spatiotemporal variation in resource availability caused by landscape gradients effects the population of bumble bees in Northern California (Williams et al., 2012). Bumble bee workers, male drones, and queen offspring number were compared across a gradient of agricultural landscapes with varying floral resource availability. This was done by quantifying flower densities in all land use types during different seasons. The data was then used to produce estimates of floral resources surrounding each colony. Results show that floral resources do not significantly affect queen production; however, the number of workers and males produced did vary, suggesting that colony growth could be effected. No landscape produced high levels of floral resources during all seasons studied. Williams et al. (2012) did not, however, investigate bee bread or the bee pollen loads so that the exact diet of each hive, as well as the distance travelled to achieve a particular diet cannot be compared. Thus, while floral diversity effects the development of bees the exact reason for this is unclear.

One study which was carried out in the tropics, specifically Papua New Guinea studied the pollen found in bee bread samples collected from hives surrounded by varying percentages of forest

(Cannizzaro et al., 2022). They found that bee bread from hives surrounded by a greater percentage of forest contained greater pollen diversity. Additionally, tree species made up the majority of the bee bread regardless of landscape, making up 52% of the abundance. Bees were shown to seek out trees even when they were scarce within the landscape. Diversity of landscape however was not taken into consideration, and only investigated forest vs non-forest landscapes.

1.2.3 The impact of pesticides on honey bees

One of the main drivers thought to be contributing to the losses of bees worldwide is exposure to pesticides (Potts, Biesmeijer, et al., 2010). The effects of a number of active substances and formulations on the honey bee are well known, although in recent years much of this attention has been drawn towards the effects of neonicotinoids (Lundin et al., 2015; Simon-Delso et al., 2015). Neonicotinoids have rapidly become one of the most popular classes of insecticides due to their wide application span and efficiency. Since their extensive use in the 1990s beekeepers worldwide have reported losses of honey bee populations, with the first report originating in France (Pham-Delègue, 2001).

1.2.3.1 Use and effects of Neonicotinoids

Neonicotinoids are used in more than 120 countries with around 140 different crop uses (Jeschke et al., 2011). Neonicotinoids are taken up by plant tissues with the aim of making plants resistant to a wide variety of pests. Studies have shown that they are not just toxic to the target pests but to other non-target organisms such as bees, even in very small quantities. Neonicotinoids have the potential to be translocated into pollen and nectar, the main constituents of a bee's diet. In the EU there has consequently been a restriction on the use of three of the most common neonicotinoids, clothianidin, thiamethoxam and imidacloprid (*EU. Regulation (EU) No 485/2013*, 2013), although this restriction was not followed worldwide, and even within the EU pesticide residues are likely to remain in the environment.

Neonicotinoids, as well as other pesticide types, can have detrimental effects on bee colonies at lethal and sub-lethal levels. Lethal effects are seen as an increased mortality rate in bees exposed in comparison to unexposed controls. A sub-lethal effect can manifest itself in a number of ways, including growth rates (Whitehorn et al., 2012), fecundity (Wu-Smart and Spivak, 2016) and behaviour (Tison et al., 2016). An example of a sub-lethal effect is shown be Faucon et al. (2005) who found that when honey bee hives were fed with different concentrations of imidacloprid in syrup solution, colonies exposed to high concentrations suffered reduced pollen carrying during the feeding period. These effects, however, stopped as soon as imidacloprid was removed from their diet (Mathieu et al., 2004).

Honey bees have been shown to be negatively impacted by pesticides in numerous ways in both laboratory and field studies, although the majority of studies have been carried out in the laboratory. The effects shown on honey bees include decreased longevity and survival (Cresswell et al., 2012; Wu et al., 2011), reduced cognitive abilities such as learning and memory (Ciarlo et al., 2012; Farooqui, 2013), and decreased immune responses or resistance to parasitic agents (Pettis et al., 2012; Retschnig et al., 2014). In addition, some semi-field studies have shown that reproductive rates may be negatively impacted after pesticide exposure (Thompson et al., 2014) and that foraging ability may be negatively affected (Henry et al., 2012; Schneider et al., 2012). While insecticides have been the most prominently studied pesticide, experiments have also shown that fungicides also negatively effect honey bee colonies (Dahlgren et al., 2013; Mullin et al., 2010).

The majority of research analysing the effects of pesticides on bees has been carried out in France, the United States, the UK and Italy (Lundin et al., 2015). As of 2015 only 2 studies had been carried out in Central America, and 17 in South America (14 in Brazil), while 82% (180 studies in total) of studies were carried out in the USA, Canada or Europe and 9% in Asia (Lundin et al., 2015). This again suggests that there is a research gap in the tropics, and particularly in Belize and neighbouring countries. This is important because although the geographical location of studies carried out on neonicotinoids is heavily skewed to just a few countries in Europe and North America, neonicotinoids are used worldwide in over 120 countries. This is particularly significant as in general, areas outside of the USA, Canada and the EU are less regulated but also host the majority of the global crop pollination value, where 10% is thought to come from Central and South America (Gallai et al., 2009).

One way in which the impact of neonicotinoids can be measured is by studying their effects on the learning and memory of bees. The cognitive abilities of bees are incredibly important as eusocial animals, in order for a colony to be successful bees need to be able to learn, remember, and communicate successful foraging locations, as well as areas representing dangers such as toxins or predators. Previous lab studies have indicated that exposure to chemicals such as pesticides or diesel fumes results in reduced learning (Frost et al., 2013; Siviter et al., 2018; Williamson and Wright, 2013). Siviter et al (2018) carried out a meta-analysis on 104 studies investigating the effect of pesticides on learning and 167 on memory were compared. They detected significant effects of pesticides on learning and memory under both chronic and acute application, and for both neonicotinoid and non-neonicotinoid pesticide groups. The majority of studies investigating cognition in bees have been carried out in the laboratory and have been based on the PER. One problem here, however, is that doses tend to be administered at higher levels than are most likely found in the environment (Frost et al., 2013), over a more concentrated period of time, often in isolation from other stressors. These tests, therefore, may not give realistic expectations of the effects observed in the field (Siviter et al., 2018).

It would be beneficial to understanding how pesticides affect bees if future studies were carried out at field realistic levels. Adaptations have recently been made to the PER protocol to make it more suitable for field environments. For example, the Free Moving Proboscis Extension Response has been developed as a field-based behavioural assay (Muth et al., 2018). Here bees are transferred to plastic cylindrical tubes with a transparent disk at one end with two 3 mm holes. Different coloured paper is then dipped into either positive or negative reinforcing stimuli and bees are tested to determine whether they learn to associate a positive or negative stimulus with a certain colour. The procedure could potentially be adapted to investigate whether learning abilities vary under exposure to different pesticides and concentrations, as well as testing other learning mechanisms than colour, such as texture or patterns.

Approximately 75% of studies have been carried out investigating the effects of neonicotinoids on bees have focused on the effects on the European honey bee (Lundin et al., 2015). This was then followed by the bumble bee species *B. terrestris* and *B. impatiens* accounting for 11% and 5% respectively, whilst few studies have focussed on the effects of neonicotinoids on either solitary or social wild bees or stingless bees (Lundin et al., 2015). Although not a neonicotinoid, an example of one study carried out on stingless bees was that by Almeida et al. (2021) who looked at the effect of a fungicide mixture of thiophanate-methyl and chlorothlonil on colour preference, respiration rates and locomotor activities of stingless bees. It was found that exposure changed the colour preference of the bees, as well as increased motor activity whilst reducing respiration rates. While there is the potential that bee species will respond in the same way to neonicotinoid presence some studies suggest that different species respond differently. A meta-analysis by Arena and Sgolastra. (2014) suggests that there is a high variability of sensitivity (oral acute LD50 and chronic LC50) among bee species, and that sensitivity is dependent on their specific life cycle, nesting activity, and foraging behaviour. Imidacloprid, for example, has been shown to have no effect on the behaviour of honey bees but reduces locomotion and feeding activity in bumble bees (Cresswell et al., 2014).

1.2.3.2 Pesticides in Belize and developing tropical countries

Agriculture in Belize is not homogenous in distribution. In Northern Belize agriculture is largely dominated by sugar cane and a variety of other food crops, whereas in the south agricultural land is heavily planted with bananas and citrus trees (Kaiser, 2011). Despite the difference in agricultural produce, application of agrochemicals is common, and is generally applied by spray through either backpacks or airplanes in some instances. Importantly, agrochemicals don’t just have the potential to impact the localised area where they are sprayed but may also experience orographic drift into surrounding environments (Kaiser, 2011; Logan et al., 1994; Van Der Werf, 1996).

While the quantities of pesticides used in developing countries such as Belize may be lower than those in fully industrialised countries, it has been suggested that they carry a higher likelihood of adverse environmental damage due to their lack of regulation and uncontrolled use (Bravo et al., 2011).

Limited information is available regarding the quantities of pesticides used in Central America and more specifically Belize; however pesticide imports for Central America were studied by Bravo et al. (2011) between 2000 and 2004. The study showed that over the four-year period, 180 thousand tons of active ingredients of pesticides were imported into Central American countries and that herbicides accounted for almost half of this, followed by fungicides, fumigants and then insecticides and nematocides. Of these pesticides 13 active ingredients made up the 77% of the total pesticide imports; mancozeb (30,477 tons), glyphosate (22,220), 2,4-D (22,154), paraquat (11,812), methyl bromide (10,225), chloro-thalonil (7,538), atrazine (4,748), tridemorf (3,563), terbufos (3,561), methamidophos (2,882), metam sodium (2,827), diuron (2,233), and ametryn (2,071) (Bravo et al., 2011). If it is assumed that imports closely reflect the amount of pesticide use, then Costa Rica and Guatemala have by far the highest usage, and Belize the lowest. Despite this Belize has the 2nd highest score in 3 of the 4 usage indicators (excluding absolute quantities) of active ingredients per inhabitant, per rural inhabitant and per agricultural worker.

More recent data from Belize supports similar patterns to those described by Bravo et al. (2011). The most commonly imported pesticide in Belize is glyphosate, followed by Mancozeb, Chlorpyrifos, 2, 4-D Amine and Diuran as of 2015 (UNEP, 2015). Table 1 shows the most common pesticides imported into Belize between 2015 and 2017 and in 2009. Generally, throughout the last 20 years the most popular pesticides have remained the same, with the most common being glyphosate and 2,4-D throughout (Table 1.1). The most frequently imported, and therefore likely the most frequently used pesticides in Belize are not neonicotinoids. It is therefore important to consider how these pesticides specifically affect bee colonies.

Table 1.1. Imports of the most common pesticides into Belize between 2015-2017 and in 2009. [1] ("Pesticide Import Data 2015-2017," 2018) [2] (UNEP, 2015).

		2009 (2)	2015-2017 (1)
Common Name	Category	Tons	Kg of Active Ingredients
Glyphosate	Herbicide	321805.643	516,954.77
2,4-D	Herbicide	86264.197	288,167.82
Mancozeb	Fungicide	196414.568	287,894.44
Atrazine	Herbicide	32586.076	99,949.18
Diuron	Herbicide	52571.356	96,797.20
Paraquat	Herbicide	31497.454	90,017.60
Chlorothalonil	Fungicide	17808.036	86,446.08
Ametryne	Herbicide	NA	73,621.20
Malathion	Insecticide	NA	59,660.80
Terbutryn	Herbicide	NA	32,662.00
Picloram	Herbicide	25473.747	NA
Chlorpyrifos	Insecticide	109034.534	NA
Terbufos	Insecticide/Nematicide	17808.036	NA

A limited number of studies have investigated the presence of pesticides in the environment in Belize. One study found a series of organochlorine pesticides in air samples taken from Belize in the 1990's, this included DDTs (dichloro-diphenyl-trichloroethane) as well as Aldrin/dieldrin among others (Alegria et al., 2000). DDTs have further been detected in Mosquitos from the towns of Dangriga and Punta Gorda in Toledo (Belize) (Somerville and Liebens, 2011). Organochlorine pesticides have also been detected in caudal scutes of crocodiles from Belize, this included dieldrin, endrin, methoxychlor, *p,p*-DDE, *p,p*-DDT, with endrin and methoxychlor being the most commonly found (Rainwater et al., 2007). Similarly organochlorine pesticides, including aldrin, dieldrin, endrin, and methoxychlor, as well as others, have been detected in the eggs of the Morelet's crocodile (*Crocodylus moreletii*) in Belize (Pepper et al., 2004), whilst other studies have found glyphosate and other organophosphates in stream water in the Mayan Mountain Protected Areas in Belize (Kaiser, 2011). Overall however few studies have been carried out, and the habitat types and organisms investigated for pesticide exposure are small, with a lot of work being carried out being centred around the contamination of crocodiles (Pepper et al., 2004; Rainwater et al., 2002, 2007; Sherwin et al., 2016; Wu et al., 2000). Belize would benefit from a wider array of studies investigating pesticide contamination of different types, particularly those used more frequently in recent years, such as those listed in Tables 1 and 2, as well as in different ecosystems.

1.2.3.3 Glyphosate uses and its effects

Glyphosate [N-(phosphonomethyl) glycine or GLY] is a low cost and broad spectrum herbicide making it popular in agriculture for weed control. As of 2018, GLY was the most widely applied agrochemical globally (Vázquez et al., 2018). It has been detected in genetically modified crops, traditional crops and resistant weeds (Guo and Rubio, 2014).

GLY targets the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway, found in plants and microorganism. As this pathway is not found in animals it was initially assumed that it would be harmless to them. Despite this numerous studies have described the negative effects of GLY on bees (Motta et al., 2018). One way this can occur is through changes in the bee gut bacteria community composition, the majority of which contain the enzyme targeted by GLY. This is important as the bacterial gut community in bees is essential for weight gain as well as immune defence. Consequently, alterations to this community can lead to an increased susceptibility to infection by opportunistic pathogens (Motta et al., 2018). For example, the pathogen *Serratia marcescens,* was shown to increasingly infect young worker bees which had been exposed to GLY, leading to greater mortality (Motta et al., 2018). This also demonstrates the synergistic effects between pathogen infections and pesticide exposure.

GLY however, does not only impact bees through the alteration of gut bacteria but also by reducing levels of β-carotene (Helmer et al., 2015). β-carotene is a carotenoid, a type of fat-soluble pigment found in plants and several bacteria (Helmer et al., 2015), and can only be obtained by bees though their diet. Carotenoids have a variety of roles and have been identified as antioxidants (Álvarez et al., 2014), and β -carotene, specifically, has been shown to be converted into vitamin A. Additionally, they are vital to the retinoic acid pathway where carotene is cleaved by the enzyme -carotene 15-15-oxygenase leading to the formation of retinaldehyde which is then reduced to retinoic acid (RA). The formation of these products is essential for a number of biological functions, including cellular differentiation, vision, reproduction, embryonic development and the immune system response (Helmer et al., 2015).

GLY has also be shown to affect the larval development of bees. A study by Giesy et al. (2000) fed honey bees with food sources contaminated by 1.25, 2.5 and 5 mg of GLY per litre of food (representing natural, agricultural and heavily exposed landscapes) (Solomon & Thompson, 2003). In all cases, in comparison to a control (no exposure to GLY) there was a higher proportion of larvae with delayed moulting and reduced weight, however at the colony level the effects varied. This could possibly be explained by genetic variation in susceptibility to GLY (Vázquez et al., 2018).

Studies have also suggested that glyphosate may cause reduced cognitive abilities in honey bees. Balbuena et al. (2015) exposed bees to sub-lethal levels of glyphosate (0.125, 0.250 and 0.500 µg per animal), and monitored the homeward flight paths of control and treated bees in an open field. Forager bees were trained to an artificial feeder, captured, and fed with sugar solution containing traces of GLY and released from a novel site either once or twice. Their routes back to the hives were tracked using harmonic radar technology. They found that bees exposed to GLY (0.500 µg) took a greater amount of time to reach their hives and had fewer direct flights than bees exposed to lower concentration of GLY. After a second release from the novel location control bees were able to reach their hives in a reduced amount of time whereas treated bees could not (Balbuena et al., 2015). This therefore suggests that GLY may impair the ability of bees to retrieve and integrate spatial and visual information allowing them to return to the hive. Other studies have also found negative cognitive influences of GLY. Herbert et al. (2014) found that olfactory PER conditioning with a sucrose reward paired with acute GLY exposure resulted in a decrease in learning and short term memory. Bees were exposed to GLY by contamination of the sucrose solution at three different levels (0 (control group), 2.5 and 5 mg l^{-1}) (Herbert et al., 2014). This was based on concentrations recommended for spraying and on those measured in natural environment (Feng and Thompson, 1990; Giesy et al., 2000; Goldsborough and Brown, 1988). In addition Gonalons and Farina (2018) found that GLY exposure (field realistic levels) resulted in reduced olfactory learning as well as reduced food uptake during rearing (Gonalons and Farina, 2018).

One limitation of these studies is that few have investigated field realistic levels of GLY and thus the quantities of GLY exposure in these studies are not necessarily accurate, and tend to verge towards the higher limits of what could be expected. Guo and Rubio (2014) found that in commercially produced honey up to 70% of samples had GLY concentrations of 0.064 mg kg−1, however as these figures were taken from commercial honey and not honey combes bees may actually be exposed to higher levels (Guo and Rubio, 2014). Another study (Thompson et al., 2014) exposed honey bees to a *Phacelia tanacetifolia* plantation which had been sprayed with 7.2 g kg^{-1} GLY in water. Nectar collected from hive combs were then monitored, after 1 day nectar collected from the hives contained 31.3 mg kg^{-1} GLY and after 6 days contained 0.99 mg kg^{-1} (Thompson et al., 2014). In addition, data collected by Feng and Thompson (1990) and Goldsborough and Brown (1988) on exposure risk levels from approximately 30 years ago, and thus may no longer be representative of current levels, whilst that of Giesy et al. (2000) is 22 years old.

1.2.3.4 Glyphosate use in Central America

There is limited information available on the use of GLY in Central America, however there are some reports focusing on both Mexico and Belize. GLY is widely used in the state of Campeche, in the Mayan zone of the western Yucatan peninsula, here tests were carried out to investigate the usage of GLY through its presence in ground water, and was detected in all agricultural communities studied (Rendón-Von Osten and Dzul-Caamal, 2017). The highest concentrations of GLY were found in the ground water. GLY was also present in bottled drinking water although the mean levels were lower than that in ground water (Rendón-Von Osten and Dzul-Caamal, 2017). These results both confirm the use of GLY in these areas as well as raise the issue of runoff and drift from the original site of application. A further study sampled 23 water body locations across southern Mexico during the dry and wet season (Ruiz-Toledo et al., 2014). They found GLY in all of the 23 samples, including those in natural protected areas, demonstrating runoff possibilities. Whilst the concentrations found in this study were below those in the regulations in Canada and the USA, they were above the concentrations allowed in Europe and Australia (Boyd, 2006).

Kaiser. (2011) investigated whether glyphosate runoff from spraying around the Maya Mountains would be detectable inside the protected areas of the Maya Mountain where spray does not occur. Samples were taken across 7 sites, where water was collected from phytotelms (plant-held water). GLY was detected in all sites sampled, and did not vary with distance into the protected area. This again demonstrates the ability for GLY to impact beyond its intended application area, as well show as its presence in Belize. Alternatively, in another study, water samples were collected from coastal areas of southern Belize across eight transects, and while led and mercury were detected in all samples GLY was below levels of detection throughout (Alegria et al., 2009). Despite the lack of GLY presence a number of other pesticides were detected, including trifluralin, dacthal, chlorothalonil, and chlorpyrifos, and less frequently dimethoate, malathion, atrazine, acetochlor, parathion, oxamyl, cadusafos and chlorpyrifos methyl (Alegria et al., 2009).

1.2.3.5 2,4- Dichlorophenoxyacetic acid, its uses and effects

2,4-Dichlorophenoxyacetic acid or 2,4-D is one of the most commonly imported pesticdes into Belize (Table 1.2, 1.2), and is a widely used pre-/post-emergent systemic herbicide that controls broad-leaved weeds and other vegetation (Islam et al., 2018). It was the first commercial herbicide to be introduced into the market to control broadleafed weeds in the 1940s and remains as one of the most cost effective herbicides available (Islam et al., 2018). Other benefits include broad selectivity and ability to desolve in water, allowing it to penetrate through leaves and roots quickly. 2,4-D is a phenoxy class of herbicides, which work by mimicking auxins, a group of plant hormones (Raghavan et al., 2006). The exact mechanisms of action as to how this herbicide effects insects has not been indentified but is thought to disrupt cellular membranes, metabolic pathways involving acetyl-CoA and uncouple oxidative phosphorylation in cells in mammals (Freydier and Lundgren, 2016). 2,4-D is a moderately persistant chemical, which has a half life of between 20 and 312 days, meaning that there are wide opportunities for it to effect non-target organsisms. In addition, due to its ability to easily disolve in water and its low absorption coefficients it is reguarly detected in ground and surface water, making it a potential environmental hazard (Gaultier et al., 2008; Shareef and Shaw, 2008).

Studies have demonstrated the bioaccumulation capacity of 2,4-D in non-target organisms exposed for only a short period of time (da Fonseca et al., 2008; Kiljanek et al., 2016) and has been shown it to cause endocrine disruption (Guerrero-Estévez and López-López, 2016), and genetic alternation (Lajmanovich et al., 2015). Toxic effects have been shown for both vertebrate and non-vertebrate non-target organisms. Adverse effects have been reported for both wild birds and mammals when applied at typical application rates of 1.11 kg/ha (Bautista, 2007). For non-vertebrates, dung beetle declines (Ataenius apicalis) have been shown to be correlated with increased use of 2,4-D (Martínez et al., 2001), while increased motility (30%-40%) of earthworms (*Eisenia foetida*) was seen after a 14 day exposure period to 10 mg of 2,4-D. 100% mortality was found at 500 mg/kg soil and morphological abnormalities were also detected (Correia and Moreira, 2010). 2,4-D has also been shown to be toxic to the lady beetle, *Coleinegilla maculate*, in which it was shown to be highly lethal to the larvae; the LC90 of this herbicide was 13% of the label rate (Freydier and Lundgren, 2016). In addition, larval development time was significantly reduced and more males than females were effected (Freydier and Lundgren, 2016).

Limited information is available on the impact of 2,4-D on honey bees. Almer-Jones (1964) suggested that following an application of 2,4-D to areas surrounding bee hives that “bees were found crawling in front of hive entrances in a distressed condition” and that hive strength tended to be below normal. This study, however, did not statistically compare their results to a control site.

Moreover many of the results were observational and not recorded in a replicable manner, reducing the validity of this paper.

1.2.3.6 Paraquat, its uses and effects

Paraquat is among one of the most commonly imported pesticides in Central America and is used in frequently (Bravo et al., 2011; “Pesticide Import Data 2015-2017”, 2018; UNEP, 2015), largely due to its affordability, accessible application, broad-spectrum and fast acting nature (Tsai, 2013; Wesseling et al., 2001). It is used for weed control for bananas, coffee, plantain, sugarcane, corn, citrus fruits, mangos, avocados as well as many other crops (Wesseling et al., 2001).

Paraquat is a quaternary ammonium herbicide of the bispyridinium family, which acts by interfering with the intracellular electron transfer systems in plants through the reactive oxygen species, which interact with the unsaturated lipids of membranes resulting in cell apoptosis (Tsai, 2013; Wesseling et al., 2001). It has been shown to have numerous deleterious effects on organisms including fish, Collembola (Choi et al., 2008), birds (Bauer, 1985), Gastropoda (Bacchetta et al., 2002), and mice (Dial and Dial, 1987), and is a non-specific inducer of apoptosis (Cousin et al., 2013). In humans Paraquat has been shown to cause lethal lung fibrosis and has been implicated in the development in Parkinson’s disease in frequent users (Cousin et al., 2013). In honey bees, Paraquat has been shown to decrease the size of the bees oenocytes even at low concentrations. Oenocytes are important in the production of lipids and lipoproteins but also in the formation of the external cuticle (Cousin et al., 2013). Other studies have found it to be between non-toxic and slightly toxic to adult bees (FAO-UN, 2013; US EPA., 1997). Additionally, Paraquat is a high contact herbicide, meaning that spraying occurs with high frequency, particularly in humidconditions, roughly every 6-8 weeks, making exposure of non-target organisms likely (Wesseling et al., 2001). Consequently, Paraquat has been banned in the EU since 2007, however despite its high toxicity it is still used at a field rate or 0.3-2 kg/ha on more than 100 crops in over 100 countries, particularly in developing countries (Cocenza et al., 2012).

Morton et al. (1972) fed a variety of herbicides to newly emerged worker honey bees in a 60% sucrose solution at concentrations of 0, 10, 100, and 1000 parts per million (ppm). They found that Paraquat was toxic at all concentrations and caused a significant reduction to the half-life.

1.2.3.7 Pesticide exposure across a landscape gradient

Bees have been shown to experience greater pesticide exposure when hives are located in an agricultural setting, as opposed to natural landscapes such as grassland or forest. One study placed 16 hives in three different agricultural landscapes of varying intensity, and natural landscapes in Tennessee, USA (Alburaki et al., 2018). Pesticide toxicity was recorded in the agricultural habitats, through the detection of neonicotinoids in dead bees, but not in the hives placed in the natural landscape (Alburaki et al., 2018). Another study collected pollen and beeswax from 45 different apiaries located across a gradient of landscapes including agricultural, mountainous, grassland and urban habitats in France. They found that pollen was predominantly contaminated by miticides and insecticides such as chlorpyrifos and acetmiprid, which were significantly higher in apiaries located in intensive farming contexts. A further study investigated wild bee contamination of pesticides in New York, USA, it was found that wild bee community abundance and species richness decreased linearly with increasing pesticide use in orchards (Park et al., 2015). They did however find that the effects of pesticides were buffered by an increasing proportion of natural habitat in the surrounding landscape.

Fewer studies have been carried out in the tropics investigating pesticide exposure of bees across a landscape gradient, however one that has, points to a different trend. A study in Indonesia investigated the effects of agricultural intensification by placing beehives in selected habitats such as gardens, forests and agricultural areas and then compared neonicotinoid contamination between these sites (Buchori et al., 2020). They found that neonicotinoids were barely present in any of the samples collected, and the samples it was detected in were from forest and garden habitats not agricultural. Alternatively a study in Columbia investigating pesticides exposure in farms found that wild bee abundance and species richness decreased as pesticide toxicity increased, but an increase in natural habitat buffered for this (Obregona et al., 2021).

Bees located in or close to agricultural habitats appear to face greater risk of exposure to pesticides than those located in natural habitats, although this is not always the case, as found by Burchori et al. (2020). It is important that more studies are carried out in the tropics as different levels of agricultural intensification, the pesticides used, and the percentages and configuration of natural habitat may impact how bees are effected by pesticides, and results maybe different to those in temperate regions.

1.3 Animal cognition and its importance for honey bees

Cognition is an important part of honey bee survival, as they must be able to learn and react to both negative and positive cues in the environment, such as predators or foraging locations (Shepherd et al., 2019). This ability however has the potential to be threatened by stressors in the environment. Cognition has received relatively little attention as an impact of stressors on bees, despite its importance. Often more attention is placed on colony mortality; however, it is important to study the non-lethal effects of stressors, as here hive weaknesses can be identified before colony numbers drop, and hive productivity can be kept high. Cognition is an essential aspect of bee foraging and thus poor cognition can lead to reduced honey production and reduced brood and thus colony numbers. This section will provide a background and context to animal cognition as well as the importance of cognition in honey bees, and the methods in which this has been studied.

The research relating to this section was carried out in the UK as opposed to Belize due to restrictions put in place by COVID in 2020.

1.3.1 A background to animal cognition

Animal cognition is defined as the ability to acquire, process, store, and act on information (Shettleworth, 2010), and is essential for a species to carry out the tasks needed to survive by allowing an organism to plastically respond to the environment around it (Gerlai, 2016).

Two theories have been put forward explaining the development of cognition. The first is known as the “ecological intelligence” hypothesis which proposes that the need for organism to locate food resources is what drives the development of enhanced cognition (Byrne, 2009; Milton, 1981). The second is known as the “social intelligence” hypothesis which proposes that cognition is favoured in species which have complex societies due to their abilities to respond to species cues and develop social relationships (Byrne, 2009; Jolly, 1966; Sol et al., 2005). Substantial evidence has been put forward to support both of these ideas, however in recent years a greater appreciation of the two systems working together to develop enhanced cognition has been established (González-Forero and Gardner, 2018).

One of the first cognitive ecological studies stemmed from the observations that the Marsh Tit (*Parus palustris*) was able to store food in specific locations and then relocate them with high accuracy (Cowie et al., 1981). Following this, species such as the blackcapped chickadee (*Parus atricapillus*) was shown to return to stored food items they preferred but not to stores from which they had already recovered the food (Sherry, 1984). Scrub jays (*Aphelocoma coerulescens*) have been shown to be able to identify when and where different food items were stored, by rapidly being able to avoid searching for foods that had perished by the time of recovery whilst continuing to search for foods that were still fresh (Clayton et al., 2001).

Spatial learning and memory has been assessed using numerous different behavioural paradigms in rodents (D’Hooge and De Deyn, 2001; Holcomb et al., 1998; Holmes et al., 2002; Koopmans et al., 2003; Kuc et al., 2006; Paul et al., 2009), including the use of the T-Maze, Y-Maze, Radial Arm Maze, Morris Water Maze and the Barnes Maze (Sharma et al., 2010). Although many of these techniques were initially designed for rats, mice have since been utilised due to their role in understanding the molecular mechanisms behind learning and memory, particularly through transgenic techniques (Gerlai, 2016). Other organisms commonly used to investigate learning include pigeons, which are able to display spatial learning abilities, by being able to locate a food source in a radial maze (Dale, 1988), as well as display visual learning through the identification of squares which had changed colour (Wright and Elmore, 2016). Perhaps the most well-known examples of learning in a non-vertebrate species is that of *Aplysia* where classical conditioning was produced in the defensive syphon and gill withdrawal (Carew et al., 1981). Here the conditioned stimulus was a light tactile touch to the syphon, and the unconditioned stimulus and electric shock to the tail, which caused defensive syphon and gill withdrawal. This produced the conditioned response, where the *Aplysia* would retract its syphon and gill through just the tactile touch (Carew et al., 1981). Other examples of non-vertebrate learning include the cuttlefish which in laboratory experiments were able to learn to exit an alley maze more quickly after successive learning trials (Karson et al., 2003). In addition, the octopus *Octopus bimaculoides* was found to be able to develop quicker movements across a novel arena over time as well as to locate a single open escape burrow across 6 locations (Boal et al., 2000).

Non-vertebrates and insects represent an important area of cognition studies and provide great opportunities for increasing the current understanding of learning and the mechanisms behind it. Insects and vertebrates have independently evolved complex behaviours such as cooperation, central-place foraging, navigation and communication abilities; understanding the process behind this, the similarities and differences will greatly enhance our picture of cognition (Simons and Tibbetts, 2019). Insects also provide mechanisms for understanding cognition not available in vertebrate species, largely stemming from the fact that insects possess small nervous systems, with a reduced number of neurons but can still carry out sophisticated and complex behaviours (Chittka and Niven, 2009). Behavioural plasticity has been shown in a number of insect species, which can learn and memorise sensory cues such as predictors of rewards (Daly and Smith, 2000; Matsumoto and Mizunami, 2000; Menzel, 1999) or punishment (Fiala, 2007; Keene and Waddell, 2007; Vergoz et al., 2007), and retrieve these in both short and long term range after learning. Consequently species such as the fruit fly *Drosophila melanogaster* (Davis, 2005, 2011; Guven-Ozkan and Davis, 2014) and the honey bee (Menzel, 1999, 2001) have become important models for the study of learning and memory for insect species.

1.3.2 Learning and memory in insects

Most research on insect learning and memory has focused on simple forms of associative learning such as Pavlovian and operant conditioning (Fearing et al., 1929; Giurfa, 2015). In Pavlovian conditioning an organism learns to associate a conditional stimulus with an unconditioned stimulus, whilst in operant conditioning an association is learned between a behavioural action with a reinforcement which results from the initial behaviour. In recent years, however, novel research has been carried out where more elaborate forms of learning techniques have been utilised. This includes literature on attention-like processes in fruit flies (Van Swinderen and Greenspan, 2003) and honey bees (Spaethe et al., 2006), observational learning in a social context in bees (Worden and Papaj, 2005) and fruit flies (Battesti et al., 2012), individual recognition in wasps (Sheehan and Tibbetts, 2011) as well as categorization and concept learning in bees (Avarguès-Weber et al., 2011). Ants have even been shown to avoid unsuitable plants for symbiotic fungus after previous exposure (Saverschek et al., 2010).

Drosophila have been used to investigate the role of attention in visual object recognition. For example, a tethered fly tracking a visual object (a black vertical bar) which moved at a constant frequency around the fly, was shown to display anticipatory behaviour (early onset local field potentials) after being trained to the image, in comparison to a non-trained imaged (Van Swinderen and Greenspan, 2003). Alternatively, another study exposed the fly to competing moving gratings which were exposed to either eye. This led to the fly alternating directly between the two moving gratings, showing a supressed response to the grating which was not being followed (Tang and Juusola, 2010). Fruit flies have also been used to demonstrate observational learning. For example, a female which was allowed to observe a successful copulation between a marked male and a female and an unsuccessful differentially marked male and a female, would preferentially choose to mate with the successful male. When the marks were removed from the males, the effect disappeared (Mery et al., 2009). Furthermore female flies who have observed demonstrator female flies choose oviposition sites would choose the same sites, even when both media were equally rewarding (Battesti et al., 2012).

Another example of learning studied in insect species is individual face recognition; this has been shown particularly in wasp species. For example, the paper wasp *Polistes fuscatus* has been shown to be able to identify individual wasps by the yellow and black patterns on their face and abdomen, and when these patterns were altered wasp aggression increased irrespective of whether the new patterns indicated higher or lower social ranking (Tibbetts, 2002). This is not true for all wasp species, however, and whilst *P. fuscatus* was shown to recognise facial features *P. metricus* did not, likely due to differences in their social structure (Sheehan and Tibbetts, 2011).

1.3.3 Learning and memory in bees

Bees, and more specifically honey bees, have been one of the most widely used species to study insect learning (Menzel, 1999, 2001). They have made substantial contributions to the general understanding of cognition over the past century, initially focused on a form of Pavlovian conditioning; appetitive learning, identified first by Karl von Frisch (Frisch, 1914). The honey bee has a repertoire of complex behaviours, and has been shown to be able to distinguish and respond to a diverse range of environmental stimuli, including distinguishing between odours, colours and other visual patterns as well as tactile cues (Frost et al., 2012). The honey bee has a relatively simple neural system, making the study of the neural mechanism behind learning and memory accessible (Frost et al., 2012), leading to the honey bee becoming ideal model species for studying cognition.

One of the first methods used to study learning and memory in the honey bee was the proboscis extension response (PER) (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Takeda, 1961). In this method a harnessed honey bee, responds to stimulation of their antennae with a sucrose solution. The sucrose is the unconditioned stimulus, which the honey bee responds to by extending is proboscis. Simultaneously, the honey bee is exposed to an odour at the same time as the sucrose solution, the odour is the conditioned stimulus. Over successive trials the bee learns to associate the odour with the sucrose reward, and will thus extend its proboscis when the odour is presented even without the sucrose solution (Bitterman et al., 1983). Learning success rate has been shown to reach around 80% by the 5th trial (Bitterman et al., 1983).

Alternatively, the sting extension reflex (SER), a form of aversive learning, can also be used to analyse bee learning. In this method honey bees are thought to associate an originally neutral odour with a mild electric shock (Vergoz et al., 2007). The bees are fixed onto a metallic holder where metal forks come into contact with their thorax. When a shock is applied, the bee reflexively extends its sting and contracts its abdomen. In the same way as PER, the bee learns to associate the odour with the electric shock and will thus extend its sting in the presence of the odour, in anticipation for the shock. Vergoz et al (2007) found this method did not have such a high learning rate, with just over 40% of bees showing a conditioned response by the 5th trial. A study by Shepherd et al. (2019) similarly found that up to 50% of control bees could learn the response. Suggesting a lower ability for a bee to learn the SER paradigm compared to PER. Another protocol for studying bee learning is a free flying proboscis extension response, which was developed to study cognition in the field rather than in a laboratory setting. This method relies on visual learning, where a bee is taught to associate a coloured strip with a sucrose reward, over a strip of a different colour soaked in a negative stimuli such as a salt solution. Over time the bee will immediately extend its proboscis towards the rewarding coloured strip, displaying learning (Muth et al., 2018). This protocol takes place in a sample tube and requires little equipment, and thus has the advantage of being mobile. In addition bees display a high learning rate in control samples where over 90% of bees learn by the 5th trial (Muth et al., 2018).

Bees have been shown to identify more than just colours; they are also able to carry out visual pattern recognition. For example, honey bees have been trained to discriminate between two gratings orientated perpendicularly to each other with both vertical, horizontal and oblique gratings (van Hateren et al., 1990). They could do this for a number of patterns including black bars, white bars, thin lines, and broken bars. This experiment confirmed that bees can utilize visual orientation information and show that bees can learn the dominant orientation of two patterns irrespective of their structural detail (van Hateren et al., 1990).

Attention-like learning shown by *Drosophila* has also been seen in both the honey bee and the bumblebee. Honey bees have been trained to choose a coloured target disk among numerous disks of different colours. It was found that decision time increased and accuracy decreased with an increasing number of disks present (distractor number), but when more targets were present the performance increased (Spaethe et al., 2006). It was noted that similar results were also found for attentional learning in primates suggesting that serial visual attention is common in both mammals as well as bees (Giurfa, 2015).

Social learning has also been demonstrated in honey bees in regards to the waggle dance, which is used to communicate the location of pollen and nectar resources, by conveying its direction and distance (Simpson and von Frisch, 1969). Whilst the initial ability to perform this dance is genetic there is also a considerable amount of learning which occurs. After the dance has occurred the bee giving the dance will provide the other bee with a drop of scented nectar from the desired flower, and subsequently an association is made been the odour and the flower (Gil and De Marco, 2005). Experiments have shown bees which have previously been exposed to flower odours in the hive, will develop a preference for feeding on the flower outside the hive (Arenas et al., 2007). Furthermore, Gil & De Marco (2005) found that honey bees exposed to odour scents via trophallaxis will respond by extending their proboscis when they were later exposed to the same scent, demonstrating the association is made by the bee between the odour and the nectar.

These methods have since been used to identify how stressors such as pesticides, pathogens, electromagnetic fields and diet can effect bee cognition, to provide an indication of bee fitness level in light of exposure to these stressors, as discussed in previous sections (Abramson et al., 1999; Arien et al., 2015; Decourtye et al., 2005; Han et al., 2010; Hesselbach and Scheiner, 2018; Kralj et al., 2007; Shepherd, 2018).

1.4 Thesis overview and objectives

Figure 1.2 gives an overview of the research presented in this thesis on the assessing the impact of stressors on honey bees in both Belize and the United Kingdom. Landscape has been shown - although with conflicting results - to affect the abundance and species richness of both wild and managed bees, where the percentage of natural and anthropogenic habitat has caused a change in bee species composition. There are numerous ways though which a changing landscape has the potential to affect the bee compositions habituated there, including a change in nutritional resources, potential pesticide exposure from agricultural habitats, as well as impacts on navigation, foraging routes and even cognitive abilities. Previous research has shown varying impacts of landscape on the pollen collected by the European honey bee and bumble bee; however this literature is limited and predominantly focused on temperate areas. This is important as the inherent differences between tropical and temperate regions including biodiversity levels, seasons, and different agricultural intensities may have different impacts on indigenous species of bees. The Africanised honey bee that is commonly found in Latin America has the potential to respond differently to landscape changes due to its increased pollen foraging effort in comparison to the European honey bee. Similarly, information regarding the pesticides present in Belize is limited and few studies have investigated their presence in the environment. Whist previous studies have investigated pesticide exposure across an agricultural gradient, finding greater contamination in more agriculturally intense areas, there is a lack of tropical representation in the literature.

This thesis therefore aims to investigate the pollen diet of honey bees in Belize, where previously no research has been carried out in regards to bee pollen diet, and determine how this diet changes across the landscape, contributing to the understanding of the stressors that affect the health of bees in the tropics. The thesis also aims to investigate if the pollen diet of the honey bee in Belize, represents a route of pesticide exposure, and to further assess if this risk varies across a gradient of landscape.

Cognition plays an important role in ensuring bee survival, allowing them to respond correctly to both positive and negative cues from the environment. Currently no studies have directly linked the landscape to the visual learning abilities of bees. Whilst some studies have investigated bee flight time across different landscapes complexities, which can be used to infer learning, no study has directly linked the two (further discussed in chapter 5). Another aim of the thesis is therefore to identify and evaluate the effect of landscape on bees. A modified version of the proboscis extension response cognition experiment was used to compare the visual learning of honey bees across a gradient of landscape complexities, to gain a better understanding of how bee cognition is influenced by the surrounding environment. Due to COVID lockdown and travel restrictions in 2020, this part of the work was carried out in the UK as opposed to Belize as data collection had not yet been completed prior to these restrictions. Consequently, the methodology for this section was adapted to work in the UK.

