Pollinator Community Homogenization and Pollination Services in Agroecosystems

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DEDICATION

I would like to dedicate my work to my wonderful advisor, Dr. Laura Russo. She has been an incredible mentor and I will never forget the lessons I have learned from her. I would also like to dedicate this thesis to everyone who has supported me along the way. It has been a long journey for me, each stop along the path as meaningful as the last.

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ABSTRACT

Pollination, or the transfer of pollen to plant stigmas, is an essential part of plant reproduction. The term "pollination system" refers to the floral phenotype and pollinator of a given plant. Although angiosperms exhibit a variety of different pollination systems, most rely partially or completely on animals, particularly insects, to vector their pollen. In agricultural systems, understanding the pollination system of the crop species is necessary to produce an economically valuable yield. Moreover, agricultural management may affect pollination systems by altering the abundance, diversity, or function of the pollinator community. In natural ecosystems, there is a great diversity of pollinating insects. This pollinator diversity may be vulnerable to global change and land use. One concern is that land use change may homogenize these pollinator communities, which in turn might affect their pollination service to angiosperms. To better understand the effect of agriculture on the homogenization of pollinator communities, we conducted a survey of pollinator diversity in different land-use types in eastern Tennessee. We sampled flower-visiting (pollinating) insects from the landscape around experimental plots of plants native to Tennessee. We found that the plots represent a subset of the pollinator diversity at the landscape level at most of our sites but found no effect of land-use type of pollinator community homogenization. To complement this landscape survey, we also evaluated pollination services in a focal agricultural crop. First, we conducted a greenhouse study to evaluate biotic effects of tree health and pollen donor on fruit set. We found that tree identity and size affected the probability of fruit set in greenhouse cacao trees. Another way to evaluate the effect of management on agricultural pollination services is to measure fruit set in different management scenarios. For example, agroforestry is a more sustainable way to grow cacao (*Theobroma cacao*), an extremely important crop world-wide; however, the effects of agroforestry on cacao pollination are unknown. In addition, pollination may affect the varietal purity of cacao, thereby affecting its market value. We conducted a hand-pollination experiment on Criollo and Trinitario cacao grown within an agroforestry setting in Punta Gorda, Belize. We examined the self-pollination and cross-variety compatibility of these cacao varieties. We found that the Criollo variety can self-pollinate whereas the Trinitario variety cannot. However, both varieties are compatible with one another, leading to implications for pure heirloom chocolate production where they are grown in close proximity.

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INTRODUCTION

Natural ecosystem services provide many benefits to society, such as the creation of soils, providing fresh water, and pollination of our foods and native plants. Although angiosperms exhibit a variety of different pollination systems, most rely partially or completely on animals, particularly insects, to vector their pollen. Agricultural management may affect pollination systems by altering the abundance, diversity, or function of the pollinator community. Pollination services performed by animals account for almost 35% of global crop-based food production (Klein et al. 2007). Crops, such as *Theobroma cacao*, depend on insect pollination (Falque et al. 1995), while several others such as strawberries, show increased benefit from insect pollination (Klein et al. 2007). While honeybees (*Apis mellifera*) are typically used to assess the value of pollination services, native or wild bees also provide essential pollination services. Crop yield in 41 cropping systems globally has been shown to have universally positive associations with pollinator diversity (Garibaldi et al. 2013). Wild bees are the primary pollinators in many cropping systems (e.g., coffee) and responsible for the pollination of many insect-pollinated native plants, some of which have been shown to decline parallel with the decline of wild bees (Biesmeijer et al. 2006).

In agriculture, many wild bees visit crops, such as coffee (Klein et al. 2003) and watermelon (Kremen et al. 2002). Populations of native, wild pollinators can also help to boost pollination services for farmers. They are an extra resource to be utilized, however, populations are often reduced due to land-use practices in agriculture-based settings (Kremen et al. 2002). Large-scale farms impact the local pollinator populations due to decreased biodiversity and the reduction of forage and nesting resources (Winfree et al. 2009). Thus, agricultural systems often have negative impacts to the very pollination services on which they depend.

In natural ecosystems, there is a great diversity of pollinating insects. This pollinator diversity may be vulnerable to global change and land use. One concern is that land use change may homogenize these pollinator communities, which in turn might affect their pollination service to angiosperms. Simplified agricultural systems often have little to no natural habitat (Ponisio et al. 2016). Monoculture systems, commonly found in agriculture, convert diverse, complex floral and faunal landscapes into a landscape that becomes overly simplified (Ponisio et al. 2016). By simplifying or homogenizing landscapes, agriculture can have an influence on many types of communities. For example, a homogenized agricultural landscape affects the floral community by reducing the system to crop itself and associated common, nonnative species. In combination with natural habitat loss, the loss of diverse floral communities can lead to a homogenization of the pollinator community (Moreira et al. 2015). Specialized species are lost or replaced by more resilient generalists. The homogenization of communities caused by agriculture can affect the distribution of species, not only within the immediate landscape, but over large scales (Ponisio et al. 2016). Negatively impacting pollinator communities over large scales can lead to a loss of pollination services (Klein et al. 2009).

To better understand the effect of agriculture on the homogenization of pollinator communities, we conducted a survey of pollinator diversity in different land-use types in eastern Tennessee (Chapter 1). Seventeen percent of Tennessee's land-use is dedicated to cropland (1997 National Resources Inventory, NRCS). While there have been several studies investigating pollinator communities on Tennessee crops (Wilson et al. 2016; Lawson 2020), there have been few studies exploring the impact of Tennessee's agriculture on the local pollinator communities. In order to further explore the impacts of agricultural land-use on pollinator services, we also evaluated land-use in a more natural, agroforestry setting on Theobroma cacao in Belize (Chapter 2).

For many years, there was confusion regarding how cacao was pollinated (Billes 1941). After many studies, cacao has been shown to be insect-dependent and pollinated by small midges (Posnette 1950). The biting midges (Forcipomyia: Ceratopogonidae) and gall midges (Cecidomyiidae) (Posnette 1950; Toledo-Hernández et al. 2017) are very small insects between 0.6 mm to 1.4 mm in length (Saunders 1959). Cacao's dependence on insect pollination could necessitate changes in modern management strategies. Cacao is generally grown in full sun, requiring the native habitat (usually rainforest) to be clear-cut and removed. An agroforestry approach to cacao would not only be more sustainable but would also create more pollinator-friendly habitat. Our goal (Chapter 2) was to determine whether cacao agroforestry might also relate to pollination services and yield.

Cacao grows under shade canopy in its natural habitat, but some commercial farmers grow cacao in the full sun after clear cutting existing forests. This practice has resulted in negative environmental impacts, including direct destruction of biodiverse rainforests. Agroforestry, the practice of intentional integration of trees and shrubs around crops in an agricultural setting, has many environmental benefits, including conservation of native habitat and reduction of soil erosion (Nair 1993). Cacao agroforestry approaches encourage small-scale farmers to lessen the ecological impacts of cacao farming by leaving the rainforest intact (Duguma et al. 2001). These approaches are more sustainable than clear-cutting. We hypothesized that cacao agroforestry may also lead to a higher economic impact for farmers both in the near and long term by improving yield through increased pollination (Young and Severson 1994).

Overall, this thesis explores concepts surrounding pollination services in agricultural systems, and how they relate to agricultural management at the landscape scale. I study the importance of pollination to yield and sustainability using a cacao-based agroforestry system and landscape level effects on pollinator abundance, diversity, and community composition in eastern TN.

CHAPTER I
THE INFLUENCE OF LAND-USE ON POLLINATOR COMMUNITY HOMOGENIZATION IN EASTERN TENNESSEE

Abstract

Human land use is making landscapes around the world more similar to one another. This homogenization leads to decreases in global (gamma) diversity through the loss of habitat-specialist species. Such losses in global biodiversity might not be apparent at small scales and are only detectable when communities of species are evaluated at landscape scales. This kind of landscape scale impact may play a role in the decline of insect populations, and in particular, the loss of valuable pollinating insects. Our goal was to determine whether certain types of land-use led to homogenization among pollinator communities in eastern TN. We spent 20.83 hours collecting approximately 1,500 specimens of flower-visiting insects at four pollinator garden plots at each of five different locations (Urban Gardens, Forage Grassland, Mixed Agriculture, Forest, and Organic Farm) over the course of six weeks in 2020. Each of the twenty plots contained six plant species native to the state of Tennessee. We concurrently spent a total of 16.67 hours surveying the landscape within a 50 m radius of each plot, collecting approximately 3,200 flower-visiting insects. We collected 116 different species or morphospecies, including 62 species of bees. Two species, *Lasioglossum fattigi* and *Lasioglossum simplex*, were both new records for the state of Tennessee. Our objectives were to determine 1) whether the pollinator communities visiting plants within the experimental plots were a subset of the pollinator communities in the surrounding landscapes and 2) if land-use affected the overlap in the flower-visiting insects between plots and landscapes (beta diversity). We found the pollinator abundance was higher in the landscape than the plots for four sites, and roughly equal at the Mixed Agriculture site. Across the landscapes surveyed, we saw significant differences in both plant diversity and plant density. We observed the highest insect abundance at the Urban Gardens and observed significant relationships between pollinator abundance and species richness and plant diversity and density. We found that the pollinator community visiting the plots were a subset of the community within the landscape. There was not a significant relationship between beta diversity and the proportion of agriculture in a 2 km radius around each site. Overall, we did not detect any evidence of homogenization within the pollinator communities at these locations in eastern TN.

Introduction

As the human population grows, so does the global production of food, both in terms of the extent and intensity of agricultural production. Many crop species rely on insect pollination and land-use intensification has been shown to reduce pollinating insect species richness and diversity (Flynn et al. 2009). For example, while native bees can provide sufficient pollination services on watermelon farms, continued agricultural intensification drastically decreases unmanaged pollination services (Allen-Wardell et al. 1998). In fact, intensive agriculture-based land-use changes have been shown to be a main driver of insect declines world-wide (Sánchez-Bayo and Wyckhuys 2019). Land-use changes not only result in a loss of biodiversity, but can also result in changes in species community composition, promoting disturbance-tolerant species over specialized

or sensitive species (Harrison et al. 2018). In this way, human land-use is homogenizing landscapes around the world (Baiser et al. 2012).

Biotic homogenization is defined as "the gradual replacement of native biotas by locally expanding non-natives" (Olden et al. 2004). This process involves unique, native species being "replaced" by common, widespread species. This often results in the loss of specialist species and instead generalist species take their place in the community (Manlick and Newsome 2021). Communities can naturally become more similar over time, but today homogenization is often driven by human disturbance (Clavel et al. 2011). Though human activity is relatively recent on Earth's geological timeline, it has had a large impact on Earth's environment. This shift in Earth's environment has suggested that Earth has entered a new epoch, the Anthropocene (Lewis and Maslin 2015). It is currently estimated that over 50% of Earth's land surface has been modified by humans (Hooke and Martín-Duque 2012). Not only has land-use intensification been shown to drive biodiversity loss on a global scale (Foley et al. 2005), but these losses in global biodiversity may be seen when communities are evaluated at landscape scales. Landscape scale impacts may play a role in the decline of insect populations, and of particular concern is the loss of valuable pollinating insects (Klein et al. 2007).

Our goal was to determine whether agricultural land-use has led to homogenization in pollinator communities in eastern TN. We compared the abundance and diversity of flower-visiting insects in 20 plots of fixed plant communities to the abundance and diversity of flower-visiting insects in five surrounding landscapes**.** Our research questions were: 1) are the flower-visiting insects in the research plots a subset of the surrounding insect diversity or a distinct community and 2) did land-use patterns affect the overlap in the flower-visiting insects between plots and landscapes?

Methods

Research Plots

Each of the research plots contained four individuals of six different perennial wildflower species native to Tennessee. In each of five sites, we planted four separate plots: 1) six species of the plant family Asteraceae, 2) six species of Fabaceae, 3) six species of Lamiaceae, and 4) a mixed plot two species of each of the aforementioned families (mixed plot) (**Figure 2**, all tables and figures located in the Appendix). These plots were planted for a previous study on pollinator preferences (Khalil 2020). These three plant families were chosen due to their attractiveness to pollinating insects. The Asteraceae family are visited by pollinators due to their open and easily accessible flowers. Asteraceae also often have long flowering durations. Fabaceae are known to have high-quality pollen and this family attracts pollinators with specific pollen requirements. Finally, the Lamiaceae have been considered to provide large quantities of high-quality nectar.

We observed flower-visiting insects on these plants in five different landscapes: 1) Urban, 2) Forested, 3) Organic Agriculture, 4) Forage Grasslands, and 5) Mixed Used

Conventional Agriculture. The University of Tennessee (UT) Gardens in Knoxville represented our Urban landscape. The UT Gardens is a highly curated and diverse landscape with heavily maintained trails of ornamentals, trees, and shrubs. Next, the UT Arboretum located in Oak Ridge was chosen as our Forested landscape. This 250-acre facility is home to over 2,500 native and exotic woody plants and areas of uninterrupted forest. With 14 acres of certified organic farming, the 90-acre UT Organic Crops Unit represents our Organic Agriculture landscape. Last but not least, Forage Grassland and Mixed-Use Conventional Agriculture were both located at the East Tennessee AgResearch and Education Center in Crossville, TN. This research and education center uses its 2,000 acres for conventional farming such as cattle, cash crops, and horticulture research. Both sites were placed on opposite ends of the East Tennessee AgResearch and Education Center, about one mile apart.

Plot Survey

We collected all insects that contacted the reproductive parts of the inflorescences within the research plots during standardized surveys. These surveys involved sampling on each plant species in bloom in each plot at a given site for five minutes, using an insect vacuum. During each collection event, we counted the number of inflorescences of each plant species. We calculated floral display size as the number of inflorescences open during a given sample multiplied by the average size of the inflorescences of each species.

Each site was visited weekly throughout the growing season. Collected insects were stored in a freezer until they could be pinned, labeled, and identified. All bee specimens were identified to the species level with the help of Sam Droege (USGS). The remaining insect specimens were identified to the highest level of resolution possible using Borror and DeLong's “Introduction to the Study of Insects” (Villet M.H. 2005). For the purposes of this comparison with pollinator diversity in the landscape, we include here only data collected during the six weeks from July 13th – August 17th, 2020 (see Khalil 2020 for further details on the plot survey).

Landscape Survey

For six weeks, July 13th – August 17th 2020, flower-visiting insects were collected in the landscape within a 50 m radius of each plot once a week (see, **Figure 2**) An insect net was used to capture the pollinating insects, which were then aspirated into a vial, and frozen until they could be further processed. The Urban and Forest sites contained many ornamental plants, and a Heavy-Duty Hand-Held DC Vacuum Aspirator from BioQuip was used to avoid damaging valuable plants. Honeybees (*Apis mellifera*) and carpenter bees (*Xylocopa virginica*) were counted and recorded, but not collected as they could be identified on sight. Collected insects were then pinned, labeled, and databased for identification. Bees were identified to genus, and species where possible, using the Discover Life dichotomous key. Identifications were verified by Sam Droege (USGS). All other non-bee pollinators were identified to family level using keys in Villet M.H. (2005) respective to their order.

We surveyed flowering plants within the 50 m radius around each research plot in the following manner. First, we ranked the flowering plant diversity on a scale from 1–10, where 1 was the least diverse survey area, and 10 was the most diverse survey area. Then, we ranked flowering density on a scale from 1–10, where 1 was the least dense flowering within a survey area, and 10 was the highest density of flowering within a survey area. Flowering plant diversity and density were ranked as such before each pollinator survey was performed. The same surveyor ranked diversity and density each time, according to their own perception. These subjective measures allowed us to qualitatively compare the survey areas to one other.

Landscape Analysis

To classify the landscape around our research sites, Khalil (2020) used ArcGIS Pro and the US National Land Cover Database (NLCD) (Dewitz, 2019). The land cover was classified within 2000 m with the Asteraceae plots as the center of each buffer zone (**Figure 2**). The 2 km buffer radius was selected for this study to be inclusive to all types of bees. While smaller bees have been documented to travel only up to 100–200 m, larger bees, such as *Bombus* spp., have been shown to forage over 2 km (Zurbuchen et al. 2010; Redhead et al. 2016).

Khalil (2020) aggregated the various NLCD land cover classes into three general land-use types: developed, agriculture, and natural. For the purpose of this study, we focused on the agricultural land-use class. Agriculture comprised of land cover categorized as pastureland, cultivated crops, or grasslands by the NLCD. Khalil (2020) then created a percentage of each land cover classification for the 2 km radius (Table 1).

Data Analysis

First, we tested for correlations between the average plant density and diversity among the landscape surveys. We compared the average abundance of pollinators (including and excluding honeybees) between the different sites. We also tested the relationship between plant density and diversity and flower-visiting insect abundance and species richness. We used a rarefaction analysis to test for differences among the species richness and diversity indices (Shannon and Simpson) at the different sites. Next, we calculated the alpha, beta, and gamma diversity of the different plots and sites. We tested for a relationship between the average beta diversity (comparing plot and landscape surveys) and the proportion of agricultural land-use at a 2 km radius around the sites.

Finally, we used Non-metric Multidimensional Scaling (NMDS) to compare the flower-visiting insect community (broadly and specifically just bees) among the sites and survey types. NMDS is an ordination technique that allows for visualization of multivariate responses to treatments. In our case, we were looking for overlap, or non-overlap in the community structure of the flower-visiting insects among these sites and survey types.

Results

Plot Survey

Khalil (2020) collected about 1,500 specimens during 20.83 hours of sampling. A total of 87 different flower-visiting insect species or morphospecies were collected with 46 (53%) of the species bees (of the families Halictidae, Megachilidae, Colletidae, Andrenidae, and Apidae). Halictidae (971 specimens) was the most abundant bee family collected within the plots during the sampling period. Apidae followed as the second most abundant bee family with 356 specimens. The most specimens were collected from the Cattle Forage (379 specimens) and the Organic Farm (318 specimens) sites.

Landscape Survey

We conducted 100 hand-net surveys and collected or observed 3,350 (3,324 considered for data analysis) flower-visiting insects during 16.67 hours of sampling. The landscape around each plot was sampled 5 times. 2,914 (88%) of the specimens were bees (Halictidae, Megachilidae, Colletidae, Andrenidae, and Apidae) and the remaining 410 (12%) of the specimens were non-bees (**Figure 3**). We collected 116 different species or morphospecies, including 62 species of bees (Error! Reference source not found.). We also collected six species of bees not found in the plot surveys: *Melissodes communis*, *Eucera* (*Peponapis*) *pruinosa*, *Hylaeus leptocephalus*, *Lasioglossum fattigi**, *Lasioglossum simplex**, and *Hoplitis producta* (Sam Droege, USGS). In addition, L. *fattigi* and L. *simplex* were both new occurrence records for the state of Tennessee.

We observed the highest abundance of pollinating insects at the Urban site with an average of 69.45 insects per sample (**Figure 4)**. It was followed by the Forest site, with an average of 36 insects per sample, and the Organic Agriculture site, with an average of 34.15 per sample. The Forage Grasslands and Mixed-Use Agriculture had an average of 28.8 and 11.25 insects per sample, respectively. After removing observed honeybees from the abundance (**Figure 5**), Mixed-Use Agriculture proportionally decreased the least, while the Forest and Organic Agriculture sites decreased by almost half.

During the survey, Halictidae represented the most speciose group, with 24 species collected. Apidae followed with 18 species, Megachilidae with 9, Colletidae with 3 species, and finally Andrenidae with 1 species collected (**Figure 6**). However, Apidae represented the most prevalent group totaling 1,924 individuals, while only 855 Halictids were collected (**Figure 7**). Out of the ~3,400 total insects, 1,310 (39%) were honeybees (*Apis mellifera*).

There was a significant difference in both plant diversity and plant density among the sites. There was a positive correlation between the average plant density and plant diversity in the landscapes around the plots (**Figure 8**). The Urban site had both the highest average plant density and diversity. There was a positive correlation with greater variance between the average plant density and average insect abundance per sample per plot (**Figure 9**). The Urban plots had the highest average abundance of insects. There is a

similar positive trend between average plant diversity and insect abundance (**Figure 10**). The pollinator communities sampled within the landscape had a higher abundance than the research plots in the Urban, Forage Grasslands, Forest, and Organic Agriculture sites (**Figure 11**). There was a similar abundance between the plots and landscapes in a Mixed-Use Agriculture landscape.

The Non-Metric Multidimensional Scaling (NMDS) ordination plots (**Figure 12**) showed community overlap between the sites and surveys. The NMDS of the different sites showed that all communities overlap in community composition, but overlap is less when only factoring in bees only. When comparing the plot survey to the landscape survey, we can see that the plot survey mostly overlaps with the much larger landscape survey (**Figure 12**). This suggests that the species diversity of the plots was a subset of the diversity within the landscape.

The rarefaction analysis showed we had high sample coverage for the landscapes. The Cattle Forage and Mixed Agriculture sites both had significantly higher flower-visiting insect species richness, Shannon diversity, and Simpson diversity than the other sites (**Figure 13**). When we evaluated just the bee species, all sites were relatively similar in terms of species richness, but the Cattle Forage site showed a significantly higher Shannon and Simpson diversity (**Figure 14**). We also compared the landscape and plot surveys (**Figure 15**) and found that the plots and landscapes did not differ in species richness. The rarefaction analysis also showed that we had a good sample coverage for both surveys and that they also differ significantly in terms of the two diversity indices, Shannon and Simpson. The difference in the diversity indices shows that the evenness was higher in the plots than in the landscape surveys. This difference was probably driven by the dominance of honeybees and carpenter bees in the landscape surveys. When we removed all non-bee insects from the analysis, we did see a small gap in the species richness between the plot and the landscape (**Figure 16**), however this gap was not significant.

Beta Diversity Comparison

As a precursor to calculating beta diversity, the alpha diversities for each plot between the plot survey and the landscape survey were calculated and compared for all insects (**Table 2**) and again for bee species only (**Table 3**). The plot survey generated an average alpha diversity of 16.5 flower-visiting insect species for each plot, while the landscape survey had an average alpha diversity of 26.8 species per plot. When looking at just bee species, the plot survey average alpha diversity was 10.45 and the landscape average alpha diversity was 16.35. The gamma diversities for each site were: Forest, 76; Gardens, 82; Organic Farm, 65; Cattle Forage, 84; Mixed Agriculture, 82.

Overall, the Forage Grassland had the highest average beta diversity (all insects), followed closely by Mixed Agriculture and Urban land types (**Figure 17**). When calculating beta diversity with bee species only, the Forage Grassland and Urban sites remained high, but Organic Agriculture had an increase in beta diversity compared to

when all insects were included(**Figure 18.**). However, these differences were not significant. We compared the average beta diversities of the five sites to the proportion of agriculture within a 2 km radius around the site (**Figure 19.** Scatterplot comparing agriculture-based land-use within a 2000 m radius around each research site on the x-axis to the average beta diversity of each plot on the y-axis. Each dot represents a different site. Beta diversity is calculated by using the diversity of all insects collected from the plot collections and the landscape survey collections.) and found no significant correlation. We excluded non-bee specimens and again compared the average beta diversities of the sites to the proportion of agriculture land use and again found no correlation (**Figure 20**). Given these results, we did not find evidence of homogenization in these communities of flower-visiting insects.

Discussion

The sites in our study were quite diverse, but across all of them we found strong relationships between plant diversity and density and insect abundance. In part, we observed higher plant densities and diversities in sites managed for gardens. The Urban site's four plots were all in well-manicured areas with many flower beds for display. The Forest site had two plots in a garden setting and two plots adjacent to the forest and they tended to have a higher plant density, diversity, and insect abundance. These were the two sites that also had the highest honeybee and carpenter bee abundances recorded, both extremely generalist bees that tend to benefit from human land-use. Many of the plants in these locations were put in place to support honeybees. The landscape of two plots near the forest, along with most of the plots at the Organic Agriculture, Forage Grassland, and Mixed-Use Agriculture, were mowed during our study. This reduced the plant density and diversity, further lowering the number of insects collected at these locations.

Overall, the sites all had relatively similar gamma diversities (**Table 2**). However, the Organic Farm site had the lowest gamma diversity at 65 compared to other sites (76, 82, 82, 84, respectively). Though not significant (**Figure 13**), one possible reason for this difference could have been due to mowing during our sampling period at the Organic Farm site. The sampling area had been mowed before our sampling took place many times, removing most of the flowers in the landscape. This would explain low collections for several dates, along with missed opportunities to collect different species of flower-visiting insects.

When we analyzed the surrounding landscape of our sites at a radius of 2 km, we did not see any effect of land-use on the homogenization flower-visiting insects collected nor overlap between landscape and plot surveys. It is possible that there were other types of land-use that may have had an influence on the pollinator communities in these agriculture systems. Also, the sites selected were in a patchy landscape. Native bees can thrive with patchy, heterogenous habitat (Winfree et al. 2009; Ruiz-Toledo et al. 2020) similar to those found in our sites. This could contribute to our results showing no correlation between land-use and pollinator communities. We also had two sites, the University of Tennessee Organic Crops Unit and the East Tennessee AgResearch and Education Center, which housed three of our field sites (Organic Farm, Cattle Forage,

and Mixed Agriculture). Research farms are more diverse than typical agricultural landscapes and contain many simultaneous research studies in various plots across the farm. Our findings might therefore not be representative of conventional or monoculture agriculture settings. Measuring landscape heterogeneity and patch structure in the landscape or extending the study to more conventional landscapes could elucidate the extent to which these constraints limited our findings.

APPENDIX

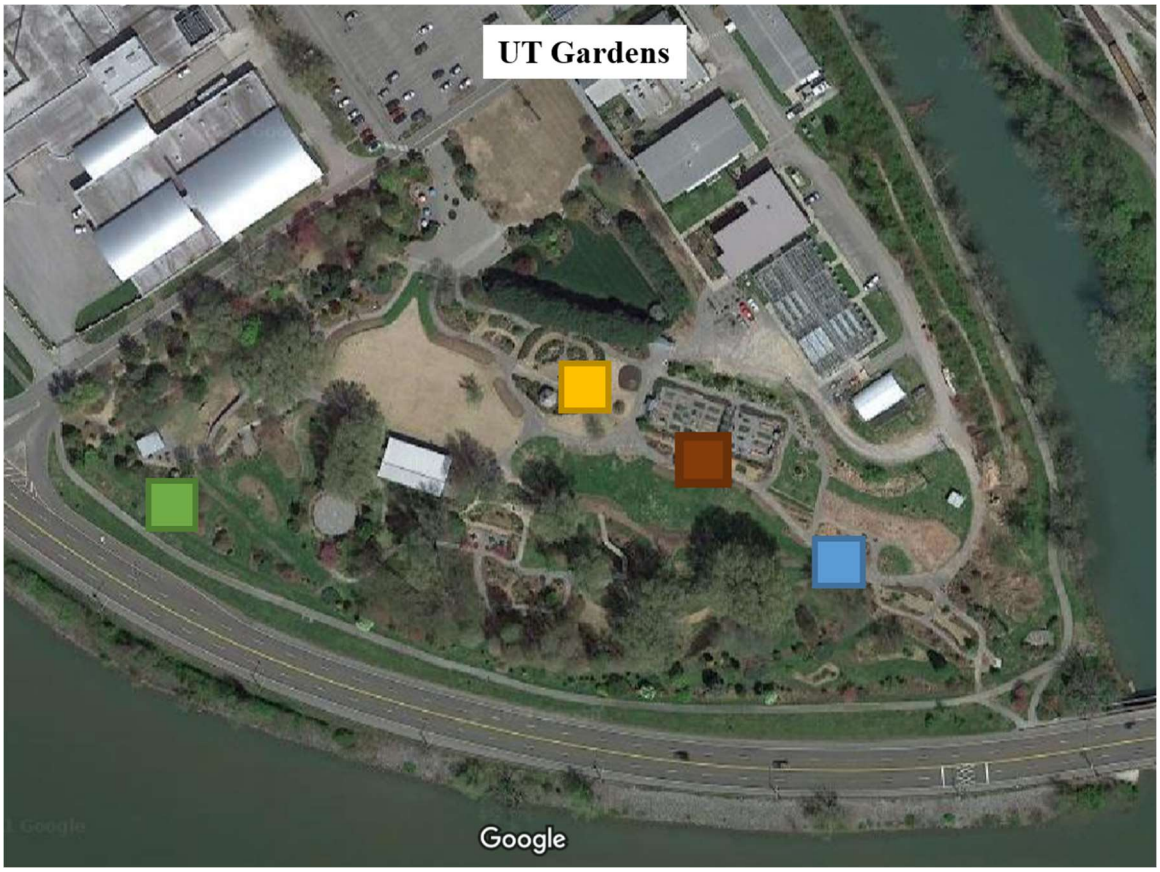

Figure 1. Diagram using Google Maps to show the UT Gardens research site. Each of the four colored squares represents a different plot. From left to right, the green square is Fabaceae, yellow is Asteraceae, brown is Mixed, and blue is Lamiaceae.

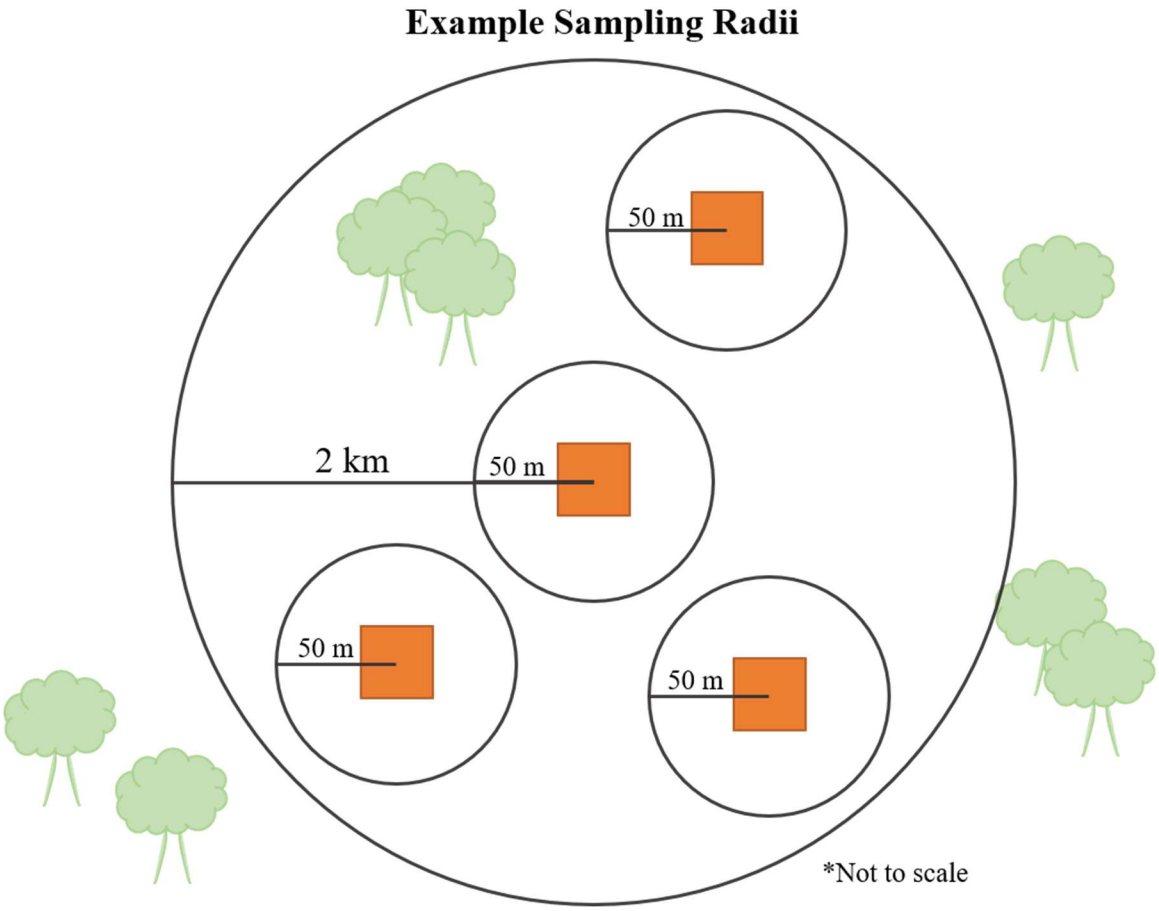

Figure 2. A heuristic diagram showing an example of the sampling that took place for the landscape survey. The four native plant plots are represented by orange squares which are surrounded by the 50-meter landscape sampling area. The 2-kilometer circle around all four plots represents the area analyzed during the GIS land-use analysis.