This thesis aims to identify and evaluate the effect of landscape on honey bee diet, pesticide exposure and cognition in both the tropics and the UK. Specifically focusing on the pollen diet of honey bees in Belize, and how this is influenced by landscape diversity, contributing to the body of knowledge on bee nutrition and the importance of certain flora to the honey bee diet. A contribution will also be made to the importance of pesticide exposure as a stressor to honey bees in the tropics (Fig. 1.2). The final data chapter looks at the potential impact of landscape on visual learning in honey bees in the United Kingdom, in order to gain a better understanding of how bee cognition is impacted in the field as opposed to laboratory studies.

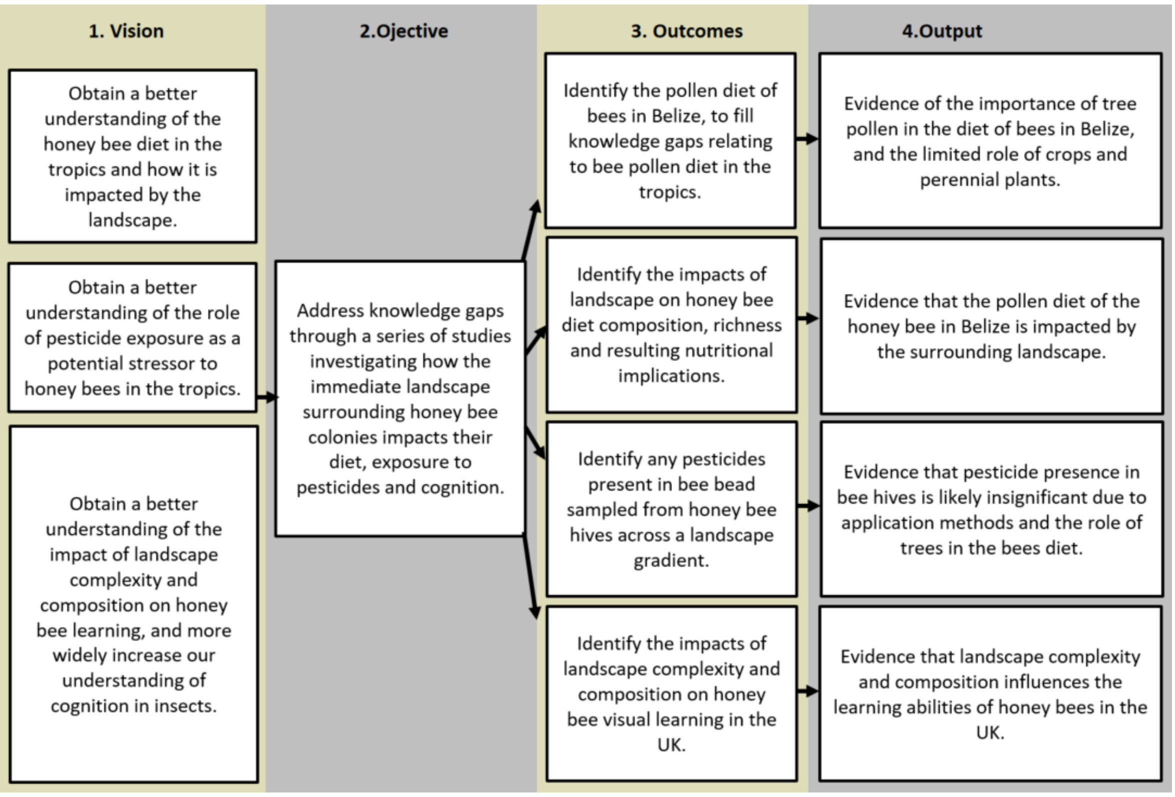


Figure 1.2. Hierarchical framework of thesis assessing the impact of stressors on honey bees in both Belize and the United Kingdom

Objective 1: Identifying the pollen diet of honey bees in the Cayo and Toledo districts of Belize

The objective of chapter 2 is to investigate the pollen diet of honey bees (*Apis mellifera scutellata Lepeletier*) from 46 different colonies in the Toeldo and Cayo districts of Belize, in terms of total abundance, weighted abundance, frequency of occurrence, species richness and composition. Pollen species composition are identified and assessed using bipartite networks as well as NMDS plots to show compositional changes across the two districts. Average crude proteins content is calculated for each pollen family present in samples using the values stated in the literature in order to provide a guide as to how protein rich each taxa identified in the samples were. This is done to investigate how bees were choosing the pollen most prominent in their diet as well as assess potential health implications regarding nutrition via their diet.

Objective 2: The impact of landscape on honey bee pollen diet in Belize

The objectives of chapter 3 are to investigate how landscape diversity impacts the species richness, species diversity and community compositions of pollen found in bee bread samples from 46 AHB hives in Belize. To do this land use in Belize is identified using the Biodiversity and Environmental Resource Data System of Belize – Belize Land Use Map 2017 by Meerman and Clabaugh. (2017). The landscape metrics package (Hesselbarth et al., 2020) in R is used to calculate various landscape metrics including Shannon’s diversity, edge density and mean patch size across four different buffer radii surrounding the hives. General linear mixed effects models as well as NMDS plots are used to assess the impact of these metrics on pollen diversity, richness and composition.

Objective 3: The impact of landscape on pesticide presence in bee bread in Belize

The objectives of chapter 4 are to investigate the presence of pesticides in bee bread from Africanised honey bees collected from 46 colonies in the Cayo and Toledo District of Belize using liquid chromatography with mass spectrometric detection (HPLC-MS/MS) in selected reaction monitoring mode carried out by Fera LMT. A specific Glyphosate scan is also carried out using liquid chromatography with mass spectrometric detection (HPLC-MS/MS).

Semi-structured interviews are carried out with the owners of each apiary in order to gain a better understanding of pesticide use in Belize as well as to help explain the presence or absence of pesticides in the bee bread samples. Identifications of pollen taxa carried out in Chapter 3 are also used to explain these results.

Objective 4: The impact of landscape on visual learning of the honey bee in the United Kingdom using the Free Flying Proboscis Extension Response

The objectives of chapter 5 are to investigate how landscape configuration and in particular edge density as well as landscape composition, impacts learning behaviour of the European honey bee (*Apis mellifera*) in Hampshire, United Kingdom. 12 different apiary sites are located across the Hampshire area, sites which differed most in landscape configurations but were most similar in terms of landscape composition/diversity are selected. A field adapted proboscis extension response is used to test how well bees learnt across these landscapes, here their ability to learn to associate a positive sugar reward and a negative salt solution with different colour paper strips was tested.

Chapter 2: Pollen diet of honey bees in the Cayo and Toledo districts of Belize

Honey bees (Apis mellifera Linnaeus) are highly dependent on pollen to ensure that the nutritional needs of the colony are met. Protein and amino acids concentrations in pollen have, however, been shown to vary between taxa, and therefore pollen diversity, as well as plant species, surrounding bee hives are important in determining colony health. Currently very little research has focused on the pollen diet of bees in the Neotropics and only a few studies have been carried out on honey bees in Belize, and none in Belize on the honey bee pollen diet. This study aims to identify the pollen taxa richness and compositions utilised by honey bees in Belize using bee bread samples collected from apiaries across the Cayo and Toledo districts. Additionally, the study explores the impact of this pollen diet on bee health from the perspective of crude protein concentrations calculated from the literature at the pollen family level. Forest tree species were found to be particularly important making up 8 of the top 10 most abundant species. Pollen community composition differed between the Cayo and Toledo districts, however, species richness and diversity remained the same. No relationship was found between the relative or weighted abundance of the pollen taxa and its protein content. These results contribute to the growing body of research identifying the importance of tree species in the bee pollen diet, as opposed to annual and perennial wildflowers and crops. This infers the importance of forest trees in the landscape surrounding honey bee hives in Belize. The preference for tree pollen taxa in the bee diet is unlikely to be based on the protein content of the pollen, but more likely due to their abundances and densities in the environment.

2.1 Introduction

Honey bee colonies are experiencing losses due to multiple stressors including but not limited to pesticide exposure (Potts, Biesmeijer, et al., 2010), increased parasitic infection (Cameron et al., 2011), climate change (Ploquin et al., 2013), habitat loss (Santos-Leal, 2006) and decreased floral diversity (Arien et al., 2015). Nutritional stress has been identified as a prominent factor of this decline and has the potential to exacerbate the effects of pathogens, viruses and parasites by compromising their immune system (Arien, et al., 2015; Huang, 2012). Identifying honey bee pollen diet is therefore critical to assess colony health (Nicolson & Wright, 2017), as discussed further in chapter 1.

To date, much of the research on bees has been carried out in temperate regions leaving a large gap in our knowledge and understanding of bees in the tropics (Maggi et al., 2016). For example, of all papers published identifying different bee species, only 7% were from Latin America, whilst 62% were from North America and 20% were from Europe (Orr et al., 2021). Whilst global concerns have prompted monitoring programs of honey bee colony losses, such as the Bee Informed Partnership, developed in the USA and the COLOSS program in Europe, Latin America lacks published data on honey bee declines (Requier, 2020). The only continent-wide data on honey bee losses from Central and South America is that published by Vandame & Palacio (2010) and more recently by Maggi et al. (2016). This highlights the need for further studies investigating bees and their health in tropical regions as bees located at different latitudes may respond to stressors in different ways, due to altering biotic and abiotic factors.

Honey bees forage on nectar and pollen from flowering plants as their primary source of nutrition (Donkersley et al., 2017). Pollen is important in providing protein, essential amino acids and lipids (Morgano et al., 2012). It is vital for brood development, regular immune responses, and underpins a successful and healthy colony (Frias et al., 2016). Honey bees need pollen containing between 23% - 30% crude protein to successfully rear brood (Forcone et al., 2011). In the hive, pollen is stored in comb cells, where it is enriched with honey, digestive enzymes and organic acids from the salivary glands of the bee causing it to ferment. In this process, the pollen sheaths are dissolved and pollen proteins are degraded to peptides and amino acid (Kieliszek et al., 2018). This process helps to protect the pollen from loss of properties over time and helps ease digestion.

Amino acid profiles and crude protein percentages of pollen vary between plant species, which means that the plant species available and their abundances may directly impact the health of honey bees (Forcone et al., 2011). Crude protein levels in pollen range from 2.5% - 61% of dry mass (Roulston, Cane, & Buchmann, 2000). In terms of amino acids, 10 have been identified as essential for honey bees (De Groot, 1953), however, while most plant species contain a full spectrum of essential amino acids, some provide these in very small quantities, and therefore may not be able to sustain a healthy colony (Weiner, Hilpert, Werner, Linsenmair, & Blüthgen, 2010). Both pollen quality and diversity are therefore considered important for bee health (Di Pasquale et al., 2013).

This study focuses on the Africanised honey bee, referring to the African and European honey bee hybrid, present in Belize since 1987 ("Honey," 2017; Quezada-Euán, 2007). Relatively little research has been carried out investigating the Africanised honey bee pollen diet in the tropics (Villanueva-Gutiérrez & Roubik, 2004) and no previous work was found in Belize. Studies which have been carried out in South America (Brazil) have found the pollen families Arecaceae, Asteraceae and Myrtaceae to be the most frequently occurring taxa, particularly *Vernonia*, *Eupatorium*, *Crotalaria* and *Mycia* (Almeida-Muradian et al., 2005). Alternatively, one study from Mexico found *Cecropia peltata* and *Metopium brownei* to be the most dominating pollen taxa collected by both European and Africanised honey bees (Villanueva G, 1999). In Belize some studies have been carried out looking at pollen taxa types, however these tend to be in the context of paleo-ecological studies relating to the Maya (Bhattacharya et al., 2011), none of which relate to bee diet. Much of the research which has been carried out has occurred in Brazil and Mexico (D'Apolito et al., 2010; Hilgert-Moreira, et al., 2014; Roubik & Buchmann, 1984; Roubik & Villanueva-Gutiérrez, 2009; Villanueva-Gutiérrez & Roubik, 2004). It is important to identify diet niche in the Africanised honey bee for multiple reasons. Firstly, as an introduced species they have the potential to interrupt local pollination networks, such as the foraging of the native stingless bees, represented by approximately 400 species of Meliponini (Hilgert-Moreira et al., 2014). Villanueva-Gutiérrez and Roubik (2004) found that Africanised honey bees exploit a greater variety of pollen sources, exploring more trees, grasses and sedges, than European honey bees in the same habitat, suggesting the impact of the Africanised honey bee may be greater than native species. Additionally, these honey bees occupy an important source of income in terms of both honey and bee products for many local farmers, helping to subsidise their income and pollinate their crops.

This study aims to identify and characterise the pollen diet of honey bees in Belize. To do this the predominant, secondary, important minor, and minor pollen taxa are identified from bee bread samples both in terms of taxa richness, relative abundance, weighted abundance, frequency of occurrence and community compositions in the Cayo and Toledo districts. Dominant pollen is defined as making up more than 45% of the total pollen grains counted, whilst secondary pollen makes from between 16% to 45%, important minor pollen from 3% to 15%, and minor pollen makes up less than 3% (Silici and Gökceoglu, 2007). Pollen-hive networks are created to visualise the pollen diet across locations (Toldeo/Cayo), sites and hives and unpick diet compositions and trends. Characteristics of the predominant pollen types, such as plant types (trees, herbs, shrubs, crops) are also identified. The information obtained on honey bee diet is then be used to assess possible implications for colony health. Average crude protein levels of pollen/plant families obtained from the literature will be used together with diet composition to provide an estimate of the protein contents within the bee's pollen diet and thus provide implications for health. Furthermore, this study identifies the number of pollen grains required to assess the predominant, secondary, important minor, and minor pollen taxa present in homogenised beebread samples.

2.2 Methods

2.2.1 Study region and sites

Bee bread was collected from 46 hives across 14 sites in Belize, Central America: 8 from the Toledo district and 6 from Cayo district (Fig. 2.1). These sites were chosen to capture the variation in forest and agricultural landscapes across Belize, though site location was constrained by sites with active apiaries. The Belize mainland as of 2010 was estimated to be 63% forested (Cherrington et al., 2010), with 43% being uninhabited by humans (Foster et al., 2016), including national, private and candidate protected areas. Belize experiences frequent natural disasters such as hurricanes and consequently flooding, as well as anthropogenic disturbances, that have resulted in the most common forest type becoming secondary moist broadleaf forest with interspersed patches of primary forest (Satter et al., 2019). The landscape, however, is a mosaic which includes a range of different habitat types, also including pine forests, savannah, wetlands, mangrove and littoral forests (Satter et al., 2019).

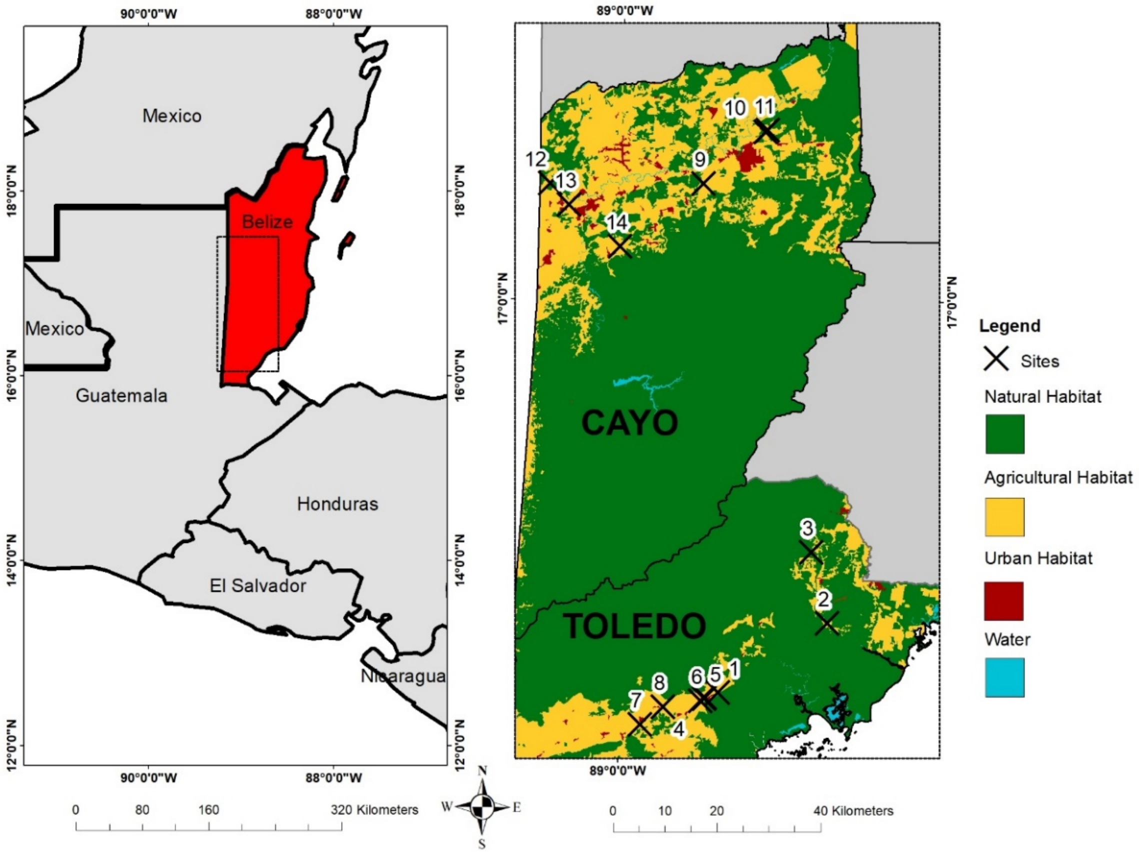


Figure 2.1. Study site locations across Belize, including a wide view of Central America, highlighting where Belize is located. Apiary locations are marked with a black cross, 8 sites are in the Toledo District and 6 are in the Cayo District.

Both the Cayo and Toledo districts were studied in order to assess honey bee diet across a greater area of Belize. The landscape and agricultural practices vary across these locations and thus the honey bee diet may be different between the districts. Cayo comprises a mosaic of lowland broadleaf moist forest, secondary forest, pine forest, short-grass savannah, shrubland, wetland and agricultural habitat (Foster et al., 2020). Toledo has a higher covering of lowland broadleaf wet forest and savannah but also includes shrubland and some pine forest (Meerman and Clabaugh 2017). Toledo encompasses the Maya Golden Landscape (MGL), a 302,259 acre mosaic of private and nationally protected areas, private lands and Mayan and Hispanic communities (Voight et al., 2019). Agricultural activities in the Cayo district include large citrus and sugar cane plantations; there are also numerous Mennonite communities such as Spanish Lookout and Springfield which undertake large scale productions, which have adapted mechanised cultivation (Haines, 2019). Alternatively, in Toledo farming is predominantly slash and burn, and comprises mostly very small scale citrus farms (Haines, 2019). The role of crops in the honey bee diet may therefore vary between these locations as well as the pollen taxa from wild species.

Within a 500 m radius of the honey bee hives used in this study, the most dominant landscape in the Toledo district was agricultural: 6 sites contained over 80% agricultural habitat; broad-leaved forests were only seen around two sites; and pine forests in one. In the Cayo district, 3 sites were dominated by agriculture, with over 65% agricultural habitat, whilst 3 sites contained at least 30% broad-leaved forest. With a 1 km radius, agriculture made up at least 70% of the habitat in 8 out of the 14 sites across Belize, 5 of which were in Toledo and 3 in Cayo. Savanah, shrubland, pine forest and urban habitat are also important in the landscapes surrounding Toledo based hives whilst broadleaved forests and agricultural habitat were most dominant around Cayo hives

Bee bread collection was carried out between May and June 2019. This season was chosen as Africanised honey bee pollen collection activity is highest during the dry season in the Neotropics (Pesante, 1985). Annual rainfall in Belize is typically between 150 – 175 cm (Wyman and Stein, 2010) with a pronounced dry season lasting from February to May (Renton, 2006).

2.2.2 Pollen sampling

Bee bread was extracted from 50 cells located in the outer frames of the brood box and stored in 50 ml sample tubes with 10 mg of Silica Gel (RS®). Each sample tube contained one hive sample. Beebread was collected as one complete block of cells; however, in some hives where the pollen across the frame was more dispersed, cells were taken from different areas of the same frame. Samples were taken as a block of cells so that the time of year the pollen was collected by the bees was known (only the most recently collected pollen was taken and so will have been collected in May/June). Additionally, as the pollen samples were collected during the bees most active foraging period, this provides a good snapshot of the pollen most important to the bees. Samples were then stored at -20°C until they were processed. Analysis of pollen taxa composition was carried out at the School of Geography and Environmental Science, University of Southampton, UK, as described in the following sections.

2.2.3 Pollen preparation

Pollen samples were initially manually homogenised by crushing the beebread so that clumps were no larger than 1 mm. Three subsamples of 0.25 g of pollen were taken from each homogenised subsample and processed using standard acetolysis procedures (Jones, 2014). Briefly, 14 ml of distilled boiling water was added to each sample which were then centrifuged at 3000 RPM for 3 min and the water was then decanted. Each sample was then dehydrated with 5 ml of glacial acetic acid and centrifuged again (3 min at 3000 RPM) and the excess water and glacial acetic acid were decanted. Next, 9 ml of glacial acetic acid and 1 ml sulphuric acid were added to each sample and stirred. The sample was then left in boiling water for 5 min before 1 ml of potassium hydroxide was added, after which the sample was again centrifuged. Finally, the sampled liquid was decanted before 7 ml of distilled water and 1 drop of saffron ink, to dye the pollen grains, was added, and the sample then centrifuged for 3 min at 3000 RPM.

One drop of each sample was mounted on a slide using glycerine jelly and then was sealed with paraffin for permanent preparations (Dimou et al., 2006). One slide was produced per subsample. A summary of the study design is shown in Figure 2.2.

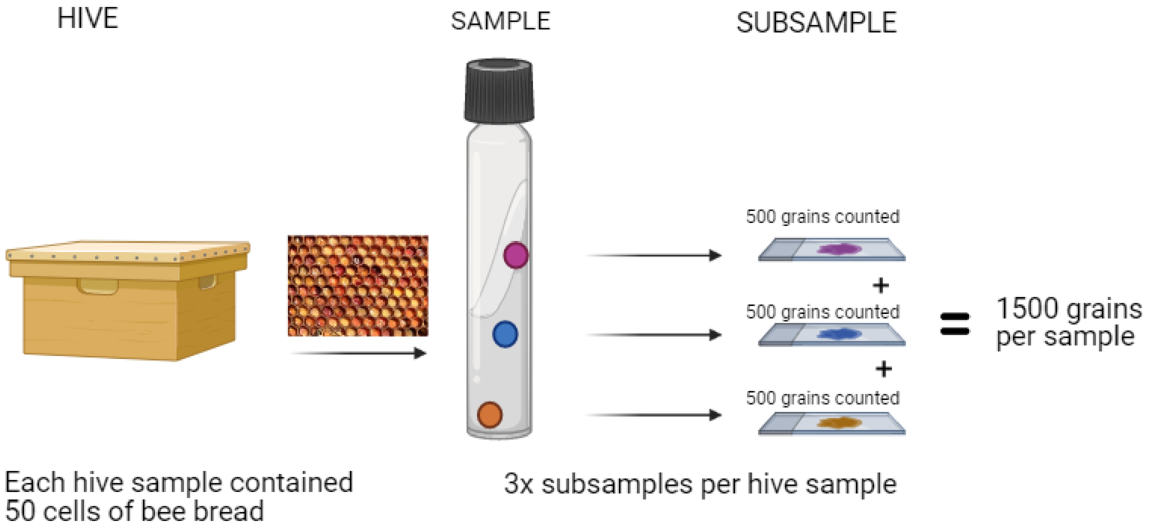


Figure 2.2. Study design of bee bread collection and identification. 14 site/apiaries were identified across Belize. Between 1 and 6 hives were sampled per site and 1 sample was collected per hive. The bee bread samples were then subsampled three times after homogenisation to gain a better representative sample. For each slide, 500 pollen grains were counted and identified making a total of 1500 grains per sample.

2.2.4 Pollen count and identification

Five sets of 100 pollens grains (500 in total) were counted from each of the three slides/subsamples, providing a total count of 1500 grains per hive (Fig. 2.2). Pollen types were photographed at x400 magnification using a Nikon Eclipse 80i microspore and identified to the lowest taxonomic level possible. Taxonomic identification was based on the ornamentation, orientation, size and surface structure of the grains, largely using the Amazon Pollen Manual and Atlas (Collinvaux, 2014) and the Atlas of Pollen and Spores of the Florida Everglades (Jensen et al., 2011).

2.2.5 The sampling strategy used to count pollen grains in bee bread samples

Initial sampling suggested subsampling was required to represent the diversity of pollen within the bee bread samples due to the high diversity and unevenly distributed nature of the samples. An investigation was therefore carried out into the effectiveness of subsampling the bee bread samples. The pollen species richness of each slide was extrapolated from the initial 500 counts to 1500. Extrapolations were carried out using iNEXT (Chao et al., 2016), where 50 bootstraps were run per slide at 40 knots, with a confidence interval of 0.95. iNEXT is based on the seamless rarefaction and extrapolation sampling curves of Hill numbers. Extrapolated species richness estimates per slide were then compared to the total observed species richness at the hive level (the species richness from all the three slides). This was done to give an indication as to whether it was best to subsample and count 500 grains from each slide, or 1500 grains from just one slide.

2.2.6 Identifying crude protein levels in pollen families

Average crude protein content (%) was determined according to published literature for each family present in the samples (Basuny et al., 2013; Forcone et al., 2011; Hassan, 2011; Liolios et al., 2015; Pamminger et al., 2019; Pernal and Currie, 2001; Radev, 2018; Roulston et al., 2000; Somerville and Nicol, 2006; Szczęsna, 2006; Taha et al., 2019).

Crude protein contents were identified for 26 different pollen families out of the 38 found in the bee bread samples. Where multiple measurements of the same species were found in a number of published papers, these values were averaged and the median crude protein levels calculated for each pollen family. As crude protein levels are generally conserved at the family level (Roulston et al., 2000), family level median crude protein values have been used as proxies for each taxa. This was done as pollen taxa were not identified to species level. Family level was determined to be sufficient to gauge the relationship between pollen nutritional content and its abundance.

2.2.7 Data Analysis

Sample completeness (the percentage of observed species from the calculated expected number) was calculated at the hive level using Chao1 species estimates produced using SpadeR (Chao et al., 2015), where 100 bootstraps were run for each slide. Total observed species richness (the number of species) per hive was compared with the Chao1 estimates to gauge how complete the samples were.

To compare species richness at the hive level between the Cayo and Toledo districts unpaired two sample T tests were run in R (R Core Team, 2017):

Species riches ~ Location

The weighted abundance of each pollen taxa was calculated following the methods of da Silveira. (1991), by classifying each taxa into three groups based on the geometric configuration which it resembled most: these were sphere, ellipsoid and triangular based prism (Fig. 2.3). For sphere and ellipsoid pollen types, the polar diameter and the equatorial diameter in the equatorial view were measured (in µm) using a Nixon Digital Sight screen, and the ruler tool; for triangular pollen type the triangle height in the polar view was also measured. Percentage weighted abundance was then calculated using the estimated volume of each pollen taxa using the following formulas:

- Ellipsoid: $4(\pi e^2 p)/3$
- Sphere: $4(\pi e^3)/3$
- Triangular Based Prism: $\frac{2e2ph}{2}$

where e = equatorial radius, p = polar radius, h = triangle height.

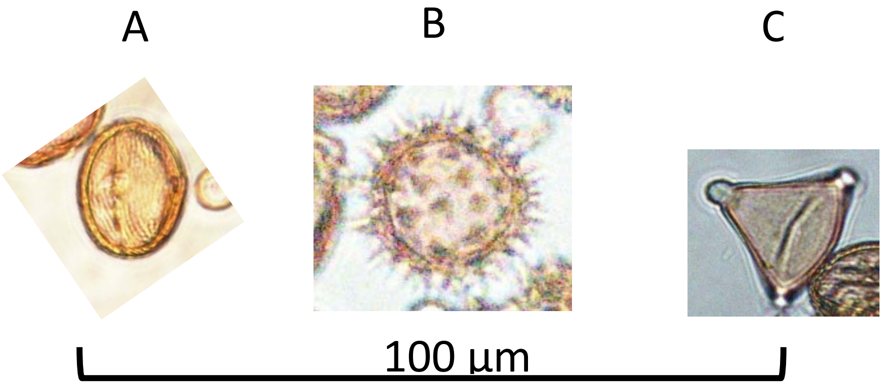


Figure 2.3. Example of pollen collected from Belize which were classed as a) ellipsoid shaped, b) sphere shaped and c) triangular shaped. All photos are shown in their equatorial view.

Both the most frequently occurring species (presence/absence of the relevant taxa in a sample) and the most abundant species (relative – the number of grains belonging to the relevant taxa) were calculated across all bee bread samples to identify the most important pollen taxa contributing to honey bee diet in the Toledo and Cayo district of Belize. In addition, a pollen taxa – hive interaction network was produced for each site to visualise pollen taxa composition at the hive and site level. The number of interactions between each hive and the pollen taxa was first converted into percentages. Web structure for each site was illustrated using a bipartite graph whereby interactions lines and boxes were scaled. The bipartite graphs were created in the “bipartite” package in R (R Core Team, 2017).

Non-metric Multi-dimensional Scaling (NMDS) plots were created to compare pollen taxa compositions between the Toledo and Cayo district using the Vegan package in R (Oksanen F et al., 2020) whilst plots were produced using ggplot2 (Wickham, 2016). The distance measure used was the Bray-Curtis dissimilarity index to assess whether the two communities were statistically different.

Spearman’s rank was used to test for a correlation between the relative abundance and the weighted abundance of the pollen taxa with the average crude protein contents for that family. This was done to determine if honey bees collected pollen based on its protein contents and was carried out using the Base R package (R Core Team, 2017) and plotted using ggplot2 (Wickham, 2016).

2.3 Results

2.3.1 Sampling strategy for bee bread

In order to identify the pollen taxa, each of the three microscope slides per hive were subsampled, as initial testing suggested a high diversity of pollen taxa in the samples that were unevenly distributed. Consequently, 500 pollens grains were counted from three different slides per hive. Species richness of each slide was extrapolated from 500 grains to 1500 grains. This allowed for the pooled species richness of each hive to be compared to the extrapolated values from each hive, testing if subsampling affected total species richness.

It was found that 90% of pooled hive samples had a greater or equal species richness count compared to the extrapolated count from the single slides (Table 2.1). Only 6 of the 134 slides had a greater pollen diversity when extrapolated than the pooled hive samples (4.5%). The greatest difference can be seen in hive S13.3 where for one slide the pooled species richness contained 10 more species than the estimated species richness. This shows that diversity was greater when the three subsamples were pooled together than when pollen was counted from a single slide. On average (mean) the estimated and pooled hive sampled differed by 3.8 species demonstrating the importance of sampling multiple slides. It is worth noting the variation per slide, for example estimated species richness ranged from between 16 to 22 at site S13.3.

Table 2.1. The observed (O) species richness of each slide at a count of 500 grains and extrapolated species richness to 1500 counts. respectively. Observed species richness (hive richness) is displayed for each complete sample (the total species richness of the 3 subsamples). 90% of hive samples had a greater species richness than the extrapolated count at the slide/subsample level, demonstrating the importance of subsampling.

Site	O Species Richness	E Species Richness	Hive Richness	Site	O Species Richness	E Species Richness	Hive Richness	Site	O Species Richness	E Species Richness	Hive Richness	Site	O Species Richness	E Species Richness	Hive Richness	Site	O Species Richness	E Species Richness	Hive Richness
1.1	8	8	14	4.2	17	21	20	8.1	18	22	27	10.3	8	8	11	13.2	7	8	11
	9	9			13	15			16	16			7	7			9	9	
	12	13			12	15			18	21			9	9			8	8	
2.1	9	9	11	5.1	8	9	14	8.2	19	20	26	10.3	16	18	19	13.3	18	18	26
	7	7			9	9			17	22			10	10			20	22	
	7	7			9	11			19	20			10	10			15	16	
2.2	8	8	13	5.2	15	16	20	9.1	9	9	9	11.1	17	20	22	14.1	23	23	25
	8	8			11	18			6	6			19	20			18	18	
	12	15			13	15			NA	NA			14	14			20	20	
2.3	10	11	13	6.1	13	18	16	9.2	4	4	5	11.2	3	3	5	14.2	7	7	18
	9	11			11	11			5	5			4	4			7	7	
	9	12			10	10			4	4			5	5			14	14	
2.4	11	11	13	6.2	7	7	12	9.3	6	6	6	11.3	4	4	6	14.3	18	18	28
	9	11			7	7			4	4			4	4			14	14	
	9	9			11	14			6	6			6	6			15	16	
3.1	10	10	13	7.1	12	19	14	9.4	13	13	20	12.1	15	15	16	14.4	11	11	13
	12	13			11	15			13	15			11	11			9	9	
	10	10			6	6			13	13			NA	NA			8	8	
3.2	10	10	10	7.2	7	7	8	9.5	10	10	18	12.2	14	14	24				
	5	7			5	5			14	14			20	20					
	5	3			NA	NA			10	12			16	16					
3.3	4	5	9	7.3	6	6	10	9.6	9	9	14	12.3	9	9	13				
	5	6			8	8			10	10			9	9					
	5	7			9	9			11	12			9	9					

3.4	14	16	18	7.4	6	6	8	10.1	14	14	17	12.4	7	7	8
	9	9			6	6			14	14			6	6	
	12	14			6	8			12	13			NA	NA	
4.1	9	9	18	7.5	9	9	12	10.2	9	9	13	13.1	16	16	21
	11	15			8	9			10	12			15	17	
	12	12			7	7			11	11			14	14	

2.3.2 Diversity of pollen in the honey bee diet

In total 82 different pollen taxa were identified from the 14 different apiary sites located across Belize and belonged to at least 38 different families. Of this, the top 4 most abundant species made up over 60% of the pollen collected with the top 10 making up 84%. Images and descriptions of all pollen found is displayed in the accompanying materials 1.

The most commonly identified families in terms of richness were Euphorbiaceae, in which 9 pollen taxa were associated, followed by Fabaceae with 6 and Asteraceae where 5 different pollen taxa were identified. For the most part there were between 1 and 3 taxa identified per family, with the rarest families including Oleaceae, Polygonaceae, Salicaceae and Arecaceae.

Hive completeness in terms of species richness ranged from between 56% to 100% complete, where on average (mean) hives were 94% complete. Furthermore, around 60% of hive samples were identified as being complete (Table 2.2), and therefore in 40% of samples there were more species present than were identified. Over 90% of hive samples were, however, at least 80% complete suggesting that the majority of species were accounted for at the hive level.

At the site level only 2 out of 14 samples were complete, however 13 out of 14 were 80% complete and 11 out of 14 samples were 90% complete (Table 2.3). Completeness ranged from between 46% to 100% (Table 2.3), again showing that for the majority of samples at the site level the pollen taxa present were largely captured.

Table 2.2. Chao1 pollen species richness estimated for each combined 1500 count sample with the observed species richness for each hive. Around 60% of hive samples were identified as being complete, whilst around 90% were at least 80% complete, showing that a combined 1500 pollen grain count is sufficient to identify the predominate and secondary pollen taxa.

	O Ttl	Chao1	% Completeness		O Ttl	Chao1	% Completeness		O Ttl	Chao1	% Completeness		O Ttl	Chao1	% Completeness
1.1	14	14	100	5.2	20	22	91	9.3	6	9	67	12.2	24	24	100
2.1	11	11	100	6.1	16	20	82	9.4	20	20	100	12.3	13	13	100
2.2	13	13	100	6.2	12	12	100	9.5	18	19	95	12.4	8	8	100
2.3	13	13	100	7.1	14	14	100	9.6	14	16	88	13.1	21	21	100
2.4	13	16	81	7.2	8	8	100	10.1	17	17	98	13.2	11	11	100
3.1	13	13	100	7.3	10	10	100	10.2	13	13	100	13.3	26	26	100
3.2	10	10	100	7.4	8	8	100	10.3	11	13	85	14.1	25	25	100
3.3	9	16	56	7.5	12	12	100	10.4	19	21	93	14.2	18	18	98
3.4	18	20	90	8.1	27	27	100	11.1	22	22	100	14.3	18	28	64
4.1	18	21	86	8.2	26	27	96	11.2	5	5	100	14.4	13	13	100
4.2	20	20	100	9.1	9	9	100	11.3	6	6	100				
5.1	14	15	92	9.2	5	9	56	12.1	16	16	100				

Table 2.3. Chao1 pollen species richness estimated for each combined 1500 count sample with the observed species richness for each site. 11/14 sites were 90% complete demonstrating that this count was sufficient at the site level to identify the predominant, secondary and important minor pollen taxa.

Site	O Total	Chao1	% Completeness
1	13	14	93
2	24	28	84
3	24	52	46
4	23	24	94
5	22	26	85
6	18	19	95
7	20	20	99
8	32	34	94
9	26	26	100
10	23	24	98
11	24	26	92
12	30	31	98
13	39	40	98
14	38	38	100

Pollen taxa richness was also compared between the Toledo district and the Cayo district due to the differing landscapes and agricultural practices between the two districts, where it was expected that crop species may play a more dominant role in Cayo due to the presence of large scale farms. 59 species in total were identified in Toledo whilst 61 were identified in Cayo. In Cayo the mean number of species found per hive was 15 (± 1.4 SE), and ranged from between 5 and 28 species. In Toledo the mean number of species identified per hive was 14 (± 1.2) ranging from between 6 and 27 species. There was no difference in species richness between Toledo and Cayo (t = 0.405, df = 43.432, p = 0.686) when compared at the hive level (Fig. 2.4). In both locations Euphorbiaceae was the most common pollen family in terms of richness, with 7 taxa from this family being identified in each. Some families were only present in one location such as Aquifoliaceae which was only seen in the Toledo district and Typhaceae which was only seen in the Cayo district.

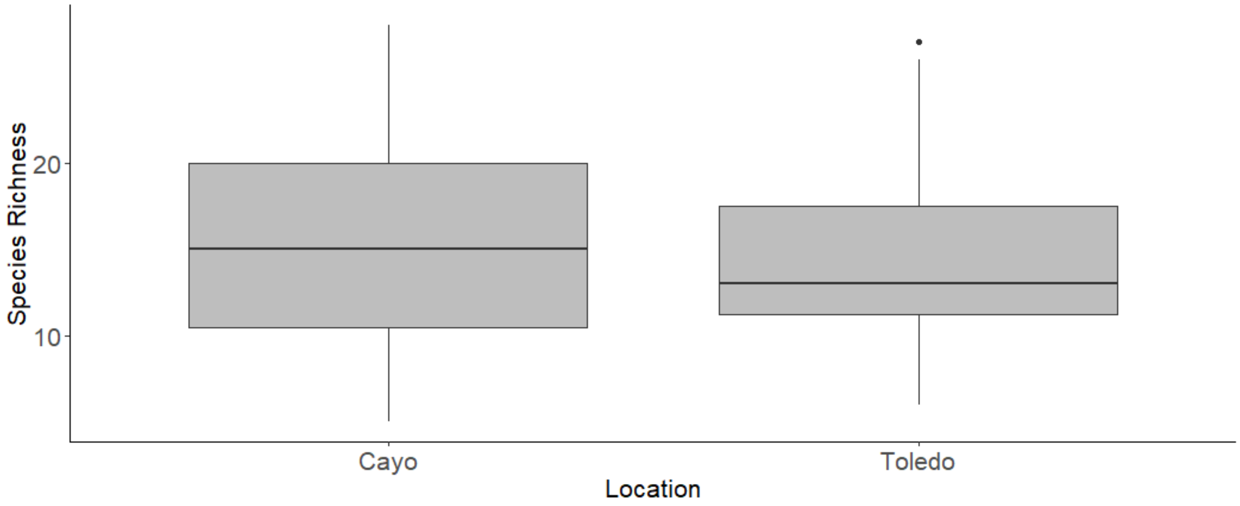


Figure 2.4. Pollen taxa richness at the hive level found in bee bread samples taken for the Toledo and Cayo districts of Belize. Displayed are the median species richness found in a hive sample, along with the inter-quartile range and minimum and maximum values, outliers are displayed as a back circle. No difference was seen between species richness in the Cayo and Toledo districts.

2.3.3 Bee bread pollen taxa compositions in Belize

Both the weighted abundance and the relative abundance of each pollen taxa was calculated across Belize in order to identify the taxa contributing most significantly towards the honey bee diet. Figures 2.5a and 2.5b show the most abundant taxa in terms of both weighted and relative abundance respectively. The taxa that contribute most towards the diet are not ranked the same when looking at both abundance types, likely due to the smaller nature of some of the most abundant taxa. When comparing relative and weighted abundance 6 species occur in the top 10 of both abundance types, and for the most part are not ranked in the same order. For example, Uritaceae 1, which in terms of relative abundance makes up almost 20% of the samples does not make up even 1% of the samples in terms of weighted abundance, as it had a diameter of less than 5µm. Arecaceae 1 contributed the most to honey bee diet in terms of both weighted and relative abundance, making up over 60% and 30% respectively.

Arecaceae 1 was further identified to genus and species, revealing that it is *Attalea cohune*, or more commonly known as cohune palm. Uritaceae 1 was identified to genus level and revealed to be a member of the *Cecropia* genus, whilst Burseraceae 1 was identified as *Bursera Simaruba* which is locally known as Gumbo Limbo. Fabaceae 6 was identified as *Dalbergia brownie*, more commonly known as Indian Rosewood. Images of these species are displayed in Figure 6. In terms of the taxa which were most abundant in weight, Euphorbiaceae 1 was identified as a member of the *Croton* genus, and Poaceae 1, *Zea*, more commonly known as Maize.

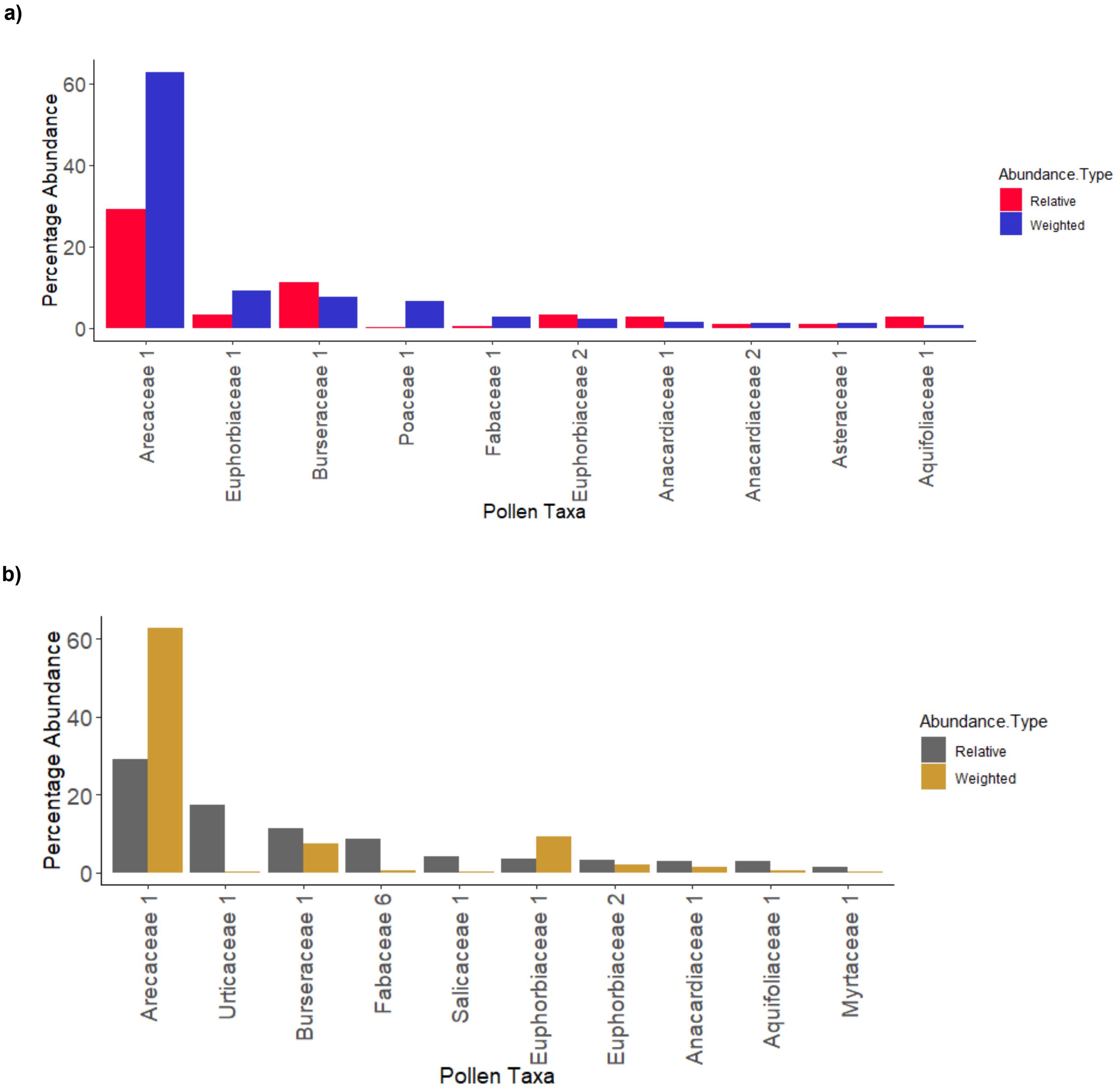


Figure 2.5. The top 10 most abundant pollen taxa across all bee bread samples ranked in terms of both (a) weighted and (b) relative abundance in Belize. a) bars are ranked in order or weighted abundance, with equivalent relative abundance for each of these taxa displayed. b) bars are ranked in order of relative abundance with weighted abundance also shown. Both figures are displayed as different pollen taxa are in the top 10 depending on the abundance type. Arecaceae 1 and Burseraceae 1 were in the top 4 most important pollen taxa in terms of both weighted and relative abundance. Uritaceae 1 and Fabaceae 6 also had high relative abundance whilst Euphorbiaceae 1 and Poaceae 1 had high weighted abundance.

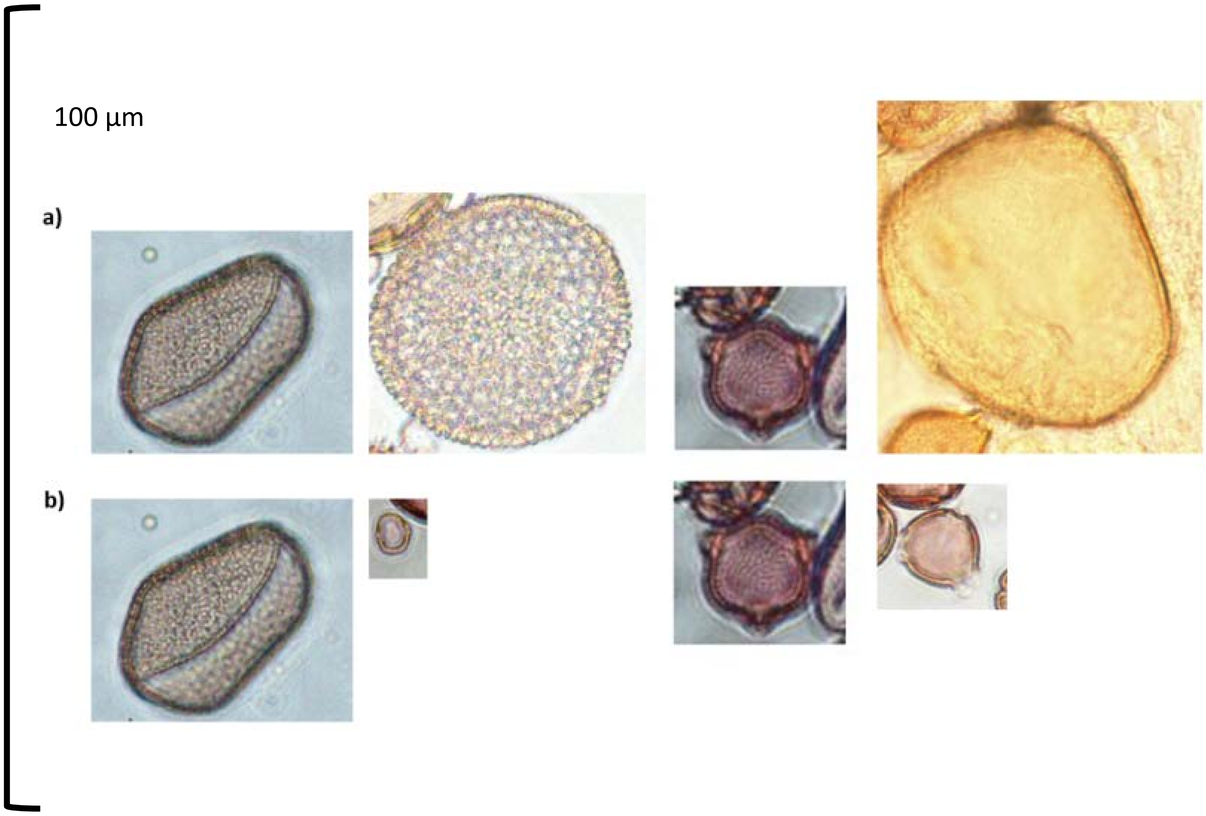


Figure 2.6. Images of the most abundant pollen taxa, a) in terms of weighted abundance (Arecaceae 1, Euphorbiaceae 1, Bursuraceae 1 and Poaceae 1), b) in terms of relative abundance (Arecaceae 1, Uritaceae 1, Burseraceae 1 and Fabaceae 6), captured across the 14 apiary sites in Belize from bee bread samples.

Percentage occurrence was also investigated to determine which pollen taxa occurred most frequently among the hive samples. This was done by identifying the number of hive samples each pollen taxa had occurred in at least once. Thirty-four different pollen taxa were found in at least 15% of samples. The top four most commonly occurring species were seen in over 70% of samples, and this is followed by a sharp drop to the 5th most commonly occurring species which was only seen in 50% of samples. By the 15th most frequently occurring species, occurrence was at 30%. Suggesting a high presence of just a few taxa, whilst the majority of taxa were only found in a few samples. As with relative abundance, Arecaceae 1, Uritcaceae 1, Moraceae 1 and Burseraceae 1 were the most frequently occurring species (Figs, 2.6 and 2.7). Following this, however, the most frequently occurring species are not necessarily the most abundant in terms of either weight or counts. For example, the 5th and 6th most occurring species are Fabaceae 5 and Salicaceae 1. Fabaceae 5 was not in the top 10 most abundant species for weight or counts, and Salicaceae 1 was only in the top 10 for relative abundance. Only 6% of pollen taxa occurred in over 50% of the samples, and just over 20% of pollen taxa occurred in 20% of the hive samples.

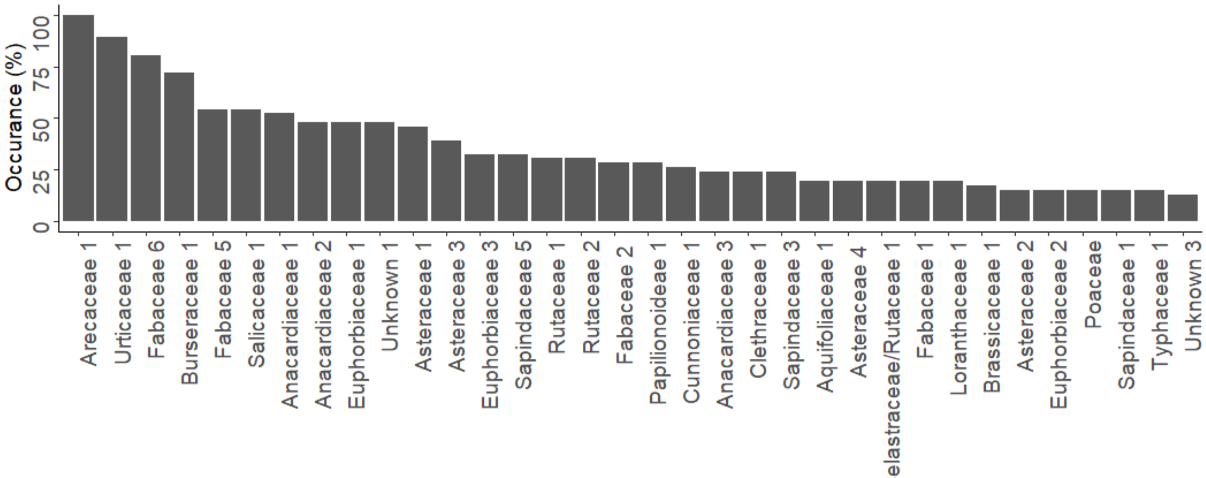


Figure 2.7. The most commonly occurring pollen taxa in bee bread samples taken from honey bee hives in Belize. The percentage of pollen samples in which each pollen taxa occurred at least once in the 46 hives sampled across Belize are displayed. Only taxa which occurred in over 15% of samples are included. The most commonly occurring species were Arecaceae 1, Uritaceae 1, Fabaceae 5 and Burseraceae 1. 34 taxa were identified as being present in at least 15% of samples.

A non-metric multi-dimensional scaling ordination was carried out to compare the pollen taxa communities between those in the Toledo district and those in the Cayo district. A clear distinction was found between the communities (Fig. 2.8), where sites in Cayo had lower NMDS1 scores than those from Toledo. Along the Y axis NMDS2 scores were similar for both locations. The clear groupings of each location suggest that there are distinct pollen taxa which occur in certain abundances in each location, and the community compositions are different between the two districts. This is further supported by Bray's dissimilarity measure which showed that the two districts were statistically different in terms of community composition (R = 0.103, p = 0.005).

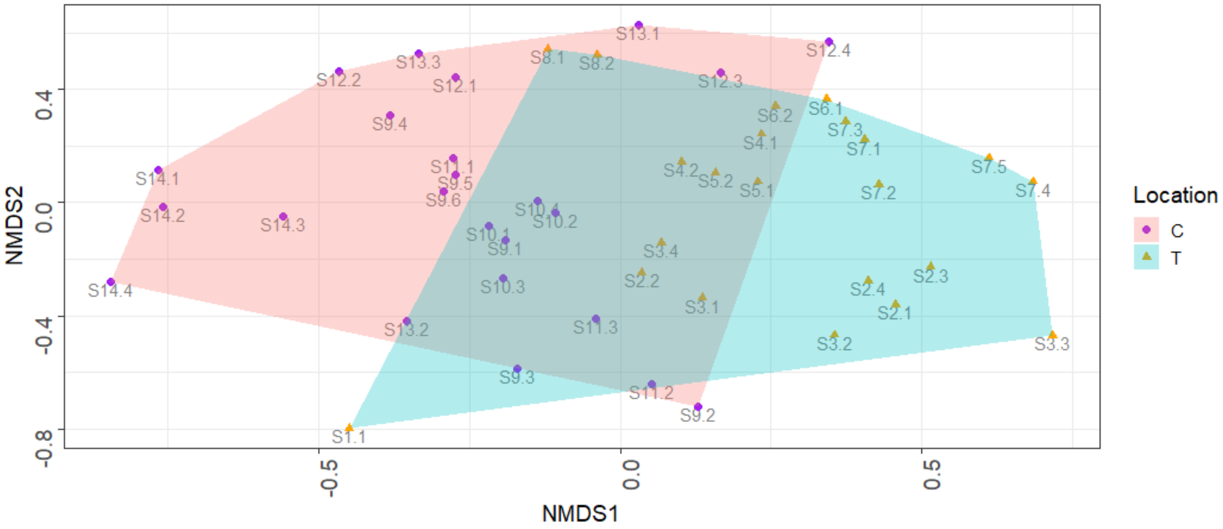


Figure 2.8. Non Metric Multi-Dimensional Scaling ordination investigating the different communities of pollen taxa found in the Cayo (C) and Toledo (T) districts of Belize. Showing distinct differences between the pollen compositions from Cayo and Toledo samples in terms of NMDS 1. Pollen was captured from bee bread from 46 different hives across 14 different apiary sites, 8 of which were located in Toledo and 6 in Cayo.

Ten pollen taxa made up over 80% (relative abundance) of the pollen identified from bee bread samples in Belize, all of which belonged to forest tree species (Table 2.4), with the exception of Anacardiaceae which may have come from a Cashew tree. These trees are often grown by farmers and the cashews sold to supplement income. No other agricultural species was detected in the most dominant pollen taxa found. Mytaceae 2 could not be identified to genus level and therefore the type of plant it came from is unknown. The top 4 most common species all belonged to trees and made up over 65% of the total pollen sampled.

Table 2.4. The 10 most abundant (relative) pollen taxa found in bee bread samples taken from hives across Belize, the associated apiary sites they were found in, and the type of plant they come from. All top 4 species were trees, the remaining taxa except for Anacardiaceae, likely a cashew plant, were either forest trees or shrubs. One taxa, Myrtaceae was unknown in terms of genus. The top 4 most abundant species were found in at least 12 of the 14 sites.

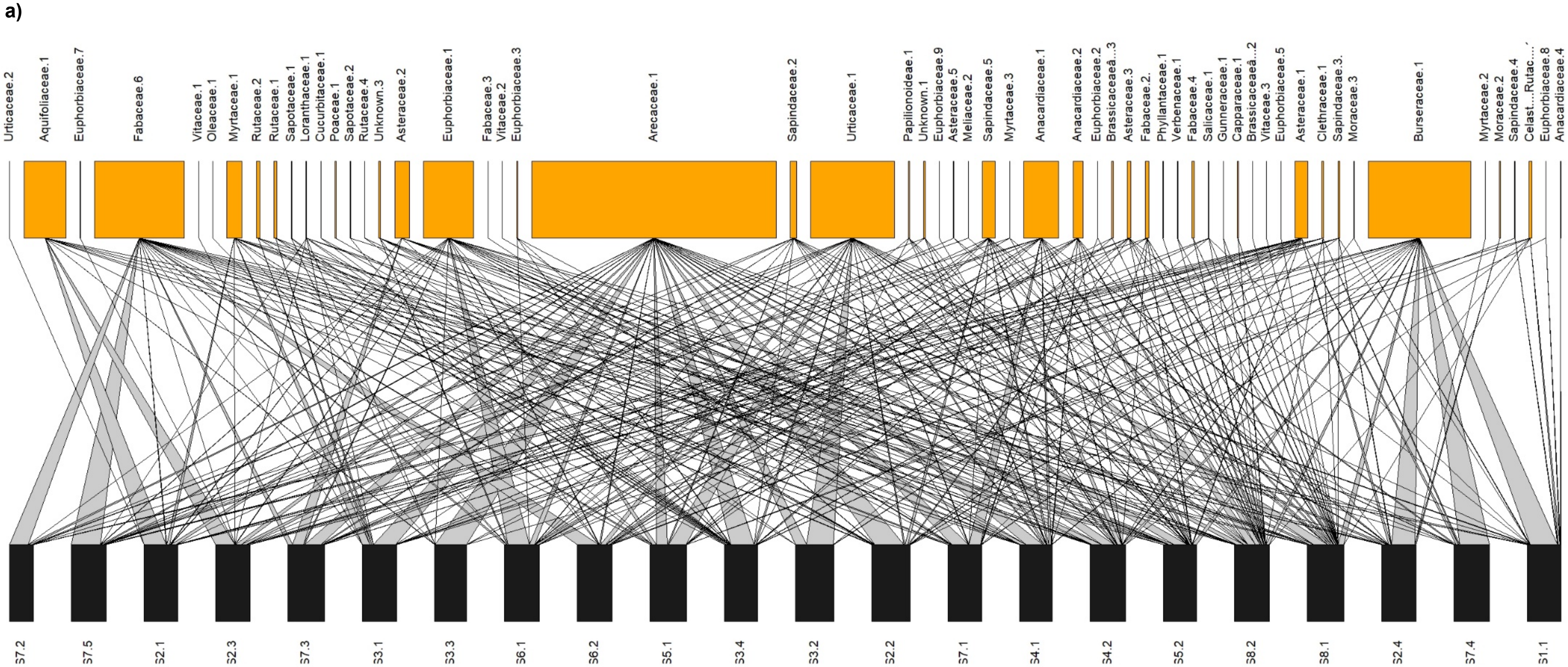
Family Name	**Genus**	**Species**	**Sites**	***Relative Abundance (%)***	**Type**
Arecaceae	Attalea	*cohune*	1,2,3,45,6,7,8,9, 10,11,12,13,14	29.2	Forest Tree
Urticaceae	Cercropia	*sp*	1,2,3,4,5,6,7,8,9, 10,11,12,13,14	17.2	Forest Tree
Burseraceae	Bursera	*simbura*	1,2,3,4,5,6,7,8,9, 10,11,12,13,14	11.3	Forest Tree
Fabaceae	Dalbergia	*brownei*	2,3,4,5,6,7,9, 10,11,12,13,14	8.7	Forest Tree
Salicaceae	Casearia	*sp*	1,2,3,4,5,6,7,9, 11,13	4.0	Forest Shrub/Tree
Euphorbiaceae	Croton	*sp*	1,3,5,6,8,9, 10,11,12,13,14	3.4	Forest Shrubs/Trees
Euphorbiaceae	Euphorbia	*sp*	7,8,10,14	3.2	Forest Shrub/Tree
Anacardiaceae	Anacardium	*sp*	4,5,6,7,8,9, 10,11,13,14	2.8	Tree
Aquifoliaceae	Ilex	*sp*	1,2,3,4,5,8	2.8	Forest Tree
Myrtaceae	Unknown	*sp*	2,3,4,5,6,9, 10,11,13,14	1.3	Unknown

To further unpick the differences in community composition between the pollen diet of bees in the Toledo district and those in the Cayo district, pollen-hive networks were created (Figs. 2.9a,b).

In the Cayo district hives were dominated by Arecaceae 1, Uritaceae 1, Burseraceae 1 and Salicaceae 1. Arecaceae 1 was found in every hive sample and, in some cases made up almost 100% of the sample, for example, in hive 11.2. Similarly, Uritaceae 1 also made up large percentages of some of the hive samples, for instance at hive 11.3. Following this, the next most dominant pollen taxa were

Euphorbiaceae 2 and Fabaceae 6. After this each pollen taxa only contributed minimally to the overall bee diet across Cayo, however, this does not mean that they were not important at the hive level. For example, Thyphaceae 1 makes up almost the half of the pollen collected from hive 9.3. While some hives were dominated by just 1 or 2 pollen taxa such as at hives 11.2, 14.4 and 10.3, others were made up of many pollen taxa contributing much more evenly to the bees diet, for instance at hives 13.1 and 9.5. It is also important to notice that there are differences between hives located at the same site, for example, hive 11.2 is highly dominated by Arecaceae 1 while 11.3 is dominated by Urtiaceae 1, and 11.1 has a much more even distribution of pollen taxa. This shows variability at the hive level as well as at the site and location level.

In the Toledo district, as with the pollen identified in Cayo, Areaceae 1 was found in all hive samples and made up almost 100% of the pollen sampled in at least 5 hives. Buseraceae was the next most dominant pollen forming almost all of the pollen collected from hived 2.4, 7.4 and 1.1. Following this Uritaceae 1, Aquifoliaceae 1, Euphorbiaceae 1 and Anacardiaceae 1 also made up significant portions of the pollen collected from the hives. The taxa were not evenly distributed throughout the hives, Aquifoliaceae 1 for example, was very abundant in site 2 and occurred in multiples hives but was seen rarely across the remaining sites. The majority of hives from the Toledo district were dominated by just a few pollen taxa, where 64% of hives had just one taxa that made up over half of the pollen grains identified. In comparison, in Cayo only 41% of hives had half or more of the pollen identified made up of only one taxa, suggesting a much more even composition. Again, as with the compositions of pollen collected in the Toledo district there were differences between hives from the same site, whilst others were more similar. For example, both hives found at site 8 had similar pollen compositions, both being made up by a diverse range of pollen taxa, while there was much more variation between the hives samples at site 5, where they were dominated by different pollen taxa as well as being made up by different secondary and important minor pollen taxa.


a)
Urticaceae.2
Aquifoliaceae.1
Euphorbiaceae.7
Fabaceae.6
Vitaceae.1
Oleaceae.1
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Rutaceae.2
Rutaceae.1
Sapotaceae.1
Loranthaceae.1
Cucurbitaceae.1
Poaceae.1
Sapotaceae.2
Rutaceae.4
Unknown.3
Asteraceae.2
Euphorbiaceae.1
Fabaceae.3
Vitaceae.2
Euphorbiaceae.3
Arecaceae.1
Sapindaceae.2
Urticaceae.1
Papilionoideae.1
Unknown.1
Euphorbiaceae.9
Asteraceae.5
Meliaceae.2
Sapindaceae.5
Myrtaceae.3
Anacardiaceae.1
Anacardiaceae.2
Euphorbiaceae.2
Brassicaceaeâ...3
Asteraceae.3
Fabaceae.2.
Phyllantaceae.1
Verbenaceae.1
Fabaceae.4
Salicaceae.1
Gunneraceae.1
Capparaceae.1
Brassicaceaeâ...2
Vitaceae.3
Euphorbiaceae.5
Asteraceae.1
Clethraceae.1
Sapindaceae.3.
Moraceae.3
Burseraceae.1
Myrtaceae.2
Moraceae.2
Sapindaceae.4
Celast....Rutac....
Euphorbiaceae.8
Anacardiaceae.4
57.2
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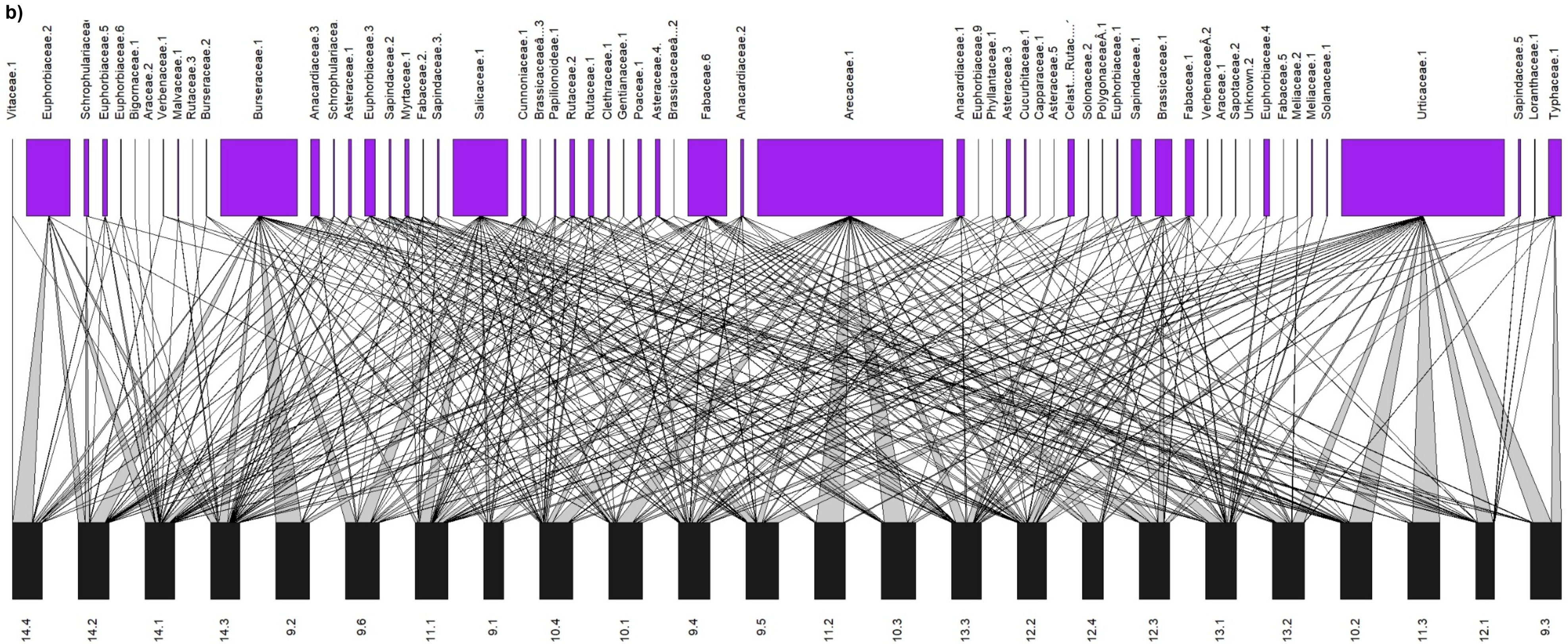


Figure 2.9. Pollen-hive bipartite network showing the weighted connections between 24 different bee hives across 14 different sites in the (a) Toledo and (b) Cayo district of Belize. Each connection represents the amount of a particular pollen taxa found in the hive bee bread samples. Arecaceae 1, Uritaceace 1, Burseraceae 1 and Fabaceae 6 were important in both Toledo and Cayo districts, but with Fabaceae 6 contributing more in Toledo. In Toledo 64% of hives had just one taxa make up over half of the pollen grains identified whereas in Cayo 41% of hives had half or more made up of only one taxa, suggesting a more even composition.

2.3.4 Nutritional content of pollen taxa sampled from hives located across Belize.

The average crude protein content of the pollen families found in the bee bread samples was investigated to determine if there was a link between the nutritional content of pollen and its frequency and abundance in the pollen samples. This was investigated across the Toledo and Cayo district looking at all pollen samples. All taxa were included where data on crude protein levels could be found for that family. The average crude protein level per pollen family is displayed in Figure 2.10.

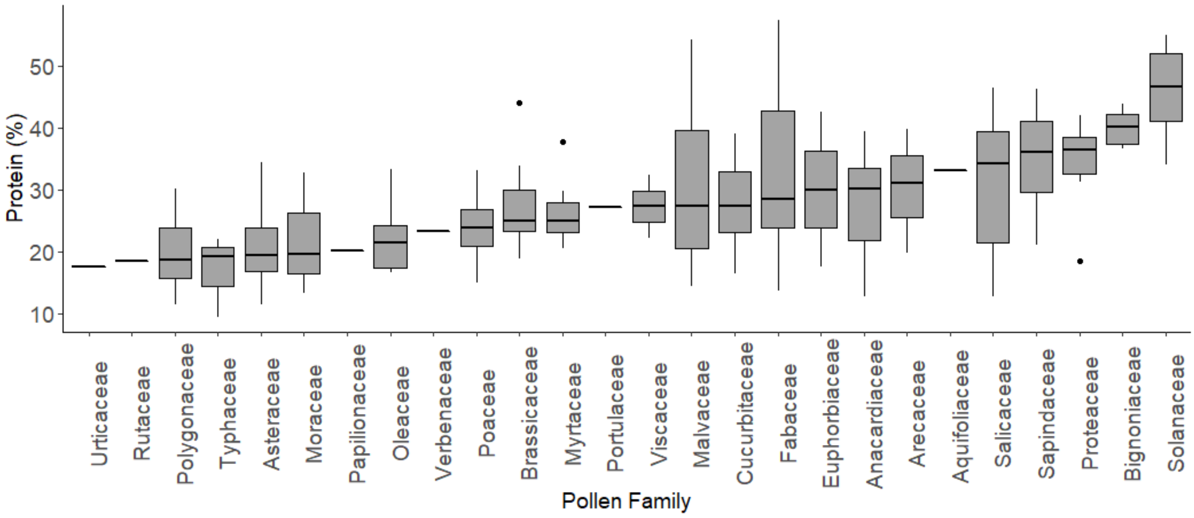


Figure 2.10. Boxplot of pollen grain crude protein content (%) per pollen family. The box signifies the upper and lower quartiles, and the median is represented by the black line within each box. Outliers are depicted as black dots. Values are calculated from crude protein contents reported in the literature. Median crude protein levels were calculated for 26 families. Solonaceae containted the highest levels of protein, followed by Bigorniaceae and Proteaceae. Uritaceae and Rutaceae had the lowest protein levels. Fabaceae, Malvaceae and Salicaceae had the biggest ranges in crude protein level.

Crude protein content varies between pollen family, where those from Solonaceae, Bignoniaceae, Proteacea and Sapindaceae have particularly high levels. Solanaceae, for example, has a median protein content of 47% and Bignoniaceae 40% (Fig. 2.10). On the other hand, crude protein contents of Uritaceae, Rutaceae, and Polygonaceae are fairly low. Whilst there is a range in protein content within pollen families, there is still a marked difference between pollen families with median low and high protein counts. Pollen families with the biggest range include Fabaceae, Salicaceae and Malvaceae. The pollen families shown here with the highest levels of protein content do not align with the most frequently occurring species, which are in the pollen families Arecaceae, Asteraceae and Fabaceae.

The relationship between the weighted abundance and relative abundance of each pollen taxa and the average crude protein level per pollen family was then compared to determine whether bees collect more or less pollen containing low or high protein levels, and if this is affected by the size of the pollen grain. No relationship was found between either the weighted abundance of each pollen taxa (s = 32204, p = 0.238, rho = 0.105) or the relative abundance (s = 30537, p = 0.248, rho =

0.151) with average crude protein content per pollen family. Weighted abundance of pollen taxa was consistent across a range of 10% to almost 50% crude protein content (Fig. 2.11a), with an exception at just over 30% crude protein, where weighted abundance was high at over 60%. In terms of relative abundance there was slightly more variation in terms of crude protein content against abundance levels, however there was still no trend (Fig. 2.11b).

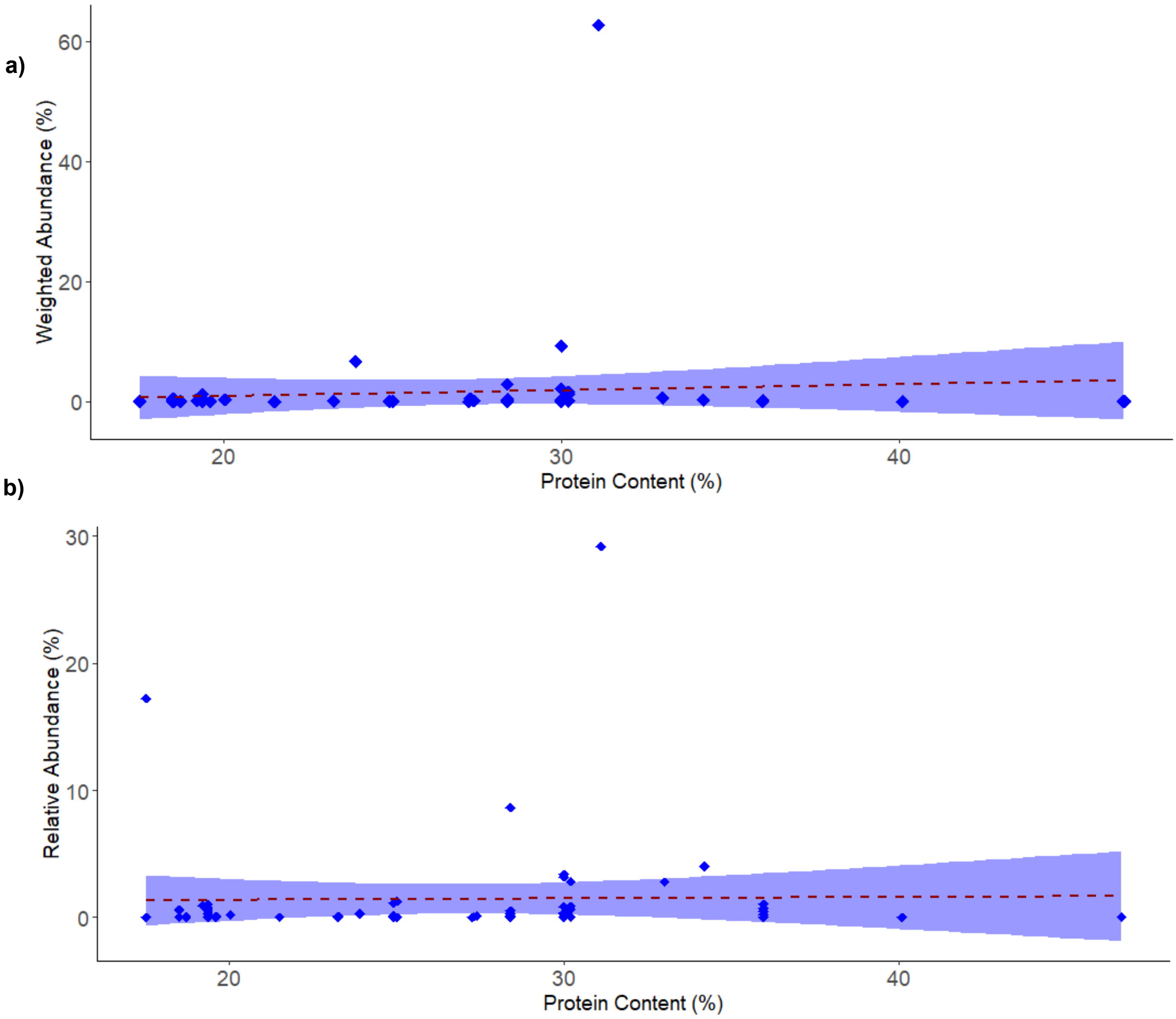


Figure 2.11. The relationship between weighted (a) and relative (b) abundance of pollen taxa found across 46 hives in Belize with the average protein content (%) of each pollen family identified in the samples. The protein content and abundance levels are plotted for 34 different pollen taxa. The regression line is displayed with a red-hashed line and confidence intervals in blue.

2.4 Discussion

This study aimed to determine the pollen richness and composition in bee bread in Belize and infer the effect of this pollen diet on bee health. Pollen samples were determined to be dominated by forest tree species. Pollen community composition differed between different districts in Belize, however, species richness and diversity remained the same. No relationship was found between the relative or weighted abundance of the pollen taxa and its protein content, suggesting that bees do not forage on the basis of the protein content of pollen.

2.4.1 Sampling strategy for bee bread

Palynology, the study of pollen grains, is widely used in archaeological, ecological, paleoenvironmental and geological studies to identify floral taxonomic groups in various substrates, such as sediments, honey, peat (Lau et al., 2018). In previous studies a 200 grain pollen count has been considered sufficient for identifying species richness of fossil pollen, largely from peat (Barkley, 1934). Studies on pollen grains in honey samples to identify the origin of the honey, reported that at least 200 pollen grains need to be counted from one sample to identify the dominant pollen taxa present (Lau et al., 2018). Whilst greater counts than this usually lead to increased species richness, often the increase in sampling effort is too great for the benefits of identifying a few minor pollen taxa (Jones and Bryant, 2001).

Of the 46 hive samples collected across Belize, 60% of hive samples were identified in terms of richness as being complete, although over 90% of hive samples were at least 80% complete suggesting that the majority of species were accounted for at the hive level. This, however, suggests that in order for important minor (<15%) (Song et al., 2012) and minor pollen types (<3%) (Song et al., 2012) to be picked up, a count greater than 500 grains per slide needs to be carried out; however this count is sufficient when trying to identify the bulk of a bees diet through the use of bee bread. This difference could be explained as previous studies were carried out on pollen pellets and honey samples rather than bee bread (Jones and Bryant, 1998; Lau et al., 2018). The pollen samples of bee bread are likely to be much more diverse than that of pollen pellets. Pollen pellets contain only the pollen taxa that the bee has been foraging on during a particular foraging trip, and while multiple pollen taxa will be present, the majority of the pellet will be from one species (Lau et al., 2018). Bee bread, however, contains pollen taxa collected over many months (Vásquez and Olofsson, 2009) and is usually stored in layers of pollen type based on collection time (Barene et al., 2014). Therefore, bee bread collected from just one cell is likely to contain a greater diversity of pollen taxa than a pollen pellet. This difference will increase further when considering sampling from multiple cells of bee bread. It has been reported that at least 35 cells need to be sampled to provide a good representation of the diet of a colony (Wróblewska et al., 2010). In addition, unlike pollen pellets, bee bread has also been mixed with honey and digestive enzymes and, therefore, is sticky, and consequently even after manual homogenisation it may be that certain pollen types remain grouped. Together this is likely to be the cause of a greater number of pollen counts needed from bee bread samples. In this study the most recently stored bee bread was subsampled as a block of 50 cells; this was done so that the time period in which the bees collected this pollen was known. As 50 cells were taken this provided a good snapshot of the pollen the bees were feeding off during the dry season. However, in reflection bee bread could have been collected from across the comb to provide a wider picture of the pollen consumed by honey bees in Belize across the year. This would have allowed for a better understanding of the bees pollen diet over time and not just captured a snapshot. As honey bees forage using recruitment methods sampling a block of cells could potentially bias the pollen samples to the taxa bees favoured over a short period of time.

Additionally, it was found that a greater diversity of pollen taxa was found in combined subsamples than if 1500 counts had been made per slide, suggesting that when bee bread samples are used, it is necessary to subsample the bee bread in order to capture the diversity and species richness of the pollen. This is again likely to be due to the high density of pollen grains and the variety of taxa contained in just one cell of bee bread (Barene et al., 2014), as well as the sticky, and thus unevenly distributed, layered nature of bee bread.

2.4.2 Diversity of pollen in the honey bee diet

In total 82 different pollen taxa were identified from 38 different families, and of these families Euphorbiaceae, Fabaceae and Asteraceae were the most common. These are all large families containing many different genera of various morphological characteristics. Within Euphorbia L. (Euphorbiaceae) there are over 8,000 accepted species, from 340 genera (Secco et al., 2012) making it the third largest genus of flowering plants, second to Astragalus (Fabaceae). They have a rich morphological variability (Ernst et al., 2015), and are one of the most complex and diverse families of Angiosperms. They are heavily distributed in the tropics and can be found in multiple types of vegetations and habitats (Secco et al., 2012). Fabaceae, the second most commonly occurring family in the pollen samples, includes legumes and is known as the pea family. It contains over 19,000 species from 730 genera, with the largest genus being Astragalus which contains over 2,400 species (Ahmad et al., 2016). Mimosa is another large genera within Fabaceae which contains over 500 species (Ahmad et al., 2016). Asteraceae, the third most frequently occurring family contains over 20,000 species from 950 genera. They are a highly specialised family of mainly herbaceous plant and are distributed throughout the tropics (Rahman et al., 2008).