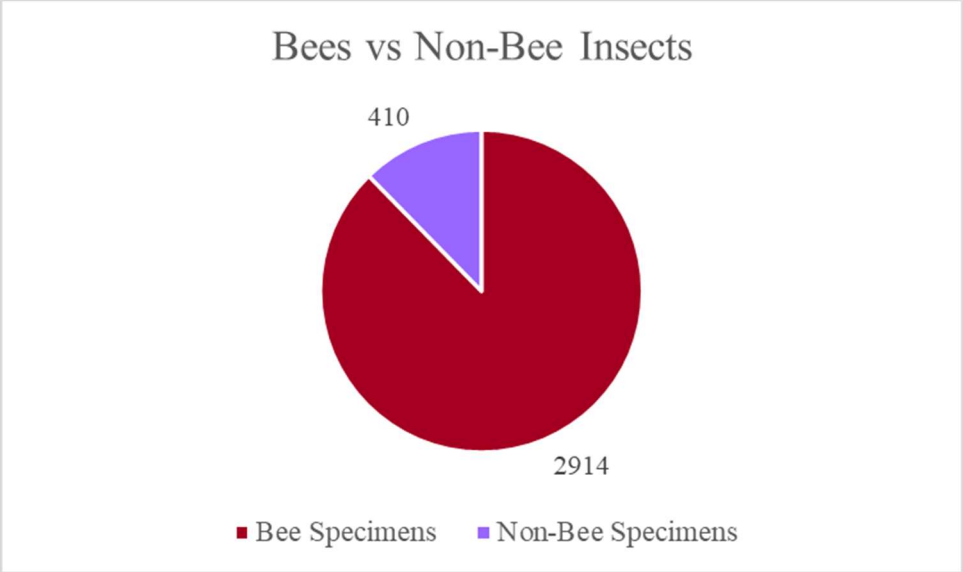

Figure 3. Pie chart showing how many bee specimens (red) were collected compared to all non-bee specimens (purple) collected during the landscape survey.

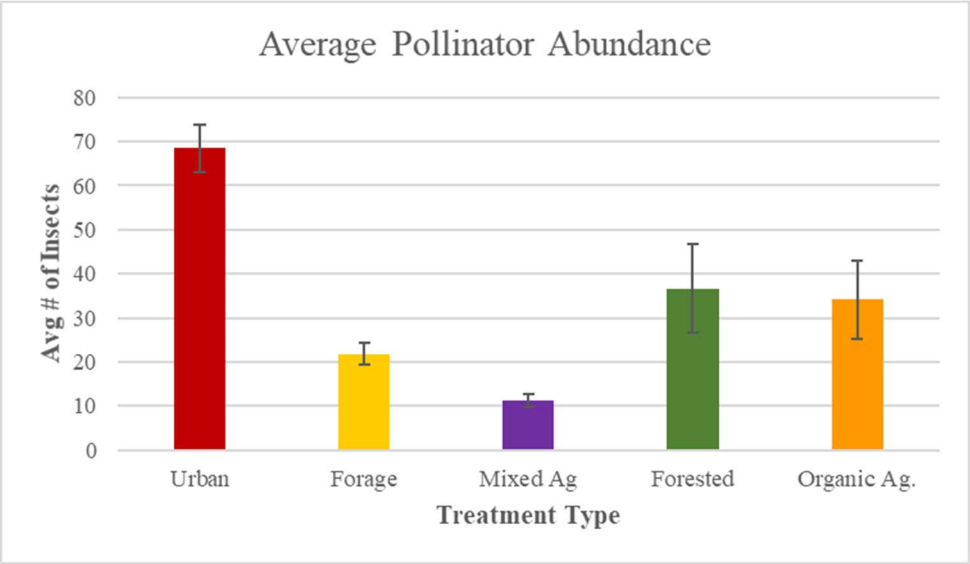

Figure 4. Average number of flower-visiting insects per individual survey compared between the different sampling sites. The error bars represent standard error.

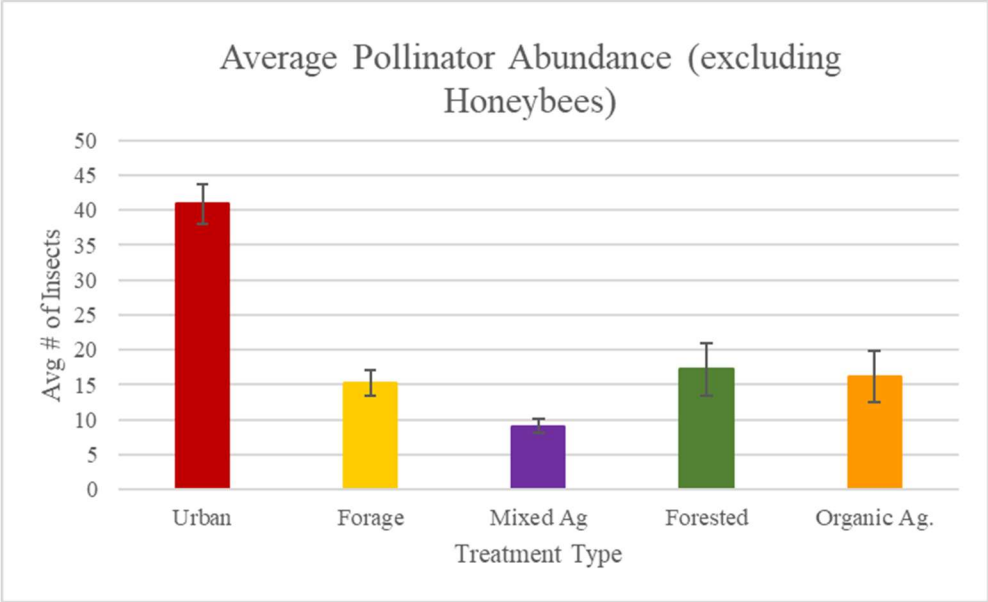

Figure 5. Average number of flower-visiting insects per individual survey, excluding honeybees (*Apis mellifera*), compared between the different sampling sites. The error bars indicate standard error.

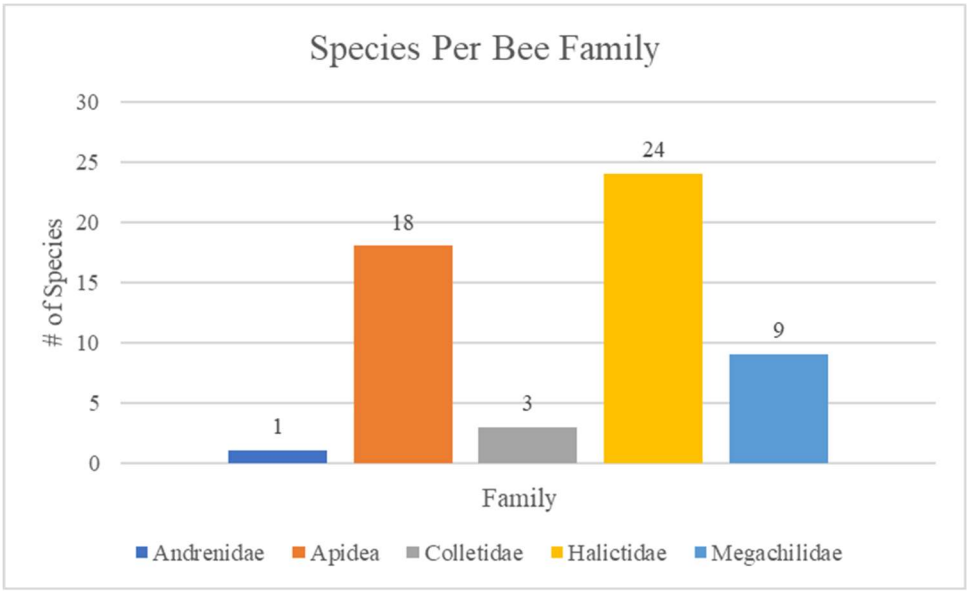

Figure 6. Bar graph showing the total number of bee species collected in each bee family (Andrenidae, Apidae, Colletidae, Halictidae, and Megachilidae) during the landscape survey.

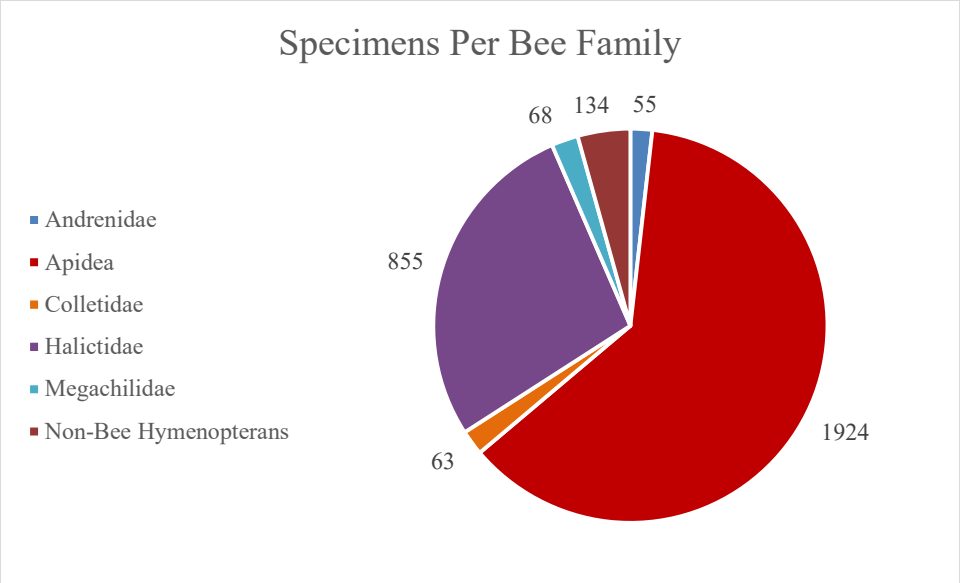

Figure 7. The number of specimens collected per bee family compared to the number of specimens collected in non-bee Hymenopteran families (orange).

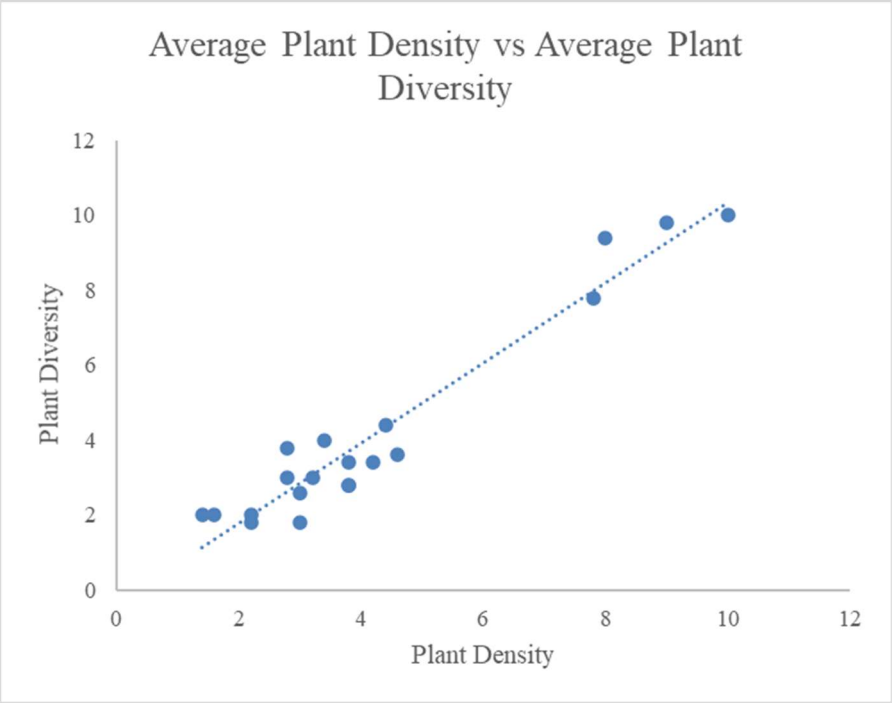

Figure 8. The relationship between the plant density score (x-axis) and plant diversity score (y-axis). Every time a location was sampled, both the density and diversity (no. of species) of flowering plants was scored on a scale of 1 to 10.

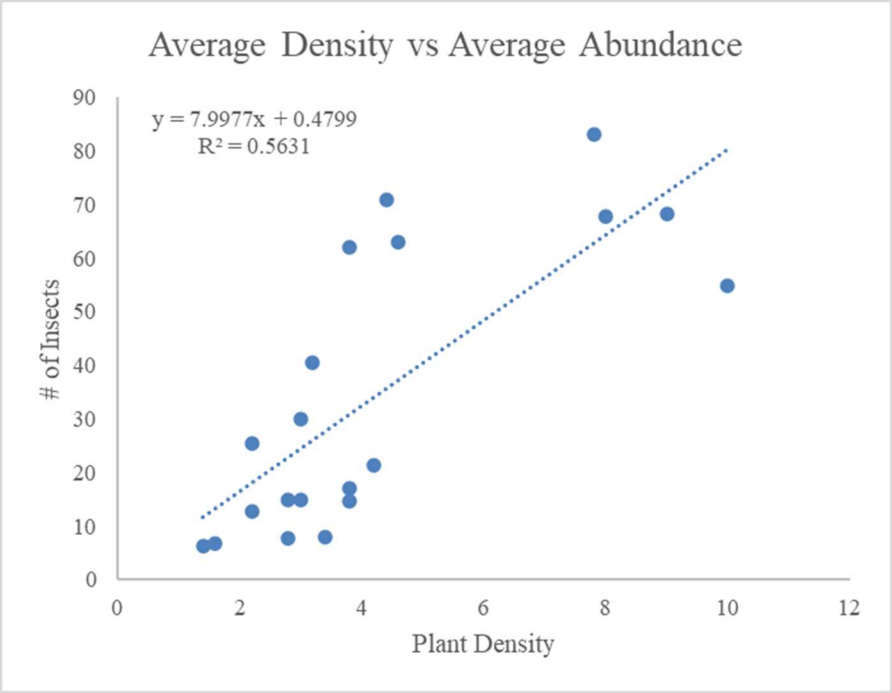

Figure 9. Average number of insects collected per sample (y-axis) compared with the average plant density of the landscape on a scale of 1 to 10 (x-axis).

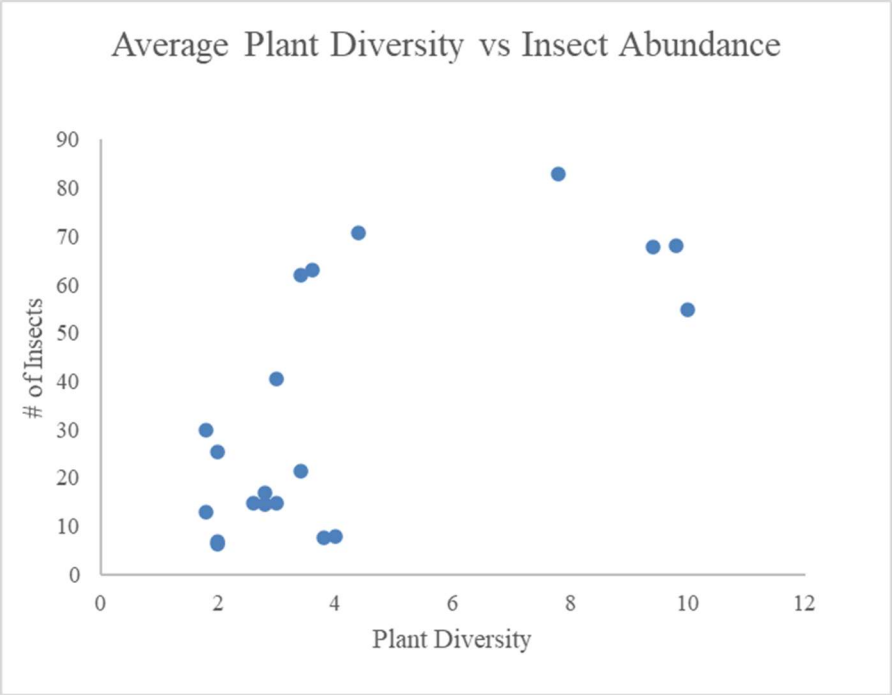

Figure 10. The relationship between plant diversity (x-axis) and insect abundance (y-axis). Insect abundance was the number of flower-visiting insects collected in each sample. Each location was given a score of 1 to 10 based on the density of flowering plants for each sample.

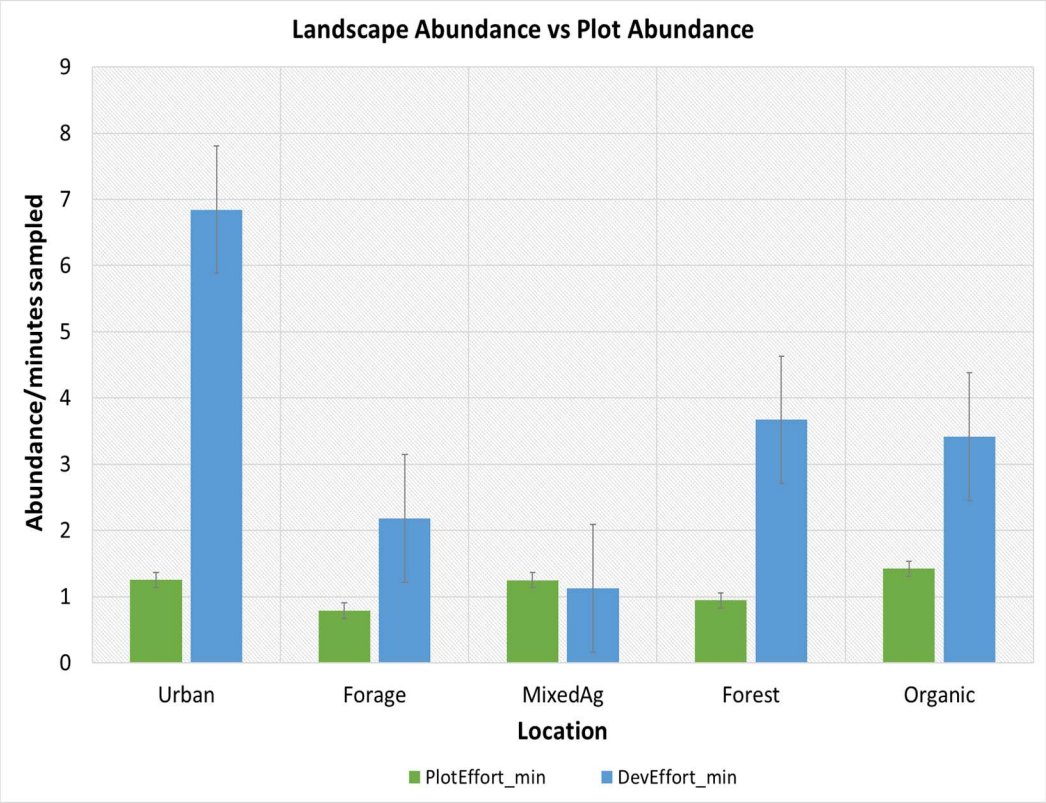

Figure 11. Each location is represented on the x-axis (Urban Gardens, Forage Grassland, Mixed Agriculture, Forest, and Organic Farm), and the average number of pollinators collected (y-axis). The raw abundance was standardized by the number of minutes sampled. Green bars represent the research plots and blue bars represent the landscape around them. Error bars are standard error.

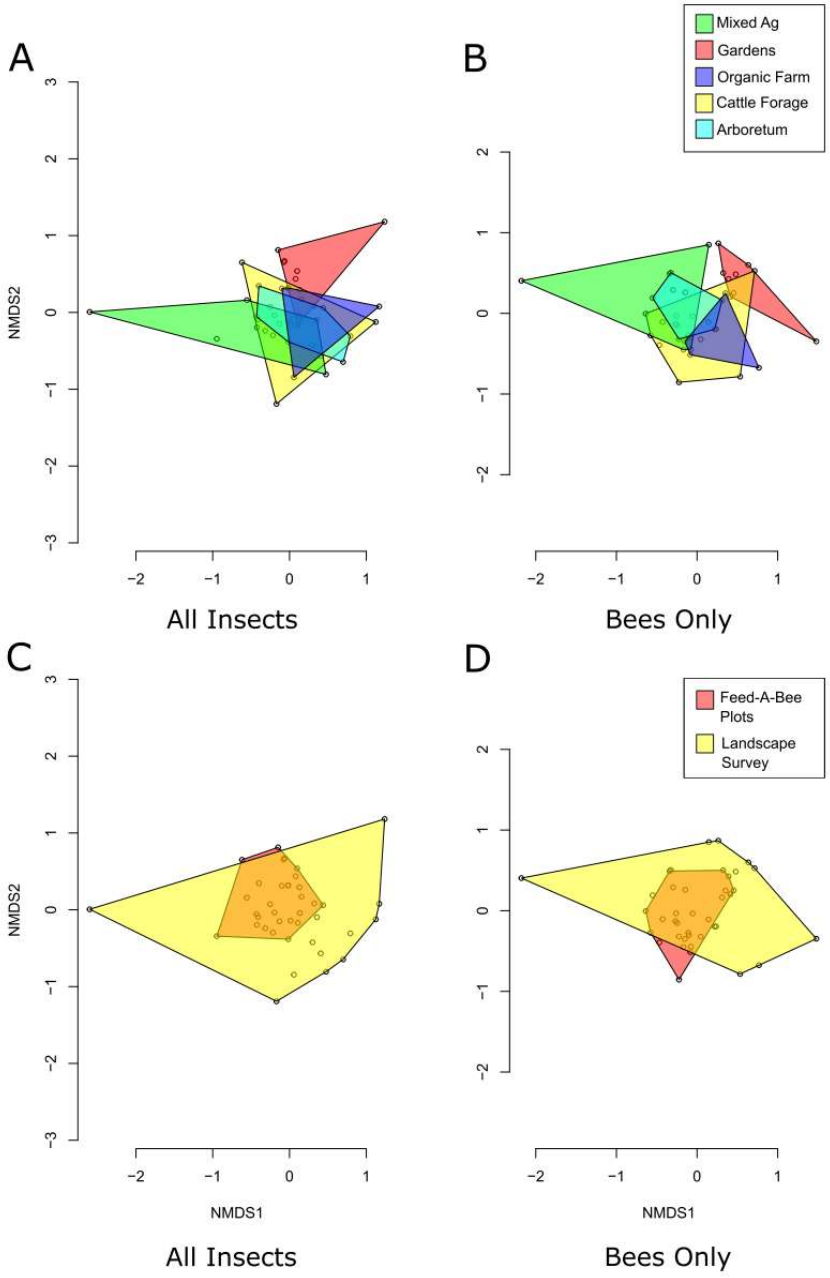

Figure 12. Non-metric Multidimensional Scaling (NMDS) ordination plots of the different sites (A, B) and surveys (C, D) conducted. Overlap in the shapes indicates overlap in the insect (A, C) or bee (B, D) communities in the sites or surveys. Ordination is a way to visualize multivariate responses.

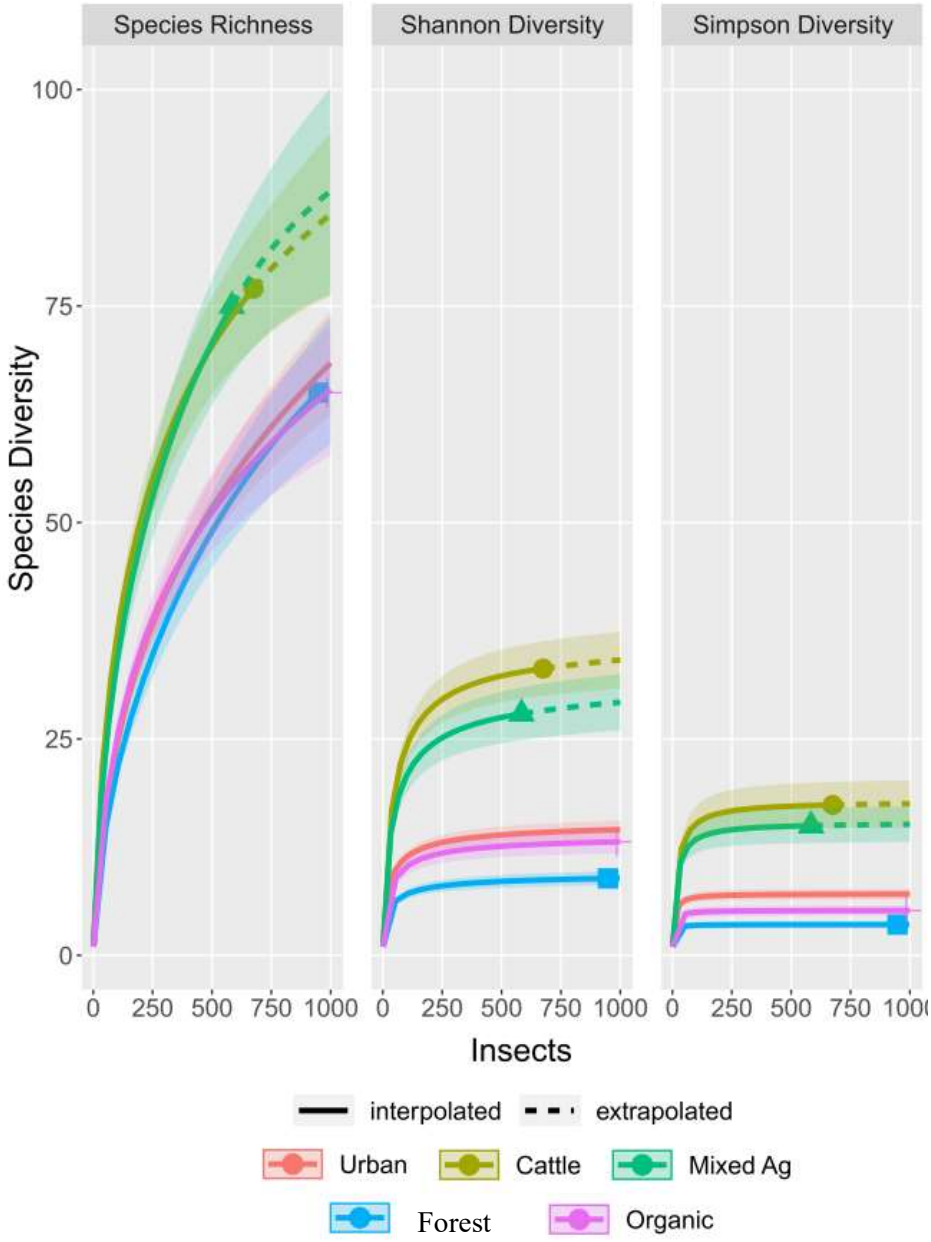

Figure 13. A rarefaction analysis of the species richness (0), Shannon diversity (1), and Simpson diversity (2) of all flower-visiting insects collected in the landscape survey at the five different land-use sites (red = Urban, yellow = Cattle Forage, green = Mixed Agriculture, blue = Forest, and pink = Organic Farm). The solid lines indicate the interpolated (observed) diversity at the different sites, while the dotted lines indicate the extrapolated (predicted) diversity.

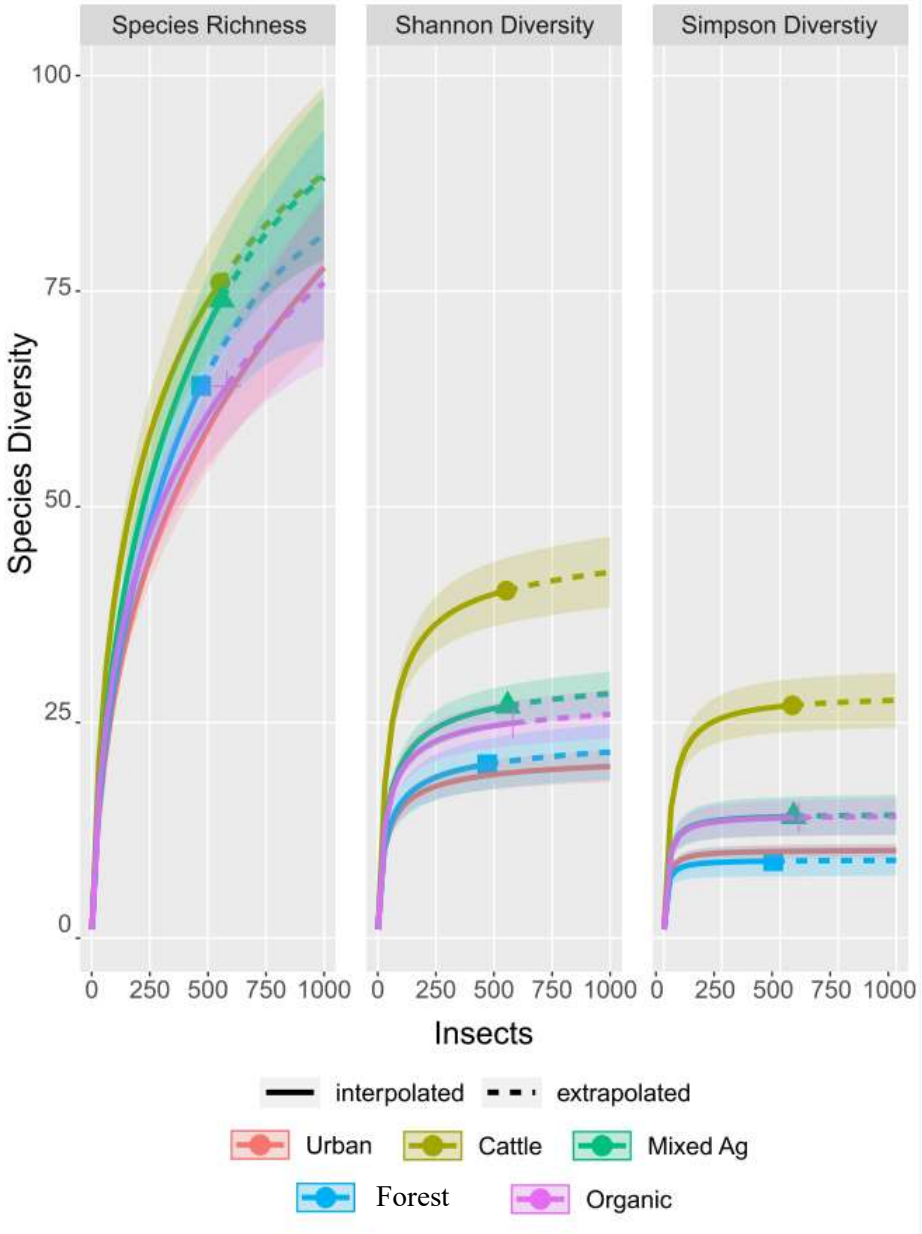

Figure 14. A rarefaction analysis of the species richness (0), Shannon diversity (1), and Simpson diversity (2) of *bees only* collected in the landscape survey at the five different land-use sites (red = Urban, yellow = Cattle Forage, green = Mixed Agriculture, blue = Forest, and pink = Organic Farm). The solid lines indicate the interpolated (observed) diversity at the different sites, while the dotted lines indicate the extrapolated (predicted) diversity.