From the 82 different pollen taxa identified it was clear that four taxa were incredibly important at contributing to the honey bee pollen diet after being shown to dominate bee-pollen networks in both Toledo and Cayo districts. These were Arecaceae 1 (*Attalea cohune*) which occurred in 100% of samples and made up almost 30% of the total pollen grains counted in terms of relative abundance; Urticaceae 1 (*Cecropia sp*), occurring in almost 90% of samples making up 17%; Burseraceae 1 (*Bursera simaruba*), which occurred over 70% of sampled and made up 11% of total pollen grains sampled, and Fabaceae 6 (*Dalbergia brownie*), occurring in around 80% of samples making up 9% of the total pollen grains. Whilst honey bees have often be described as "super-generalists" feeding from a wide range of flowering plants (De Vere et al., 2017), other studies have similarly found that they focus on core species, such as that by Hawkins et al. (2015), who found that honey bees actual resource use was represented by a comparatively small number of central species, as found in this study.

In terms of weighted abundance both Arecaceae 1 and Burseraceae 1 were still in the top four due to their large size and high relative abundance with Arecaceae 1 contributing to over 60% of the pollen collected. *Attalea cohune* is a large pollen taxa with an equatorial diameter of 45 µm and was also found in all samples contributing to a high relative abundance. Uritaceae 1 whilst found in great quantities, is small, with an equatorial diameter of just 4 µm. This means that although it occurs in a high relative abundance and frequency, it does not contribute highly to the total volume of pollen consumed by the bees, and therefore may offer less in terms of nutrition. Also in the top four species was Poaceae 1 (*Zea sp*), a species of Maize, the only agricultural species that was seen in either high relative or weighted abundance. Maize pollen is reported to contain low amounts of protein and is deficient in some essential amino acids that bees require (Pernal and Currie, 2001; Somerville and Nicol, 2006), where it has been reported that maize pollen contains between 14% and 26% crude protein depending on the variety and other environmental conditions (Höcherl et al., 2012). Maize, therefore, may not provide the bees with all the nutritional requirements they need. Additionally, mass-flowering crops are a potential exposure pathway for bees to be exposed to pesticides applied for agricultural purposes (Danner et al., 2014).

Interestingly, all 4 of the most dominant (relative abundance) pollen taxa were tree species, highlighting the important role of trees in honey bee pollen diet. Commonly, although largely in temperate regions, honey bees have been reported to have a preference towards annual and perennial wildflowers (Ballantyne et al., 2017; Hicks et al., 2016; Hitchmough et al., 2017; Saunders, 2018), and that wildflowers are preferentially visited over crop species (Donkersley et al., 2017). Less attention has been drawn towards the role of tree species in honey bee diet (Donkersley, 2019) but clearly they have a major role in the diet of honey bees in Belize. Donkersley et al. (2017) also found that contrary to the predominant literature that honey bees collected a large proportion of their pollen diet from woody species. In addition, honey bees continue to forage on tree pollen even when their abundance in the landscape in decreasing, i.e. foraging of less abundant species in terms of land cover. Requier et al. (2015) also pointed out the important role of trees in honey bee diet. After investigating the roles of cropped plants, horticultural plant species, herbaceous plants and woody plant species, they found that woody and herbaceous plant species contributed to more than 60% of the annual pollen diet. Horticultural plant species were only marginally used, whilst cropped plant represented the second most dominant source of pollen accounting for 30% of the total pollen collected. Studies carried out in the tropics such as that by Wilms et al. (1996) also suggested the dominant role of trees in bee tiet, along with Villanueva-Gutiérrez and Roubik. (2004) who round that Africanised honey bees exploted a greater variety of resources the European honey bee, particularly trees grasses and sedges. These studies together show the important role that trees have in contributing to the pollen diet of honey bees, and suggests that the contribution trees play in bee diets should be investigated more, particularly in the tropics where they appear to have an increased role.

There was little difference in species richness in the samples between the Cayo and Toledo districts, however, there compositions were distinct. Whilst the same 4 taxa, described above, made up a substantial contributions to the pollen collected at each site, there were some differences between their presence, as well as the distribution and abundances of secondary and important minor pollen types. For example, Fabaceae 6 had a relative abundance of almost twice higher in Toledo as opposed to Cayo. This is likely due to a difference in species distribution between the two regions. Fabaceae 6 was further identified as rosewood *Dalbergia brownei*, and whilst they are found dispersed through Belize, Guatemala and Mexico, the largest remaining populations are found in the forests of southern Belize, particularly in the Mayan Golden Landscape ("Tackling illegal rosewood logging in Belize", 2020), where some of the Toledo apiary sites were located. It is, therefore, not surprising that rosewood pollen would be more abundant in the Toledo samples, and points towards the bees being opportunistic foragers, feeding from the pollen abundant in the landscape. Other differences include Aquifoliaceae 1, *Ilex sp,* which was ranked in the top 10 taxa in terms of relative abundance but was only found in hives from Toledo and not at all from hives in Cayo. The distribution of this plant in Belize in not known, but it is possible this difference in presence between Toledo and Cayo sites relates to it distribution in Belize. Differences in species presence was not the only factor separating Cayo and Toledo pollen communities, but also species dominance and generalism. For example, in Toledo 64% of hives had just one taxa make up over half of the pollen grains identified. In comparison in Cayo only 41% of hives had half or more of the pollen identified made up of only one taxa, suggesting that bees in Cayo had a more generalised and diverse diet than those in Toledo, where bee pollen stores were highly dominated by just a few species. The NMDS plots also provided support to the show the differing pollen community compositions between the Toledo and Cayo district. The fact that in different locations bee pollen composition changed, suggests that it might be the abundance of these plants in the environment which lead to their abundance and occurance in the bee bread.

Alternatively, the frequently occurring and abundant pollen taxa have also been shown to be important in bee diets in other studies. This could either be because these species are abundant throughout the tropics, or due to bee preference. For example *Cecropia sp*, one of the most dominant species in this study was identified by a Brazilian study as making up 13.68% of pollen samples (Dórea et al., 2010). In this study the most frequently occurring species was *Attalea cohune*, a member of the Arecaceae family, other studies have found different genre of Arecaceae to be highly dominant. For example, a study in Brazil found *Cocos nucifera* in 100% of the samples (De França Alves and De Assis Ribeiro DosSantos, 2014). A study carried out in Mexico looking at both Africanised and European honey bee found that *Cecropia peltata* and *Bursera simaruba* were the pollen species with the largest percentages of occurrence (Villanueva-G, 2002), both of which were highly dominant in this study.

Differences in both the minor and major pollen taxa were not just seen between sites, but also within site. The differences between pollen diet between hives located at the same site could be related to foraging recruitment. This is where bees communicate via the waggle dance the most rewarding patches of flowers, thus signally foraging locations (Carr-Markell et al., 2020). Bees from different hives at the same site may therefore be signalled to forage on different flowers. In this study bee bread was sampled from one block on the comb, therefore it is possible that different recruitment phases were picked up for each hive, and that if a wider area of the comb had been studied, diet within site may have been more similar. A possible modification to study could be to sample from across the comb in order to gain a wider picture of the honey bee diet across a greater period of time.

The season in which the bee bread was collected may have influenced the preferred pollen taxa of the honey bees. The flowering period of the top 10 most occurring taxa is listed below in table 2.5 (Myrtaceae not included as only identified to family level, and Euphorbia could not be found in the literature). It is also worth noting that the flowering time for Belize specifically could not be found and so flowering time of other Latin American countries has been used. Additionally, when not all the taxa could be identified to species level, an example at the genus level has been included. This therefore only acts as a guide to the preferred pollen flowering times, due to limitations in the literature and limitation with identification.

Table 2.5. The flowering time of the most dominating pollen taxa making up honey bee pollen diet in Belize. Sampled between May and June. Flowering times taken for known flowering times for species across the region.

Family Name	**Genus**	**Species**	**Flowering Time**	***Location and Reference***
Arecaceae	Attalea	*cohune*	Throughout the year	Text identifies as Central America (Martin et al., 1987)
Urticaceae	Cercropia	*sp*	Throughout the year	Columbia (Zalamea et al., 2008)
Burseraceae	Bursera	*simbura*	April to May	Costa Rica (Stevens, 1987)
Fabaceae	Dalbergia	*brownei*	March to June	Mexico (Infante Mata et al., 2012)
Salicaceae	Casearia	*sp*	March to May	Panama (Perez and Condit, 2021)
Euphorbiaceae	Croton	*sp*	June to October	Mexico (Steinmann, 2021)
Anacardiaceae	Anacardium	*sp*	July to October	Brazil (Freitas and Paxton, 1998)
Aquifoliaceae	Ilex	*sp*	February to April	Panama (Hahn, 1993)

Both *Attalea cohune*, and *Cercropia sp* flower consistently across the year, and therefore it is it likely that these species may remain a prominent part of the bees diet all year round. These two species make up 46% of the bees pollen diet in terms of relative abundance, and therefore it is possible that almost half of their diet stays consistent throughout the year. The remaining dominant species appear to flower mostly between March and June, with a few species flowering until October. Therefore if the bee bead was sampled at different times of the year a different picture as to the most dominating pollen taxa in their diet may emerge. The sampling carried out in this study is a good reflection of honey bee diet during the dry season, and into the early wet season. However, more work will be needed to identify honey bee pollen diet in Belize during the wet and very early dry season.

Honey bee preference for different pollen taxa did not appear to be linked to the percentage of crude protein predicted to be present for each family. Urticaceae was actually predicted to have the lowest levels of crude protein at less than 20% despite its high abundance in the samples. This is important as it has been reported that honey bees need pollen containing between 23 and 30% crude protein in order to successfully rear brood (Forcone et al., 2011). Of the most dominant species occurring in the bee bread Arecaceae was predicted to have the highest levels of protein at a median of 32%. Fabaceae 6 was also a commonly occurring taxa in terms of relative abundance, with a median crude protein level of just over 30%, however, it has the widest range of protein content of any family, reaching between 24 and 43%. Predictions of crude protein content could not be obtained for Burseraceae.

Plant families with the highest predictions of crude protein found in bee bread samples such as Solonaceae, Bigoniaceae and Proteacae were not seen in particularly high or abundance or frequencies. Salicaeae which was found in around 5% of samples had a high predicted crude protein level of 35%, however, there was a wide range of reported crude protein levels in the literature for this family. This supports the idea that bees do not choose pollen taxa/plants based on crude protein levels. It is possible that the taxa chosen are based purely on the abundances and occurrence in the environment or that bees adapt to crude protein levels by altering their foraging frequency. Some studies have shown honey bees avoid pollen of low crude protein contents (Requier et al., 2015), and that bees will forage greater distances to achieve a more diverse pollen diet (Danner et al., 2014; Steffan-Dewenter and Kuhn, 2003). However, studies carried out in Western Australia, for example, suggest bees do not have a preference for high protein or amino acid content and instead are more attracted to local plants in high abundances (Chemaly, 2002), supporting the results of this study. Corby-Harris et al. (2018) found that nurse honey bees were unable to differentiate between pollen of high protein and that of low protein. Pernal & Currie (2001) argued that bees will increase the gross amount of pollen returned to the colony when protein levels are low, rather than specialising in collecting pollen with higher protein contents (Pernal and Currie, 2001). In this study there appears to be little link between the abundance, in terms of either weight or its relative frequency, and its protein content, where the majority of highly dominant taxa have a low to middle crude protein content, not reaching over 32%. This suggests that the abundance of the pollen in the environment may be the main driver behind abundance. This is supported by instances where the floral distribution is known. For example, greater levels or Fabaceae 6 were found in the Toledo district in comparison to the Cayo district, where rosewood populations are more sparse (“Tackling illegal rosewood logging in Belize”, 2020). To further the work carried out in this study it may be beneficial to measure the abundances of the plant taxa surrounding the hives. As this study has identified tree species as the most dominant component of the honey bee diet, one way to do this would be the record the tree species within 1 km of the hives (the most informative distances as identified by the model) and record their densities within a number of quadrats or along a transect. The data produced from this would help to inform whether it is the most dominant trees in the environment which make up the majority of the pollen diet. As discussed in chapter 1, amino acids also play an essential role in pollen nutritional qualities; in the future, it could therefore be informative to investigate the essential amino acids present in pollen, either through a literature search or chemical analysis of the pollen, to see if this could help explain pollen abundances.

Tree species were found to dominate the pollen diet of bees in Belize, with just four species making up over 60% of all pollen collected, demonstrating the importance of a few particular species, *Attalea cohune*, *Cecropia sp*, *Bursera simaruba* and *Dalbergia brownei*. Whilst these species remained constantly important across the region, the overall compositions changed between the Toledo and Cayo districts due to the presence of different minor pollen taxa and changes of evenness amongst the pollen taxa. No relationship was found between pollen abundance and crude protein content, and therefore their dominance and attractiveness in the environment is more likely to be the cause of their importance rather than protein levels.

Chapter 3: The impact of landscape on honey bee pollen diet in Belize

The conversion of natural landscapes to crop systems has been cited as one of the primary drivers of both wild and managed bee declines. Land use change, where diverse natural habitats are converted to crop lands and agricultural sites is occurring rapidly in the tropics, and raises concerns as to how tropical fauna, and in particular bee species, will respond to these changes in landscape. Bees require pollen in their diet to ensure that the nutritional needs of the colony are met; however this is dependent on the availability and abundance of high quality pollen resources in the environment. The use of honey bees in Belize to support the incomes of local people is growing in popularity, with beekeeping schemes being implemented at local and national levels. For this to be successful healthy bee colonies are needed. This chapter therefore aims to investigate honey bee pollen diet over a gradient of landscapes to assess potential effects on bee health. Bee bread samples were collected from across the Cayo and Toledo districts of Belize and pollen content was identified. Pollen richness and diversity were then compared across a gradient of landscapes ranging in diversity (Shannons Diversity) within 500 m and 1 km radii of the hives. Pollen richness and diversity were found to be negatively related to increasing landscape diversity. This was thought to be due to the specific relationship between each plant taxa and the changing environment along with bee preferences for certain species. The dominance/abundance of preferred pollen taxa in different landscapes has a strong influence over how that landscape influences diversity and species richness of pollen diet. A high presence of preferred species, would lead to a lower diversity in pollen, even if in a mixed landscape where the diversity of available floral resources is high. This perhaps suggests that honey bee preference and subsequent recruitment is more important than landscape diversity, at least in situations where no landscapes are short of floral resources.

3.1 Introduction

Land use change is largely associated with the expansion of cropland, and leads to loss and degradation of natural habitat, it is one of the main drivers of biodiversity loss (Grau et al., 2013). Today over one third of the world's land surface is used for agropastoral production, including plant and animal based systems, monocultures and mixed farming, with small land holders (<2 ha) making up approximately 24% of agricultural land (Balvanera et al., 2019). Conversion of natural landscapes to crop systems is cited as a primary driver of wild and managed honey bee decline (Potts, Biesmeijer, et al., 2010). Currently the impact of land use change is concentrated in the tropics where diverse natural habitats are being converted to agricultural land uses (Gibbs et al., 2010; Meyfroidt et al., 2010). This raises concerns as to how tropical fauna, and in particular bee taxa, will respond to this land conversion (Cely-Santos and Philpott, 2019). This study therefore asks how a change in landscape can influence honey bee diet, in the tropical country of Belize.

At the landscape level, land use diversity, landscape connectivity, and proximity to undisturbed forest fragments have been shown to benefit bees, due to the availability of abundant and high quality nutritional resources (Cely-Santos and Philpott, 2019). The benefits include increasing species richness, diversity and abundance (Quistberg et al., 2016), and have been shown to alter community compositions (Brosi et al., 2008). Landscape quality has been demonstrated to differentially impact the health of both individual bees and their colonies (Smart et al., 2017). For example, in tropical environments tree nesting meliponines (social stingless bees) have been associated with larger forest fragments, whereas *Apis* populations showed the opposite pattern, and were more prevalent in pastures (Brosi et al., 2007, 2008). Similarly, Saturni et al. (2016) found native bee abundance, richness and diversity was positively impacted by forest cover but honey bee abundance was negatively affected. Specialised bees with low-dispersal ability are thought to be more strongly affected by agricultural intensification and subsequent fragmentation compared to generalised species with high-dispersal ability (Cely-Santos and Philpott, 2019).

Honey bees forage on flowering plants as their primary source of nutrition (Donkersley et al., 2017) from which they acquire amino acids, vitamins, proteins and carbohydrates from the pollen and nectar (Morgano et al., 2012). The previous investigation carried out in Chapter 2 demonstrated the importance of trees in the honey bee diet in Belize, and thus the bees preference for these species. Bees will experience nutritional stress when their choice of host plants is limited or when only sub-optimal plants are available (Vaudo et al., 2015). Malnutrition from poor pollen diet has been shown to lead to smaller hypopharyngeal glands (DeGrandi-Hoffman et al., 2010), decreased learning abilities

(Arien et al., 2018), increased susceptibility to deformed wing virus (DeGrandi-Hoffman et al., 2010), a compromised immune system (Di Pasquale et al., 2013) and a shorter life span (Wang et al., 2014). This chapter will investigate whether the landscape which surrounds honey bee hives impacts their pollen diet and therefore infer the potential effects of landscape on honey bee health.

Due to the variation of crude protein content and amino acid presence in different plant families, as discussed in chapter 2, the quality of floral resources available to bees has the potential to vary with the landscape. The floral resources within a landscape are heterogeneous in space and time, and thus the resources bees have access to will vary over the year (Ogilvie and Forrest, 2017). A complex landscape is much more likely to ensure a continuous source of pollen and nectar than a simple one, where there may be huge quantities in some seasons and low levels in others. Complex landscapes also enhance resource diversity available to its inhabitants (Donkersley et al., 2017) and thus the proteins and amino acids available. A landscape maybe complex if it either had few ecosystems types but which contain great resource diversity or if it contains many ecosystems types. For example, areas highly dominated by only agriculture may offer a more simplified diet due to a high dominance of certain crop species, however in a landscape containing forest and natural grasslands, small amounts of agriculture will act to increase resource diversity. For example, Kaluza et al. (2017) found that for the social generalist stingless bee *Tetragonula carbonaria*, Meliponini, pollen diversity increased in floristically rich landscapes, gardens and forests, when compared to orchards. This is particularly important if homogenous landscapes are dominated by plants with pollen low in crude protein, such as maize (Höcherl et al., 2012), as it may drastically reduce the crude protein content available to the bees, or cause them to increase their foraging distance/intensity, possibly resulting health effects on the colony. With Africanised honey bees it is also important to consider their ability to store large amount of pollen, even greater than the European honey bee (Danka et al., 1987), and therefore they have the potential to buffer themselves from landscape simplification. This means that if pollen becomes a limited resource they have the ability to access past stores, an ability not all bee species have. Landscape composition can be characterised not only by the habitat types from which it is formed but also by landscape diversity which considers both the richness and evenness of the landscape (both the number of different habitat types, and the contribution made by each, in a given radius). It is assumed that landscape diversity (based on the Shannon index) will provide a good indication as to the potential pollen resources available to the honey bees (Danner et al., 2017).

Importantly, honey bees are able to recruit foragers to particular resources through the waggle dance, a complex behaviour in which nestmates direct each other to profitable food resources via communication or direction and distance from the hive (Simpson and von Frisch, 1969). This may impact how the diversity of pollen in the landscape is seen in their pollen diet. For example, it is possible that even with a high variety of resources available, this recruitment behaviour may mean that this is not utilised and that high density areas of a particular species is favoured, even if a more varied diet would be more beneficial nutritionally.

This chapter was carried out in Belize where the main forest type is tropical broadleaf (Wainwright et al., 2015), often dominated by *Manilkara zapota, Vitex gaumeri, Schizolobium parahyba, Sebastiania tuerckheimiana , Manilkara chicle, Swietenia macrophylla, Spondias mombin* and *Protium copal* (Penn et al., 2004). Belize is the only country remaining in Central America where there is still over 60% forest cover, however, it is predicted that by 2045 forest cover will have fallen below 50% (Latha et al., 2016). The current conversion of forest to agriculture across Belize makes it a prime location to study the effects of land use change on bee health as it has created clear gradients between these environments. Additionally, the use of apiculture as an additional source of income for local farmers is increasing in popularity, due to the diverse range of bee products including honey, wax and royal jelly, increasing agricultural produce, as well as the increased pollination of farms. Currently, there is a push towards beekeeping in order to supplement incomes and contribute to a growing economy, with the Ministry of Agriculture launching the Honey Production Redevelopment Support Project to support farmers in 2009 ("Ministry of Agriculture launches Honey Production support project", 2019). Placing hives in suitable locations, will help to ensure a strong and healthy colony and thus high production is therefore of great importance.

Limited research has been carried out on the effects of landscape on bee pollen diet. That which has been carried out conflicts in results and covers a wide array of methods and bee species (refer to chapter 1). Furthermore, research carried out in the tropics is particularly limited. The chapter consequently aims to investigate whether the Africanised honey bee (*Apis mellifera* Africanised) pollen diet varies across gradients of high to low landscape diversity, in terms of species richness, diversity, and composition. The most common and frequently occurring pollen taxa will be studied independently to determine how their presence is impacted by the surrounding landscape both in terms of diversity and forest/agricultural dominance.

3.2 Methods

3.2.1 Study Sites

Bee bread samples were collected from 14 apiaries in the Toledo and Cayo districts of Belize (Fig. 3.1). Agricultural land and human settlements cover 20% of Belize, while the rest is formed of natural habitats including savannahs, scrublands, wetlands as well as lowland and submontane broadleaf and pine forests (Flowers et al., 2020). Lowland broadleaved forests are a diverse forest type and are characterised by tropical tree species such as *Simarouba glauca, Calophyllum brasiliense* and *Terminalia Amazonia* (Fleming et al., 2011). The submontane broadleaved forest shows characteristic vegetation of the Maya mountain and includes species such as *Podocarpus guatemalensis, Swietenia macrophylla* and *Virola brachycarpa.* Another common habitat is savannah which consists of open vegetation of which typical trees include *Acoelorraphe wrightii, Quercus oleoides* and *Gliricidia sepium* (Fleming et al., 2011). Belize has a tropical climate with pronounced wet and dry seasons, although temperatures vary with proximity to the coast and elevation. Average coastal temperatures range from 24° C in January to 27° C in July, whilst inland temperatures are slightly higher. Average rainfall ranges from 1,350 mm/yr in the north and west, but can reach 4,500 mm/yr in the south. Seasonal differences in rainfall are greatest in the northern and central regions, where between January and May less than 100 mm of rain occurs per month (Fleming et al., 2011). The Cayo district is in central Belize and is a mosaic of lowland secondary broadleaf moist forest, short-grass savannah, shrubland, wetland and agriculture (Foster et al., 2020). Agricultural activities include livestock farming, citrus and sugar cane plantations as well as multicrop slash and burn farms. Toledo is the southernmost district in Belize, where the west boarders Guatemala and the east the Caribbean Sea. The northern boundary of Toledo runs along the main divide of the Maya Mountains (Emch et al., 2005), and encompasses the Maya Golden Landscape (MGL), a 302,259 acre mosaic of private and nationally protected areas, private lands and Mayan and Hispanic communities (Voight et al., 2019). It is comprised of a diverse range of forest habitats, including upland and lowland broadleaf forests and mangrove swamps ("Mayan Golden Landscape", 2017). Land in Toledo is predominantly farmed through slash and burn agriculture, but more recently had experienced growth in large scale citrus and banana plantations (Voight et al., 2019).

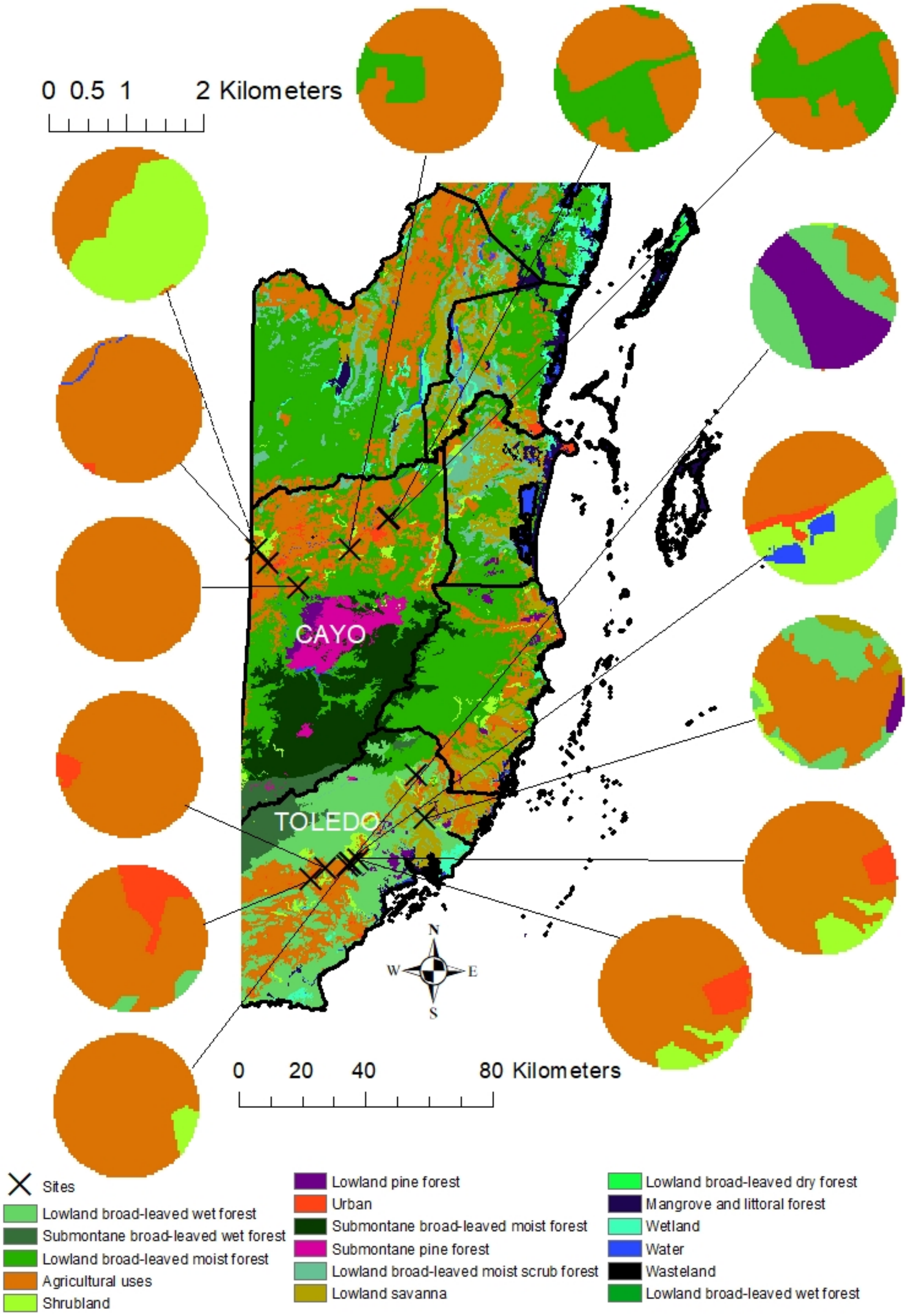


Figure 3.1. Map of the study area, apiary sites are represented by back crosses, where there are 8 sites in the Toledo District (South) and 6 in the Cayo District (North). Each site is displayed with a 1 km buffer radius to show the landscape surrounding each site more closely. Ecosystems are based on the 2017 land use map provided by Meerman and Clabaugh (2017).

3.2.2 Landscape data

Landscape data were based on LANDSAT remote sensing data (30 m spatial resolution) from 2017 provided by Meerman and Clabaugh (2017). Meerman and Clabaugh (2017) classifies land cover into 16 landscape classes as described in Table 3.1. For the purpose of analysis lowland broad-leaved wet forest, submontane broad-leaved wet forest, lowland broad-leaved moist forest, lowland pine forest, submontane broad-leaved moist forest, submontane pine forest, lowland broad-leaved moist scrub forest, lowland savannah, lowland broad-leaved dry forest, mangrove and lowland broad-leaved wet forest were aggregated into one category, forest. Alternative models were run where pine forest types and savannah were also kept separate as well as shrubland; however this did not change the outcome of the results, and so the initial combination was kept.

For this study resource density in the areas surrounding the hives was not studied due to time restrictions. Ideally, a second visit would have been made to access the abundances and densities of the plants within a 1 km area of the hives. However due to COVID restrictions a second visit was not possible. Limited information is available in the literature in regards to the penology of the area, with only a checklist of the vascular plants of the Chiquibul forest Cayo and lowlands savannah available (Bridgewater et al., 2006; Goodwin et al., 2013).

Table 3.1. Descriptions of land use types present in the 2017 map of Belize by Meerman and Clabaugh (2017). Descriptions are largely based off Meerman and Sabido (2001).

Land use type	Description
Lowland broad-leaved wet forest	Located at less than 50 m and experiences near 4000 mm of rainfall a year, with a dry season between February and May.
Submontane broad-leaved wet forest	This forest type is at the medium altitude (> 500 m). Experiences high rainfall of near 4000 mm a year, with a dry season between February and May.
Lowland broad-leaved moist forest	Located at less than 50 m. Experiences rainfall of between 2500 - 4000 mm a year, with a dry season between February and May.
Agricultural uses	Land modified to grow or raise biological products for human consumption. Includes cropland, pasture and orchards.
Shrubland	Composed mainly of woody phanerophytes, 0.5 - 5 m tall. Crowns may be touching but cover at least 30% of the sky.
Lowland pine forest	Located at less than 50 m. A coniferous forest predominated by pine trees.
Urban	Human settlement with high population density.
Submontane broad-leaved moist forest	This forest type is at the medium altitude (> 500 m). Experiences rainfall of between 2500 - 4000 mm a year, with a dry season between February and May.
Submontane pine forest	This forest type is at the medium altitude (> 500 m). A coniferous forest predominated by pine trees.
Lowland broad-leaved moist scrub forest	Located at less than 50 m. Experiences rainfall of between 2500 - 4000 mm a year, with a dry season between February and May. Vegetation is sparse, dominated by low trees and woody shrubs.
Lowland savannah	Typical Belizean savannahs are found on gently sloping alluvial deposits in the coastal plain. Generally low in nutrients and experience extremes in water availability. Species poor but highly specialised. Often short-grass savannahs to pine woodland.
Lowland broad-leaved dry forest	Located at less than 50 m and experience less than 2500 mm of rainfall a year, with a dry season between February and May.
Mangrove and littoral forest	Forests along the coasts and cays featuring salt-tolerant vegetation, and mangroves growing in coastal saline water.
Wetland	An ecosystem flooded by water for prolonged periods of time, includes swamps and marches
Water	Area covered by water, includes lakes, rivers and sea.
Wasteland	Barren and uncultivated land.

3.2.3 Landscape Metrics

This study aimed to investigate the impact of the landscape on honey bee diet. To characterise the landscape and its composition (1) percentage forest cover, (2), landscape Shannon Diversity and (3) percentage agricultural cover were calculated. Further metrics were calculated to assess the impact of landscape configuration, including (4) mean patch size and (5) edge density. Descriptions of these landscape metrics are given in Table 3.2. Metrics were calculated using the landscapemetrics package (Hesselbarth et al., 2020) in R (R Core Team, 2017) and Meerman and Clabaugh's (2017) Belize Ecosystem map. Landscape metrics were quantified in circular buffers surrounding the centre of the apiaries with radii of 500 m, 1 km, 2 km, and 10 km. These buffer extents were considered appropriate because they encompass the minimum and maximum foraging distances of honey bees (Al-Ghamdi et al., 2016; Beekman and Ratnieks, 2000; Couvillon et al., 2014; Ratnieks and Shackleton, 2015; Sande et al., 2009; Schneider, 1989). Model selection was used to identify which metrics best explained the greatest variation in the response variables, pollen richness and diversity.

Table 3.2. Landscape metrics describing the composition and configuration of landscapes surrounding the 14 apiary sites. Metrics are based on the 16 landscape types described by Meerman (2017). For all metrics, lowland broad-leaved wet forest, submontane broad-leaved wet forest, lowland broad-leaved moist forest, lowland pine forest, submontane broad-leaved moist forest, submontane pine forest, lowland broad-leaved moist scrub forest, lowland savannah, lowland broad-leaved dry forest and mangrove were aggregated into one category, forest.

Landscape Metric	Description
Composition	
Forest cover (%)	Percentage of forest cover in the buffer radii. $CA = sum(AREA[patch_{ij}])$ *where $AREA[patch_{ij}]$ is the area of each patch in hectares.*
Agricultural Cover (%)	Percentage of agricultural cover in the buffer radii. $CA = sum(AREA[patch_{ij}])$ *where $AREA[patch_{ij}]$ is the area of each patch in hectares.*
Shannon's Diversity Index – Landscape (H)	Shannon's Diversity Index for each buffer radii, measuring richness and evenness of the land covers. $SHDI = -\sum_{i=j}^{m} P_i * lnP_i$ *where Pi is the proportion of class i.*
Configuration	
Mean patch area (ha)	Mean area of continuous land covers in the buffer radii. $AREA_{MN} = mean(AREA[patch_{ij}])$ *where $AREA[patch_{ij}]$ is the area of each patch in hectares*
Edge Density (m/ha)	All edges in the landscape in relation to the landscape area. $E A = E/A * 10000$ *where E is the total landscape edge in meters and A is the total landscape area in square meters.*

3.2.4 Bee Bread Sampling

Bee bread samples were collected between the 23rd May and the 25th June 2019. These months were chosen as it has been shown that the honey bees in the neotropics are most active in pollen collection during the dry season, (Pesante, 1985), corresponding with seasonal peaks in flowering (Augspurger, 1983; Frankie et al., 1974; Roubik, 1979). In Belize the dry season occurs between February to May (Renton, 2006). The late dry season was chosen for collecting bee bread as was towards the end/immediately after heavy pollen collection, and thus provided a good representation of the pollen collected. Apiaries were visited twice during this time, once at the start and once at the end of the field season, to capture the breadth of pollen taxa collected during this time frame.

Bee bread was collected from between 1 and 6 hives per apiary. Each apiary was visited twice, where on the second visit bee bread was collected from a different hive to the first visit. The number of hives sampled depended on the number of hives available at an apiary as well as the health of the hive. Bee bread was not collected from hives where it covered less than 2 frames, so that the bees still had access to pollen stores. To ensure the most recent bee bread was taken from inside the hive, it was always sampled from the brood box and taken from furthest frames containing bee bread on either side, or from new combs as this is the most recently acquired bee bread. 50 cells containing fresh bee bread were removed from the frame. Samples collected were dry and grainy, whilst, samples of entombed pollen (pollen covered by propolis), or pollen that appeared moist and dull in colour (in comparison) were not taken (USDA APHIS, 2018). Bee bread was collected as one complete block of cells, however, in two hives where the pollen across the frame was more dispersed cells were taken from different areas of the same frame. In one case (site 5) where little bee bread was present in the hives, fewer cells were removed to ensure enough pollen was left for the colony. The removed combes were placed in separate sterile ziplock bags for each hive. A pin was used to remove the pollen from the cells and collected into a 50 ml polypropylene sample tubes. A different pin and sample tube was used for each hive sample to avoid cross-contamination. Inside the airtight sample tubes were placed 10 mg of Silica Gel (RS ®) to dry out the pollen.

3.2.5 Pollen Identification

Analysis of pollen species composition was carried out at the School of Geography and Environmental Science, University of Southampton, UK. Pollen samples were initially manually homogenised by crushing pollen so that the maximum sized clumps were no more than 1 mm. Following this, three sub-samples of 0.25 g were weighed into 15 ml centrifuge tubes to best represent the initial samples taken.

For each sub-sample, 14 ml of distilled boiling water was added, the sample was then subsequently centrifuged at 3000 RPM for 3 min, after which the water was decanted. Following this, each sample was dehydrated with 5 ml of glacial acetic acid and then centrifuged for 3 min and 3000 RPM, and the water and glacial acetic acid were then decanted. Next, 9 ml of glacial acetic acid and 1 ml sulphuric acid was added to the sample and stirred. The sample tube was then placed in boiling water for 5 min before the reaction was disabled by adding 1 ml of potassium hydroxide, after which it was again centrifuged for 3 minutes. The sample was decanted and then 7 ml of distilled water and 1 drop of saffron ink to dye the pollen grains were added, and the sample was then centrifuged for 3 minutes.

One drop of each sample was then mounted with glycerine jelly and sealed with paraffin for permanent preparations (Dimou et al., 2006).

At least 500 pollen grains were identified per sub-sample under the microscope (Nikon Eclipse 80i), providing a total of 1500 grains per complete sample. According to Lau et al (2016), this number is sufficient for accurate identification of plant taxa present, however as this is based on pollen pellets rather than bee bread, analyses were carried out to confirm this number is sufficient (see Chapter 2). Pollen identification was made primarily using the Atlas of pollen and spore of the Florida Everglades (Jensen et al., 2011), and the Amazon Pollen Manual and Atlas (Collinvaux, 2014). Identification was based on the ornamentation, orientation, size and surface structure of the grain, and identified to the lowest taxonomic level possible.

Pollen taxonomic diversity in each hive sample was calculated using the Shannon-Weaver diversity index to characterise taxonomic richness and evenness for each site. The Shannon-Weaver diversity index (*H'*) was calculated using the equation:

$$\boldsymbol{H^i = \sum_{i=1}^{S} P_i ln P_i}$$

where *pi* is the proportion of each pollen type (*i*) is the sample and *ln* is the natural logarithm. A greater *H'* value indicates greater taxonomic diversity (Lau et al., 2019). In addition, pollen taxa richness was calculated.

3.2.6 Data analysis

Data were analysed using generalized linear mixed effects models. Pollen species richness and the diversity of pollen species were taken as response variables. Pollen species richness was fitted with a binomial distribution to account for over dispersion whilst pollen diversity was fitted to normal distributions. A model-selection framework (Burnham and Anderson, 2002) was used to assess the most appropriate and parsimonious models. The main effects used were percentage forest, percentage agriculture, total edge density, forest edge density, agricultural edge density, mean patch area and location. Location was included due to the differences in floral species and agricultural practises between the regions (Fleming et al., 2011; Voight et al., 2019). The random effects used were site and day. Full models are provided on the following page:

Pollen Species Richness~Location*Average Patch Size*Agriculture Edge*Forest Edge*Percentage Agriculture*Percentage Forest*Landscape Diversity + (1|Site) + (1|Day)

Pollen Diversity~Location*Average Patch Size*Agriculture Edge*Forest Edge*Percentage Agriculture*Percentage Forest*Landscape Diversity + (1|Site) + (1|Day)

Candidate models were compared using automated AIC (Akaike's Information Criterion) model selection occurred using the "MuMIn" package (Barto, 2016) in R (R Core Team, 2017). Only models with substantial support (ΔAICc < 2) were considered, and were then ranked by AIC weight to determine the best fitted model (Burnham and Anderson, 2004). AIC values were chosen as they balance model fit with model complexity (Miljanic et al., 2019). Landscape metrics were checked to insure that collinearity did not occur between them (Spearmans Rank Correlation Coefficient > 0.7). This process was carried out for all buffer radii to ensure that both the best performing buffer radius was chosen along with the most appropriate explanatory variables. Where there was collinearity between variables one was removed from the model, for example percentage forest and percentage agriculture were not included at the same time.

After model selection the best models were assessed to determine whether they met key statistical assumptions, including normality of model residuals, homoscedasticity and whether linear relationships held using DHARMa (Hartig, 2020) in R (R Core Team, 2017). The best fitted models met all key assumptions for linear models including homoscedasticity, normality of residuals and linearity.

Pollen richness and diversity models were run with and without outliers (site 1), the results remained the same for each. In the final model site 1 was kept to avoid reducing the rage of landscape diversity.

The most abundant and frequently occurring pollen taxa, Areaceae 1, Urticaceae 1, Burseraceae 1 and Fabaceae 6 were investigated further to determine how the landscape at a 500 m buffer radius surrounding the hives impacted their abundance (%). A 500 m buffer zone was chosen as all models had substantial support (ΔAICc < 2) and consistency was kept with previous models analysing species richness. A model selection process was carried out using the "MuMIn" package (Barto, 2016) in R (R Core Team, 2017) using a generalized mixed effects model with a Quasi-Poisson distribution. The model selection included "Location", "Percentage Forest", "Percentage Agriculture", "Forest Edge", "Agricultural Edge", "Average Patch Size", "Landscape Diversity (Shannons)" and "Total Edge Density", as well as "Site" and "Day" as random factors, and was run for each pollen taxa. Models with substantial support (ΔAICc < 2) were chosen and were ranked by AIC. Models were checked so that there was no collinearity between the metrics (Spearmans Rank Correlation Coefficient > 0.7).

To assess for differences in pollen taxa composition across a gradient from low to high landscape diversity at 1 km and 500 m scales in the Toledo and Cayo districts, non-metric multidimensional scaling was performed based on the Bray-Cutis similarity matrix (Clarke and Warwick, 1994). This was done using the vegan package (Oksanen F et al., 2020) in R (R Core Team, 2017). Contour lines were included to display landscape diversity at each site. To assess strength of correlation between pollen community composition and landscape diversity a Mantel test was used. The environmental (landscape diversity) matrix was constructed based on Euclidean distance using Shannon's diversity. Standardised Mantel statistics were based on Spearman's rank correlation coefficients, and significance was assessed using randomization tests with 1000 permutations (Prada et al., 2017).

3.3 Results

3.3.1 Model selection

The most parsimonious model used to understand how landscape influenced pollen diversity and species richness from hives across Belize contained landscape diversity as the only main explanatory variable. Further landscape metrics were not included in either model as their inclusion resulted in the ΔAIC being greater than 2 in all cases. The impact on landscape diversity on pollen species richness had the lowest AIC value at a 500 m radius whilst the impact on pollen diversity was lowest at 1 km:

- Pollen species richness <- landscape diversity (Shannon's) + location + (1|Site) + (1|Day)
- Pollen diversity <- landscape diversity (Shannon's) + location + (1|Site) + (1|Day)

The following four models were identified as the most parsimonious. They all best explained at a 500m radius how landscape affected the abundance of Uritaceae 1, Areaceae 1, Burseraceae 1 and Fabaceae 6:

- Urticaceae 1 <- Urticaceae 1 ~ Percentage Agriculture + Forest Edge + Total Edge + (1|Site) + (1|Day)
- Areaceae 1 <- Areaceae 1 ~ Percentage Forest + (1|Site) + (1|Day)
- Burseraceae 1 <- Burseraceae 1 ~ Percentage Agriculture + Landscape Diversity (Shannon's) + (1|Site) + (1|Day)
- Fabaceae 6 <- Fabaceae 6 ~ Agricultural Edge + Average Patch Size + Forest Edge + (1|Site) + (1|Day)

Models at 500 m and 1 km buffer areas were suspected to be the most informative as although bees can forage up to 10 km or more, the mean foraging distance for the Africanised honey bee is estimated to be just over 1 km (Schneider and Hall, 1997), and thus it is likely that the majority of bees were foraging around this range.

3.3.2 Pollen species richness and abundance

In total, 82 different pollen taxa belonging to 38 different plant families were identified from the 14 apiaries. On average 15 (± 0.890, SE) different pollen taxa were present in each hive sample, but ranged from between 5 taxa at site 11 and 28 taxa at site 14. The most frequently occurring taxa were Areaceae 1, Urticaceae 1, Burseraceae 1 and Fabaceae 6. Pollen diversity at each hive ranged from between 0.113 and 2.320, and had a mean of 1.386 (± 0.082), where the highest pollen diversity was found at a hive in site 9, Cayo, the lowest pollen diversity was found at site 3, Toledo.

The 10 most abundant pollen taxa made up over 80% of the pollen taxa identified, all of which belonged to forest species with the exception of two, Myrtaceae 1 of which the type is unknown and Anacardiaceae 1 which is likely cashew and grown for agricultural purposes. These 8 tree species made up at least 70% of 70% of hive samples, and over 60% of all but 3 hive samples. The most abundant pollen taxa were Areaceae 1, Urticaceae 1, Burseraceae 1 and Fabaceae 6, and together made up over 60% of the pollen collected.

3.3.3 The landscape at a 500 m and 1 km radius around each hive

Within a 500 m radius of the hives landscape diversity ranged from between 0 to 1.21 with a mean of 0.372 (± 0.095) (Table 3.3). Site 1 was the most diverse and contained 4 different habitats including shrubland, agricultural land water and urban. Sites 8, 13 and 14 were the least diverse and only contained one habitat type (Table 3.3). The percentage of forest averaged (mean) 19% (± 8.791) across the sites whilst percentage agriculture averaged (mean) 68% (± 9.373).

Table 3.3. Landscape diversity, percentage forest, percentage agriculture and the number of habitats within a 500 m radius of the honey bee hives located at each of the 14 sites. Landscape diversity ranged from between 0 and 1.210 on the Shannons diversity index, percentage forest and percentage agriculture ranged between 0% and 99%. The number of habitats at each site ranged from between 1 and 4. Sites 1-8 are from Toledo, and 9-14 from Cayo.

Site	Landscape Diversity	Percentage Forest	Percentage Agriculture	Number of habitats
1	1.210	0.000	46.129	4
2	0.399	13.55975	85.794	2
3	0.549	99.22697	0.228	2
4	0.193	0.000	94.918	3
5	0.063	0.000	98.086	2
6	0.000	0.000	99.734	1
7	0.433	0.000	83.766	2
8	0.000	0.000	99.480	1
9	0.615	30.411	69.193	2
10	0.598	70.967	28.387	2
11	0.680	58.041	41.567	2
12	0.474	0.000	17.995	2
13	0.000	0.000	99.227	1
14	0.000	0.000	99.480	1

Within a 1 km radius of the hives landscape diversity ranged from between 0 to 1.170 and had a mean of 0.546 (± 0.092) (Table 3.4). Site 1 was again the most diverse and contained 5 different habitats including forest, shrubland, agricultural land, water and urban. Site 14 was the least diverse and only contained one habitat type (Table 3.4). Percentage forest had a mean of 16% (± 7.071) across the sites whilst percentage agriculture averaged (mean) 70% (± 7.226).

Table 3.4. Landscape diversity, percentage forest, percentage agriculture and the number of habitats within a 1 km radius of the honey bee hives located at each of the 14 sites. Landscape diversity ranged from between 0 and 1.170 on the Shannons diversity index, percentage forest ranged between 0 and 87% whilst percentage agriculture ranged from between 12% and 99%. The number of habitats at each site ranged from between 1 and 4. *Sites 1-8 are from Toledo, and 9-14 from Cayo.*

Site	Landscape Diversity	Percentage Forest	Percentage Agriculture	Number of habitats
1	1.170	4.177	46.237	5
2	0.895	33.482	62.500	3
3	0.997	87.691	12.181	3
4	0.567	0.000	83.546	3
5	0.553	0.000	83.546	3
6	0.196	0.000	95.026	2
7	0.605	4.145	80.038	3
8	0.156	0.000	96.620	2
9	0.416	14.605	85.459	2
10	0.693	48.788	51.339	2
11	0.666	38.584	61.862	2
12	0.63	0.000	32.526	2
13	0.101	0.000	98.214	3
14	0.000	0.000	99.809	1

Whilst the gradient in landscape diversity is not huge, the following results are unlikely to be due to the presence of urban and water habitats at some sites. Firstly, water was only present at site 1 at 500 m, where only 1 hive was present and pollen diversity and species richness represented median values; for example out, of the 46 hives it had the 26th highest species richness. At 1 km only two sites contained water, again site 1 and site 13, here species richness ranged from the 8th lowest to the 2nd highest so is again unlikely to have impacted the results. Alternatively, urban habitat was seen in just under half the sites at 1 km, some of which had low pollen richness other of which had high richness.

Consequently, these results are likely true to the real variation in landscape seen at the ground level between these sites and not the presence or absence of water/urban habitat.

3.3.4 Impacts of landscape diversity on pollen taxa diversity and species richness

To determine how landscape influenced the bee pollen diet in Belize, the impact of landscape diversity on both pollen diversity and species richness was analysed. There was no impact of location on either pollen diversity or species richness.

When landscape diversity was at its lowest, 0, pollen diversity had confidence intervals of between 1.6 and 2.1 with a mean of 1.9, whilst at a landscape diversity of 1.2, pollen diversity had a mean of 1 and confidence intervals of between 0.5 and 1.3 (Fig. 3.2). Within a 1 km radius of the hives landscape diversity significantly influences pollen diversity found in bee bread, whereas lower landscape diversity resulted in greater pollen diversity (t = -3.907, df = 43, p <0.001).

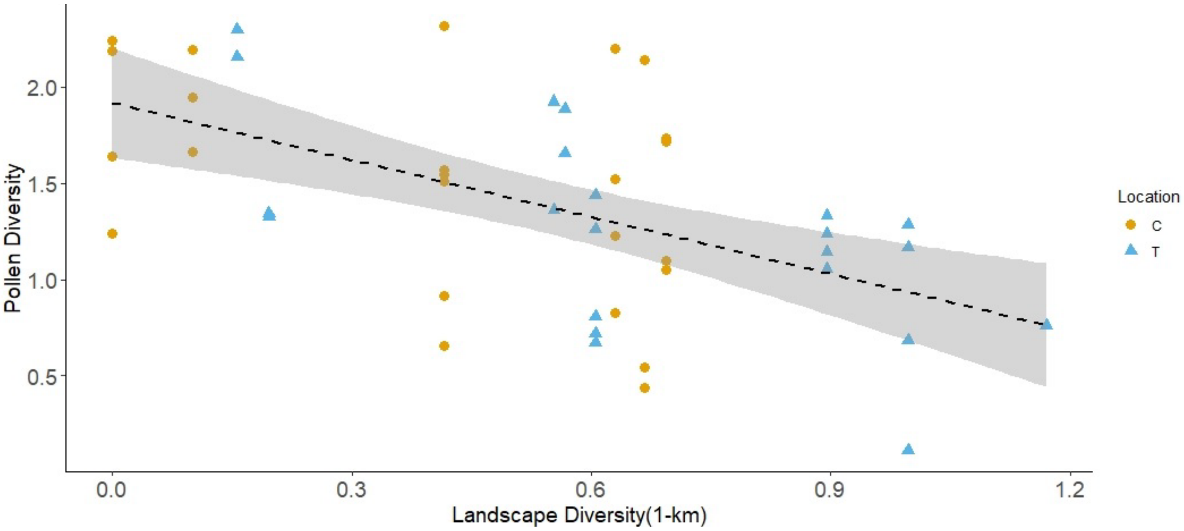


Figure 3.2. The effect of landscape diversity on pollen diversity at a 1 km buffer radius. Toledo (T) hives are shown in blue triangles and Cayo (C) hives in orange circles. There was a negative effect of landscape diversity on pollen diversity within a 1 km radius.

There was a negative effect of landscape diversity on pollen species richness at a 500 m radius. Pollen species richness was greatest when landscape diversity was lowest (z = -3.549, df = 43, p < 0.001). When landscape diversity was at its lowest, 0, pollen species richness had a mean of 18 species, with confidence intervals of between 16 and 20 species. By contrast, when landscape diversity was at its highest 1.20 pollen species richness had confidence intervals of between 2 and 16 species, with a mean of 8 (Fig. 3.3).

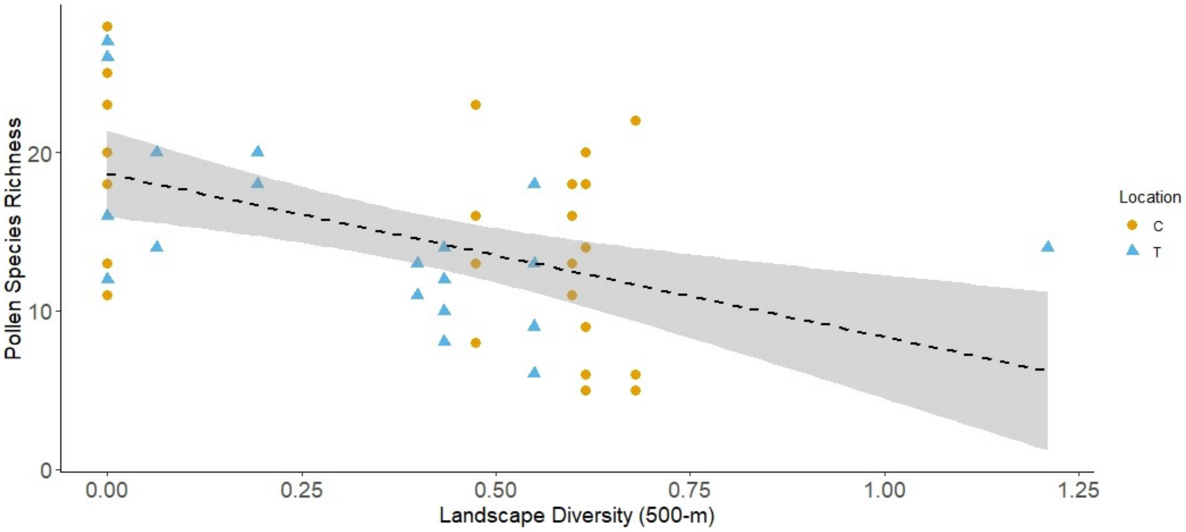


Figure 3.3. The effect of landscape diversity on pollen species richness at a 500 m buffer radius. Toledo (T) hives are shown in blue triangles and Cayo (C) hives in orange circles. There was a negative effect of landscape diversity on pollen species richness within a 500 m radius of the beehives.

The negative impact of landscape diversity on pollen species richness and diversity raised the question as to whether one landscape in particular, forest or agriculture, could affect either pollen species richness or species diversity, driving the impact of landscape diversity. Pollen diversity however was not impacted by either percentage forest (t = 1.450, df = 43, p = 0.177) or percentage agriculture (t = 0.488, df = 43, p = 0.500). Pollen richness was also not impacted by either percentage forest (t = -0.337, df = 40, p = 0.327) or percentage agriculture (t = 0.981, df = 40, p = 0.736).

3.3.5 Impact of landscape on the abundance of the most frequent and most abundant pollen taxa; Areaceae 1, Urticaceae 1, Burseraceae 1 and Fabaceae 6

To further unpick the relationship between the landscape and pollen taxa found in the bee bread samples, the effect of various landscape metrics on the abundance of the dominant four species, Areaceae 1, Urticaceae 1, Burseraceae 1 and Fabaceae 6 at the hive level was studied.

The model selection procedure identified the impact of percentage forest at a 500 m buffer radius on the abundance of Areaceae 1 as the most parsimonious model with substantial support (ΔAICc < 2). Percentage forest significantly increased the abundance of Areaceae pollen found in bee bread, where a lower percentage forest resulted in low Areaceae abundances ($z = 2.754$, $df = 41$, $p < 0.001$). When percentage forest was at 0% Areaceae 1 abundance was also low, with a mean of 20% and confidence intervals of between 14% and 25% (Fig. 3.4). At 100% forest the mean abundance of Areaceae 1 was just over 50% with confidence intervals between around 40% and 70% (Fig. 3.4).

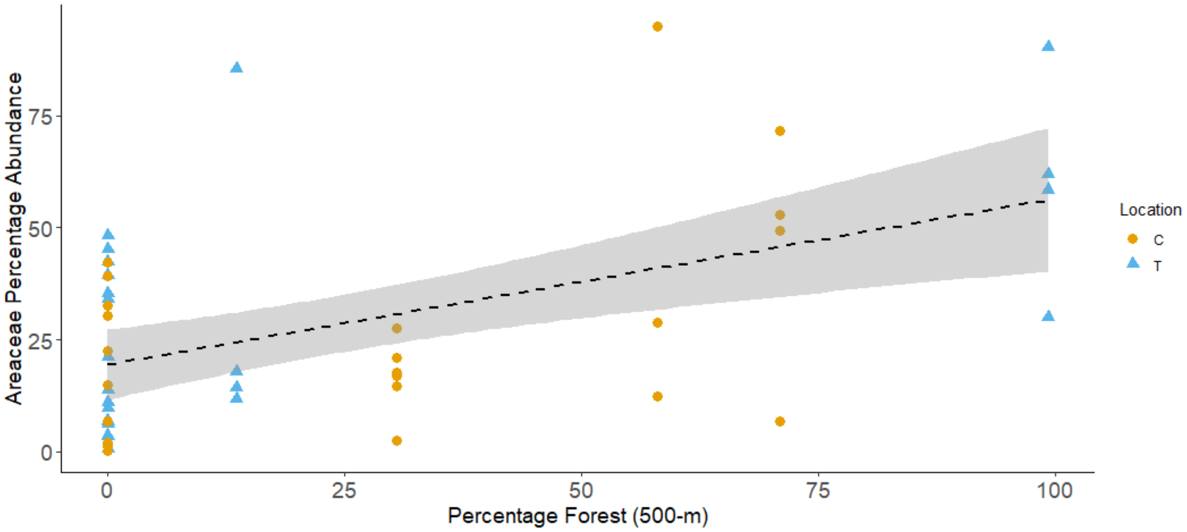


Figure 3.4. The effect of percentage forest on Areaceae (Attalea cohune) abundance (%) at a 500 m buffer radius. Toledo (T) sites are shown in blue triangles and Cayo (C) hives in orange circles. As percentage forest increased so did the abundance of Attalea Cohune.

The most parsimonious model with substantial support for the impact of landscape on the abundance of Uritaceae 1, included total edge, forest edge and percentage agriculture as the main variables. There was a positive impact of total edge (z = -3.356, df = 41, p < 0.001) and forest edge (z = 3.115, df = 41, p = 0.001) on the abundance of Urticaceae 1, where as forest edge and total edge increased so did the abundance of Uritaceae 1 (Fig. 3.5). Where total edge density was at 0 m/ha mean Urtitaceae abundance was at 12.5% with confidence intervals of between 0% and 25%, and by 20 m/ha mean Uritaceae abundance reached 20% with confidence intervals of between 10% and 25%. A similar relationship was found between forest edge and Uritaceae abundance, however the relationship appears stronger with a mean of over 25% Uritaceae abundance with confidence intervals of between 12.5% and 40% at a forest edge density of 30 m/ha. There was a strong negative relationship between percentage agriculture and Uritaceae 1 abundance (z = -3.499, df=41, p <0.001) (Fig. 3.5). At 0% agriculture Uritaceae 1 abundance reached around 30% with confidence intervals of between 23% and just under 50%, whilst at 100% agriculture there was a mean Uritaceae abundance of 20% with confidence intervals of between around 10% and 25%.

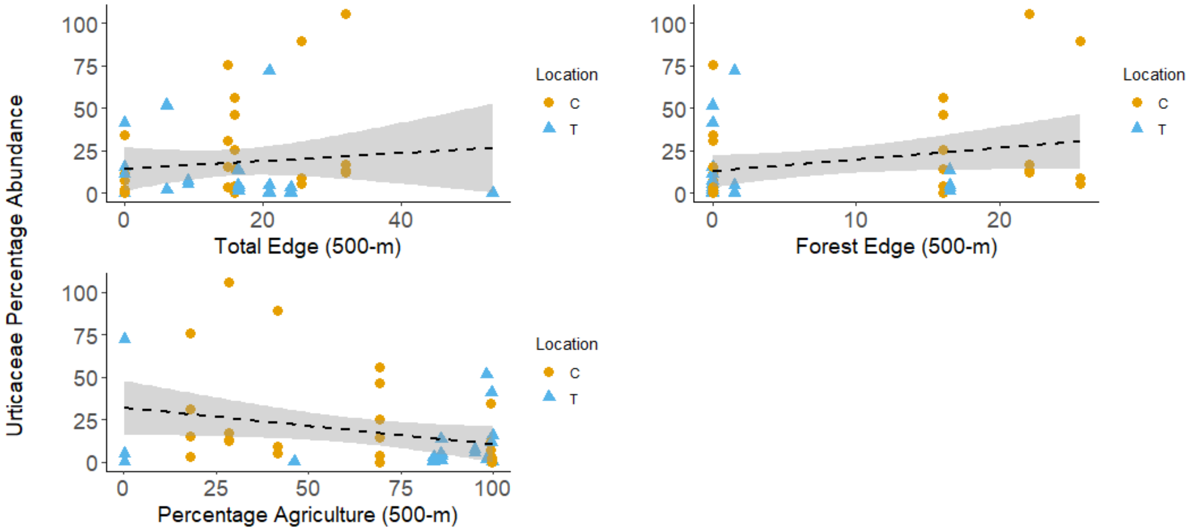


Figure 3.5. The impact of total edge, forest edge and percentage agriculture on Urticaceae 1 (Cecropia sp) abundance (%) at a 500 m buffer radius. Toledo (T) hives are shown in blue triangles and Cayo (C) hives in orange circles. There was a positive effect of total edge and forest edge on Urticaceae abundance and a negative effect of percentage agriculture.

The most parsimonious model, with substantial support, for the effect of landscape on Burseraceae 1 abundance included percentage agriculture and landscape diversity as main factors. Both percentage agriculture (z = 4.547, df = 41, p < 0.001) and landscape diversity (z = 3.443, df = 41, p < 0.001) had positive effects on the abundance of Burseraceae 1. The mean Buseraceae abundance increased from 10% to 20% when agricultural cover increased from 1% and 100%, whilst abundance increased from 5% to 25% as landscape diversity increased from 0 to 0.75 (Fig. 3.6). Confidence intervals were large when landscape diversity was highest at 1.25, where Burseraceae abundance range from between 10% and 60%.

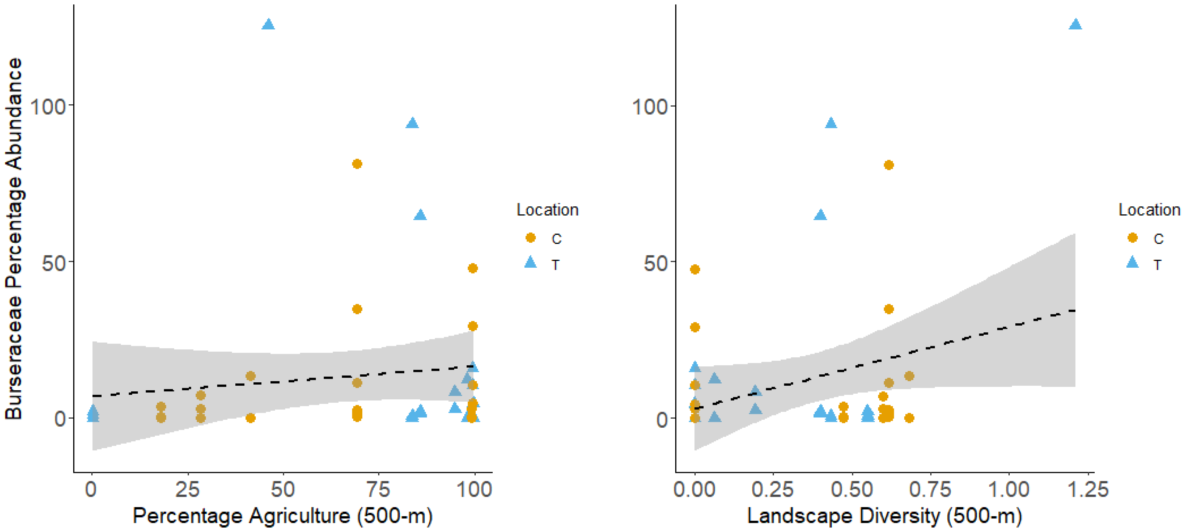


Figure 3.6. The impact of percentage agriculture and landscape diversity on Burseraceae (Bursera Simaruba) abundance (%) at a 500 m buffer radius. Toledo (T) hives are shown in blue triangles and Cayo (C) hives in orange circles. As both percentage agriculture and landscape diversity increased so does Burseraceae abundance.

The most parsimonious model with substantial support for the impact of landscape on the abundance of Fabaceae 6, included agricultural edge, average patch size and forest edge as main variables. As agricultural edge (z = -1.210, df = 41, p <0.001) and average patch size (z = 2.417, df = 41, p = 0.02) increased so did the abundance of Fabaceae 6 (Fig. 3.7). The mean abundance of Fabaceae when agricultural edge was at 0 m/ha was 5%, and this increased to 15% when agriculture edge reached just over 30 m/ha, showing a positive relationship. Confidence intervals ranged from between 0% and 12.5 % at 0 m/ha and 0% and 25% at just over 30 m/ha. There was also a positive trend between average patch size and Fabaceae 6 abundance, but to a lesser extent, with mean Fabaceae abundance increasing from 12% to 13%, and confidence intervals increasing from between 0% and just under 20% to between 0% and just under 25% as average patch size increased from 10 to 80 ha. As forest edge increased the abundance of Fabaceae 6 decreased (z = -2.686, df = 41, p =0.007), showing a negative relationship (Fig. 3.7). Mean Fabaceae abundance decreased from 12.5% to almost 0% as forest edge increased from 0 to 30 m/ha. Confidence intervals ranged from between 10% and 20% with a forest edge of 0 m/ha edge to between 0% and 20% at 30 m/ha forest.

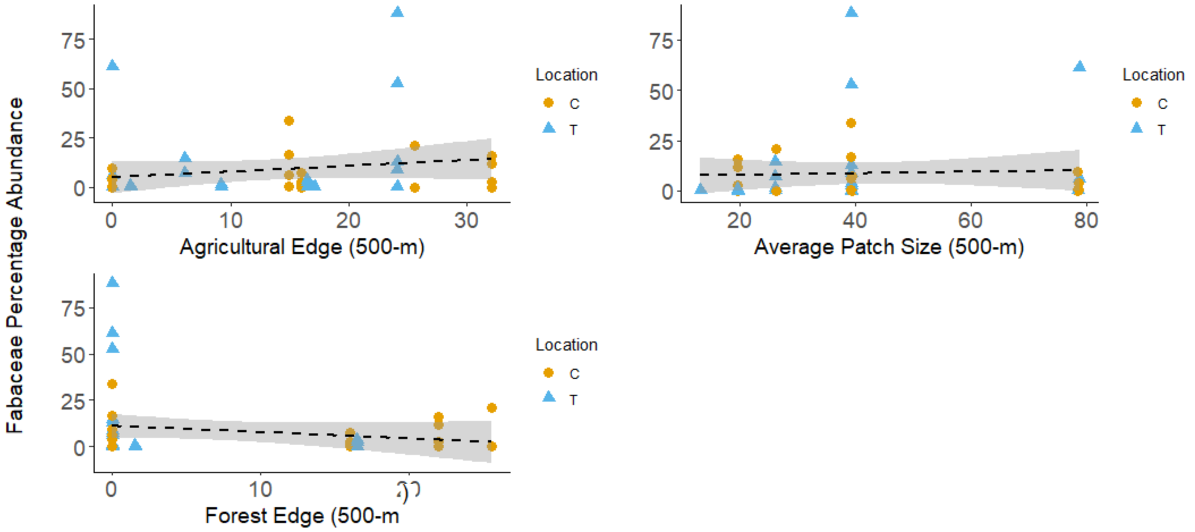


Figure 3.*7. The impact of average patch size, forest edge and agricultural edge on Fabaceae 6 (Dalbergia brownei) abundance (%) at a 500 m buffer radius. Toledo (T) hives are shown in blue triangles and Cayo (C) hives in orange circles. As agricultural edge and average patch size increased so did Fabaceae 6 abundance, however there was a negative relationship between forest edge and Fabaceae 6 abundance.*

3.3.6 Impacts of the surrounding landscape on pollen taxa compositions

To determine the impact of landscapes on the pollen composition found within the bee bread non-metric multidimensional scaling plots were created and tested using the Bray-Curtis dissimilarity matrix. This showed that the landscape diversity matrix at a 1km radius had a strong relationship with pollen species compositions (Mantel statistic: 0.210, $p = 0.002$). Similar results were found at a 500m radius (Mantel statistic: 0.119, $p = 0.017$). As the hive samples become more dissimilar in terms of landscape diversity, they also become more dissimilar in terms of pollen community composition. In the Toledo district, at a 500 m radius hives with higher landscape diversity appear to have higher NMDS2 scores (Fig. 3.8). Hives in Cayo have greater NMDS1 scores and landscape diversity is higher with low NMDS2 scores. Hives with medium landscape diversity appear to have middling NMDS1 and 2 values. At a 1 km radius (Fig 3.9) Toledo hives to have two groupings, one with higher NMDS2 scores and one with lower NMDS2 scores. This seems to separate hive with lower and higher landscape diversity. Cayo sites appear to be less grouped together in terms of landscape diversity. There is a strong effect of location (Cayo/Toledo) on the community compositions of the pollen, with pollen samples from the Toledo district having lower NMDS1 values than those in the Cayo district at both a 500 m buffer radius (Fig. 3.8) and 1 km buffer radius (Fig. 3.9).

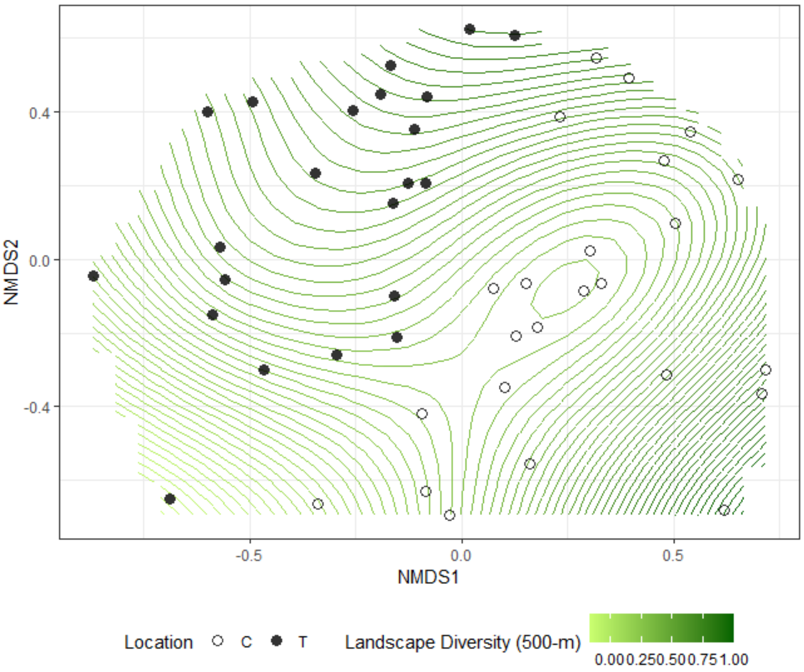


Figure 3.8. Non-metric multidimensional scaling of honey bee diet composition (pollen taxa) across landscapes gradients in the Cayo and Toledo district of Belize. Contour lines of landscape diversity at a 500 m buffer radius surrounding the hives are plotted, black dots represent hives in the Toledo district, open circles represent hives in the Cayo district.

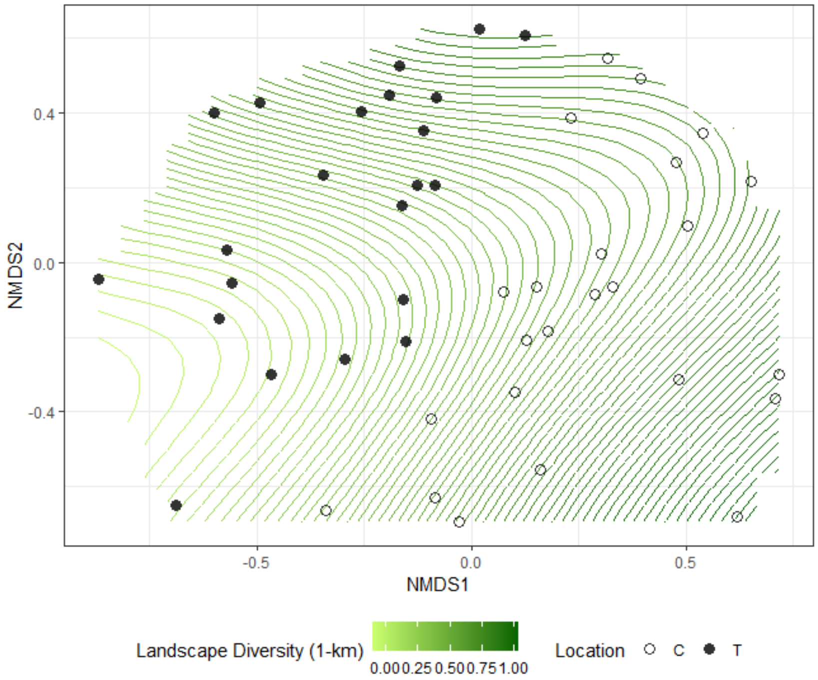


Figure 3.9. Non-metric multidimensional scaling of honey bee diet compositions (pollen taxa) across landscapes gradients in the Cayo and Toledo district of Belize. Contour lines of landscape diversity at a 1 km buffer radius surrounding the hives are plotted. Black dots represent hives in the Toledo district, open circles represent hives in the Cayo district.

3.4 Discussion

This study provides insights into the pollen resources used by honey bees across varied landscapes in Belize. Sample hives were located across the Toledo and Cayo Districts and were surrounded by a mix of different habitat types, including lowland broadleaved forest, agricultural land, shrubland, urban and savannah. This study provides evidence as to the importance of trees in honey bee diet, regardless of the surrounding landscape, and the complex relationship between honey bee diet and landscape diversity driven by both bee foraging behaviour and the relationship between pollen species and the landscape.

A total of 82 different pollen taxa were identified from 34 different families. The 10 most abundant pollen taxa made up over 80% of the pollen taxa identified, all of which belonged to forest species with the exception of two species, Myrtaceae 1 of which the type is unknown, and Anacardiaceae 1 which is likely cashew (cannot be confirmed due to the amount of species/genus's with similar morphological features) and planted for agricultural purposes. The most common species were *Attalea cohune, Cercropia sp, Bursera simbura* and *Dalbergia brownei,* all of which are forest tree species.

Landscape diversity was found to impact honey bee pollen diet, where higher landscape diversity results in lower pollen diversity and species richness. Pollen community composition was also found to be influenced by landscape diversity. Percentage forest and percentage agriculture did not have an impact.

These results are surprising as previous studies carried out in the neotropics found that whilst honey bees foraged more greatly on anthropic pollen species, both forest and all other pollen species found in honey bee pollen diet were positively correlated with the proportion of forest area surrounding each apiary (Montoya - Pfeiffer et al., 2021). Montoya-Pfeiffer et al. (2021) also found that pollen diversity was positively correlated with landscape diversity. This was thought to be because in more heterogeneous landscapes, the honey bees were able to forage easily on forest species, not only present in forest patches but also in unmanaged transitional areas found between anthropic land cover types and natural types (Montoya - Pfeiffer et al., 2021). Machado et al. (2020) analysed how landscape composition affected pollen collection by stingless bees (*M. quadrifasciata anthidioides)* and found that stingless bees collect a greater diversity and richness of pollen when in an environmentally diverse landscape with low human interference. *M. quadrifasciata anthidioides*, has a smaller foraging radius than the honey bee, and therefore is less likely to be able to compensate for low pollen diversity around their nests by foraging greater distances, as has been reported in honey bees (Machado et al., 2020). Alternatively, Kaluza et al. (2017) found that hive pollen species richness and diversity of stingless bees (*Tetragonula carbonaria*, Meliponini), was related to season rather than land use. One potential explanation for this was that bees across different land use's aim for similar plants. Hives surrounded by lower floral diversity may have increased foraging ranges or compensated for the lower diversity of plants by consuming less preferred pollen taxa.

Similarly, contradictory results have been found between the relationship between pollen and landscape diversity in temperate agricultural regions. For example, Danner et al. (2017) found no relationship between pollen diversity in pollen loads collected from honey bee colonies in Germany and the diversity within the surrounding landscape. They observed an increase in foraging distances with decreasing landscape diversity, suggesting that the honey bees compensated for lower diversity within the landscape by increasing their foraging range so that they could maintain the diversity of their pollen diet. Whilst Piroux et al. (2014), found that a high landscape diversity does not necessarily result in a wider diversity in the pollen diet of honey bees.

There are two distinct reasons as to why a negative relationship may have been found between landscape diversity and pollen diversity/pollen species richness in this study. This includes pollen preferences and interactions with wild bee species, as well as the interactions between plant species and the landscape, and how their abundance and presence changes with the landscape.

When considering bee foraging behaviour, honey bees have been shown to prefer mixed floral resources from semi-natural habitats over mass flowering crops even when semi natural resources decrease within the landscape (Danner et al., 2016). Donkersley. (2019) found a clear disparity between the composition of forage available in terms of local land cover composition and what honey bees were foraging from. It was found that there was a constant presence of tree derived pollen in the landscape even as the amount of grassland in the landscape increased and forest decreased. This suggests that bees prefer to forage on tree pollen regardless of their abundance in the environment, and will forage at greater distances to obtain their preferred pollen. Similar results were found in this study, where trees were dominant in all samples regardless of landscape diversity, percentage forest or percentage agriculture. Of the top 10 most dominant species, 8 were identified as tree species, and made up a total abundance of over 80% of the pollen collected. The high abundance of tree pollen in the bees diet could be due to foraging preference, which can be influenced by nutritional value but also visual attractiveness of plants and addictive qualities (Nicholls and Hempel de Ibarra, 2017). Alternatively trees offer advantages other than just pollen resources, offering bees refuge during wind and rain during foraging trips, increasing their chances of survival and thus colony strength (Donkersley, 2019). As bee foraging is more complex than foraging solely based on the abundance and occurrence of pollen in the landscape, as shown by Donkersley (2019) and Danner et al. (2017), this may explain the negative relationship seen between landscape diversity and pollen diversity and species richness. Bee foraging preferences may have resulted in higher pollen diversity and species richness in low diversity areas, as these habitats and subsequent pollen availability may align better with their preferences. Alternatively, these patterns may relate to the distribution of wild bee species, and potential competition over pollen resources. Wild bee species in Belize include social stingless bees (Meliponines) (Schüepp et al., 2012). These bees are associated with larger forest fragments with smaller edge:area ratios whilst honey bees have been shown to display the opposite pattern (Brosi et al., 2008). Brosi et al. (2008) showed bee community composition to be substantially different between forest and pastures despite close spatial proximity. In forests, even in small patches stingless bees comprised the majority of the apifauna, and orchid bees (Euglossa) were common, whilst in pastures the honey bee was much more abundant and no orchid bees were found. Further examples of this were seen by Brosi et al. (2007) who found that at forest edges stingless bees comprised approximately 50% of bee samples whilst honey bees made up 5%. Away from forests, stingless bees dropped to 20% of those samples whilst honey bees made up 45%. This suggests that landscapes with a high percentage of forest may be better suited for stingless bees over honey bees, however the high use of tree pollen collected in hive samples shows the importance of forests and tree flora to honey bees. These niche partitions may help to explain to complex relationship between pollen species richness and diversity and landscape diversity due to interactions between preferred pollen taxa, competition over forest resources, the relationship between these taxa and different landscape types, and well as competition with other bee species.

At the species level numerous different landscape metrics influenced the abundance of the four most prominent pollen taxa. Either percentage forest or forest edge respectively had a positive effect on the abundance of *Cecropia sp* and *Attalea Cohune,* whilst *Dalbergia brownei* and *Bursera Simaruba* abundances increased with percentage agriculture. *Bursera Simaruba* was also positively impacted by landscape diversity. Total edge had a positive impact on the abundance of *Cecropia sp*, and average patch size on the abundance of *Dalbergia brownie.* The four most common pollen taxa consumed by the bees responded to changes in the landscape diversity differently. It is likely that the interaction between these common species and the landscape cause the interesting relationship between pollen and landscape diversity. One example of this can be demonstrated using Cohune Palm, the most dominant species. Low landscape diversity sites in this study have less forest as they are predominantly dominated by agriculture; this can decrease the abundance of trees such as cohune palm.

Simultaneously, there is a honey bee preference for forest tree species such as cohune, and therefore sites which are dominated by cohune have a lower mix of pollen diversity. This means that in the mixed landscapes where forest trees are available there is more of the preferred floral resources, which in turns lowers the overall pollen diversity in the sample. In the low diversity landscapes these trees are still present but they are less abundant and therefore do not dominate the pollen samples as much as in the landscape with forest area, resulting in higher pollen diversity. Figure 3.10 shows a schematic demonstrating this effect of having more forest tree species/cohune in mixed landscapes as opposed to landscapes of low diversity which were dominated by agriculture.

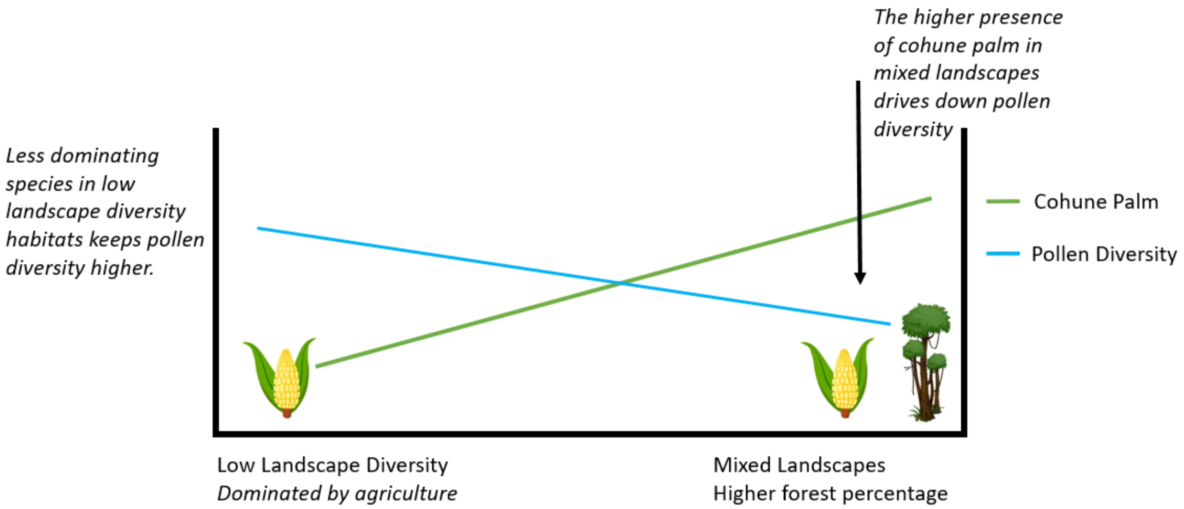


Figure 3.10. The effect of low diversity landscapes being dominated by agricultural habitats on pollen diversity. The higher presence of bee preferred tree species such as cohune palm in mixed landscapes as opposed to landscapes of low diversity results in pollen driven down.