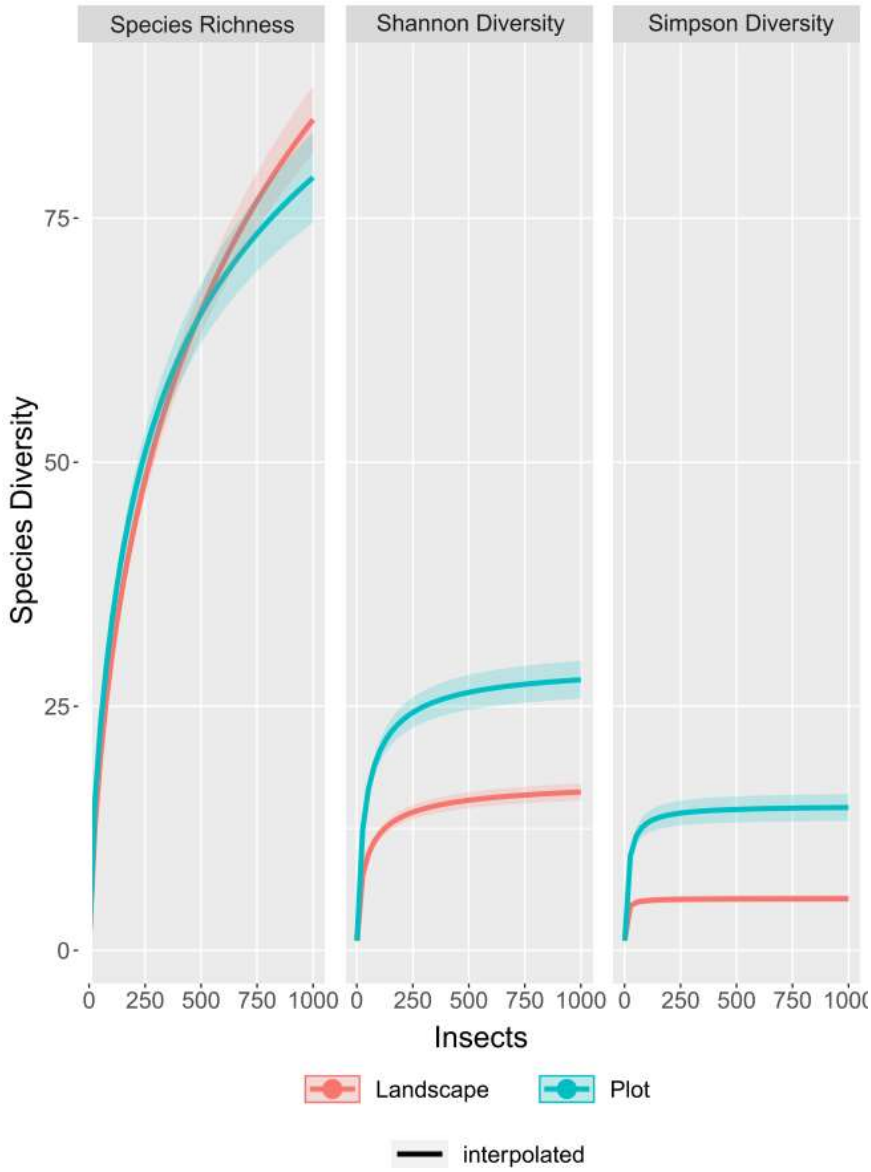

Figure 15. Rarefaction analysis of species richness (0), Shannon diversity (1), and Simpson diversity (2) of all flower-visiting insects compared between the landscape survey (red) and the plot-based survey (blue).

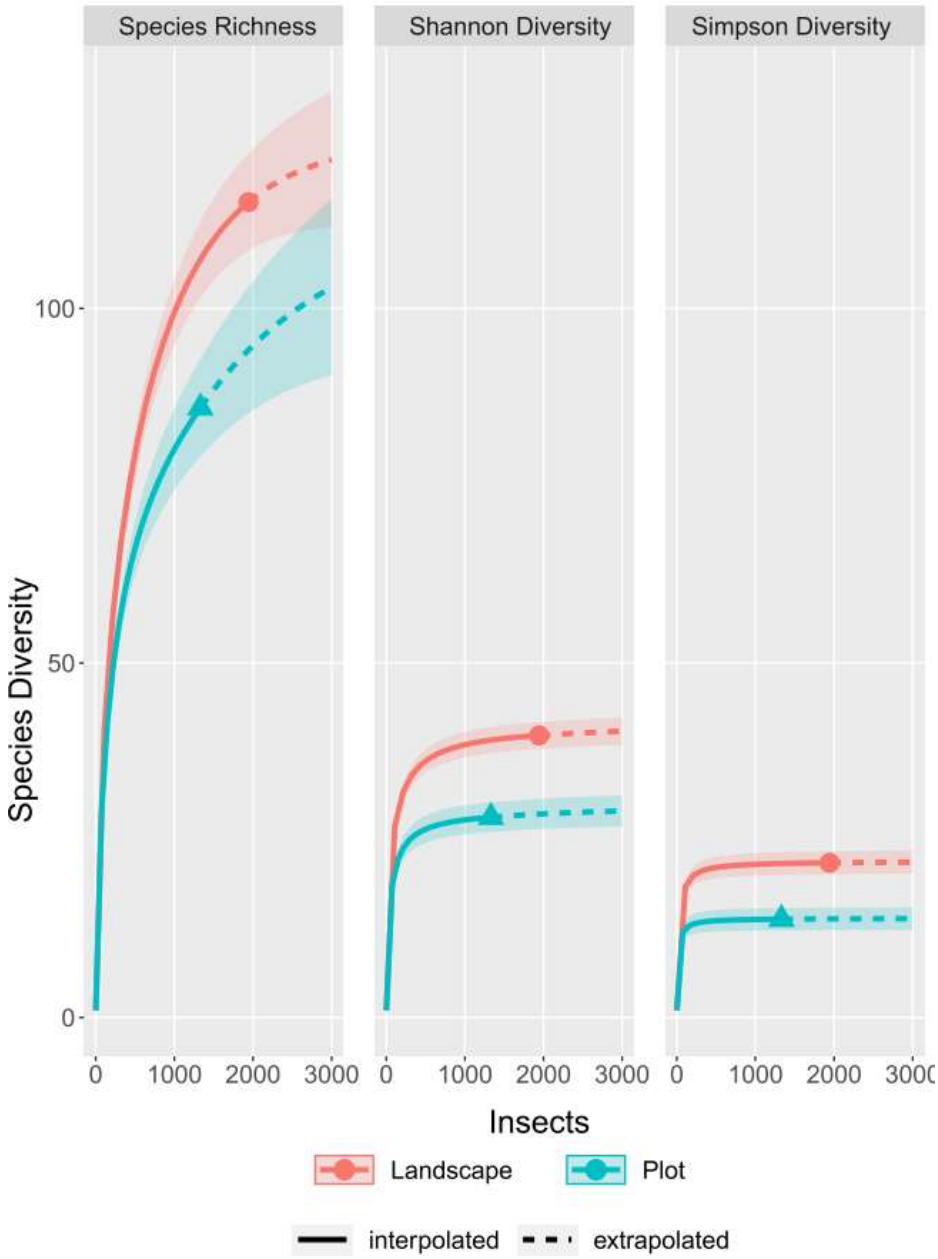

Figure 16. Rarefaction analysis of species richness (0), Shannon diversity (1), and Simpson diversity (2) of *bees only* compared between the landscape survey (red) and the plot-based survey (blue).

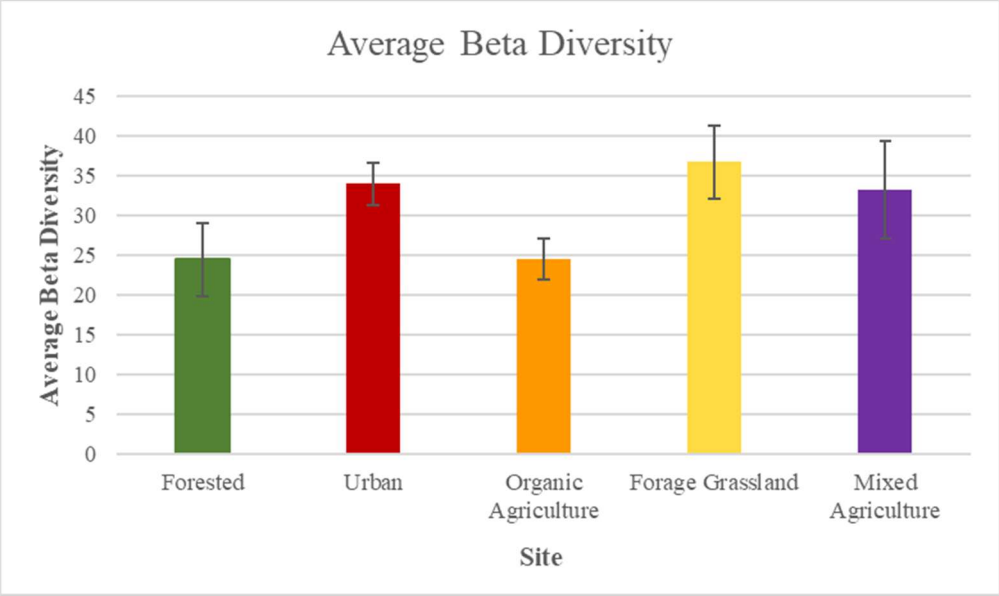

Figure 17. Bar graph showing the average beta diversity of all insects collected at each site comparing the plot and landscape surveys. Average beta diversity is on the y-axis and each site (Forested, Urban, Organic Agriculture, Forage Grassland, and Mixed Agriculture) is represented on the x-axis. The error bars represent standard error around the mean.

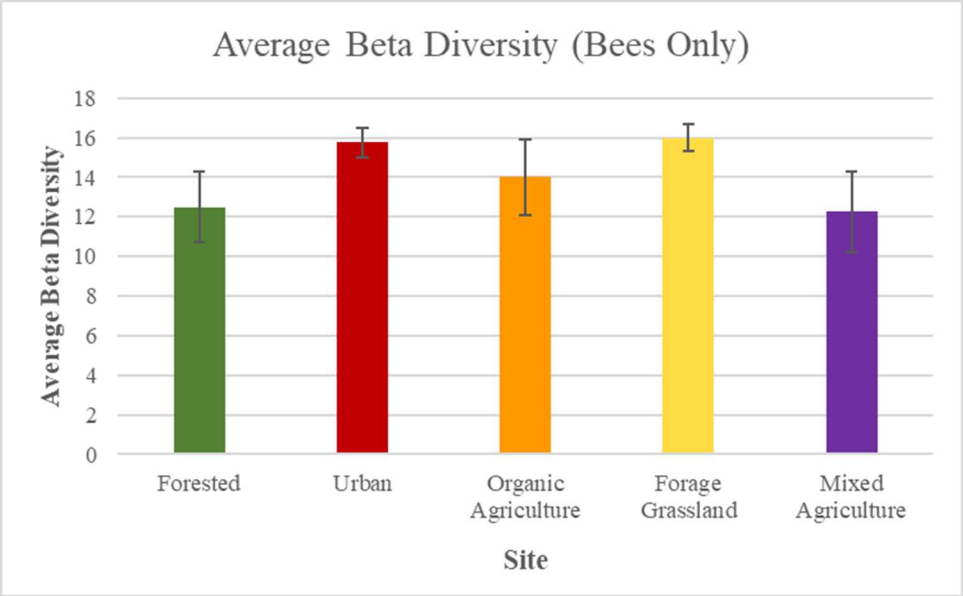

Figure 18. Bar graph showing the average beta diversity of bees collected at each site comparing between the plot and landscape surveys. Average beta diversity is on the y-axis and each site (Forested, Urban, Organic Agriculture, Forage Grassland, and Mixed Agriculture) is represented on the x-axis. The error bars represent standard error around the mean.

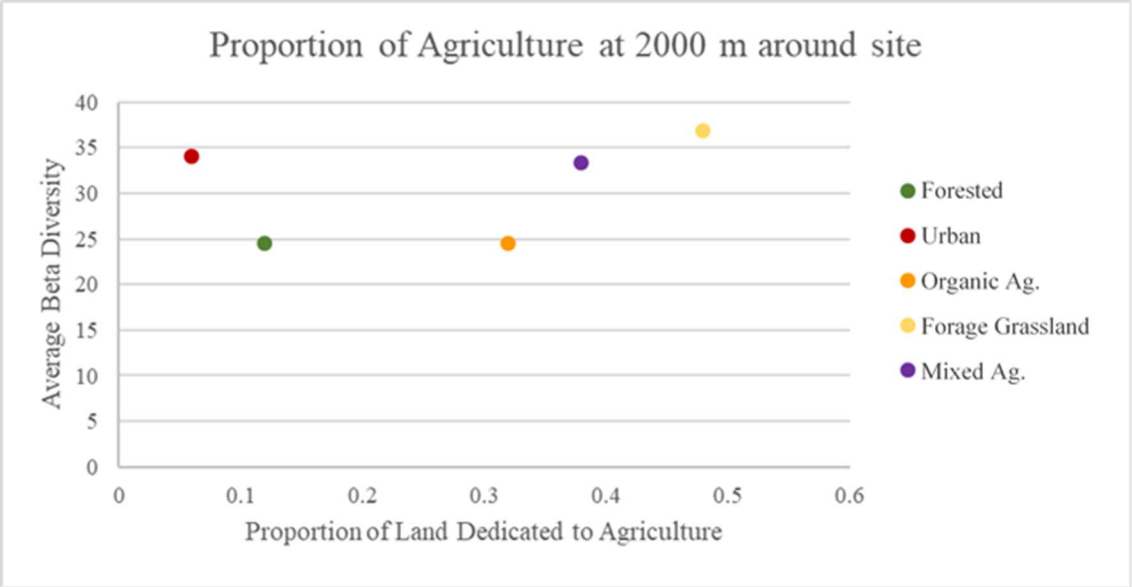

Figure 19. Scatterplot comparing agriculture-based land-use within a 2000 m radius around each research site on the x-axis to the average beta diversity of each plot on the y-axis. Each dot represents a different site. Beta diversity is calculated by using the diversity of all insects collected from the plot collections and the landscape survey collections.

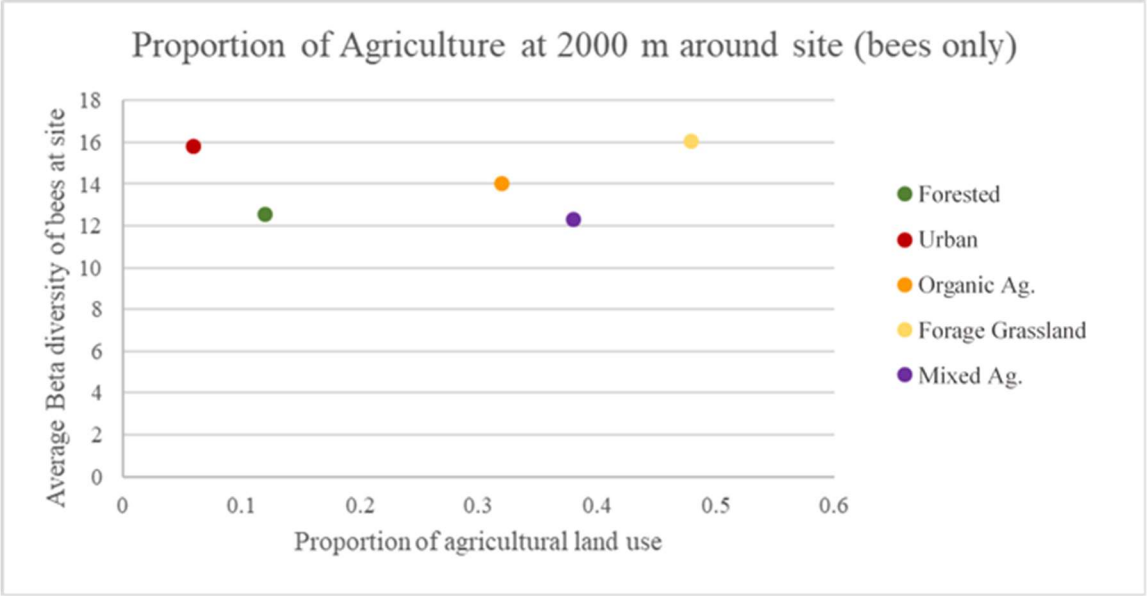

Figure 20. Scatterplot comparing agriculture-based land-use on the x-axis to the average beta diversity of bees only of each plot on the y-axis. Each dot represents a different site. Beta diversity by using the diversity of only bees collected from the plot collections.



Figure 21. Species list of bees collected during the Landscape Survey.

Table 1. Proportion of land-use types in the surveyed areas. Using GIS, the land-use types for each site have been measured within 2000 meters of each site (Khalil 2020). The land-use types have been broken down into three classes: Agriculture, Developed, and Natural. The first column titled "Site" contains each site (Gardens, Cattle Forage, Mixed Agriculture, Forest, Organic Farm, and Gardens). Finally, the right-most column titled "Prop" gives the proportion of the land-use class for each field site.

Site	Meters	Class	Prop
Gardens	2000	Agriculture	0.06
Cattle Forage	2000	Agriculture	0.48
Mixed Ag	2000	Agriculture	0.38
Forest	2000	Agriculture	0.12
Organic Farm	2000	Agriculture	0.32
Gardens	2000	Developed	0.6
Cattle Forage	2000	Developed	0.06
Mixed Ag	2000	Developed	0.05
Forest	2000	Developed	0.3
Organic Farm	2000	Developed	0.15
Gardens	2000	Natural	0.23
Cattle Forage	2000	Natural	0.43
Mixed Ag	2000	Natural	0.55
Forest	2000	Natural	0.55
Organic Farm	2000	Natural	0.49

Table 2. Diversity of all collected insects per plot. The types of diversity for all insects collected at each block. Alpha diversity (α) is shown for each block from both the plot and landscape survey. The Beta diversity between each block from both collections is displayed in the β column. Finally, the Gamma diversity of the sites, as a whole, is in the right most column titled "γ". Each site is listed (Forest, Gardens, Organic Farm, Cattle Forage, and Mixed Ag) with its accompanying gamma diversity in the following row.

Plot Diversity:		**Landscape Diversity:**		**Beta Diversity:**	**Gamma Diversity:**
Plot	α	**Plot**	α	β	Γ
A4A	9	**A4A**	40	35	Forest
A4F	7	**A4F**	11	19	76
A4L	11	**A4L**	16	15	
A4M	26	**A4M**	29	29	
G1A	22	**G1A**	39	37	Gardens
G1F	4	**G1F**	30	26	82
G1L	25	**G1L**	25	36	
G1M	22	**G1M**	39	37	
O5A	10	**O5A**	19	21	Organic Farm
O5F	11	**O5F**	33	32	65
O5L	16	**O5L**	32	24	
O5M	26	**O5M**	29	21	
P2A	15	**P2A**	27	40	Cattle Forage
P2F	9	**P2F**	22	25	84
P2L	35	**P2L**	23	47	
P2M	12	**P2M**	35	35	
P3A	8	**P3A**	19	25	Mixed Ag
P3F	1	**P3F**	24	21	82
P3L	25	**P3L**	22	41	
P3M	36	**P3M**	22	46	

Table 3. Diversity of bees collected per plot. The types of diversity for just bees collected at each block. Alpha diversity (α) is shown for each block from both the plot collections and landscape survey. The Beta diversity between each block from both collections is displayed in the β column. Finally, the Gamma diversity of the sites, as a whole, is in the right most column titled "γ". Each site is listed (Forest, Gardens, Organic Farm, Cattle Forage, and Mixed Ag) with its accompanying gamma diversity in the following row.

Plot Diversity:		**Landscape Diversity:**		**Beta Diversity**	**Gamma Diversity**
Plot	α	**Plot**	α	β	Γ
A4A	7	**A4A**	23	16	Forest
A4F	7	**A4F**	10	10	32
A4L	11	**A4L**	10	9	
A4M	16	**A4M**	21	15	
G1A	14	**G1A**	20	14	Gardens
G1F	3	**G1F**	20	17	31
G1L	13	**G1L**	17	17	
G1M	10	**G1M**	21	15	
O5A	8	**O5A**	15	15	Organic Farm
O5F	8	**O5F**	21	19	34
O5L	11	**O5L**	20	11	
O5M	19	**O5M**	20	11	
P2A	9	**P2A**	17	18	Cattle
P2F	8	**P2F**	14	16	32
P2L	18	**P2L**	15	15	
P2M	9	**P2M**	12	15	
P3A	5	**P3A**	8	7	Mixed Ag
P3F	1	**P3F**	15	16	34
P3L	14	**P3L**	11	15	
P3M	18	**P3M**	17	11	

CHAPTER II
CACAO POLLINATION SYSTEMS: AGROFORESTRY IN PUNTA GORDA, BELIZE AND GREENHOUSE STUDIES OF FRUIT SET

Abstract

Pollination systems of crop species, or the floral morphology and pollinators, determine the production of an economically valuable yield and may be affected by management. In many crops, pollination systems rely on insect vectors to transport pollen. One crop species that depends entirely on insect pollination for fruit set is cacao (*Theobroma cacao*). Although some cacao is grown in clear-cut areas, agroforestry is a more sustainable way to grow cacao; however, the implications of agroforestry on cacao pollination are unknown. In addition, in cacao production systems where variety affects market value, cacao pollination may have implications for chocolate quality by affecting varietal purity. For example, Criollo is considered an heirloom variety of cacao and sells at a much higher market value than the more commonly cultivated (and higher productivity) Trinitario variety. Where these two varieties are grown in close proximity, it is possible that they can cross-pollinate. To determine whether Criollo and Trinitario varieties of cacao can cross-pollinate, and to establish the pollen limitation of an agroforestry system, we conducted a hand-pollination experiment on Criollo and Trinitario variety cacao grown within an agroforestry setting in Punta Gorda, Belize. We examined self-pollination, pollen limitation, and cross-variety compatibility. We found that the Criollo variety was able to self-pollinate, although self-pollinated cherelles were aborted before the 30-day check, and the Trinitario variety was self-incompatible. However, both varieties were compatible with one another, with implications for pure variety chocolate production where they are grown together. Finally, we did not find evidence of pollen limitation in either variety of cacao in this system.

Introduction

Humans have been farming cacao, the source of chocolate, for thousands of years (Zarrillo et al. 2018). Ancient Mayans were among the first to cultivate cacao, consuming it in various forms (Young 1994). They called cacao the "food of the gods", which later influenced its scientific name, *Theobroma cacao*. Cacao became an important commodity in South America, often prepared by fermenting the sweet pulp and served in the form of an alcoholic drink. It is this cacao "beer" that is believed to be the stepping stone to the elaborate process of creating the chocolate drink popular in Mesoamerica (McNeil 2009). Not only was cacao consumed, but the beans were used as one of the earliest forms of currency (Dand 1999).

Cacao domestication began with the cultivation of wild trees and then the management of wild cacao forests. Soon, cacao was planted and managed in enriched-forest orchard gardens. With the discovery of the cacao drink by the Spanish conquerors, the cultivation of cacao intensified. The European invention of chocolate soon increased the demand for cacao even more and drove the cultivation higher in Latin America (Dand 1999). The invention of chocolate led to the introduction of the cacao tree to various parts of the world, including the west indies, Asia, and west Africa. Today, 70% of cacao, the third largest legal crop commodity (2004), is grown in West African countries: Ivory Coast,

Ghana, Nigeria and Cameroon. Cacao is typically grown by small-scale farmers (Rice and Greenberg 2019). While large-scale productions do exist, a large amount of cacao beans are from in small-scale farms (Duguma et al. 2001).

Historically, there was some confusion surrounding the pollination system of cacao. Early on, cacao was believed to be wind and water pollinated, however these methods were disproven (Billes 1941). Along the way, cacao was hypothesized to be insect pollinated. The flowers of cacao are small, with the stamens occurring under petal hoods. Due to the flower-morphology, the pollinator in question must also be small to reach the pollen. Studies examined various insects in attempts to determine the infamous cacao pollinator. Common insects found near cacao flowers were investigated, such as ants, thrips, small bees, and flies (Glendinning 1972). Through several studies, the current general consensus is that cacao is primarily pollinated by small species of biting midges (*Forcipomyia*: Ceratopogonidae) and gall midges (Cecidomyiidae) (Posnette 1950; Toledo-Hernández et al. 2017). These small flies are small enough to access the stamens located under the stamen hood and are large enough to transport pollen between cacao trees.

Cacao has been grown using several different methods. For example, cacao Cabruca involves growing of cacao in a thinned native forest under the shade of native trees (Gama-Rodrigues et al. 2011). However, to promote cacao productivity, farmers have been shifting to methods that involve clearing intact forest to plant the cacao orchard either with introduced shade trees or in full sun (Gama-Rodrigues and Willy 2012). The practice of clearing native forests to grow cacao has led to the deforestation of intact, biodiverse rainforests (Norris et al. 2010). For example, in Ivory Coast, the world's leading producer of cacao (Ruf et al. 2015), 14% of intact forestlands have been cleared for cacao orchards (Gogué 2008). This method of growing cacao is not a sustainable practice. Fortunately, cultural practices for growing cacao may be shifting. Agroforestry concepts have begun to be encouraged and implemented across the globe in cacao orchards. Agroforestry, the practice of integrating trees and shrubs around crops in an agricultural setting (Nair 1993), can be used to help offset the current deforestation rate. Cacao is described as an understory species and requires various amounts of shade, depending on where it is in its growth cycle. Growing cacao in an agroforestry setting can help to lessen the negative ecological impacts of growing cacao by leaving much of the forest intact. Not only does cacao thrive in a shaded environment (Tscharntke et al. 2011), but so do the small midges that pollinate cacao, which require decomposing plant matter, rotting banana husks, or epiphytic bromeliads to complete their life cycles (Winder and Silva 1972). Based on this information, our hypothesis was that, by implementing an agroforestry approach to cacao farming, it would be possible not only to farm in a more sustainable way, but also to increase pollinator habitat leading to an increase in yield in pollen limited systems.

One such example of a cacao agroforestry project is at the Belize Foundation for Research & Environmental Education (BFREE) in Punta Gorda, Belize. BFREE was

founded in 1995 to develop a conservation program for the Bladen Nature Reserve, one of the three nature reserves in Belize. The Bladen Nature Reserve is a species-rich landscape home to undisturbed old growth rainforest. It is known as a crown jewel in Belize and contains many endemic species of flora and fauna. BFREE is a 1,153-acre private reserve which not only borders Bladen Nature Reserve, but three other protected areas. These protected areas are all a part of the 1.25 million acres of continuous tropical forest known as the Mayan Mountain Mastiff. This area has been nationally recognized not only as a Key Biodiversity Area but it is also known as the center of ancient Mayan civilization. For example, BFREE has discovered Ancient Mayan archeological sites within its rainforests such as house mounds, a terraced hillside, and an heirloom variety of cacao believed to have been used by the ancient Maya. The wild cacao population found within BFREE forests has been submitted for genetic testing to the Heirloom Cacao Preservation Fund (HCP) and it was determined to be 100% pure Criollo cacao. This heirloom variety is believed to have been "The Mother" of cacao, an ancient wild cacao used and grown by the ancient Maya. BFREE and HCP have worked together to grow Criollo cacao in an agroforestry setting to promote this "heirloom fine flavor" cacao. BFREE uses an agroforestry approach and grow cacao in a shaded environment, leaving much of the rainforest intact and allowing native fauna to travel through the forest as if no orchard was present. By working to perfect their agroforestry methods, BFREE then plans to pass this information on to local cacao farmers. By changing the way local farmers grow cacao, they can help to restore and protect local, biodiverse rainforest.

BFREE grows two varieties of cacao, Criollo and Trinitario. In some of BFREE's orchards, both varieties are grown in close proximity, in some cases they are side-by-side. However, it is not known if the two varieties can cross-pollinate. If cross-compatibility did occur between the two varieties, then beans harvested from the trees possibly would not genetically be composed of entirely one variety. The beans that come from these trees may not be genetically pure of either variety, leading to complications if BFREE continues to grow the offspring in their nurseries. To show the success of BFREE's agroforestry-grown cacao, the chocolate is sold, and the proceeds help continue the agroforestry project into future years. BFREE's cacao agroforestry project helps to promote and educate local cacao farmers about agroforestry and how it can not only benefit conservation, but the grower as well.

Cacao yield has been shown to suffer from pollen limitation; in some cases, only 5-10% of flowers are successfully pollinated and form bean pods (Falque et al. 1996). While cacao is often thought to be self-incompatible, there are many systems where self-pollination is possible or likely (Posnette 1940). There is also a gap in our understanding of cherelle wilt that has a significant impact on optimizing cacao production. Cherelle wilt is the early abortion of developing pods (cherelles) before they have reached maturity. In some cases, up to 75% of pods can be lost to cherelle wilt, a large concern for cacao farmers (Melnick 2016). Cherelle wilt has been shown to be affected by a number of factors, physiological and biotic (Nichols 1964). For example, one study found

that nutritional stresses, causes by competition between leaf shoots and young fruits, correlated with cherelle wilt on cacao (Alvim 1954). It is possible that the branch and trunk size could also play a role in resource distribution and cherelle wilt.

My goal was to improve our understanding of the pollination system of cacao in both an orchard and greenhouse setting. Our study aimed to help answer some of these questions surrounding the pollination ecology of cacao and, specifically, the Criollo cacao variety within an agroforestry landscape. I explored these concerns about pollen limitation, compatibility, cherelle wilt, and tree health play out in a cacao agroforestry setting. My main questions were:

1) Are different varieties of cacao able to cross pollinate in an agroforestry setting in Belize?
2) Is there pollen limitation in cacao agroforestry?
3) Are cacao varieties self-compatible?
4) Does tree or branch size affect the probability of cherelle formation or wilt?