The effect of these preferred species can be exacerbated by recruitment, where honey bees communicate the best foraging patches to other foragers within the hive (Dornhaus et al., 2006). This then leads to mass foraging in the directed areas. A single tree offers a significant amount of pollen resources, and even more so if there is a dense area of a given tree species, therefore many foragers may be recruited to forage from these species. It is therefore likely that in the mixed landscapes where there is a greater dominance of these preferred species, honey bees are recruited to these patches of cohune palm for example, further increasing its dominance in their pollen diet, and lowering the pollen diversity.

Interestingly despite the importance of forest tree species, and their dominance within the honey bee pollen diet, there was no effect of percentage forest on pollen richness or diversity. This would have been expected as it is the presence of these species which reduce the diversity and richness within the bee bread samples. This could be explained as although these species will be of higher dominance within forest habitat, they are not exclusive to it, and likely also occur in other habitat types, such as in and around agricultural landscapes. This will act to reduce the overall effect of forest, leading to its insignificance. To further study this, it would again be useful to collect abundance and presence/absence data as to the location of the preferred floral species in the environment in Belize.

Attalea Cohune has been described as Belize's most abundant and distinctive tree (Furley, 1975), and is common throughout Belize in both lowland forest and agricultural systems. It is found throughout its distribution in high density stands within more species rich forest, but is also found in varying densities over deep, well drained soils (McSweeney, 1995). The higher density of cohune palm in forested areas explains the positive impact of forest an *Attalea Cohune* pollen abundance, as higher forest means higher *Attalea Cohune* density, and thus a more attractive pollen resource. *Bursera Simaruba* are also common across Belize, and grow in a wide range of habitats, they can be found in, upland, lowland and riverine forests (Foster, 2007), and can survive well in highly disturbed areas (Brandeis, 2006), potentially explaining the positive relationship with agriculture percentage. *Cecropia sp* are pioneer trees and are widespread throughout Belize (Zalamea et al., 2012). They often dominate in Neotropical understories after disturbances (Shiels et al., 2015), they are light demanding and rapidly invade disturbed areas such as forest canopy gaps, roadsides, agricultural areas and urban locations (Binggeli, 1999). They form dense stands (Binggeli, 1999) which may contribute to their popularity as a pollen source by honey bees. *Dalbergia sp also* known as rosewood, is a slow growing, highly valued tropical hardwood. The largest populations in Belize grow across the wet forests of the southernmost district Toledo (Wainwright and Zempel, 2018). Rosewood, although a forest species, suffers poaching and illegal trade. The demand for rosewood, along with population increase and limited enforcement, has caused a rise in the deforestation in the Toledo area, land which can then be subsequently used for agricultural purposes. This potentially explains how an increase in agricultural land leads to greater Rosewood abundance, as these areas may be situated close to dense areas of rosewood (Folkard-Tapp, 2020). All of these species have high densities and are common in Belize. It is possible that due to their densities in the environment, that they are more easily foraged by honeybees. Where densities are high, less effort is required by the bees to fly between conspecific plants and the waggle-dance is used less frequently to communicate further resource locations (Montoya ‐ Pfeiffer et al., 2021).

In conclusion, the species richness and diversity of pollen from bee bread samples was negatively influenced by the landscape diversity surrounding the bee hives, with the most dominant pollen taxa being influenced in different directions by various landscape metrics, including percentage forest and agriculture. This indicates a complex relationship between the landscape and pollen foraging, and suggests that the bees pollen diet is a product of both the abundant species in the surrounding landscape and the bees innate preferences and foraging behaviours. The preferred pollen taxa in the honey bees diet has been shown to be tree species, which though occurring in both forest and agricultural landscapes, occur more frequently and in higher dominance in forest and natural habitats, as opposed to agricultural ones. The presence of these taxa act to drive down the diversity and species richness within the honey bees pollen diet, a process which is likely exacerbated by foraging recruitment strategies. The honey bee's method of recruitment means that when these preferred pollen species are present, they will be foraged in large quantities by many foragers, and will thus dominate pollen samples. This therefore suggests that honey bee preference and subsequent recruitment is more important than landscape diversity, at least in countries such as Belize where few landscapes are devoid of resources. In order to assess the best location for beehives to be placed by local farmers/beekeepers in the tropics to obtain healthy colonies in terms of nutrition, more work needs to be carried out assessing not just the surrounding landscape but also foraging distance, abundance of the most dominant pollen taxa in the landscape, as well as potential differences between the bee pollen diet across seasons.

Chapter 4: The impact of landscape on pesticide presence in bee bread

Pesticides are thought to be one of the main drivers contributing to the losses of bees worldwide, and have been shown to decrease both longevity and survival of honey bees (Apis Mellifera). The tropics support a minimum of two thirds of the worlds biodiversity, however, much is becoming a mosaic of agriculture and forest patches. Agricultural expansion in the tropics is the main driver behind deforestation and is predicted to increase at an accelerated rate. This growth in agricultural land use had caused an increase in pesticides use, which has the potential to not only impact crops and weeds, but also non-target organisms such as bees. One of the primary routes though which bees could be impacted by pesticide application is by contamination of the food sources, such as pollen. This chapter aims to investigate pesticide contamination of bee bread collected from 14 different sites across Belize across a land use gradient using a Liquid Chromatography Mass Spectrometry Pesticide Multi Residue and a separate Glyphosate screen during May and June of 2019. No pesticides were detected in any of the bee bread samples, even those collected in close proximity to agricultural land. Interviews with the beekeepers of each apiary revealed that the most likely reasons for this are the use of a knapsack sprayer for application which limited non-target exposure, and the bees pollen diet, which are mainly tree species, and does not overlap with the target species. In addition to differences between the seasons where the bees are most actively foraging (when this study took place) and the period where pesticides are sprayed in the highest quantities. Together this all suggests the bees in this area are not negatively affected by pesticide application.

4.1 Introduction

The use of agrochemicals, such as pestisides, has contributed substanitally to global food security over the past 50 years. It has been estimated that almost one third of the agricultural products worldwide are produced with the use of pesticides (Rainwater, 2003; Wenjun, 2018); however, its use and presence has led to negative effects on the environment (Tang et al., 2021).

4.1.1 Pesticide exposure as a stressor to honey bees

Pesticide exposure is thought to be one of the main drivers contributing to honey bee colony losses (O'Neal et al., 2018). Exposure in honey bees has been shown to cause decreased longevity and survival (Cresswell et al., 2012; Wu et al., 2011), reduced cognition (Ciarlo et al., 2012; Farooqui, 2013), and decreased immune response or resistance to parasitic agents (Pettis et al., 2012; Retschnig et al., 2014). Colony growth is also negatively impacted as reproductive rates have been shown to slow after pesticide exposure, threatening the continual development of the colony (Thompson et al., 2014). A reduced workforce can lead to decreased foraging efficiency (Henry et al., 2012; Schneider et al., 2012), reducing the amount of available nutrients, and thus further limiting brood development. Despite the high diveristy of fauna in the neotropics and the largely unregulated use of pestidices, little literature is available or reported on the accumulation of pesticides on non-target species in the tropics (Rainwater et al., 2007). This study will therefore analyse the presence of pesticides in bee bread, a main food source of honey bees, and potential pesticide exposure routes across landscapes in Belize, to assess potential stressors on honey bees in the tropics.

In recent years many studies have investigated the impact of neonicotinoid pesticides (Amaral-Rogers, Belzunces et al., 2015; Lundin et al., 2015), which are used in more than 120 countries on around 140 different crops (Lundin et al., 2015). In developing countries such as Belize neonicotinoids do not pose the greatest threat to bees, but instead herbicides, such as Glyphosate, 2,4-D and Paraquat, are frequently used and therefore their effects on bees in these areas are equally as important (Bravo et al., 2011). Whilst the quantities of pesticides used in developing countries such as Belize may be lower than that in fully industrialised countries, it has been suggested that they carry a higher likelihood of adverse health risks and environmental damage due to the lack of regulation and uncontrolled use (Bravo et al., 2011).

Whilst many studies have investigated the effect of pesticides at the individual level, few have studied the effects at the colony level. At the colony level negative impacts of pesticide exposure include decreased foraging and hygienic activities of worker bees and reduced colony development, including brood production and pollen collection (Wu-Smart and Spivak, 2016). These effects were weaker as colony size increased, suggesting that an already strong and large colony have a better ability to buffer the effects of pesticides (Wu-Smart and Spivak, 2016). Thiamethoxam at high exposure levels have been shown to cause honey bee colony losses (adult bees and brood), although bees were able to recover after 2-3 brood cycles (Thompson et al., 2019). Another study, although focusing on bumblebees found that combined pesticide exposure impaired natural foraging behaviour, increased worker mortality and reduced brood development (Gill et al., 2012). It is however important to consider the increased ability for honey bees to buffer these effects in comparison to bumblebees due to their large colony size. Because of the effects caused by pesticides on overall colony health and bee development, it is important to study potential routes honey bee colonies may be exposed to them.

4.1.2 Pesticides present in Belize

According to the Belize Pesticide Control Board ("Register of Pesticides Active Ingredient 26 March 2020", 2020) 170 different active ingredients are registered in Belize. While there is no public reports or documentation of quantities of pesticide used in Belize, information is available on pesticide imports. Pesticide imports into Belize are dominated by herbicides and fungicides, with lower imports of insecticides (Refer to Table 1.1). In both 2009 and 2015-2017 Glyphosate was the most commonly imported pesticide into Belize, while 2,4-D and Mancozeb were also highly imported between 2015-2017, as well as Chlorpyrifos in 2009. Whilst herbicides and fungicides were the most widely imported pesticides into Belize, Malathion, Chlorpyrifos and Terbufos were the only insecticides imported. Pesticide application to crops is common across Belize, however, land use is not homogenous in its distribution (Kaiser, 2011). In the north sugar cane is farmed extensively, whilst further south agricultural land is heavily planted with bananas and citrus (Kaiser, 2011). Local small holder farms in both the north and south are predominantly focused on growing beans, plantain and corn (Wikström, 2014), and these farms are dispersed and interwoven with the natural environment and often near semi-urban environments. In the south larger farms operate nearby, focusing mainly on growing bananas. Farms in this area rely heavily on the application of pesticides (Kaiser, 2011). The spread of these farms among the natural environments increases the likelihood of pesticide run off and contamination of non-target species. As the majority of farms are owned and run by local farmers, the spraying of pesticides largely goes undocumented but also means that pesticide application is largely limited to knapsack spraying as opposed to more wide scale aerial application.

Glyphosate [N-(phosphonomethyl) glycine or GLY] is a low cost and broad spectrum herbicide making it popular in agricultural settings for weed control (Vázquez et al., 2018). It has been detected in both genetically modified crops, traditional crops and resistant weeds (Guo and Rubio, 2014). Glyphosate affects bees through various mechanisms including the alteration of gut bacteria which can lead to an increased susceptibility of infection (Motta et al., 2018), as well as affecting the larval development of bees through delayed moulting and reduced weight gain (Vázquez et al., 2018). Studies have also suggested that GLY may cause reduced cognition in honey bees, where bees exposed to GLY take a longer time to reach their hives and have a less direct flight route when released away from their hives (Balbuena et al., 2015). Other studies have found that GLY exposure (at field realistic levels) results in reduced olfactory learning as well as reduced food uptake during rearing (Gonalons and Farina, 2018).

2,4-Dichlorophenoxyacetic acid or 2,4-D is a widely used pre-/post-emergent systemic herbicide that controls broad-leaved weeds and other vegetation (Islam et al., 2018). Studies have demonstrated the bioaccumulation capacity of 2,4-D in non-target organisms exposed for only a short period of time (da Fonseca et al., 2008; Kiljanek et al., 2016) can cause endocrine disruption (Guerrero-Estévez and López-López, 2016), and genetic alternation (Lajmanovich et al., 2015).

Manconzeb is an ethylene bisdithiocarbamate fungicide, and was first registered in the United States in 1948. It is used in the production of major crops including potato, tomato and citrus and can protect against over 400 different plant pathogens (Runkle et al., 2017). Mancozeb production is forecasted to continue to grow at an increased rate across the early 2020's due to a low purchasing price and increasing global demands for fruit and vegetables (Runkle et al., 2017). Whilst Mancozeb has been reported to cause reduced survival and reproductive ability of non-target species such as collembolans and enchytraeids (Camargo Carniel et al., 2019), so far studies on Manconzeb have found it to be nontoxic to honey bees (Holmes and Ruhman, 2007).

4.1.3 Routes of pesticide exposure

In agricultural environments bees can be exposed to pesticides in several ways, including via contaminated air particles and consumption of food and water (Toselli and Sgolastra, 2020). While there are various routes of pesticide exposure, pollen is a particularly important route as it provides an essential source of amino acids and crude protein, and is crucial in bee development (Li et al., 2012). Pesticides (insecticides, fungicides, herbicides) are transported back to hives by foraging bees, and then stored as bee bread, and used as a source of nutrition for the developing brood. Once in the hive pesticides accumulate, and can affect brood development (O'Neal et al., 2018), threatening the future development of the colony. Whilst it is possible to measure honey bee pesticide exposure directly through analysis of the bee body, bee bread was chosen so that the route of exposure could also be identified. Nectar can also be an important route of contamination; however bee bread was chosen due to its role in brood development and thus overall impact on colony health (Xiao et al., 2022).

Furthermore multiple studies have found bee bread to often be the most contaminated matrix (Daniele et al., 2018; Xiao et al., 2022). Depending on the foraging patterns of the colony the sequestration of pesticides may occur all year round, and thus expose all members of the colony (Quigley et al., 2019). A global review found that of 31 studies, almost two thirds of pollen samples collected from bees were contaminated with pesticides. Fluvalinate and Coumaphos were the most commonly detected pesticides, found in 21 and 20 of the studies respectively (Toselli and Sgolastra, 2020).

4.1.4 Honey bee pesticide exposure in the tropics

The tropics are diverse and support a minimum of two-thirds of the worlds biodiversity (Giam, 2017), however, this is threatened by agricultural expansion (Rudel et al., 2009). Much of the tropics now exists as a mosaic of agricultural and forest patches which are becoming increasingly isolated as deforestation proceeds (Norris, 2016). Agricultural expansion in the tropics is the main driver behind this deforestation, and is predicted to continue as global demand for food is expected to rise by 70 to 100% by 2050 (Laurance et al., 2014). As the percentage of agricultural increases, this also means pesticide use to control weeds and pests will also increase. Importantly, pesticides do not just have the potential to affect the localised area where they are sprayed but may also experience orographic drift into surrounding environments, and subsequently negatively impact non-target organisms (Sanchez-Bayo and Goka, 2016).

While honey bee species have been extensively studied in temperate regions, largely in Europe and the USA (Sánchez-Bayo and Wyckhuys, 2019), research carried out in tropical regions is limited, particularly in Africa, Latin America and Asia (Matias et al., 2017). Of the research carried out in the tropics so far most has occurred in Brazil, where pesticides have been reported to be responsible for bee declines in many regions (Maggi et al., 2016). The reported effects of pesticides on honey bees from Latin America include reduced learning abilities (Herbert et al., 2014), impaired bee development (Tavares et al., 2015) and negative effects on Malpighian tubules of Africanised bees (De Almeida Rossi et al., 2013). Only two studies have been carried out in South American and none in Central America investigating pesticide contamination of pollen (Toselli and Sgolastra, 2020), although some have looked at direct effects on bee respiration rates, locomotion and colours preferences (Almeida et al., 2021). These studies were carried out in Brazil (de Oliveira et al., 2016) and Uruguay (Niell et al., 2015) and used Gas and Liquid Chromatography Mass Spectrometry respectively to investigate pesticide contamination in bee pollen. Pesticides detected included Bioallethrin and pendimethalin in Brazilian pollen and thiacloprid, imidacloprid, methomyl as well as a further 5 pesticides in Uruguay. It is particularly important that now, while agriculture is expanding readily in the tropics, the consequences of this on honey bees in the tropics are understood, as responses may not be the same as in temperature regions due to differences in abiotic conditions and the surrounding flora and fauna.

Here the presence of pesticides in bee bread collected from hives in Belize, Central America is investigated. Belize is one of the most forested counties within Central America, however in recent years has undergone rapid development of agriculture through the clearance of primary and secondary lowland forests and increased usage of intensive cultivation practices (Bonmatin et al., 2019). This increase in agricultural activity may increase the likelihood of bees experiencing pesticide contamination within the hives. In addition beekeepers were interviewed on pesticide application and plants commonly foraged on by honey bees in the area in order to provide context to each apiary in terms of agricultural practice and the potential exposure and the foraging activities of the bees.

4.2 Methods

4.2.1 Study sites

Bee bread pollen samples were collected from 14 apiaries across the Cayo and Toledo districts of Belize (the same hives as chapters 2 and 3) (Fig. 4.1). The apiaries were managed by beekeepers who were part of the Cayo Quality Honey Producers Cooperative in the Cayo District (apiaries 9-14, managed by 5 beekeepers) and the Maya Golden Landscape Beekeepers Association in the Toledo District (apiaries 1-8, managed by 7 beekeepers). Belize is covered by agricultural and human settlements which make up approximately 20% of the land cover, with the remaining cover being made up of natural habitats which include savannahs, scrublands, wetlands as well as lowland and submontane broadleaf and pine forests (Flowers et al., 2020). In the Cayo district agricultural activities include livestock farming, farming of vegetable crops, citrus and sugar cane plantations as well as multicrop slash and burn farms ("AYO Farmers Adapting to Climate Change and COVID-19", 2020). The Cayo district also comprises of Mennonite communities which carry out more intense agricultural practices, such as that in Spanish Lookout, which farm using bulldozers and caterpillars, here there is a greater potential for increased pesticide use (Güemes-Ricalde et al., 2006). Toledo, the southernmost district in Belize, encompasses the Maya Golden Landscape (MGL), a 302,259 acre mosaic of private and nationally protected areas, private lands and Maya and Hispanic communities. Agricultural land in Toledo is predominantly farmed through slash and burn practices, however more recently had experienced growth in large scale citrus and banana plantations (Voight et al., 2019).

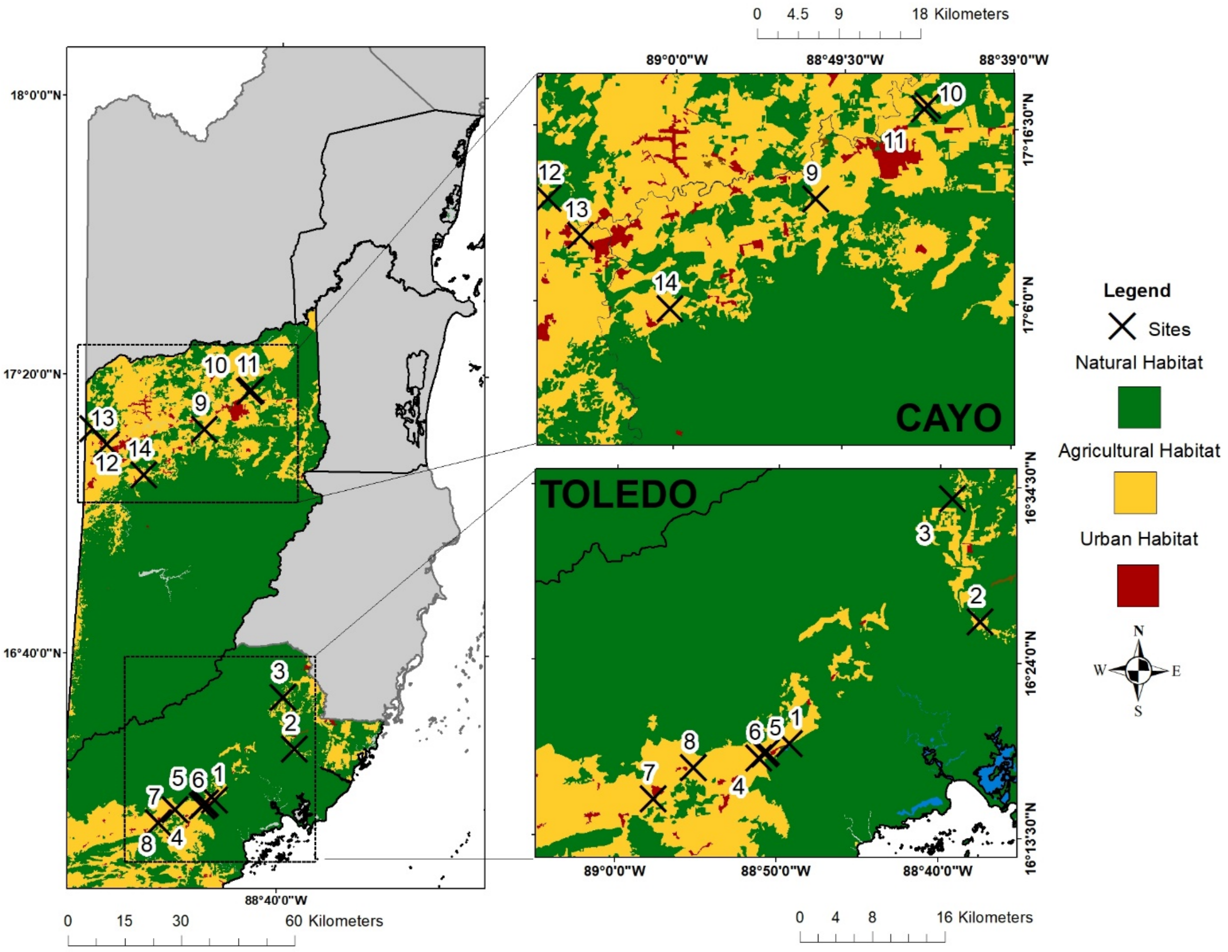


Figure 4.1. Map of the study area. Apiary sites are represented by back crosses. There are 8 sites in the Toledo District and 6 in the Cayo District. District boarders are marked across Belize, districts not studied are shaded out. Ecosystems are based on the 2017 land use map provided by Meerman and Clabaugh (2017).

4.2.2 Semi-structured interview with beekeepers

Semi-structured 15 min face-to-face interviews were carried out with beekeepers who owned and managed apiaries in the Cayo and Toledo districts, except for apiaries 12 and 13 due to lack of contact with the beekeeper. The beekeepers interviewed were all male and were first-generation beekeepers who each operated between 1 and 2 apiaries. These beekeepers were chosen in order to link their responses directly to any or no pesticide presence in the hives where the bee bread was collected, as well as the pollen taxa present, as discussed in Chapters 1 and 2. Apiaries had all been established from between 6 months to 5 yrs. The interviews were divided into three parts. The first focussed on the history of the apiary and the beekeepers background, and included the length of time they had been beekeeping, when the apiary was established, how often they visit the hives, why they began beekeeping, challenges they face, and the importance of beekeeping to them. The second part focused on the bee's diet and potential pesticide exposure, and asked what pesticides were sprayed in the area, how they were sprayed and how often, and what plants they have seen the bees feeding from. The final part assessed any other stressors they believed the bees might have experienced including mites and viral infection, impact of the forest on the bees, and their current biggest threats. The interviews were conducted and recorded in English. Where the beekeepers did not speak English, a translator from Yaaxche Conservation Trust translated the questions and answers to and from Q'eqchi Mayan. Informed consent was obtained on paper from all participants prior to the study and ethics approval acquired (ERGO/FEPS/47809). The full questionnaire is provided in the accompanying materials 2.

4.3.2 Pollen collection

Pollen collection occurred at the end of the dry season, between the 23rd May and the 25th June 2019, as Africanised honey bee pollen collection activity is highest during the dry season in the Neotropics (Pesante, 1985). Therefore, this is the most likely time when bees will be impacted by pesticides in the bee bread. After speaking with beekeepers in Belize during the investigation, pesticide application around this time was likely, although application occurs throughout the year with a higher level of application during October/December.

Pollen samples were collected following the same methods used in Chapters 2 and 3. Refer to chapter 3.2.4 for further information on how the bee bread was sampled. Bee bread samples were used to study pesticide exposure over studying the bee bodies themselves in order to identify the route of pesticide exposure. Whilst nectar samples could also be used, pollen in bee bread would allow any pesticide presence to be related to the pollen taxa in the samples identified in chapter 2.

4.2.3 Sample preparation and pesticide analysis

Pollen samples were homogenised in individual plastic bags, of which equal proportions of pollen were taken from each hive sample to make up 2 g of bee bread per site. This totalled 14 samples. Each 2 g sample was stored in the freezer until ready to be sent for analysis to reduce the likelihood of pesticide breakdown. Samples were packaged and sent to Fera Science ltd (Fera Science, 2020) for a Liquid Chromatography Mass Spectrometry Pesticide Multi Residue screen, which tested for 338 different pesticide residues (Table 4.1), and a LCMS Glyphosate screen.

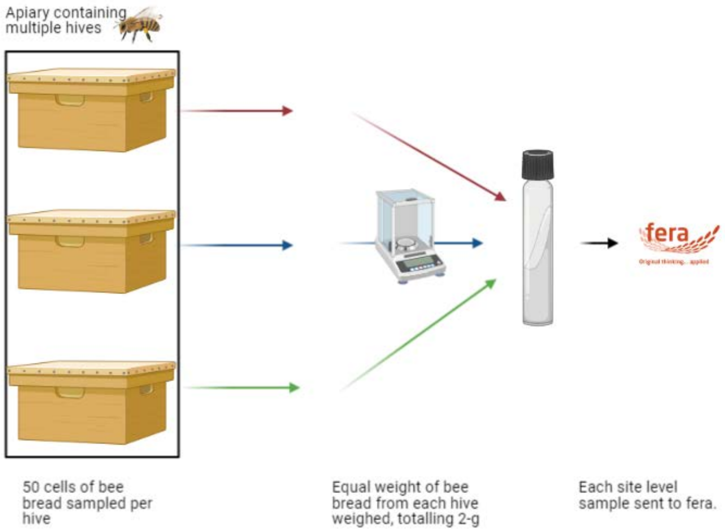


Figure 4.2. Study design of bee bread collection and analysis. 14 site/apiaries were identified across Belize. Between 1 and 6 hives were sampled per site and 1 sample was collected per hive. Equal weights of bee bread from each hive sample were taken totalling 2 g per site and placed in a new sample tube. Bee bread was stored in a freezer until analysis could take place. Samples were sent to Fera Science Ltd (an executive agency of Defra (Department for Environment, Food and Rrual Affairs), carrying out analysis on cross plant and bee health, crop protection, sustainable agriculture, food and feed quality, and chemical safety in the environment) for Liquid Chromatography Mass Spectrometry analysis.

For the Liquid Chromatography Mass Spectrometry Pesticide Multi Residue screen site samples were mixed with acidified methanol/water and then a subsample was extracted in selected reaction monitoring mode, alternatively for the GLY screen each sub-sample was extracted with acetonitrile, in the presence of salts. Analysis was carried out using liquid chromatography with mass spectrometric detection (HPLC-MS/MS) in selected reaction monitoring mode. The presence of residues was confirmed using the same technique in multiple reaction monitoring mode. Limits of detection ranged from between 0.0002 and 0.05 mg/kg (Table 4.1).

Table 4.1. Pesticide residues screened for by a Liquid Chromatography Mass Spectrometry Pesticide Multi Residue screen and GLY screen. A total of 339 different residues were screened for. Reporting limits, recovery (%) and pesticide type are provided for each for residue. Residues marked with a star are reported on the 2020 register of pesticides in Belize (“Register of Pesticides Active Ingredient 26 March 2020”, 2020).

Pesticide Residues	Reporting Limit (< mg/kg)	Recovery (%)
	Antiseptic/Disinfectant	
DDAC	0.050	116
	Fungicide	
*acibenzolar-S-methyl	0.002	105
ametoctradin	0.002	86
*azoxystrobin	0.002	96
BAC10	0.050	83
BAC12	0.050	121
BAC14	0.050	73
BAC16	0.050	75
benthiavalicarb-isopropyl	0.002	80
benzovindiflupyr	0.002	75
bitertanol	0.002	90
bixafen	0.002	83
*boscalid	0.002	90
bromuconazole	0.002	87
BTS 40348	0.002	85
BTS 44595	0.002	95
BTS 44596	0.002	86
bupirimate	0.002	106
*carbendazim	0.001	97
carboxin	0.002	83
cyazofamid	0.002	76
cyflufenamid	0.002	101
*cymoxanil	0.002	90
cyproconazole	0.002	88
*fluopyram	0.004	91
diclobutrazol	0.002	89
*diethofencarb	0.002	93
*difenoconazole	0.002	82
dimethomorph	0.002	81
dimoxystrobin	0.002	83
diniconazole	0.002	94
dodine	0.002	83
*epoxiconazole	0.002	95
ethirimol	0.002	86
famoxadone	0.002	80
fenamidone	0.004	75

fenarimol	0.002	62
fenbuconazole	0.002	86
fenbutatin oxide	0.004	71
fenhexamid	0.002	87
*fenpropidin	0.002	84
*fenpropimorph	0.002	83
fenpyrazamine	0.002	92
fentin acetate	0.003	94
fluazinam	0.002	83
*fludioxonil	0.002	97
*fluopicolide	0.002	92
*fluopyram	0.002	83
fluoxastrobin	0.002	73
fluquinconazole	0.002	78
flusilazole	0.002	83
flutriafol	0.002	88
*fluxapyroxad	0.002	85
furmecyclox	0.002	87
heptenophos	0.002	84
hexaconazole	0.002	82
*imazalil	0.005	95
iprovalicarb	0.002	74
isoprothiolane	0.002	78
*isopyrazam	0.002	92
kresoxim-methyl	0.002	86
mandipropamid	0.002	94
mepronil	0.002	81
*metalaxyl	0.002	90
metconazole	0.002	83
metrafenone	0.002	86
*myclobutanil	0.002	82
nuarimol	0.002	89
ofurace	0.002	87
oxadixyl	0.002	88
paclobutrazol	0.002	85
penconazole	0.002	89
pencycuron	0.002	91
penflufen	0.002	91
pentachlorophenol	0.002	83
*penthiopyrad	0.002	92
picoxystrobin	0.002	89
prochloraz	0.002	90
propamocarb (free acid)	0.002	77

*propiconazole	0.002	91
proquinazid	0.002	88
*prosulfuron	0.002	88
prothioconazole-desthio	0.002	87
*pyraclostrobin	0.002	83
pyrazophos	0.002	89
pyrifenox	0.010	93
* pyrimethanil	0.002	84
quinoxyfen	0.002	85
*spiroxamine	0.002	88
*tebuconazole	0.002	84
tetraconazole	0.002	88
TFNA	0.010	79
*thiabendazole	0.002	80
*thiophanate-methyl	0.001	83
triadimefon	0.002	82
*triadimenol	0.002	89
tricyclazole	0.002	85
*trifloxystrobin	0.002	88
triflumizole	0.002	82
triforine	0.002	82
triticonazole	0.002	75
zoxamide	0.002	85
Herbicide		
2,4,5-T	0.004	80
*2,4-D	0.004	84
2,4-DB	0.010	118
6-benzyl aminopurine	0.002	83
alachlor	0.002	89
amidosulfuron	0.002	88
aminopyralid	0.010	100
*asulam	0.002	89
*atrazine	0.002	93
*bentazone	0.002	95
bicyclopyrone	0.002	87
bifenox	0.002	93
*bispyribac-sodium	0.002	93
bromoxynil	0.002	83
butachlor	0.004	81
carbetamide	0.002	90
chloridazon	0.002	80
chlortoluron	0.002	88
cinidon-ethyl	0.002	94

*clethodim	0.002	77
clopyralid	0.002	91
cycloxydim	0.002	92
*cyhalofop butyl	0.002	83
desmedipham	0.002	75
dichlorprop	0.002	72
dinoseb	0.002	80
disulfoton	0.003	118
disulfoton sulfoxide	0.001	93
*diuron	0.002	92
fenoprop	0.002	68
*fluazifop (free acid)	0.002	79
*fluazifop-p-butyl	0.002	86
flufenacet	0.002	86
fluometuron	0.002	85
flurochloridone	0.002	91
fluroxypyr	0.002	75
*halosulfuron-methyl	0.005	86
haloxyfop (free acid)	0.001	99
*hexazinone	0.002	92
*Glyphosate	0.050	102
imazapic	0.002	88
imazaquin	0.002	77
iodosulfuron-methyl	0.002	85
ioxynil	0.002	86
isoproturon	0.002	90
isoxaben	0.002	88
*isoxaflutole	0.002	91
lenacil	0.002	90
linuron	0.002	89
MCPA	0.002	75
MCPB	0.010	75
mecoprop	0.004	78
mepanipyrim	0.002	87
mesosulfuron-methyl	0.002	91
mesotrione	0.002	87
metamitron	0.002	94
metazachlor	0.002	90
methabenzthiazuron	0.002	93
metobromuron	0.002	83
*metolachlor	0.002	86
metosulam	0.002	84
metoxuron	0.002	85

metribuzin	0.005	83
*metsulfuron-methyl	0.002	86
monolinuron	0.002	93
monuron	0.002	94
napropamide	0.002	107
*nicosulfuron	0.002	85
oxadiargyl	0.002	82
oxadiazon	0.002	88
oxasulfuron	0.002	84
phenmedipham	0.002	82
*picloram	0.010	79
picolinafen	0.002	87
prometryn	0.002	93
propaquizafop	0.005	85
propham	0.010	70
propyzamide	0.002	97
quinmerac	0.005	71
quinoclamine	0.002	98
quizalofop P	0.002	94
*rimsulfuron	0.002	87
saflufenacil	0.002	110
simazine	0.002	89
sulcotrione	0.002	77
tebuthiuron	0.002	90
tepraloxydim	0.002	88
*terbufos sulfone	0.001	83
*terbufos sulfoxide	0.001	92
terbuthylazine	0.002	94
*terbutryn	0.002	94
triallate	0.002	103
triasulfuron	0.002	92
*triclopyr	0.002	100
triflusulfuron-methyl	0.002	83
tritosulfuron	0.002	88
Insecticide		
*abamectin	0.002	74
acephate	0.001	79
*acetamiprid	0.002	91
acrinathrin	0.002	87
aldicarb	0.002	74
aldicarb sulfone	0.002	82
aldicarb sulfoxide	0.002	82
*allethrin	0.002	93

azinphos-ethyl	0.002	85
azinphos-methyl	0.002	90
*bendiocarb	0.002	90
*buprofezin	0.002	88
butocarboxim	0.001	86
butocarboxim sulfoxide	0.002	85
butoxycarboxim	0.002	86
*carbaryl	0.002	87
carbofuran	0.000	86
carbofuran (3-hydroxy)	0.000	90
*chlorantraniliprole	0.002	93
chlorfenapyr	0.004	139
chlorfluazuron	0.002	81
chromafenozide	0.002	86
*clofentezine	0.002	80
*clothianidin	0.002	83
coumaphos	0.002	70
cyromazine	0.002	92
*diafenthiuron	0.002	94
*dichlorvos	0.010	88
dicrotophos	0.002	83
*diflubenzuron	0.002	90
DMF	0.002	81
DMPF	0.002	95
DMSA	0.002	93
*emamectin benzoate	0.002	86
ethiofencarb	0.002	94
ethiofencarb sulfone	0.002	81
ethiofencarb sulfoxide	0.002	87
ethiprole	0.002	92
etofenprox	0.004	85
fenamiphos	0.002	84
fenamiphos sulfone	0.002	96
fenamiphos sulfoxide	0.002	85
fenoxycarb	0.002	85
fenpyroximate	0.002	88
fensulfothion	0.002	94
fensulfothion sulfone	0.001	86
fensulfothion-oxon sulfone	0.001	82
fensulfothion-oxon-sulfone	0.001	88
fenthion	0.004	123
fenthion sulfone	0.002	92
fenthion sulfoxide	0.002	94

*fipronil	0.000	107
fipronil de-sulfinyl	0.000	72
fipronil sulfone	0.000	105
flonicamid	0.002	96
*flubendiamide	0.002	79
flufenoxuron	0.002	82
fonofos	0.004	74
formetanate-HCl	0.002	104
furathiocarb	0.000	75
halofenozide	0.002	88
hexythiazox	0.002	87
*imidacloprid	0.002	92
*indoxacarb	0.002	81
isazofos	0.001	130
isocarbofos	0.001	78
isofenphos	0.002	77
isofenphos-methyl	0.002	82
isoprocarb	0.002	88
*lufenuron	0.002	91
malaoxon	0.002	84
matrine	0.002	63
mecarbam	0.002	95
mephosfolan	0.002	95
metaflumizone	0.002	88
methamidophos	0.006	77
*methomyl	0.002	89
methoxyfenozide	0.002	83
mevinphos	0.002	82
monocrotophos	0.001	89
nitenpyram	0.002	86
*novaluron	0.002	84
omethoate	0.001	81
oxydemeton-methyl	0.001	78
oxymatrine	0.004	94
paraoxon-methyl	0.002	81
phenthoate	0.002	82
phosmet	0.002	90
phosphamidon	0.002	80
phoxim	0.010	94
*picaridin	0.002	91
pirimicarb	0.002	83
pirimicarb-desmethyl	0.002	80
*profenofos	0.002	94

promecarb	0.002	90
propargite	0.002	90
propetamphos	0.002	82
propoxur	0.002	89
pymetrozine	0.002	96
*pyrethrins	0.002	86
pyridalyl	0.002	80
*pyriproxyfen	0.002	85
quassia	0.002	89
rotenone	0.002	81
spinetroram	0.002	85
spinosad	0.002	87
spiromesifen	0.005	83
*spirotetramat	0.002	84
spirotetramat enol	0.002	79
*sulfoxaflor	0.002	85
tebufenozide	0.002	91
tebufenpyrad	0.002	96
*teflubenzuron	0.002	68
terbufos	0.001	111
*tetramethrin	0.002	87
TFNG	0.002	64
thiacloprid	0.002	90
*thiamethoxam	0.002	92
*thiodicarb	0.002	77
tolfenpyrad	0.002	93
triazamate (free acid)	0.002	82
triazophos	0.001	81
trichlorfon	0.002	82
*triflumuron	0.002	92
Insecticide/Acaricide		
chlorfenvinphos	0.002	82
demeton-S-methyl	0.001	122
demeton-S-methyl sulfone	0.001	91
dimethoate	0.001	82
flucythrinate	0.002	95
methiocarb	0.002	93
methiocarb sulfone	0.002	100
methiocarb sulfoxide	0.002	91
metolcarb	0.002	99
*oxamyl	0.001	85
phorate	0.004	85
phorate sulfone	0.002	84

phorate sulfoxide	0.002	94
phosalone	0.002	90
Nematicide		
cadusafos	0.001	88
Pesticide Synergist		
fosthiazate	0.002	86
piperonyl butoxide	0.002	88

4.3 Results

4.3.1 Reported pesticides use and pollen foraging behaviour

The 10 beekeepers interviewed in Belize discussed pesticide application within a 10 km radius of their hives, the pesticides used, and plants which are known to experience pesticide application. Pesticide use was reported for the whole year, though there was a distinctly higher frequency of pesticide use in the wet season, which in Belize runs from June to January (Renton, 2006). In October and December 80% of beekeepers reported that pesticides were applied, whilst 70% reported application occurred between July and August (Fig. 4.3a). In November 20% fewer beekeepers reported pesticide application in comparison to October and December. In the dry season which runs from between February to May (Renton, 2006) between 20% and 60% of beekeepers reported that pesticides were applied (Fig. 4.3a).

According to all beekeepers at least one pesticide was used within the area of the apiaries with a total of six different pesticides reported (Fig. 4.3b). The most frequently mentioned chemicals were GLY and Paraquat, mentioned by 70% of the beekeepers, 2,4-D was also frequently mentioned, and noted by 60% of the beekeepers (Fig. 4.3b). The three most frequently mentioned pesticides, Round up, Gramoxone and 2,4-D are all herbicides, along with Torodn, and Milagro. Merinsa is a fungicide and Nuemectin an insecticide. The active ingredients in all of these pesticides were included in the register of pesticides in Belize 2020 (“Register of Pesticides Active Ingredient 26 March 2020”, 2020). Paraquat was not included in the Multi-residue screen. The beekeepers all reported that knapsack sprayers were used for pesticide application.

Four crops were reported by the beekeepers as receiving pesticides application (Fig. 4.3c). 70% of beekeepers said that corn crops were sprayed with pesticides, 60% said beans were sprayed, and 10% of the beekeepers said that citrus and tomatoes were sprayed (Fig. 4.3c). No other crops were mentioned in relation to pesticide application. These crops however were seen in extremely low levels within the bee bread sampled collected in Chapters 2 and 3 (Fig. 4.3d).