Methods

Field methods – BFREE, Belize

This study was conducted between December 2nd to 15th, 2019 at the Belize Foundation for Research & Environmental Education (BFREE) in Punta Gorda, Belize in Central America. BFREE was established to conserve the biodiversity and cultural heritage of Belize, including cacao-based agroforestry as one solution to halt rainforest destruction. The wild cacao on BFREE's property is an ancient Criollo variety, economically high in value because of its fine flavor. BFREE's nursery also includes another locally grown (and higher yielding) cacao variety, Trinitario. It is not known if Trinitario can cross-pollinate with Criollo, potentially impacting the genetic composition of the offspring. BFREE's cacao is shade-grown under a diverse forested environment and located within BFREE's privately protected rainforest. Approximately 15 acres, the orchard consists of the two varieties planted in rows.

Both cacao varieties at BFREE were used in this experiment. We hand-treated 241 Criollo flowers and 70 Trinitario flowers. The day before pollination, five budding flowers per tree were randomly selected and assigned one of five treatments: 1. closed, no pollen, 2. closed, self-pollinated with flowers from the same tree, 3. open, ambient pollination, 4. open, pollinated with Criollo pollen from another tree, and 5. open, pollinated with Trinitario pollen (or opposite variety of mother tree). Closed flowers were first labelled with a metal tag secured to the branch or trunk with metal wire. Colored pushpins were pinned into the tree to the left of each flower to designate the flower's respective treatment. Treatments 1 & 2 (closed) flowers were covered with a plastic vial and remained closed for 48 hours after opening or until cherelle formation. The vial was placed over a closed flower and tied to the tree using rubber bands. The base of the vial was then sealed to the tree using Crayola modelling clay to ensure no arthropods could

enter the vial. A small organza bag was attached to the vial via rubber bands that were further wrapped around the tree to provide support (**Figure 22. 2**, all tables and figures are located in the Appendix).

The following day, we checked all marked flowers to see whether they had opened. If the flowers were open, we applied the assigned treatment. Covered flowers were uncovered for pollination, then re-covered. When pollinating, three staminodes were removed to access the anther and flowers were pollinated using all 5 anthers from the donor tree (treatment specific flower). Donor trees were identified as either Criollo or Trinitario (depending on treatment) trees in flower which were not being pollinated on that day. Self-pollinated flowers for treatment 2 were selected from the same tree that was being pollinated. If flowers did not open the day after marking, we continued to check them daily. Once opened, they were treated as above. All treatment effects were recorded in terms of days since bud opening.

Each flower was checked daily and status (open, pollinated, dropped, or cherelled) was recorded. Flowers were designated as "cherelled" if flowers reached "swollen ovary state" (Sukha et al. 2017). If there was a cherelle, long flagging tape was tied around the branch to improve visibility of the cherelles. We followed this protocol for the duration of the study in Belize (Dec 2 – 15). After that time, we conducted a 30-day check (January 13th, 2020) and 90-day check (March 13th, 2019) on flowers that remained on the trees after our departure. The flowers that were marked, but not open as of Dec 14 were excluded from the experiment.

Field methods – Greenhouse, Knoxville, TN

Nineteen CCN51 F1 self generation cacao trees were selected for use in this experiment, labelled A through W. The cacao trees were grown and kept in a greenhouse at an average 25°C temperature, an average 61 percent relative humidity, in a (pot size) using Pro-Mix (Premium Horticulture Inc., Quackertown, PA), and were watered daily. The trees were fertilized with Southern Ag 20-20-20 Water Soluble Fertilizer every two weeks during the pollination period and at XX concentration after pollinations had finished to promote cherelle growth.

Between May 4th and June 9th, 2020, the circumference of each tree was recorded at soil level, and again at 30 cm. If the trunk had split into multiple branches, the circumference of each branch was taken at 30 cm. Floral abundance was ranked on a 1 to 10 scale, in 10 flower increments. Flowers were hand-pollinated using the same methods as described above between 8am and 12pm. Five open flowers were randomly selected on each of the nineteen trees and were tagged with a paper tag using string and rubber bands; at the time of pollination, branch circumference at the height of the flower was recorded. Due to availability of flowers, once all trees contained 5 pollinated flowers, trees with large numbers of flowers received more pollination treatments in increments of 5. Two trees (A & S) did not flower over the course of this study. Flowers were monitored daily and

cherelle formation was assumed after 48 hours or once the flower reached swollen ovary state (Sukha et al. 2017). Once pollinations were completed, remaining cherelles were monitored every other day.

Results

BFREE, Belize

A total of 335 flowers were treated, with 128 developing into cherelles. When field pollinations in Belize ended on December 15th, 2019, 80 cherelles remained on the trees. After the 30-day check, 18 cherelles remained and after 90 days, only 8 cherelles remained (**Figure 23**). Twenty-four flower buds selected for the experiment did not open and were excluded from the data.

A total of 241 flowers were treated and pollinated on Criollo and 46 to 50 flowers were pollinated per Criollo treatment (1-5). Criollo formed a total of 106 cherelles (**Figure 24**). Treatment 5 (open, pollinated with Trinitario) produced the most cherelles (30). Both treatments 1 and 2 (closed, no pollen & closed, self-pollen) produced cherelles, but self-pollinated (treatment 1) flowers only produced 4 cherelles, and none remained at the 30-day check.

On Trinitario cacao, 70 flowers were treated, with 13 to 16 pollinated per treatment (1-5). Treatment 1 (closed, no pollen) did not produce cherelles. Treatment 2 (closed, self-pollen) produced the most cherelles on Trinitario, with a total of 9 cherelles; however, none of these remained on the tree at the 30-day check (**Figure 25**). When pollinations ended on December 15th, 80 cherelles were present between both Criollo and Trinitario cacao trees. The 30-day check was performed on January 13th, 2020 and only 18 cherelles remained, with 14 and 4 on Criollo and Trinitario, respectively. By the time the cherelles were checked on March 13th, 2020 for the 90-day check, only 8 cherelles remained between the two varieties (**Figure 26**).

Overall, the treatment with highest fruit set was treatment 5 (open, opposite variety pollen) on both the Trinitario and Criollo varieties (**Figure 24** & **Figure 25**). This was followed closely by treatment 4 (open, same variety pollen). Treatment 3 (open, ambient pollen) differed very little from the two open and supplemental pollen treatments (treatments 4 & 5). The similarity between the supplemental and ambient pollen treatments shows that neither Criollo or Trinitario varieties are pollen limited.

Greenhouse, Knoxville, TN

In the greenhouse, a total of 221 flowers between 16 trees were hand-pollinated from May 5th and June 9th, 2020. Two trees in the study did not flower over during pollinations. We recorded the CCN51 F1 self mother-father pairings along with the following successful and unsuccessful pollinations from each pair (**Table 4**, **Figure 27**). Out of the 221 pollinations, 152 (69%) were successful and 68 (31%) did not form cherelles. Overall, we had a 69% successful pollination rate. The average trunk

circumference at soil level was 16.91 cm with the average branch circumference at 30 cm was 9.18 cm. The average circumference of branch at the flower level was 9.22 cm. Finally, the average floral abundance of all trees was 3.04 out of a scale of one to ten. Four (1.8%) cherelles successfully reached mature pods and were harvested.

Unsuccessful pollinations had an average branch width of 9.08 $\pm$ 0.37 cm, while successful cherelles had an average of 9.25 $\pm$ 0.18 cm (**Figure 28**). Unsuccessful pollinations had a lower average trunk circumference at soil level of 16.77 $\pm$ 0.23 cm and successful cherelles had an average of 16.91 $\pm$ 0.13 cm (**Figure 29**). Finally, unsuccessful pollinations had an average trunk circumference at 30 cm of 10.54 $\pm$ 0.26 cm and successful cherelles had an average of 10.91 $\pm$ 0.14 cm (**Figure 30**). We found no evidence of trunk circumference at soil level, trunk circumference at 30 cm, or branch width having effect on cherelle formation.

We saw no trend of floral abundance on cherelle formation (**Figure 31**), though floral abundance 4-5 saw the highest proportion of successful cherelles. Finally, there was no correlation between the number of days to flower drop (cherelle wilt) and the tree girth at soil level or branch diameter at flower (**Figure 32**, **Figure 33**).

Discussion

BFREE was established to conserve the biodiversity and cultural heritage of Belize, including cacao-based agroforestry as one solution to halt rainforest destruction. The wild cacao on BFREE's property is an ancient Criollo variety, economically high in value because of its high-quality flavor. BFREE's nursery also includes another locally grown (and higher yielding) cacao variety, Trinitario. Here we have demonstrated that Trinitario can cross-pollinate with Criollo, potentially affecting resulting offspring from these trees leading to unintentional hybrids.

The two varieties are grown together in the same orchards at BFREE. Although in most cases, morphology differed substantially between Trinitario and Criollo varieties, in some cases, trees were missing labels and had similar morphology and it was not clear as to what variety the tree represented. We selected only trees that were clearly labelled and could be specified to variety. However, this could pose issues with pod harvesting. The number of trees for this study, and number of flowers per tree, depended on availability of recipient and donor flowers. Due to a severe drought the summer of 2019 before our pollinations in December, many of the cacao trees were not flowering well or at all, limiting the number of flowers and buds per tree. Furthermore, due to the decreased flowering, most available buds were young. This caused a delay between marking for treatment, and an open flower for pollinations. At the end of the experiment, several buds had not yet opened and were excluded from this experiment.

Our overall cherelle formation for the two varieties in Belize was approximately 38 percent, consistent with published literature (N-Zi et al. 2017). For cherelle development

into mature pods, there were several significant marks within the development time (Sukha et al. 2017). We saw a substantial loss in cherelles at the two checks, however this loss was consistent with reports of unsuccessful pollinations dropping after 48 hours and aborted cherelles (cherelle wilt) in two waves: after 30 days and 90 days (Falque et al. 1995; Sukha et al. 2017). A majority of cherelle wilt occurs up to 90 days after pollination (Falque et al. 1995).

Cacao has often been thought to be self-incompatible and unable to self-pollinate. However, there have been several reports of different varieties able to self-pollinate (Cope 1961; Falque et al. 1996; Sukha et al. 2017). It was previously not known if the ancient Criollo variety at specifically at BFREE is able to self-pollinate, though Criollo in general is thought to be self-compatible (Lanaud et al. 2017). In our study, flowers enclosed and not hand-pollinated with external pollen produced 4 cherelles on Criollo cacao, though these did not last to the 30-day check. This is noteworthy; it shows that BFREE's Criollo cacao is able to self-pollinate its flowers without supplemental pollen. However, due to the low success rate (8.7%) of self-pollination, it is possible that the tree was able to abort the selfed flowers at a later stage. Moreover, when Criollo flowers were enclosed and hand-pollinated with pollen from the same tree, 18 cherelles were produced (37%). This demonstrates that Criollo is self-compatible with its own pollen delivered by a pollinator. We have thus shown that Criollo cacao at BFREE is self-compatible and potentially able to self-pollinate. However, because these cherelles did not last to the 30-day check, more research could be done to show that BFREE's Criollo cacao can produce mature fruit and seeds when self-pollinated.

Adding Criollo pollen to Trinitario flowers was successful, resulting in the fruit set of 6 cherelles. The success of adding pollen from a different variety on both varieties shows that Criollo and Trinitario are cross-compatible in both directions. This cross-compatibility has implications on bean purity and chocolate production. Even though pods may be harvested from a BFREE Criollo cacao tree, the beans within the pods may not be 100% pure Criollo variety. The chocolate then made from these pods would not be pure Criollo chocolate, as previously thought. We saw no cherelles produced from the closed, no external pollen added treatment. Because of this, there is no evidence that Trinitario can self-pollinate. However, we had only treated 13 flowers in this way on Trinitario, so our data are preliminary. On the other hand, adding supplemental Criollo pollen was a successful treatment, showing that Criollo can cross-pollinate Trinitario.

The ambient and supplemental pollination treatments on both varieties were all very similar in fruit set. This shows that the cacao trees grown in this setting were not pollen limited. Supplemental pollen did not produce more cherelles than the open flowers that were visited by pollinators. Cacao farmers are hesitant to grow cacao in an agroforestry setting due to the concern of the trees produce fewer pods. In BFREE's agroforestry orchard, the cacao trees produced about the same amount of cherelles in both pollination scenarios (ambient and supplemental). This shows that pollination would probably not be a limiting factor in cacao agroforestry production. This study could be expanded to

compare the cacao yield in an agroforestry orchard to a more conventional cacao orchard grown in full sun.

In the greenhouse, the cacao trees were CCN51 F1 self trees. We observed little to no effect of branch and trunk circumference on fruit set or cherelle wilt. Fruit set depended more on the pollen receiver and pollen donor combinations than it did the physical size of the tree. A similar observation was made in a thesis looking at hybridization between T. *cacao* and *Herrania* sp. (Doodnath 1996). Doodnath (1996) found that the maternal parent had significant effect on embryo abortion of cacao beans. The paternal parent also was found to have significant effect, however less than the maternal effect. This is particularly interesting in our study, as all trees used in the greenhouse study were CCN51 F1 self siblings, grown together in the same conditions. The genetic differences between the trees should be minor, yet we continued to see compatibility issues with certain combinations. For example, the mother-father pair, D-I, formed zero cherelles out of the 5 flowers pollinated (Table 1). The same mother, but different father (D-W) formed 3 cherelles out of 5. Other pairings, such as O-B and O-L, were all successful, forming 10 and 5 cherelles, respectively. It seemed as though some trees were better pollen "receivers", while other trees were better pollen "donors".

Overall, our complementary field and greenhouse studies illustrate the importance of fully understanding the pollination system of cacao. Effective pollination has large impacts on cherelle formation, retention, and overall yield. We illustrated that cross-varietal pollination is possible, and likely, that agroforestry cacao is not pollen-limited, and that the identity of the tree has a large impact on fruit set. The results of this study will have implications for cacao agroforestry, such as the agroforestry project at BFREE and small-scale farmers in Belize.

APPENDIX



Figure 22. Set-up for closed flower treatments. In the image on the right, the pink pin designated treatment type. A metal tag noting the flower ID number was attached to the tree near the treated flower. The plastic vials were used to protect the flowers the day before they opened, and the mesh bag prevented insects from pollinating the flowers.

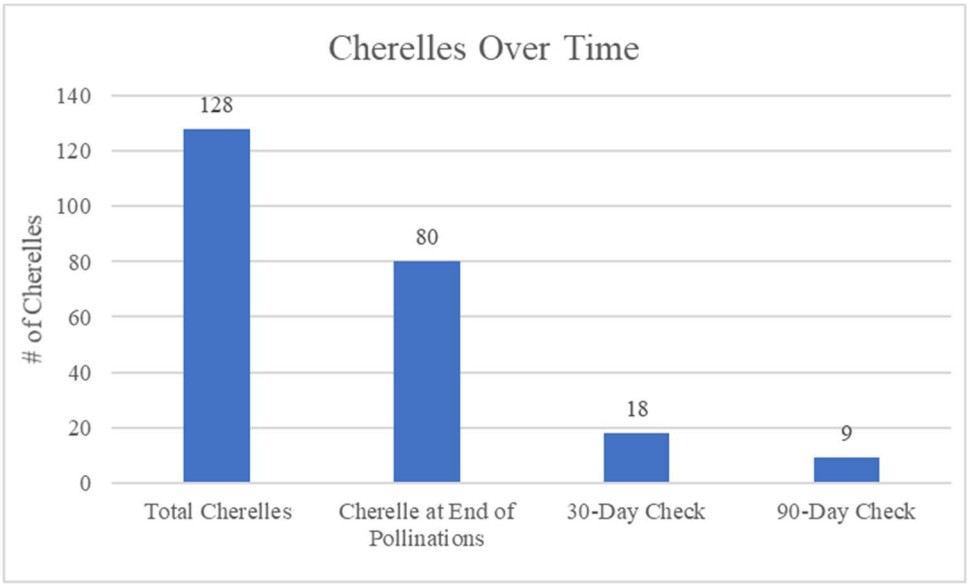

Figure 23. Bar graph comparing the number of cherelles remaining on the cacao trees in BFREE over time. Cherelles were counted at the end of pollinations on December 15, 2019, at 30 and at 90 days later.