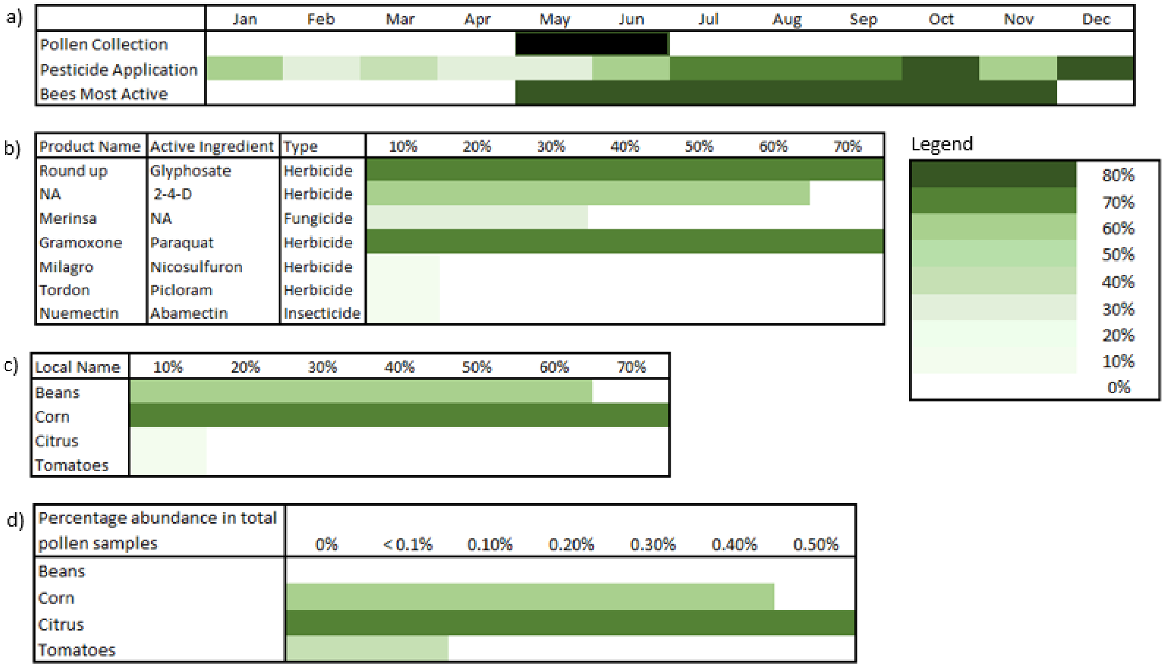
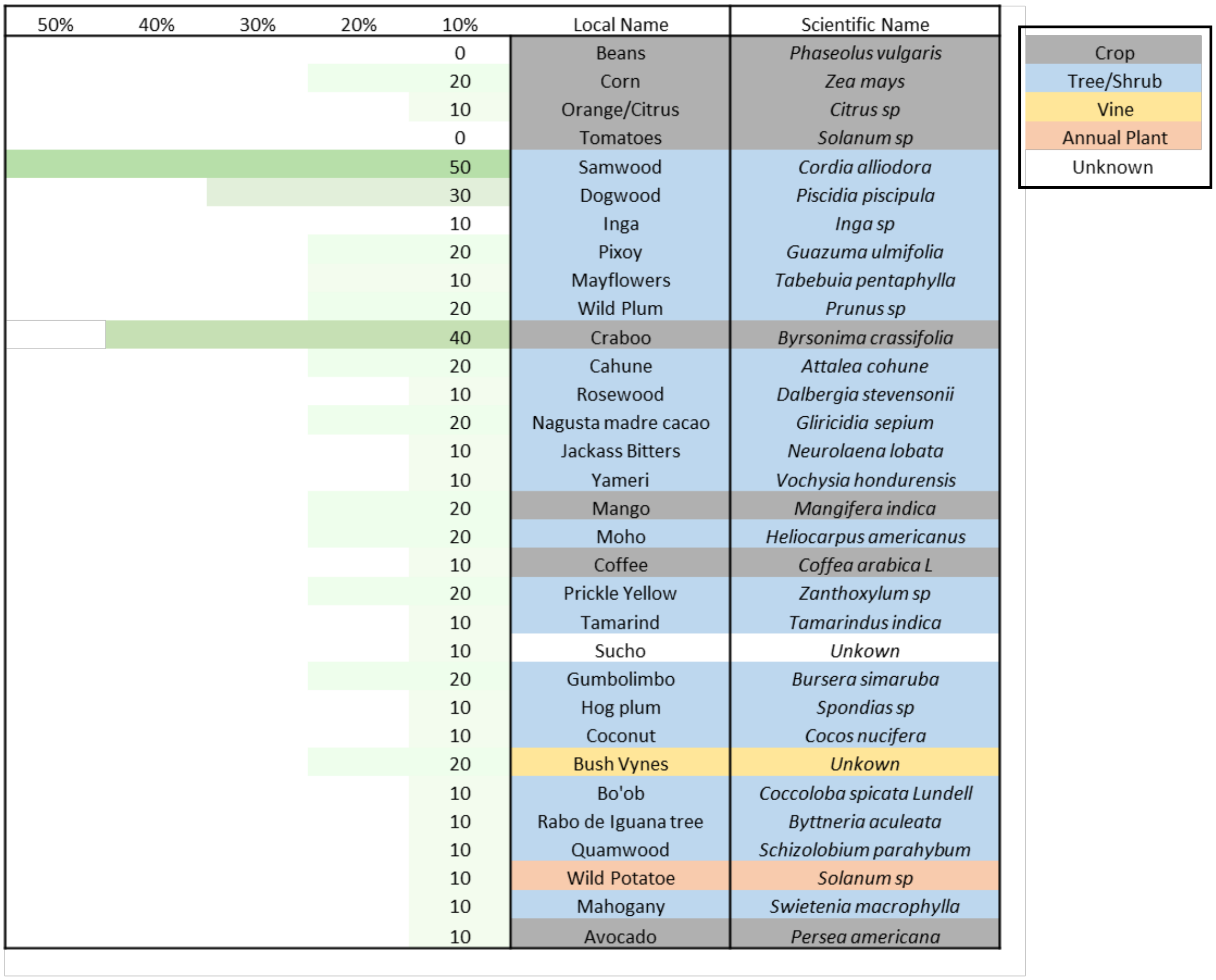


*Figure 4.3. Beekeeper surveys (10 Beekeepers were interviewed). a) The percentage of beekeepers who listed each month when asked when pesticide application occurred. Also displayed are the months when pollen was collected (black filled), and the time of year the honey bee is most active (Pesante, 1985). b) The percentage of beekeepers who mentioned each pesticide when asked which pesticides were applied within a 10 km radius of their hives. c) Plant species which according to the beekeepers were sprayed with pesticides, the percentage each plant was mentioned is displayed. d) For the plants the beekeepers listed as experiencing pesticide application, the percentage abundance of their pollen found in all bee breads samples (Chapter 1) is provided. *Tomatoes belong to the family Solanaceae which was present in the pollen samples collected. These morpho-species could not be identified to genus or species and so these pollen grains could be tomato, but it is not certain.*

There was a wide range of plant species reported by the beekeepers when asked what plants they could see honey bees foraging pollen from (Fig. 4.4). It was found that 22% (7 species) were crop plants, with Craboo (*Byrsonima crassifolia*), corn (*Zea mays)* and mango (*Mangifera indica)* the prominent taxa mentioned. Beekeepers did not mention beans or tomatoes as plants which bees forage pollen from, whilst citrus was mentioned by 10%.



50%	40%	30%	20%	10%	Local Name	Scientific Name
				0	Beans	*Phaseolus vulgaris*
				20	Corn	*Zea mays*
				10	Orange/Citrus	*Citrus sp*
				0	Tomatoes	*Solanum sp*
				50	Samwood	*Cordia alliodora*
				30	Dogwood	*Piscidia piscipula*
				10	Inga	*Inga sp*
				20	Pixoy	*Guazuma ulmifolia*
				10	Mayflowers	*Tabebuia pentaphylla*
				20	Wild Plum	*Prunus sp*
				40	Craboo	*Byrsonima crassifolia*
				20	Cahune	*Attalea cohune*
				10	Rosewood	*Dalbergia stevensonii*
				20	Nagusta madre cacao	*Gliricidia sepium*
				10	Jackass Bitters	*Neurolaena lobata*
				10	Yameri	*Vochysia hondurensis*
				20	Mango	*Mangifera indica*
				20	Moho	*Heliocarpus americanus*
				10	Coffee	*Coffea arabica L*
				20	Prickle Yellow	*Zanthoxylum sp*
				10	Tamarind	*Tamarindus indica*
				10	Sucho	*Unkown*
				20	Gumbolimbo	*Bursera simaruba*
				10	Hog plum	*Spondias sp*
				10	Coconut	*Cocos nucifera*
				20	Bush Vynes	*Unkown*
				10	Bo'ob	*Coccoloba spicata Lundell*
				10	Rabo de Iguana tree	*Byttneria aculeata*
				10	Quamwood	*Schizolobium parahybum*
				10	Wild Potatoe	*Solanum sp*
				10	Mahogany	*Swietenia macrophylla*
				10	Avocado	*Persea americana*

Crop
Tree/Shrub
Vine
Annual Plant
Unknown

Figure 4.4. The percentage of beekeepers who mentioned each plant species when asked to list the plants most frequently foraged for pollen by bees. Crop species are highlighted by grey shading, trees/shrubs by blue shading, vines in yellow and annual plants in orange. The percentage of times each species was mentioned by a beekeeper is shown *in the first column.*

4.3.2 Pesticide presence in bee bread samples from Belize

No detectable pesticides were present in any of the bee bread samples collected from the 14 sites. Samples were tested at a detection level of between 0.0002 mg/kg and 0.05 mg/kg depending on the pesticide and active ingredients being screened for (Table 4.1). The Multi Residue and GLY screen did not cover all potential residues listed by the Belizean Pesticide Control Board (“Register of Pesticides Active Ingredient 26 March 2020”, 2020), which listed 170 different active ingredients, of which the multi residue scan covered 85.

4.4 Discussion

The results of this study show that pesticides were not detected in any of the bee bread samples taken from the 14 apiaries in the Cayo and Toledo districts. While surprising this is not completely unexpected as studies carried out at both national and regional scales investigating the field realistic exposure of bees to pesticides have found varying results, relating to location and year. Additionally varying analytical and collection methods made it difficult to compare results between studies (Toselli and Sgolastra, 2020). Whilst it is difficult to compare results between studies, our detection range for pesticides is between 0.0002 and 0.05 mg/kg, which is similar to other studies which ranged from between 0.0001 to 0.02 mg/kg (Kasiotis et al., 2014). Of the 31 studies within the Toselli and Sgolastra. (2020) review, a third of studies did not find pesticide contamination in pollen and bee bread samples. Pesticide contamination was thought to be related to the number of pesticides permitted by each country. In 2019 the greatest importers were Brazil ($3.75B), France ($2.01B), United States ($1.58B), Germany ($1.57B), and Canada ($1.43B), while Belize imported $11.4M of pesticides, making it the 113th largest importer of pesticides globally ("Pesticides In Belize", 2019). This low level of pesticide importation, may therefore suggest low pesticide use, and help to explain the lack of pesticides in the pollen samples.

Strikingly, from the Toselli and Sgolastra (2020) review no studies investigating pesticide contamination of pollen were carried out in Latin America, and only 2 in South America from Brazil (de Oliveira et al., 2016) and Uruguay (Niell et al., 2015). A worldwide review analysing pesticides in bees found that in total 155 compounds were detected from 59 different chemical classes of pesticides in 1,369 samples. The most frequently detected group were insecticides, accounting for 55% of the samples under analysis. Fungicides were observed in 26% of the samples and herbicides in 4.6% (Hrynko et al., 2021). A US national survey of pesticide residues in bee bread samples found that only 18% of samples were pesticide free (Traynor et al., 2021). Most commonly, Varroacides were found making up to 41.8% of the pesticides detected. This was then followed by fungicides, insecticides, and then herbicides which made up 22.2%, 21% and 11.7%, respectively (Traynor et al., 2021). These studies highlight the difference in pesticide occurrence between studies, but also the types of pesticides more likely to be detected.

The beekeepers surveyed said that pesticide application occurred all year. The lowest months of application were February, April and May, where only 30% of beekeepers agreed pesticides were applied. For 8 months of the year, 60% or more beekeepers agreed that pesticide application occurred. Bee bread collection occurred in May and June, where 40% and 60% of beekeepers agreed pesticide were being applied to crops. This means that pesticides were likely to be present in the environment over the period of pollen collection, and therefore, suggests that despite pesticide being present in the landscape it does not result in contamination of bee bread. Pesticides are more likely to be found in bee bread samples over the pesticide application season (Villalba et al., 2020). This suggests that in Belize pesticide contamination of bee bread is likely to be highest between July and December. It would, however, still be expected that some pesticide residues would have been detected in the bee bread samples if the pesticide spraying was contaminating pollen collected by bees, as pesticides were likely applied in the months before bee bread collection. Bee activity in terms of pollen collection is high over the months of May and June (Pesante, 1985, Lawson and Rands, 2019), and the fact that no pesticides were brought into the hives over this period is therefore a positive situation for the bees. Even when pesticides are applied in the environment, and bees are actively collecting pollen, there was still no detectable levels are of pesticides found in their food.

The beekeepers also identified knapsack spraying as the main pesticide application method in Belize. This means that pesticides are applied directly and in close proximity to specific species/crops. Whilst there will be some pesticide drift to non-target species (García-Santos et al., 2011), this will not occur at the same level as more widespread pesticide application methods such as via aerial application or large crop sprayers. This is important because the species listed by the beekeepers as those which receive pesticide application do not correspond with either the species they recognised as those from which they have seen the bees foraging pollen from, or the plant taxa identified from the pollen grain analysis in Chapters 1 and 2 of this thesis. Corn and beans were identified as the crops which experience the greatest amount of pesticide application followed by citrus and tomatoes, however all of these species made up equal to or less than 0.5% of the total pollen sampled. The species which occurred in highest abundances making up over 60% of the pollen collected were all forest tree species, *Attalea Cohune, Bursera simaruba*, *Cepropia sp* and *Dalbergia brownei (*Chapters 1 and 2) and therefore unlikely to receive non-target pesticide application as residual spray is more likely to impact ground level plants.

It is unlikely that the pesticides may have broken down to undetectable levels before collection or analysis. In this case, pesticides would have been brought into the hives on the pollen pellets but would not have been present at detectable levels at the time of bee bread collection. This could be a positive situation for the bees as it would suggest that pesticides do not last long in bee bread form. However, during collection the freshest bee bread was sampled from each hive and therefore would have unlikely been in the hive for more than a few days, as recent studies suggest that over pollen harvesting periods, freshly stored pollen is consumed within days of deposition and that older pollen stores represent excess pollen (Carroll et al., 2017). Additionally, when local beekeepers where interviewed they reported that in May-June it would take “a strong hive 2-3 days to fill 50 cells with bee bread”. It is therefore unlikely that the pesticides broke down to undetectable levels as the half-life of the major pesticides most likely to be found in these bee bread samples, GLY, 2,4-D and Picloram, have long half-lives. Glyphosate is reported to have moderate persistence in the environment although its degradation depends on microbial activity in contaminated substrates, but is reported to have less persistence in water than soil with a half-life of a few to 91 days (Farina et al., 2019), and in soil is reported to be 7 - 60 days (Kanissery et al., 2019). 2,4-D may be less like to be picked up with an average half-life of 10 days in soils and less in water but can be significantly longer in cool, dry environments (Jote, 2019). The average half-life for Picloram is 50-100 days in soil (Parent, 2005) and Chlorypyrifos between 7 and 120 days (Christensen et al., 2009). Alternatively, the pesticides may have broken down between the collection and screening process. Bee bread collected from the hives was immediately stored with silica gel to keep the sample dry and stored in freezer conditions as soon as possible to preserve any pesticides present. Degradation is therefore unlikely and based on the half-lives of the dominant pesticides used in Belize, there would not have been sufficient time for the pesticides to break down between collection and freezer storage.

While the focus of this study was the pollen diet of the honey bee, it is also important to consider nectar, the main source of carbohydrates for the bee (Huang, 2010). Nectar is used to make honey through a number of reactions with different enzymes, including invertase and glucose oxidase (Huang, 2010). After the moisture in the honey drops to 17% - 18% the bees seal the cell (Huang, 2010). This honey then acts along with the stored bee bread as a continuous source of food and represents another source of exposure to pesticides along with the nectar directly itself (Sanchez-Bayo and Goka, 2014). Pesticides have been previously detected in both nectar, honey and pollen samples (Wen et al., 2021). Bees do not always forage for pollen and nectar simultaneously (Someijer et al., 1983) and therefore different host plants may be utilised for different roles and thus exposure to pesticides through pollen and nectar may vary. While there are different views over a bees ability to detect how good nutrient rich a pollen source is, it is agreed that bees are able to detect the sugar content and flow rate of nectar provided by flowers (Nicholls and Hempel de Ibarra, 2017), potentially leading to the utilisation of different plant species. It is therefore recommended that in the future nectar and honey sources are also investigated as a potential exposure route of pesticides to bees in Belize. Future work could also involve studying the contamination of pollen detected at various times of year. While pesticides have been shown to be applied all year round, pesticide application is likely more intense at specific points during the year, and therefore an annual assessment would be beneficial.

Within the main pollen collecting period (May-June), pesticides do not seem to be a major stressor to Africanised honey bees in Belize via contamination of pollen and consequential ingestion of bee bread, despite the likely application of pesticides in the environment. This is likely because the target species for pesticides use are not the same as the plant species that the bees are predominantly collecting pollen from. This, combined with the fact pesticide application occurs through knapsack sprayers where contamination of non-target species and in particularly trees is unlikely, substantially reduces the chance of bee bread contamination with pesticides.

Chapter 5: The impact of landscape on visual learning of the honey bee in the United Kingdom using the Free Flying Proboscis Extension Response

Honey bees have been recognised as model organisms for studying cognition for a number of years as they show complex behaviours, associative and non-associative learning and have accessible nervous systems which have provided a means to determine underlying mechanisms. Since the development of bee learning assays, methods such as the proboscis extension reflex have been utilised to study the effects of stressors such as pesticides and parasites on bee learning and consequently, their fitness. One of the major stressors facing bees currently is land use change, where natural landscapes are decreasing and often are converted to either agricultural or urban land. This chapter for the first time links landscape complexity and composition with honey bee cognition directly, through investigating in the field how honey bee visual learning abilities vary across different landscape complexities. It was thought that bees from hives based in different landscape complexities may vary in visual learning abilities due to different experiences and neural plasticity, and so bees which have experience in more complex learning environments may do better in learning tasks. To test this, bees were taught to associate a coloured yellow paper strip with a positive sugar reward and a blue coloured strip with a negative salt reward. Results showed that as edge density increased bee learning reduced, and while landscape diversity increased so did bee learning. This is important as bees must learn foraging routes, find profitable flowers, develop spatial maps as well as recognise intruders.

5.1 Introduction

The honey bee has been recognised as a model organism for the study of insect cognition, and has made substantial contributions to our understanding of learning and memory (Takeda, 1961). This is largely due to the honey bees complex range of behaviours and ability to respond to a diverse range of environmental stimuli (Frost et al., 2012). Land use change and associated decreases of natural habitat have been put forward as one of the major contributors towards bee declines (Otto et al., 2016), however, very little of the literature connects the effects of land use change to bee health/fitness and in particular bee cognition, with just a few studies investigating the effect on honey bee flight and landscape (Degen et al., 2016; Menzel et al., 2019). This study, for the first time, investigates the visual learning of honey bees across a gradient of landscape complexity.

The stability of managed honey bee populations varies across the globe and while overall honey bee stocks increased by approximately 45% between 1961 and 2008, due to an increase in the number of hives in countries such as China and Argentina (Goulson et al., 2015), there have also been widespread reports of declines (Goulson et al., 2015). In Central Europe honey bee declines of 25% were reported between 1985 and 2005, whilst in North America the number of managed honey bee colonies declined steadily over the past 60 years, from 6 million colonies in 1947, to 4 million in 1970, and to 2.5 million in 2014 ("Fact sheet: The economic challenge posed by declining pollinator populations", 2014). Numerous causal factors for this decline have been identified, this includes both socioeconomic reasons such as a decline in beekeeper numbers (Potts, Roberts, et al., 2010) and also environmental reasons including pathogens, pesticide exposure, and loss of foraging and nesting sites through habitat degradation (Potts, Biesmeijer, et al., 2010). Whilst few of these stressors can be considered novel, their increased intensity over the past decade has led to worsening effects on bees (Klein et al., 2017). Many of these stressors can be linked to a changing landscape, particularly the intensification and expansion of agriculture (Laurance et al., 2014).

Land use change and associated increase of agricultural and urban land, along with an associated decline of natural habitat is thought to be one of the major contributors of bee declines (Potts, Biesmeijer, et al., 2010). The effects of land use change are considered in terms of resource availability and its impacts on bee diet, nesting availability and pesticide exposure (Goulson et al., 2015). The effects, both in terms of landscape composition (Garibaldi et al., 2013; Kennedy et al., 2013; Ricketts et al., 2008), and landscape configuration (Lonsdorf et al., 2009; Winfree et al., 2008), are largely considered in terms of how they affect bee abundances and diversity (Clos et al, 2020). Few studies have, however, investigated how differences in landscape composition and configuration could affect bee behaviour, and no studies have investigated how difference in landscape composition/configuration directly impacts honey bee learning. Menzel et al. (2019) compared honey bee flight in a feature-less landscape and one rich in close and far distant landmarks. In their study three different forms of learning were tested; learning during orientation flight, learning during training to a feeding site, and learning during homing flights. They found that bees used elongated ground structures, such as a field boundary separating two pastures and hedgerows to orientate themselves. Furthermore, Degen et al. (2016) showed that bees learn the landscape and site structures during long-range orientation flights, leading to faster homing from explored areas. When bees were displaced before carrying out their first orientation flights they were not able to find their way back to the hive. Bees are not the only insect species where landmarks have been shown important in navigation. The digger wasp (*Philanthus Triangulum*), for example, has been shown to use landmarks to orientate their way back to their nest, this was demonstrated by displacing pine cones around the wasps next, the wasp then returned to the pine cones rather than the nest entrance (Tinbergen, 1953). Desert ants (*Ocymyrmex robustior)* have been shown to take 'snapshots' of their nest entrance whilst carrying out learning walks where landmarks were shown to be particularly important in a featureless environment (Müller and Wehner, 2010). The studies by Menzel and Degen propose the importance of landscape features in honey bee learning and suggest that they play a crucial role in the ability for bees to located resources. They do not, however, compare how different landscape configurations and composition can directly impact leaning/cognitive abilities.

These findings from Menzel and Degen led to the idea that the complexity of the landscape, particularly its configuration such as natural boundaries and edges in the environment might expose bees to different visual learning opportunities; this may then result in bees which develop different visual learning abilities. Experience changes brain structure by neural plasticity, and these structural changes to the brain have further consequences for information processing and thus effecting further experiences and learning, although the exact neural mechanisms for this in bees is largely unknown (Cabirol et al., 2018). It is therefore hypothesised that bees which have been exposed to more complex environments will have developed better learning abilities, as they are essentially trained more frequently.

Honey bees (*Apis mellifera*) were an early model for studying visual learning and memory due to their ability to learn and retain information (Rusch et al., 2017; Takeda, 1961) despite their relatively small brain size (Schultheiss et al., 2017). There complex social, navigational and communication behaviours along with relative simplicity of their central nervous system make them excellent models for studying learning and memory in response to environmental changes (Abramson et al., 1999; Reitmayer et al., 2019; Shepherd, 2018; Taylor et al., 1987). Honey bees provide a compelling system for sensing changes within the landscape and can represent responses of wild bees (Simcock et al., 2018). The advantages of using honey bees as a model system include a central nest structure of a known location where thousands of individuals are housed, with a single nest entrance and a global availability (Quigley et al., 2019).

A common feature of the stressors that affect bees is that they lead to a decrease in the cognitive abilities of bees, which in turn can affect the health of the colony (Shepherd et al., 2019). In their natural environment bees are exposed to a variety of both positive and negative cues. It is critical for bees to be able to respond to these cues appropriately to ensure survival (Shepherd et al., 2019). Negative cues include dangerous weather (heavy rain), toxins (Wright et al., 2010), and biotic threats such as parasites (Goulson et al., 2018), predators and invading robber bees. Bees need to both be able to detect these stimuli (Cappa et al., 2016) as well as learn their association with a negative effect, and then perform an appropriate response. They must also learn positive stimuli such as foraging routes to find profitable flowers and develop spatial maps, as well as communicate this to other foragers (Tsvetkov et al., 2019). Foraging bees are able to navigate complex environments, and are able to repeatedly visit rewarding food sites whilst avoiding those with little reward (Schultheiss et al., 2017). Studies carried out on visual learning in honey bees have demonstrated a wide range of capabilities from classical association of colour, pattern or orientation with a reward (e.g. Giurfa et al., 1996), to more complex tasks such as the delay-matching-to simple task (Giurfa et al., 2001; Rusch et al., 2017). Previous laboratory studies have found that exposure to chemicals such as pesticides or diesel fumes results in reduced learning (Frost et al., 2013; Siviter et al., 2018; Williamson and Wright, 2013). Siviter et al (2018) carried out a meta-analysis where 104 studies investigating the effect of pesticides on learning and 167 on memory were compared. They detected significant effects of pesticides on learning and memory under both chronic and acute application, and for both neonicotinoid and non-neonicotinoid pesticide groups. Other stressors effecting bee learning and memory include exposure to extremely low frequency electromagnetic fields, which at exposure levels commonly found in the environment (20–100 µT) reduced learning performance (Shepherd, 2018). Infection by *Nosema ceranae* had also been shown to reduce learning and memory retention in honey bees (Gage et al., 2018), a further study found that infected bees suffered further reductions in learning ability when fed on a low quality pollen diet (Gage et al., 2020).

Until recently the majority of studies investigating bee cognition have been carried out in laboratory and on honey bees, using either wild individuals brought into captivity or laboratory-reared bees (Muth et al., 2018). Studying cognitive traits in the field has proved to be problematic due to numerous confounding variables and little control, and thus carefully designed experiments are needed to measure variation between colonies (Morand-Ferron et al., 2015; Muth et al., 2018; Rowe and Healy, 2014). Muth et al. (2017) developed the protocol "Free-Moving Proboscis Extension Response" where wild honeybees and *Bombus vosnesenski,* the yellow face bumblebee*,* were successfully taught to associate colour with both positive and negative rewards. This method has the potential to be used in the field with managed and wild bee species with limited equipment and costs.

No previous studies have been carried out investigating directly the effects of landscape complexity on honey bee learning. This study aims to investigate how landscape configuration impacts honey bee (*Apis mellifera*) visual learning through the use of the “Free-Moving Proboscis Extension Response” across 12 colonies from different apiaries across Hampshire. Learning will be compared across a gradient of landscapes, ranging from low to high complexities in terms of configuration, and changes in landscape composition measured.

5.2 Methods

5.2.1 Study area

Twenty-six potential apiaries were identified from across southern Hampshire, UK (Fig. 5.1). The apiary sites are located predominantly around Southampton, Winchester, Portsmouth, Lymington and Lyndhurst. The landscape is dominated mostly by improved grassland, coniferous woodland, arable and horticultural land as well as urban/suburban habitat (Fig. 5.1). Data collection occurred between the 6th May and the 20th of July 2021, where the average daily maximum temperatures ranged from 15 to 22 (°C) (“2021 Weather History in Southampton”, 2021). Across Southampton 103.6 mm of rainfall occurred in May, 108.5 mm in June and 110.7 mm in July (“This Stations Monthly Rainfall Summary (mm)”, 2021). From 26 potential apiaries, a systematic process was used to select twelve suitable sites based on criteria relating to landscape composition and configuration, and minimum distance between apiaries. Replication was carried out at the landscape level, rather than at the hive level as while hive replication contributes to the precision of each data point, increasing the number of landscapes increases the number of data points and therefore contributes to the primary objective of understanding how landscape heterogeneity impacts honey bee learning (Fahrig et al., 2011).

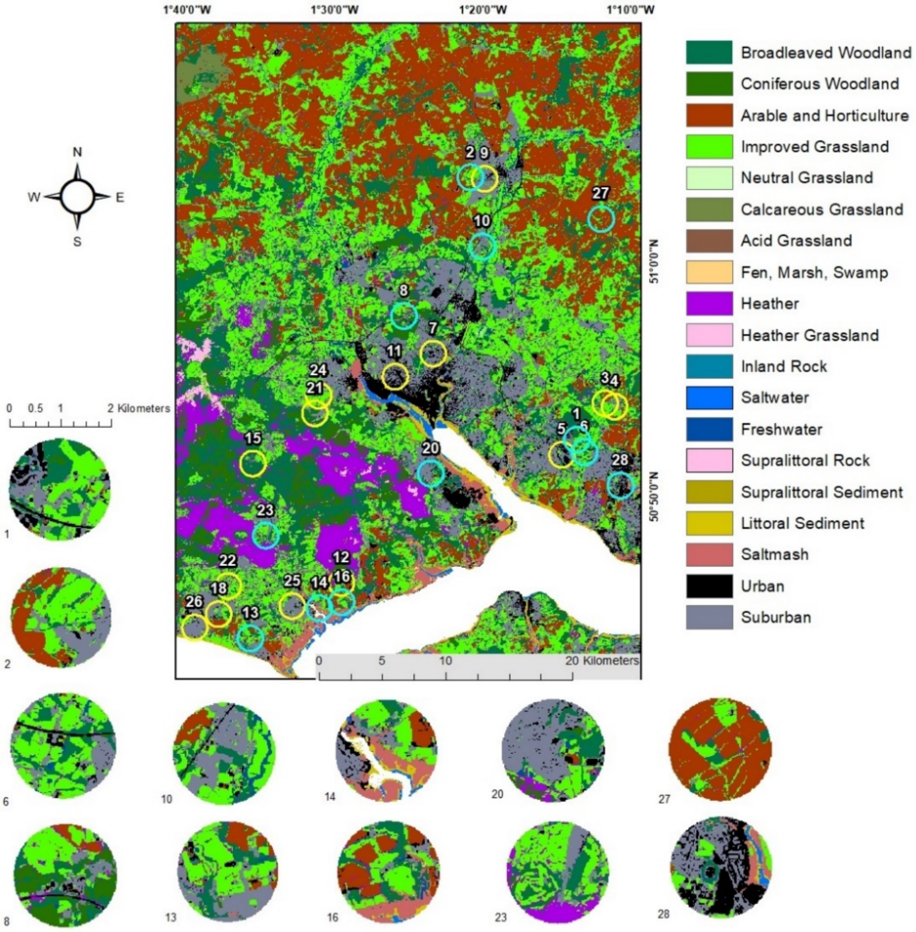


Figure 5.1. Twenty-six apiaries located across Hampshire with the potential to be used in this study. The 12 used in this study can be identified by a blue circle, which indicates the 1 km buffer zone around each apiary. Close ups are displayed of these sites. Yellow circles represent apiaries which were not chosen. Habitat types were obtained at a 25-m resolution, collected by the Landsat 5 Thematic Mapper, from EDINA Environment Digimap Service (Digimap, 2019).

5.2.2 Landscape Metrics

Landscape metrics were based on the 2019 EDINA land cover map at a 25 m resolution, which used data from the Landsat 5 Thematic Mapper (Digimap, 2019). Land cover was classified into 21 different categories (Fig. 5.1). A short description of each land cover classification is given below in Table 5.1, however for a full description see Centre for Ecology & Hyrdology (2015).

The landscape surrounding each apiary was evaluated at a 1 km buffer radius. A 1 km buffer radius was chosen as this represents the environment that the bees will most regularly be foraging/travelling through. Whilst bees do forage further than this, they will always need to be travelling directly through the landscape in close proximity to the hive. At a 500 m scale although this produces an even more focused area that they have to travel through, due to the scale of the landscape map, this begins to confine the landscape diversity and edges more. Therefore, 1 km was chosen as sensible scale taking these factors into consideration.

Seven different landscape metrics were calculated, and these could be divided into those reflecting landscape composition, (1) landscape Shannon Diversity, (2) percentage woodland (3) percentage agricultural cover (4) percentage urban/suburban cover; as well as landscape configuration (5) total edge, (6) mean patch size and (7) cohesion. Metrics were calculated using the 'landscapemetrics' package (Hesselbarth et al., 2020) in R (R Core Team, 2017). Definitions of the metrics used are provided in Table 5.2.

Table 5.1. Descriptions of land use cover provided by the EDINA land cover map. Full descriptions at Centre for Ecology & Hyrdology, 2015.

Land Cover Classification	Description
Broadleaved Woodland	Vegetation dominated by trees > 5m high when mature, with tree cover > 20%. Stands of native and non-native broadleaved trees.
Coniferous Woodland	Vegetation dominated by trees > 5m high when mature, which forms a canopy having a cover of > 20%. 'Includes semi-natural stands and plantations and both native and non-native coniferous trees.
Arable and Horticulture	Includes annual crops, perennial crops, woody crops, intensively managed commercial orchards and commercial horticultural land.
Improved Grassland	Grasslands either managed as pasture or mown regularly for silage production or in non-agricultural contexts for recreation and amenity purposes. Characterised by a few fast growing grasses and on fertile neutral soil.
Natural Grassland	Vegetation dominated by grasses and herbs on a range of neutral soils usually with a pH of between 4.5 and 6.5.
Calcareous Grassland	Vegetation dominated by grasses and herbs on shallow, well-drained soils. Soil pH tends to be greater than 6.
Acid Grassland	Vegetation dominated by grasses and herbs on a range of lime-deficient soils. pH less than 5.5.
Fen, Marsh, Swamp	Fens are peatlands which receive water and nutrients from groundwater and surface run-off. Marsh is a general term usually used to imply waterlogged soil. Swamps are characterised by tall emergent vegetation.
Heather	Vegetation that has > 25% cover of plant species from the heath family.
Heather Grassland	Vegetation that has > 25% cover of plant species from the heath family. "Heather Grassland" is less dense than "Heather".
Bog	Includes ericaceous, herbaceous and mossy swards in areas with a peat depth of at least > 0.5 m.
Inland Rock	Natural and artificial exposed rock surfaces which are > 0.25ha.
Littoral Rock	Those in maritime zone and rocky coastline, generally more extensive than supra-littoral rock.
Saltwater	Water containing salt, the sea.
Supralittoral Rock	Occurs above the high water mark. Features include vertical rock, boulders, gullies, ledges and pools.
Supralittoral Sediment	Occurs above the high water mark. Includes shingle beaches, sand dunes and machair.
Littoral Sediment	Includes features such as beaches and sand banks.
Saltmarsh	Coastal grassland regularly flooded by seawater.
Urban	Dense urban, such as town and city centres, little vegetation.
Suburban	A mix of urban and vegetation signatures.
Saltwater	Mapped around UK coastline, limited to land and tidal areas but no sea.

Table 5.2. Landscape metrics describing the composition and configuration of landscapes surrounding the 12 apiary sites.

Landscape Metric	Description
Composition	
Woodland Cover (%)	Percentage of Woodland Cover (Broadleaved and Coniferous) present in the buffer radius. $CA = sum(AREA[patch_{ij}])$ *where AREA[patch$_{ij}$] is the area of each patch in hectares.*
Agricultural Cover (%)	Percentage of agricultural cover in the buffer radius. $CA = sum(AREA[patch_{ij}])$ *where AREA[patch$_{ij}$] is the area of each patch in hectares.*
Shannon's Diversity Index – Landscape (H)	Shannon's Diversity Index for each buffer radius, measuring richness and evenness of the land covers. $SHDI = -\sum_{i=1}^{m} P_i * \ln P_i$ *where Pi is the proportion of class i.*
Configuration	
Mean patch area (ha)	Mean area of continuous land covers in the buffer radius. $AREA_{MN} = mean(AREA[patch_{ij}])$ *where AREA[patch$_{ij}$] is the area of each patch in hectares*
Total Edge (m)	A measure of all edges in the landscape *where eik is the edge lengths in meters.* $TE = \sum_{k=1}^{m} e_{ik}$
Cohesion	An aggregation metric measuring the physical connectedness of the corresponding patch types. $COHESION = 1 - \left(\frac{\sum_{i=1}^{m}\sum_{j=1}^{n} P_{ij}}{\sum_{i=1}^{m}\sum_{j=1}^{n} P_{ij}\sqrt{a}_{ij}}\right) * \left(1 - \frac{1}{\sqrt{Z}}\right)^{-1} * 100$ *where pij is the perimeter in meters, aij is the area in square meters and Z is the number of cells*

5.2.3 Site Selection

To select which sites were most appropriate to study two initial selection criteria were applied. Firstly, to reduce variation in habitat types between the landscapes, sites which contained over 75% urban and suburban landscape were not selected. Rural to semi-rural habitats were chosen as substantially more sites fit these criteria than those heavily dominated by urban and suburban habitat types. This led to the removal of 6 sites. The minimum distance between sites that were chosen was 1 km. This was chosen to prevent complete overlap of the sites. Two further sites were not used due to bee colony collapse which occurred between the time the sites were identified and when the experiment was carried out, leaving 18 sites.

To keep consistency between sites all hives had been established for at least a year and all had one queen. All hives were successfully producing brood as well as honey and bee bread stores. No hives used were from recent swarms or had recently experienced a changed in queen. Additionally, no hives were either currently infected by *Varroa destructor* or had been in the previous 6 months.

One of the primary tasks when considering the study sites was to reduce, or avoid, frequently occurring correlations among the landscape variables, such as between total landscape edge and landscape diversity. Here landscape configuration, rather than composition, has been deemed most important. This was based on the hypothesis that bees more frequently experiencing visually complex landscapes will have better visual learning abilities, due to their greater exposure to these landmarks and their more frequent use in navigation. The important role of landscape configuration in bee navigation has been highlighted by Degen et al. 2016 and Menzel et al. 2019. Therefore it was decided that landscape composition would be kept to a minimum whilst landscape configuration a maximum, allowing for greater clarification in the results as to the mechanism behind any potential change in learning. Landscape diversity was used in the models below to describe landscape composition.

Spearman's Rank correlation was calculated for all combinations of the best 12 sites which could be made out of the 18 sites available for landscape diversity and total edge/average patch size (identical selection for each). Cohesion of habitat types against landscape diversity within each 1 km buffer zone was also checked and yielded almost identical results except for two sites. The range in landscape diversity was kept to a minimum whilst the range in configuration metric was kept to a maximum to maintain landscape diversity as consistent as possible while capturing the greatest range in total edge. After all combinations of sites were run, the best combination was checked to make sure that the sites were not within 1 km of each other. If they were, the selection process was run again, this time asking for 13 sites; the 13th site was used to replace one of the sites too close together. The site which was removed was randomly selected. The selected sites are shown in Figure 5.2. In the analysis only total edge is used to describe landscape complexity due to correlations between total edge, mean patch area and cohesion. Total edge was decided to be the most relevant based on the studies by Degen et al. 2016 and Menzel et al. 2019.

The site selected to the far left of figure 5.2 was included after a planned site could not be accessed last minute. At this point, the majority of hives had already been sampled and thus the model could not be re-run. This hive was chosen for accessibility reasons. Whilst it increases the range in landscape diversity, it was decided to not remove it from the analysis.

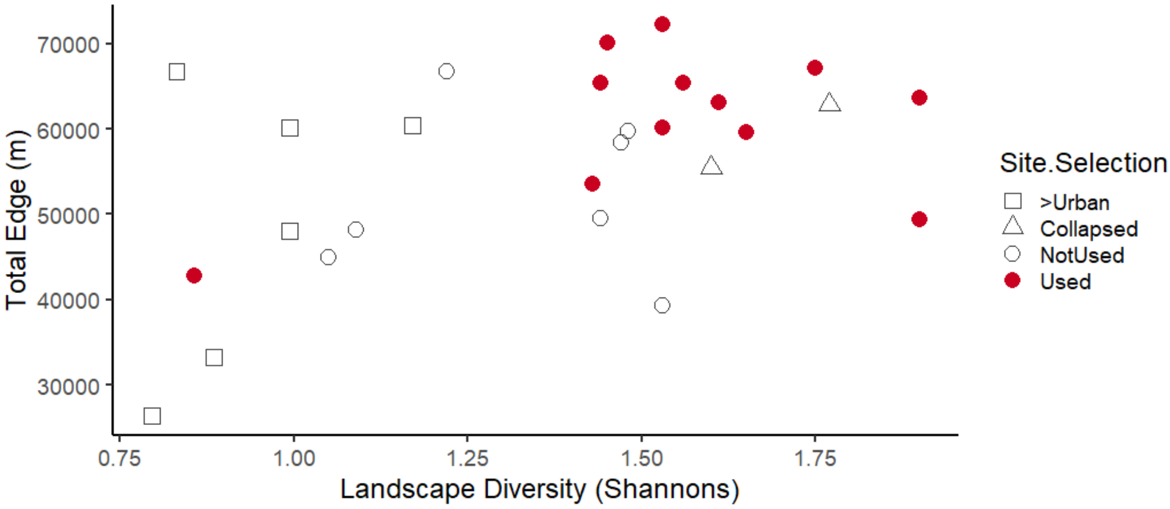


Figure 5.2. The total edge and landscape diversity within a 1 km buffer zone of the 26 potential hives which could be used in the study. The 12 sites selected to be used in this study can be identified by filled circles, sites which were not chosen are also indicated, including the reason, either the colony collapsed, there was too great a percentage of urban habitat within the area, or the model did not pick them out. Total edge includes all edges within the 1 km buffer (m), landscape diversity uses Shannon's diversity index (H) and is a measure of diversity, taking into account both the number of habitats and their evenness within the landscape.

5.2.4 Free Flying Proboscis Extension Response

The Free Flying Proboscis Extension Response (FFPER) method used to test cognitive responses was developed from a protocol described by Muth et al (2018). For each set of trials, 15 foraging active bees were collected from a single hive in each apiary. Bees were collected by blocking the entrance to the hive and forager bees identified via the presence of pollen in their pollen basket. Bees were collected into individual 50 ml sample tubes, in which thirty-two 5 mm diameter holes had been evenly placed to allow air flow. Two holes were drilled into the lid end allowing for the colour strips to be inserted. Bees were left stationary in these tubes for 15 mins to allow them to acclimatise to this environment. Each bee then experienced the learning trials as described below, which took a total of 2 hrs, and occurred outside in a shaded and sheltered location at least 20 m from the hives. Two sets of trials were carried out each day with the first initiated in the morning starting at 9am and the second in the afternoon starting at 1pm, with each set using clean sample tubes. Bees from the initial set were not released until the second set of bees had been collected. All bees were returned to the hive. More than 15 bees could not be collected at a time due to the length of time they could survive in the sample tubes against the time the trials took.

For learning trials, each bee was presented with a positive control stimulus (CS+, coloured strip with a 50% sucrose reward) followed by a negative stimulus (CS-, a coloured strip with 10% sodium chloride). The CS+ used was a yellow strip and the CS- a blue strip. The CS+ was always presented first and the bee was allowed to drink from it for 5s. This was repeated twice and was carried out as an initial teaching phase. This teaching phase was carried out to give an opportunity for the bees the learn the associated reward from each colour strip before being tested. For a further five test trials the bee was presented with the CS+ and the CS- strips simultaneously, the strip the bee fed on first was recorded. After the bee had sampled either of the two strips, one strip was removed, and the bee was allowed to sample the remaining strip. This was to reinforce the negative or positive reward from the remaining strip.

5.2.5 Statistical Analysis

All data analyses were carried out in R (R Core Team, 2017). To determine how successful the learning procedure was across all samples at all locations, a difference in the frequency of bees choosing to feed of yellow strips over blue strips was tested using X^2 tests. This was carried out for both the first test and the final 5th test.

A binomial mixed effects general linear model using the glmer() function from the lme4 package (Bates et al. 2015) in R was carried out to determine if landscape impacted the ability for bees to learn visually using the Free Flying Proboscis Extension Response. The response was binomial, where bees learned correctly a “1” was used and incorrectly a “0” during trial 5. Landscape diversity and total edge at a 1 km radius around the hives and the interactions between them were included as fixed factors, whilst date was treated as a random effect. Spearman's rank was used to check correlations between total edge (total edges (m) in the buffer zone) and landscape diversity (Shannon’s), where little correlation was found (s = 9774066, ρ = 0.052, p=0.130), (Fig. 5.3). The model used is provided below:

Bee Learning (Trial 5) ~Landscape Diversity*Total Edge (m) + (1|Date)

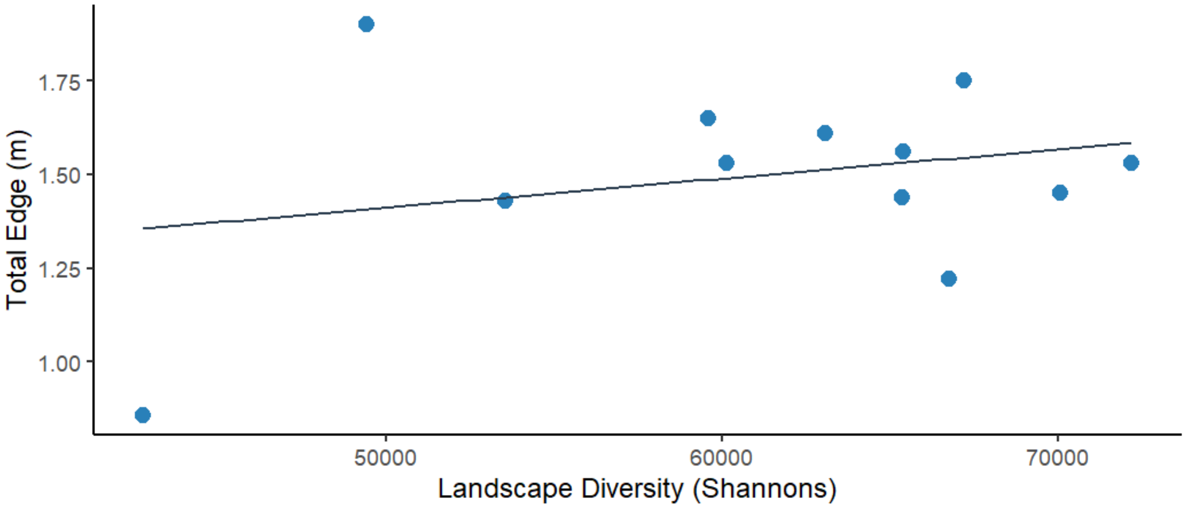


Figure 5.3. Spearman correlation coefficients between total edge (m) and landscape diversity (Shannon’s) at the chosen 12 apiary sites across Hampshire. Little correlation was found between landscape diversity and total edge (s = 9774066, rho = 0.052, p = 0.130).

5.3 Results

5.3.1 Honey bee learning

Honey bees increasingly choose the positively rewarded strip (yellow) across successive learning trials, where over twice the number of the bees chose the sugar reward over the negative strip (Fig. 5.4). At trial 1 just over 400 bees learned the yellow sugar reward, less than half the bees tested, whereas by trial 3 over 500 had, and by trial 5 just over 600 bees learned the yellow reward. The biggest increase in learning occurred between trials 1 and 2, whereas there was only a small increase between 4 and 5, showing a plateau of learning by this point. During trial 1 there was no difference between the number of bees which chose the colour blue and those that chose the colour yellow, showing no preference for the strip treated with sugar ($X^2 = 1.388$, df = 1, $p < 0.239$). At trial 2 ($X^2 = 16.998$, df = 1, $p < 0.001$), 3 ($X^2 = 83.431$, df = 1, $p < 0.001$), 4 ($X^2 = 112.320$, df = 1, $p < 0.001$), and 5 ($X^2 = 156.24$, df = 1, $p < 0.001$), there was a preference towards the yellow strip, which contained the positive sugar reward, successfully demonstrating learning.

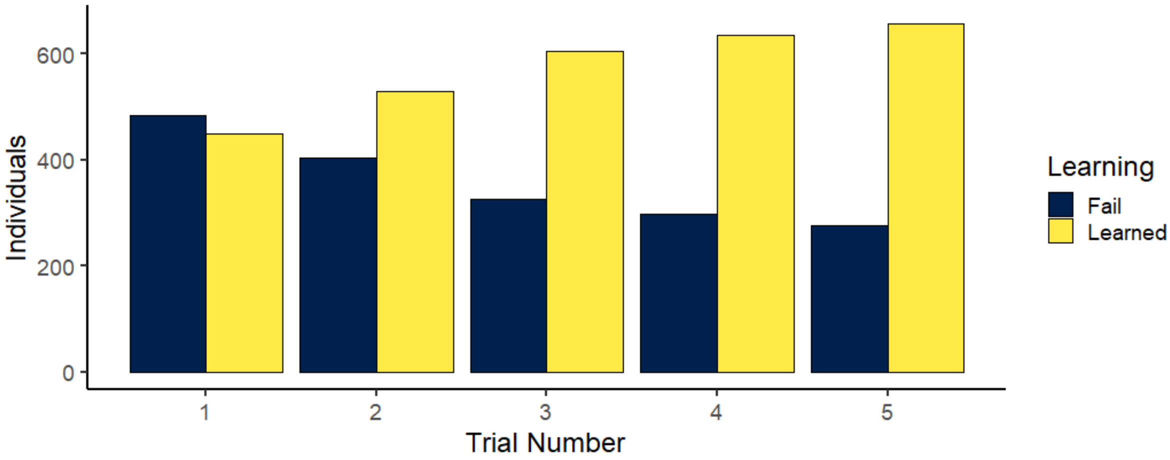


Figure 5.4. The number of bees which learned the conditioned response vs those that did not across 5 consecutive learning trials. Learning improved across trails where the greatest number of bees successfully chosing the sugar reward at trial 5. From trial 2 onwards significantly more bees chose the positive reward than the negative. Dark blue bars represent the bees which did not learn the conditioned response, and thus chose the blue negative strip containing salt water. Yellow bars represent bees which learned the conditioned response, and thus chose the yellow strips containing the positive sugar water.

5.3.2 The effect of habitat edge and landscape diversity on bee learning

Bee learning was found to range between approximately 50% - 80% across a gradient of different landscape complexities and compositions. Both total habitat edge and landscape diversity were found to affect the learning ability of honey bees. There was a positive effect of landscape diversity on the ability for individual honey bees to learn at the 5th trial (z= -4.221, df = 929, $p < 0.001$). At higher diversity levels, around 1.75 (H) over 80% of bee learned, this then reduced to almost 50% at a landscape diversity level of 1.25 (H) (Fig. 5a). One site did not follow this trend where at a low landscape density of almost 0 (H), just less than 80% of bees learned (Fig. 5.5a). Bees learned better in areas with less habitat edge (edge z= -61.914, df = 929, p = < 0.001) than bees whose hives were situated in an area of more edges. As habitat edge increased, learning ability decreased. When total edge was between 42,000 and 60,000 m per 3.1 km^2 (13,546 and 19,354 per 1 km^2) almost 80% of bees learned at trial 5, whereas this decreased to closer to between 50 and 70% where the edge values were between 64,000 and 74,000 m per 3.14 km^2 (20645 and 23.870 per 1 km^2) (Fig. 5.5b).

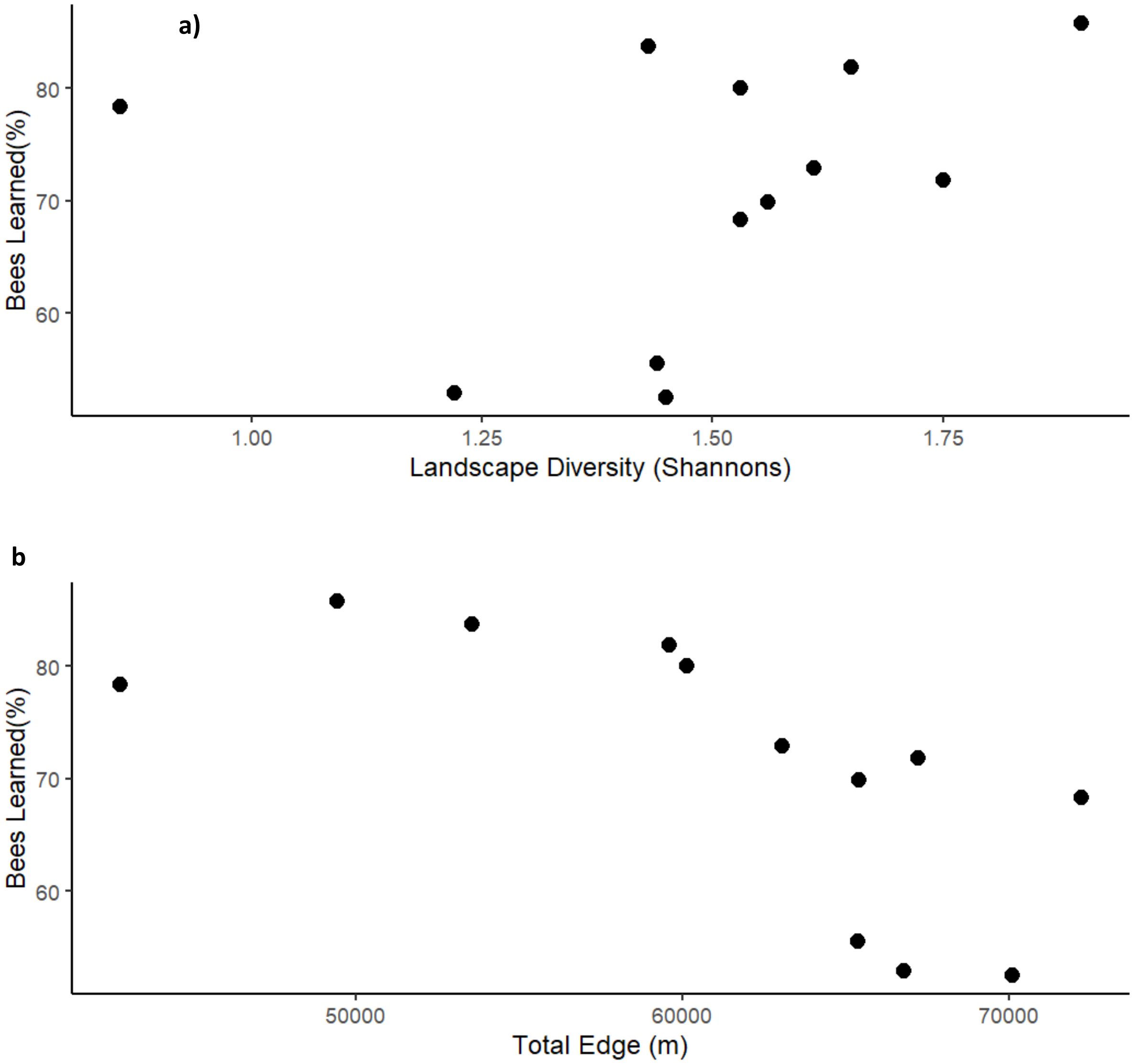


Figure 5.5. The effect of landscape diversity (a) and total habitat edge (b) on the percentage of bees which chose the sugar reward. There was a positive effect of landscape diversity (Shannon's) on the percentage of bees which learned the sugar reward, whereas landscape diversity increased, so did the percentage of bees that learned. Bees in landscapes with low total edge learned the reward better than bees in landscapes with high total edge.

5.4 Discussion

Foraging bees are able to navigate in complex environments in order to locate nectar and pollen sources (Schultheiss et al., 2017). The efficiency in which bees are able to learn and remember these food sources contributes to overall colony fitness (Muth et al., 2018). Vision is one of the important sensory modalities for organisms to perceive environmental information, and there is ample evidence of honey bees learning colours, shapes and patterns (Schultheiss et al., 2017), including the ability being able to discriminate complex forest scenes (Dyer, Rosa, et al., 2008).

The results show that honey bees can be successfully taught to associate a positive sugar reward with a coloured stripe over successive trials, and to learn to avoid a coloured strip containing a negative stimulus, salt. By the 5th trial just over 70% of bees had learned the positive reward, compared to just under 50 % during the first trial. This is in line with results found by Muth et al. (2018) where the percentage of bees choosing the correct colour varied between 0.75 and 0.95 at trial 5 depending on the difficulty in colour discrimination and adverseness of the unconditioned stimulus. Other studies looking at visual learning via bee responses to colour stimuli found that 60% of bees were able to learn the conditioned response (Dobrin and Fahrbach, 2012), whilst 80% of bees trained have been able to recognise facial cues (Dyer et al., 2005). Previous studies investigating olfactory learning in honey bees found similar levels of learning, where 70-80% of control bees were able to learn through the PER (Behrends and Scheiner, 2009; Giurfa and Sandoz, 2012; Matsumoto et al., 2012) and around 50% for SER (sting extension reflex) techniques (Shepherd et al., 2019; Vergoz et al., 2007). Importantly during the first test trial bees were shown to have no preference to either the blue on yellow strip, even after the initial two learning trials. This is important as it was possible that due to the colouration of the flowers they had been feeding from in the environment they may have entered the experiment with a preference, and therefore this may have influenced future learning. However, this was not the case.

This is the first study to directly investigate the impact of the landscape surrounding honey bee hives on their cognitive abilities. A previous study by Menzel et al. (2019) found that bees use elongated ground structures and boundaries in the landscape to learn flight routes back to their hives whilst a study by Degen et al. (2016) concluded that bees localised themselves according to learned ground structures. This led to the idea that the complexity of the landscape, particularly the configuration and thus natural boundaries and edges in the environment might expose bees to different visual learning opportunities, and thus bees may develop different learning abilities in relation to the complexities of the environment their exposed to. An adapted version of the proboscis extension response through visual learning was considered the best method to test any differences in learning across landscape. PER most commonly uses olfactory learning, however this adapted version utilised visual learning which is more relevant to the identification of edges and landmarks. Bees simplify a complex visual scene by extracting features such as size, orientation, distance and colour and therefore this method is suitable (Kheradmand and Nieh, 2019). Other options could be to look at pattern-based learning, as this may be the closest technique related to changes in landscape, however a field version of this method would need to be developed.

The total number of habitat edges within a 1 km of each bee hive was shown to impact the learning ability of the honey bee. My model showed that fewer edges resulted in greater visual learning per individual honey bee, and thus a more simple landscape may host colonies which greater learning capabilities.

This outcome could be explained in a number of ways. Firstly, the relationship of a landmark to the goal (e.g., food source) is significant in terms of both distance, specific orientation of the order of appearance between the goal and the landmark (Chittka et al., 1995; Huber et al., 1994). In a complex landscape increased edges and boundaries between landscape types may make a goal conspicuous in comparison to landmark features. In a simple landscape of continuous habitat, however, a goal may be relatively inconspicuous when compared to landmarks in the surrounding landscape and thus make visual learning of these landmarks more difficult and result in bees which are better learners. Alternatively, foraging distances have been shown to be greater in simple landscapes in comparison to complex ones (Steffan-Dewenter and Kuhn, 2003), and thus colonies surrounded by continuous landscape with fewer edges may need to forage further. This may require the bees to be able to learn a greater number of landmarks in order to make a foraging journey.

It is also important to consider that bees do not rely solely on landscape cues in order to navigate (Vladusich et al., 2005). Bees on a foraging flight may travel serval kilometres to visit their goal, and may visit multiple different locations, and to conduct these flights efficiently they rely on multiple methods. These include visual odometry where the bee estimates the distance travelled by the flow of spatial information sensed by the visual system as well as landmark cues (Dyer, Rosa, et al., 2008). While landscape cues are used across short distances, odometry is also used as a long range cue (Vladusich et al., 2005). Bee odometry does not rely on colour, using exclusively the signal from the their green receptors for determining image velocity (Chittka and Tautz, 2003). Rather than colour, it is the level of intensity contrast present in the landscape which influences the bees estimation of distance (Chittka, 2004). The visual properties of land terrain can vary considerably, depending on both the nature of the vegetation as well as the existence of manmade structures such as pavements or roads (Tautz et al., 2004), and therefore the accuracy and reliance a bees has on visual odometry will vary depending on the landscape. It is possible that in simple landscapes consisting of continuous habitat and few edges that the bees visual odometry is less useful due to fewer changes in contrast across the terrain, and thus landscape cues become more important in helping them to navigate to food sources, making them better visual learners. This theory, however, may be contradicted by the result that honey bees surrounded by a higher landscape diversity were able to learn at a higher success rate than bees whose hives were surrounded by less diversity. An increase in landscape diversity will lead to more contrast across the environment and this greater variety in land terrain may lead to bees relying more on odometry techniques and thus result in reduced learning abilities. This result, however, was not seen. Whilst changes in landscape diversity were kept to a minimum, an effect was still found, suggesting that landscape diversity may have a large impact on visual learning development and cognitive ability of a honey bee. When it comes to landscape diversity, it is important to consider potential secondary effects of the surrounding landscape. For example, as stated in previous chapters, malnutrition is a major stressor for bees, and nutritional stress will be experiences when sup-optimal plants are available for foraging (Vaudo et al., 2015). Studies have found malnutrition to have effects on honey bee learning where bees exposed to a pathogen, but also a low quality pollen diet, suffered reduced learning abilities compared to those just infected by the pathogen (Gage et al., 2020). It is possible that bees whose hives were located in a landscape of higher diversity had greater access to a wider source of nutrition from the surrounding environment and were fitter and had better learning capabilities. Other factors to consider are the pesticide levels within the landscapes surrounding the hives and well as exposure to diesel fumes and powerlines, as these factors have also been shown to effect honey bee cognition (Frost et al., 2013; Girling et al., 2013; Shepherd et al., 2019; Siviter et al., 2018; Williamson and Wright, 2013).

For future work some adjustments could be made to the study design if more time is available to complete the study. Firstly, rather than choosing pre-existing hives to work from it would be more beneficial for experimental hives to be placed in chosen environments. This way the exact age, history and management of each hive can be kept consistent, and the landscapes can be chosen to match the exact aims of the study, for example, creating the biggest range in habitat edge. Furthermore, other variables can either be measured or controlled between hives, such as honey production, brood production or colony population size to gauge an idea on the health and strength of the colony. Other data that would be beneficial to collect includes examples of the pollen diet, as this could be used to infer whether the bees in any location are experiencing nutritional stress. It could also be useful to measure pollution levels in the surrounding environment, either pesticides, diesel fumes or electromagnetic field strength as these could all effect bee learning. By controlling these factors, it will be easier to pin point the reason behind any differences in learning.

The landscape within a 1 km radius of honey bees hives has been shown to impact the ability for honey bees to learn visually, where colonies situated in landscapes containing fewer habitat edges were able to learn colours better than colonies placed in areas of greater edges. This is of importance due to the relationship between colony health and the ability for bees to learn. Bees must learn foraging routes, find profitable flowers, develop spatial maps as well as recognise intruders, if their cognitive abilities are reduced and they are unable to carry out these tasks, this will be detrimental for the continuous development of the colony. This increased ability to learn may be down to the need to learn and identify inconspicuous landmarks in areas of continuous habitat or a more limited use of visual odometry in these landscapes. This work contributes to the understanding of the working of visual learning in honey bees.

Chapter 6: Discussion

This study aimed to investigate the impact of landscape on honey bees, through changes in their pollen diet, pesticide exposure and cognitive abilities. Honey bees have been experiencing colony losses (FAOSTAT, 2009), relating to a number of synergistic stressors including pesticide exposure, increased parasitic infection, climate change, habitat loss and decreased floral diversity as a result of land use change (Goulson et al., 2015). Simultaneously the spread of the Africanised bee across the tropics has led to a decrease in beekeeping (Caron, 2001; Guzman-Novoa, 1996; Hellmich, 1991) due to the increased aggressiveness, nest abandonment and lower pollen stores (Ellis, 2008). Due to the success of new management strategies such as the placement of hives, protective clothing and abundant smoke use (Reátegui, 2020), beekeeping remains of great importance in the Latin America, where 7 of its countries are in the top 20 leading countries for honey production (Requier et al., 2018). In Belize there has recently been a push towards increasing beekeeping by the Ministry of Agriculture (“Ministry of Agriculture launches Honey Production support project”, 2019) with the aim of increasing rural livelihood options, particularly for the women and youths, as well as increase the national production of honey.

This study has shown that honey bees in Belize primarily forage on forest tree species, with annual and perennial wildflowers, and crop species playing a far less dominant role, even within agricultural landscapes. These findings contribute to a growing consensus about the importance of woody plants as pollinator resources (Donkersley, 2019; Mach and Potter, 2018), which have previously been overlooked in favour of annual and perennial flowers. Trees and large woody shrubs offer greater benefits over patches of annual and perennial flowers; a single large tree can produced thousands of flowers, and offer copious amount of pollen and nectar over a short distance (Mach and Potter, 2018), allowing bees to forage on extensive resources whilst expending relatively little energy. Landscape has been shown to impact the pollen make up of the honey bee diet, effecting pollen diversity, richness and composition, as well as honey bee cognition. This is thought to relate back to the bees preferred species; in this study more of the bees preferred pollen taxa, tree species, were likely present in the mixed landscapes, and acted to drive down pollen diversity due to recruitment strategies. This suggests that the presence of these preferred species is likely the factor most influencing the bees pollen diet, and is more important than landscape diversity.

Until now few studies have investigated the effects of bee stressors in the field, particularly looking at effects on individuals in relation to habitat (Havard et al., 2020). As of 2020 only 1% of the 293 studies investigating colony and individual bee health in response to stressors investigated the impact of habitat, and by far the majority of studies looking at stressors were carried out in the lab rather than the field, according to a meta analysist by Harvard et al. (2020). This thesis found that honey bee learning in the UK was better in landscapes of greater compositional diversity, but lower complexity in terms of configuration. This study, is the first to investigate how landscape affects honey bee learning directly, by comparing learning abilities from bees located in different landscape types. It is important to study these effects on bees as in the long term decreased learning ability will lead to decreased colony performance and long term fitness (Shepherd et al., 2019) and thus a decrease in bee abundance and populations. With this information beekeepers will be able to place their hives in areas more suitable for the honey bees, and offer them the best chances of survival. In order for a colony to be strong it must meet its nutritional requirements, as well as be able to learn foraging routes and other positive and negative stimuli.

A strong hive is important for beekeepers, as this will lead to increased honey production, a decreased risk of abscondment, as well as a hive that is likely to fight off any pathogen infection successfully, and therefore be a more reliable source of income. Beekeeping has the potential to greatly support families and local people, increasing their income and contributing to the economy of a country. Maximizing the success of each colony, however, will contribute substantially to the impact made. Whilst Africanised bees, in particular, need to be placed away from people, many beekeepers have a choice as to the exact location that there hives are located, it is hoped that information provided by this study into the effects of landscape on bees, will help guide farmers to the more suitable locations for their hives, producing stronger and more stable colonies. Further investigations into the floristics of Belize, impact of stressors of bee health as well as abundances whilst understanding the implications of honey bees on local species will help to provide more guidance towards beekeeping and promoting strong colonies.

6.1 Limitations and Improvements

Characterisation of the flora in Belize is still limited, and while descriptions of specific ecosystems are available, such as the savannah (Goodwin et al., 2013), or for specific forest regions such as the Chiquibul forest (Bridgewater et al., 2006), these studies do not cover the entirety of Belize. Moreover, neither do they cover the densities, distribution or abundances of the flora. Identification of the pollen species collected by bees could be identified to a higher classification if more knowledge was available as to the species of trees in the areas surrounding each hive. Furthermore, a greater understanding of the bees chosen pollen diet could be obtained with this information. If abundances and densities of the pollen species in the landscape were known, this could confirm why the bees choose to fed on certain plants. The protein contents of the pollen grains chosen where shown not to correlate with either there relative or weighted abundance in the bee bread samples, and therefore there must be a further explanation as to why the bees choose these pollen grains; a high abundance in the environment may be an explanation. In addition, the distribution of the pollen grains in the environment may help to explain the relationship of pollen diversity and richness with landscape diversity and richness. It is likely the abundance of each plant species in relation to the changing landscape that leads to these patterns of pollen abundance in bee bread. Further investigation in the field to the distribution and densities, particularly of the 10 most dominant pollen taxa consumed by the bees, would be essential information in regards to understanding bee diet.

The majority of previous studies investigating the effects of landscapes on bee pollen, although all in temperate regions, were carried out in a similar way to this study, using maps of the surrounding habitats to calculate landscape diversity (Danner et al., 2017; Donkersley et al., 2014; Kämper et al., 2016). Williams et al. (2012), however, investigated bee colony growth across landscapes, and used land cover maps to identify broad habitat types for non-crop land cover, but also quadrat sampling of flower densities of all plant species. This allowed an estimate of total floral resource availability for each colony. They found that floral availability strongly correlated with natural landscape types identified through mapping, suggesting that land cover provides similar information. This method allowed for a more direct link between the flora in the landscape and the pollen the bees were feeding on.

Previous to the information learned in this thesis, the identification of important plants within a 5 km radius of bee hives would have been extremely challenging. Firstly, the majority of previous studies of bee foraging identified annual and perennial flowers as being most commonly used by bees to obtain pollen (Donkersley, 2019), and therefore would likely have been the focus of species identification within the landscape. By contrast, Chapters 2 and 3 clearly show that bees in Belize primarily foraged pollen from tree species and not from the annual and perennial flowers as expected, and therefore these species may have been missed. Secondly, given that bees are foraging from trees they will forage high up in the canopy, potentially making the identification of the tree species bees are actually using difficult, particularly in high density forests where it is likely that only the trunk of the tree can be seen. Now we know the common pollen species used by bees, the densities and abundances of these plants in the areas surrounding the bee hives can be focused on, to help further explain bee foraging patterns.

To investigate the pollen diet of bees in Belize, bee bread samples were taken. Bee bread was sampled over pollen pellets from bees returning to the hive as bee bread captures the pollen a bee consumes over a wider period of time, whilst pollen pellets capture only that day of foraging. However only the most recent bee bread samples were taken, and so pollen representation will still be limited to the dry season. Previous studies, however, have shown that the season effects the pollen diet of honey bees (Danner et al., 2016, 2017) and, therefore, it would be interesting to determine how bee pollen diet changes over time in Belize, and if there is any interaction with landscape. Danner et al. (2017) found a strong seasonal variation in the amount and diversity of collected pollen, whereby the highest dry weight of pollen was collected in April and May, but richness was significantly lower in April and May compared to June and July. Similarly, Danner et al. (2016) found strong correlations between pollen diversity and season, but also that foraging range reduced in summer months when there was greater flowering in semi natural habitats. When looking at the common pollen taxa in the honey bee diet in Belize from this study, the majority were flowering between March and June, and therefore will not be as important to the honey bees diet outside of this season. Therefore it would be benefitial to collect more pollen samples during the wet season.

Another point of interest is honey bee foraging distance in Belize, as it would be beneficial to know the distance the bees travel in different landscape diversities, due to its impact on their energy expenditure, either through radio tagging (He et al., 2013) or studying waggle dances (Danner et al., 2017). There is the potential that in less diverse landscapes bees will travel further to acquire pollen sources (Danner et al., 2017), however this has a potential negative effect on bee health due to the increased energy needed to forage the same pollen requirements.

In terms of studying the potential pesticide contamination of the bee pollen diet it could have also been advantageous to study nectar contamination, in addition to the bees bodies themselves. Both pollen and nectar can be contaminated both directly through spray in the air but also through pesticides being translocated from the soil and through plant tissue (Zioga et al., 2020). Furthermore, honey bees are known to forage on different plants for both nectar and pollen resources (Robinson and Page, 1989); it may be that the honey bees consume nectar from plants which do experience pesticide exposure, and thus creating a different exposure pathway. A way round this could be to study the bees body directly, and measure any pesticide accumulation here, as this would provide the information as to if the bees themselves are experiencing pesticide contamination. A limitation here however is that the mechanism of exposure is lost, and can no longer be related to any specific area of the environment. It would perhaps therefore be most beneficial in the future to study both the bees body, pollen and nectar at the same time, providing both information as to the route of exposure as well as if this transfers through to the bee itself. Furthermore, in this study only the most recent pollen was collected. Whilst this was decided so that the collection period of the pollen could be identified, and to reduce the chance of pesticides which were present degrading before collection, it does mean that this study can only describe pesticide exposure during the dry season in Belize. Therefore, another study during the wet season may be beneficial.

When it comes to studying the effect of landscape on cognition of honey bees in the UK, further work could be carried out here too. One of the main limitations of carrying out this type of the study in the field was the number of uncontrolled variables. Therefore, a repeat of this study however conducted using experimental hives would be beneficial. If hives could be set up at the same time across numerous different landscapes and managed across the experiment by the same team, this would allow for greater consistencies between the hives. It would also be beneficial to gain a map of the linear landmarks with the buffer zone of the hives. In the study carried out in this thesis only a habitat map was used as this is what was available for the given locations. However, if a map of the hedgerows, paths and rivers for example could be created this would allow for more information on the linear paths around the hives, as well as the habitat boundaries used in this thesis.

6.2 The importance of honey bees to beekeepers in Belize

The beekeepers in Belize were interviewed in order to gain a better understanding of potential pesticides that the bees may have been exposed to. However, during this process, information as to the importance of these bees to the beekeepers was also learned.

All of the beekeepers had been beekeeping for at least 6 months, however the majority had been for 3 years or more, with the most experienced having kept bees for 5 years. When asked about the importance of beekeeping to them the majority described a source of financial income as the main reason, and for a few was there only source of income. However, two mentioned the nutritional value of both the pollen and the honey as consumables for themselves.

The beekeepers described the limitations to beekeeping in Belize, with a number mentioning access to materials and equipment as the main limitation. Others described the fear of working with the Africanised honey bee, as there aggressive nature makes working with the species difficult, and potentially hazardous without the correct protective gear. Consequently, whilst beekeeping in Belize is growing in popularity particularly due to its financial incentive, the industry is still limited due to its accessibility. In order for further rises in beekeeping at the same level as seen in neighbouring countries such as Mexico, the knowledge of how to keep bees, and the availability of the equipment needs to become more widespread and affordable.

Furthermore, beekeepers were asked what they believed were the biggest threats towards beekeeping in Belize. Surprisingly only two beekeepers believed that deforestation proposed a threat. Climate change was mentioned the most, as the beekeepers felt that the dry season was lasting too long and this was causing a change to the floral resources available as opposed to effects of deforestation and land use change. In addition, flooding and fire was mentioned by one beekeeper as a product of climate change, they claimed that they had been seeing an increase in both fires and floods at different times of the year, and that this was reducing the forage available for the honey bees. The presence of army ants was described by two different beekeepers as a potential threat, as they found that the bee brood was being predated upon, and that this was resulting in the collapse of their hives. These beekeepers described multiple ways they were trying to reduce these attacks, including placing their hive off the ground of tires containing oil, preventing the ants from reaching the hives. This however was not always successful.

From talking to these beekeepers it is clear that beekeeping plays an important role in there lives, and consequently more efforts need to be made in order to improve both the accessibility to beekeeping in Belize, as well as provide more information which could help improve the success and productivity of the hives. In the future, the issue of predation by army ants, may be an important and beneficial area to study.

6.3 Honey bees as a model system

Whilst this study focused on honey bees, not only for their uses in honey production supporting livelihoods and important role in pollination, they are also considered effective model species. This is due to their central nest structure, single nest entrance, known location and global availability, as well as their generalist nature which positions the honey bee to provide information on plant species other pollinators interact with (Quigley et al., 2019).

Whilst there is an estimated 5000 species of bees in the neotropics (Freitas et al., 2009), in Belize very little information is available on the species present and there abundances. To our knowledge only one study has been published on wild bee species in Belize (Schüepp et al., 2012) and consequently, even less is known about how these bees are affected by changes in land use and subsequent stressors. A review by Belsky & Joshi (2019) found evidence of numerous wild bee species including *Bombus terrestris*, *Tetragonula carbonaria*, *Osmia* bicornis, *Andrena flavipes*, *A haemorrhoa*, and *Meliponini sp* all being affected by land use change, either through a decrease in abundance, changes in foraging behaviour or a decrease growth rate. Data as to how wild bees in Belize are effected by land use change would therefore also be extremely valuable. If the honey bee is applied as a model system in Belize, wild bees may also not experience huge negative effects of land use change. As honey bees are extreme generalists, their diet breadth is noted to overlap considerably with many other nectar- and pollen-feeding insects (Huryn, 1997). Wild bees may also be utilising tree species over crops and annual flowers in the same way as the honey bee, and thus reducing the risk of exposure to pesticides. Alternatively, the use of the same resources may increase the likelihood for competition and thus there may be potential negative effects on wild bee abundances and species richness. However, whilst honey bees offer a model as to how wild bees may be affected by stressors, they in some instances been shown to respond differently to certain stressors such as pesticides. Almeida et al. (2021) for example found that the stingless bee *Partamona helleri* was more severely affected by pesticide exposure than the honey bee, likely due to its smaller size, although both species were impacted. Therefore recognition needs to be made when extrapolating the experiences of honey bees onto other bee species, as they may not necessarily respond the same in all instances.

These differences are also true when it comes to cognition, and therefore it important to be aware of some of the differences between honey bees and other wild bee species. For example, honey bees have better colour discrimination that bumblebees, whilst bumblebees shower greater acuity in the detection of colour stimuli (Dyer, Spaethe, et al., 2008). This is potentially due to the origin of the species where the temperate zone origin of bumblebees placed greater selective advantage to small colour targets, whilst the honey bee, which first evolved in tropical conditions, needed to be able to identify thousands of flowers in a highly localised area (Dyer, Spaethe, et al., 2008). There is also a difference between bumblebee and honey bee attentional processes. Bumblebees show slow but accurate parallel searches compared to the fast but less accurate serial searches of honey bees (Morawetz and Spaethe, 2012). Furthermore colour preferences have been shown to change throughout different seasons in stingless bees but not in honey bees (Almeida et al., 2021). Consequently, changes in the environment may affect these species in different ways. Both wild bee species and honey bees have been taught to differentiate coloured strips based of a sugar reward, as Muth et al. (2018) successfully carried out this procedure with honey bees as well as *Bombus appositus, Bombus melanopygus, Bombus bifarius, Bombus vandykei, Bombus flavifrons, Bombus flavidus* and *Bombus insularis.*

Together this all highlights that where there is a lack of data on wild bees is available, data collected from honey bees may be used to help interpret potential effects of different stressors on multiple bee species. However, this does not negate the need to collect data on how landscape, diet, pesticides and pathogens affect wild bees, as their responses may differ. Studies are needed covering all bee species, in their respective habitats to better understand both their contributions at local levels as well as how regional stressors affect them. Therefore whilst the data collected in this study with the aim of furthering beekeeping in Belize and increasing our understanding of stressors on bees can be interpreted to also help fill missing gaps on wild bee species, data should also be collected directly for these species.

6.4 Impacts on wild bees

Whilst improving the colony strength of honey bees is beneficial for beekeepers, it is important to note that this may not be the case for the wild bee species within the country. Both wild bees and managed honey bees feed on pollen and nectar from floral resources, and consequently there is the potential for competition over these resources, particularly in simple homogenous landscapes with less variability of nesting and feeding sites available. This may result in decreased fecundity, abundance and survival of wild bees (Goras et al., 2016). As competition depends on numerous local factors and conditions, no consensus has been reached regarding the importance of competition of honey bees towards wild be species (Geldmann and González-Varo, 2018; Kleijn et al., 2018; Mallinger et al., 2017). It is, however, agreed that at high densities honeybees can inflict adverse effects on wild bee populations (Kleijn et al., 2018), with studies mostly reporting negative influences of managed honey bees on wild bees (Mallinger et al., 2017).

During the colonisation of the Africanised honey bee across and the Americas the Africanised honey bee expanded across greater than 16 million Km^2 (Roubik, 2009). Previously, much of this area had been devoid of honey bees as the European honey bee did not establish many feral populations, but by the end of the last century over one trillion honey bee colonies were present across Central and South America. Many studies, particularly those carried out in Europe and North America, have reported negative effects of the honey bee on wild bee populations, particularly at high densities (Guzman-Novoa et al., 2020). Long-term studies from Central America and Southern Mexico have, however, provided evidence that the presence of Africanised honey bees does not upset the balance of native wild bees and plant interactions. Evidence of the high resilience of native bees towards the presence of the honey bee comes from a long term study in Quintana Roo, Mexico (Roubik and Villanueva-GutiÉrrez, 2009). That study showed that while native bees changed their pollen preferences after the arrival of the honey bee, they were not impacted in terms of species richness, and in fact saw an increase in abundance some years after the establishment of the honey bee. It was suggested that indirect benefits from the honey bee could be occurring, such as greater pollination resulting in an increase in abundance of native plant species (David W. Roubik & Villanueva-GutiÉrrez, 2009). Interestingly the dominant pollen detected in the honey bees diet in this thesis, has also been documented in the diet of other bees. For example the Mayan stingless honey bee *Melipona beecheii* has been shown to feed on *Bursera simaruba* pollen in Mexico (Villanueva-Gutiérrez et al., 2018). Whilst *Cercropia* species were found in high abundances in the pollen diet of *Melipona beecheii,* and *Scaptotrigona Mexicana*, also in Mexico (Ramírez-Arriaga et al., 2018). Therefore, the pollination of these species may also act towards helping other species of bee which feed from these plants, by increasing these resources in the environment. It is not only the other bee species which benefit from the pollination carried out by the honey bee, for example both the Gibnut (*Cuniculus sp)* and Agouti (*Dasyprocta sp)* have been shown to feed from the nuts of *Attalea cohune* (Ya'axche, 2018). Demonstrating the benefits honey bees can potentially have to other species within the ecosystem.

Another long-term study carried out in Panama measured levels of wild bee species over 17 years, including the 10 years after the introduction of the Africanised honey bee. There was no measurable population level impact of completion between the native wild bees and the Africanised honey bee despite observational indications of competition at flower patches and at colony levels (Roubik, 2009). Similar results were revealed in Brazil, where the population of female solitary wild bees were monitored with trap nests (Roubik, 2009). Shortly after the study began the Africanised honey bee was introduced into Brazil, and while this study found that natural disasters and severe weather impacted solitary bees, the introduction of the honey bee did not.

6.5 Conclusion

This thesis provides evidence as to the importance of forest tree species in the pollen diet of bees in Belize, as well as further contributing to the literature over the importance of trees in bee diets in general. Landscape was shown to influence the pollen diet of the bees; however, this was largely due to the resulting locations of the bees preferred pollen species. The location and thus availability of these species then led to the resulting diversity and richness of the pollen samples. The presence of preferred species is therefore likely more important than the overall landscape diversity when considering honey bee diet, at least when resources are not limited. This can inform the local beekeepers in Belize as to the importance of trees in the bee's diet when considering the location for their hives. This cannot only be used to help them make decisions within beekeeping but also for other ventures which may impact the tree floral resources. Furthermore, no pesticides were identified in any of the pollen samples, this is good news for honey bee colony growth, as it means the brood is likely not exposed to pesticides, at least through pollen during the dry season. This information can also provide the beekeeper with confidence that in the current location of hives the bees are not being exposed to pesticides during the dry season via pollen contamination. In the final chapter of this thesis, for the first time the effect of landscape on honey bee cognition was tested and demonstrated that honey bee visual learning was better in more simple landscape. Whilst improvements to reduce the number of variables can be made to this study, to allow a further dissection of the results, it contributes to the growing knowledge of cognition in honey bees.

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