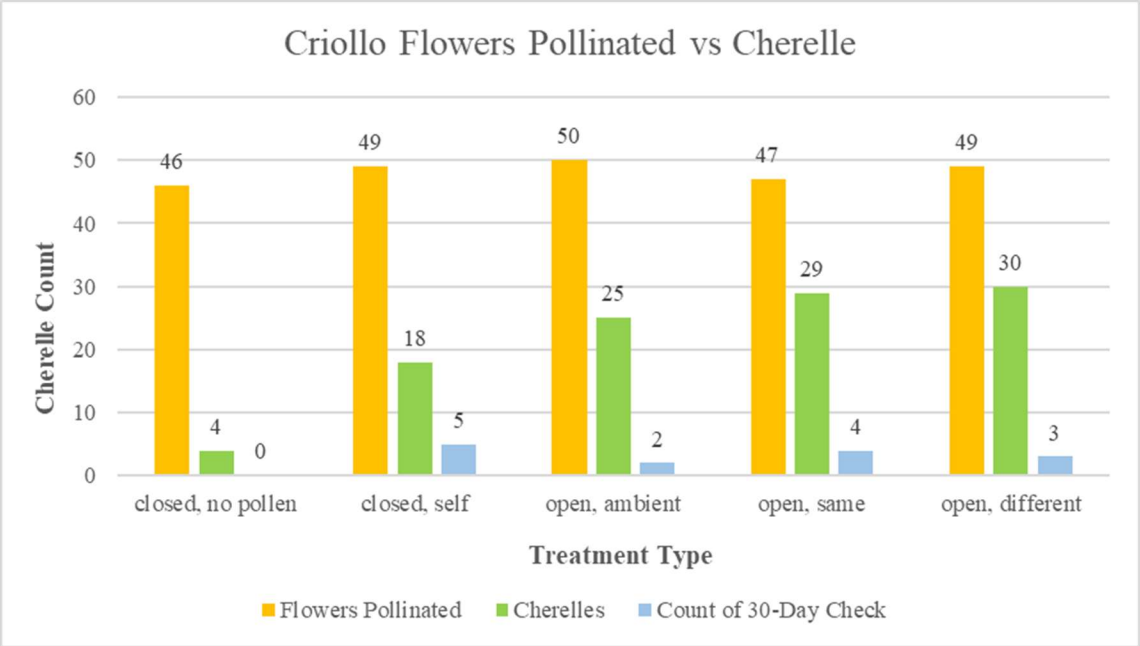

Figure 24. Comparison of total flowers pollinated, the total number of successful pollinations forming cherelles, and the cherelles remaining on the trees after 30 days. Separated by treatment type: 1. closed, no pollen, 2. closed, self-pollen 3. open, ambient pollen, 4. open, Criollo pollen, and 5. open, Trinitario pollen.

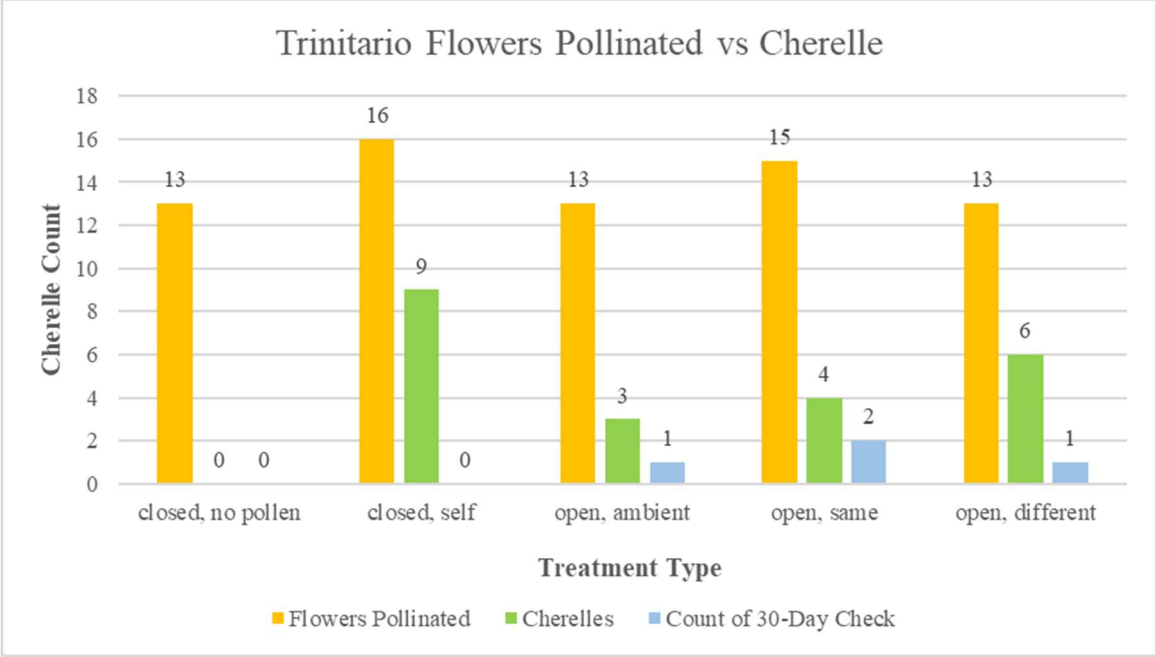

Figure 25. Comparison of total flowers pollinated on Trinitario, the total number of successful pollinations forming cherelles, and the cherelles remaining on the trees after 30 days. Separated by treatment type: 1. closed, no pollen, 2. closed, self-pollen 3. open, ambient pollen, 4. open, Trinitario pollen, and 5. open, Criollo pollen.

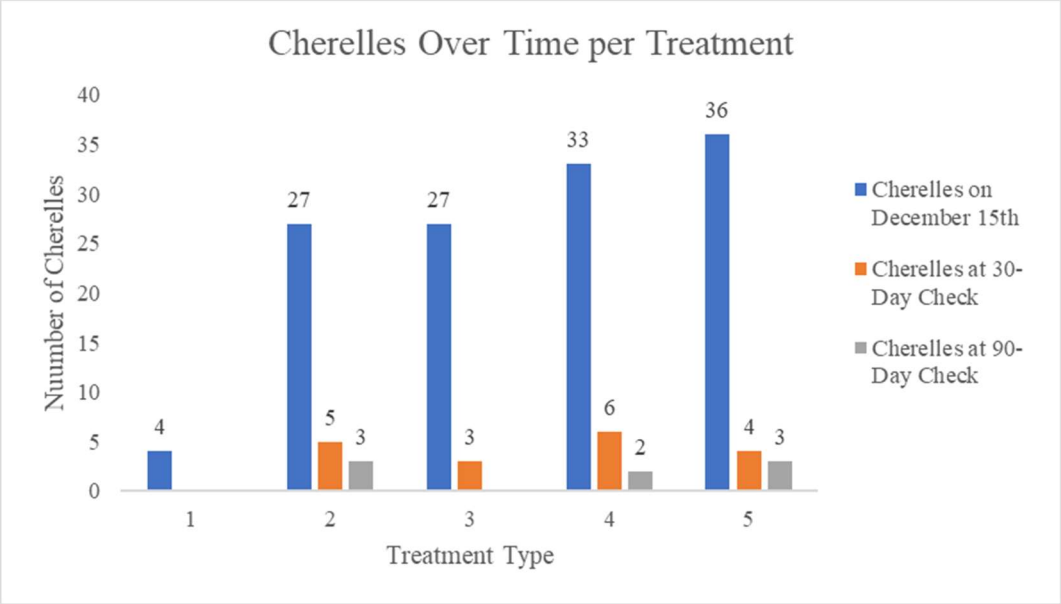

Figure 26. The number of cherelles per treatment, including both Criollo and Trinitario varieties, at the end of sampling on December 15th, 2019, at the 30-day check, and at the 90-day check.

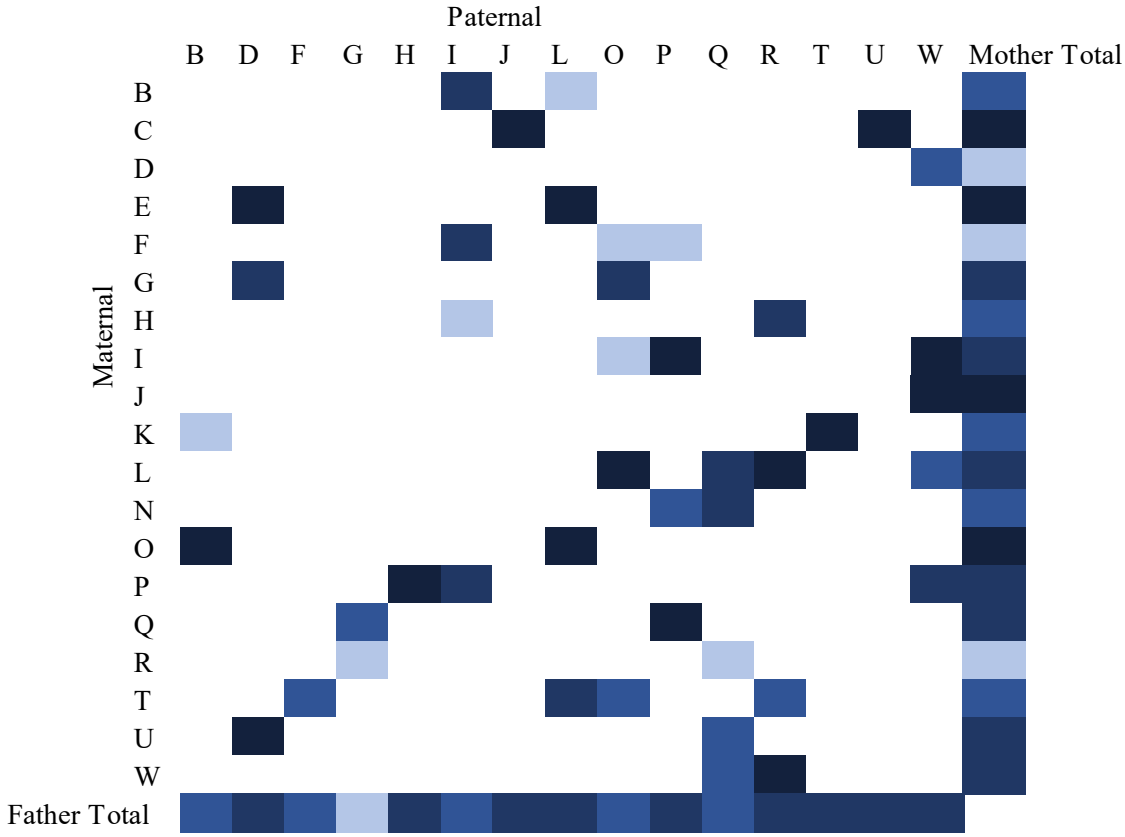

Figure 27. Heatmap displaying the proportions of successful fruit set between pairs of cacao parents. The paternal parent is delineated by columns and the maternal parent is delineated by rows. The darker the color on the heatmap, the higher proportion of successful pollinations between the pair. The color increase in darkness in increments: 0 (white), 0.001-0.5, 0.5-0.8, 0.8-0.99, 1 (very dark). The proportion of total successful pollinations for the respective individual is in the final column and rows labeled "Mother Total" and "Father Total".

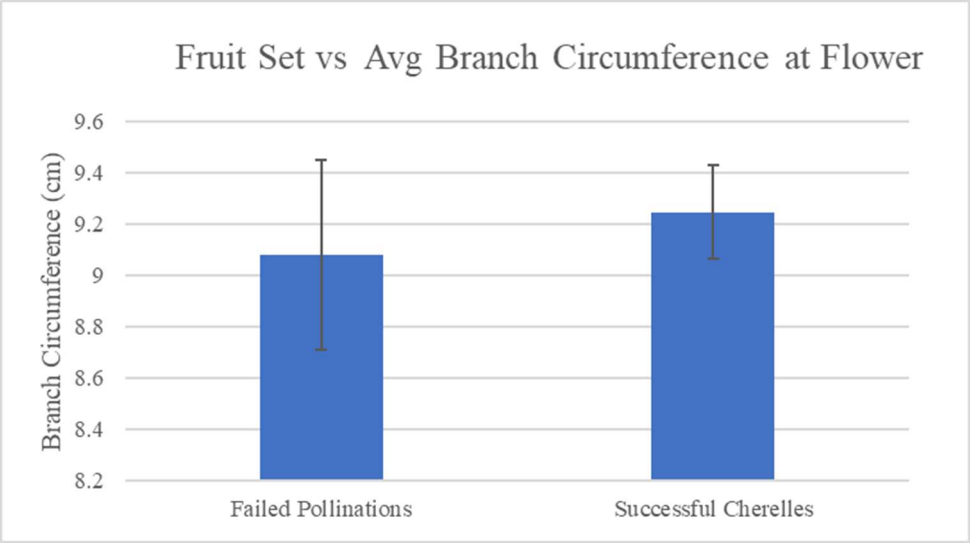

Figure 28. Bar graph showing the relationship between fruit set and the circumference of the cacao branch at the location of the flower. Error bars represent standard error.

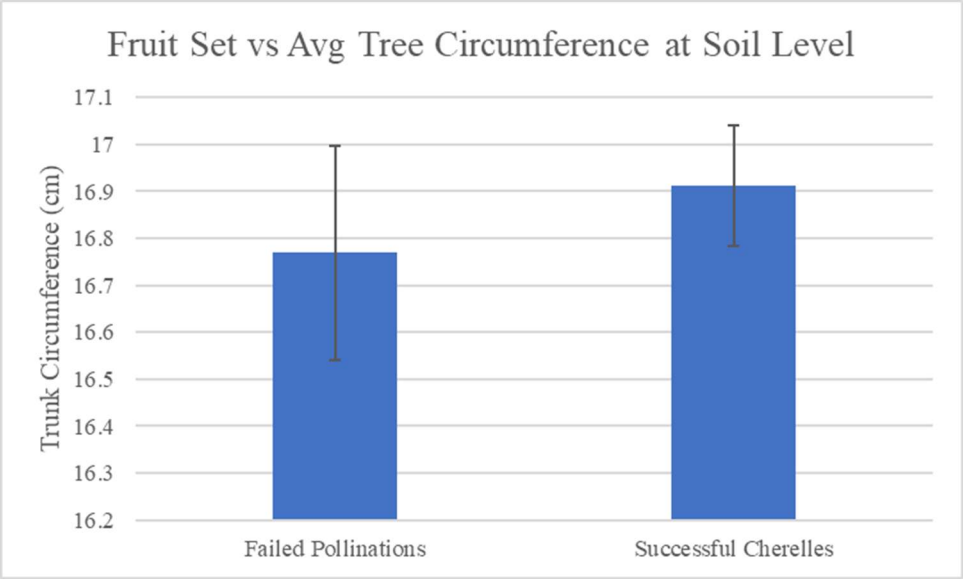

Figure 29. Bar graph showing the relationship between fruit set and the circumference of the cacao trunk at the soil level. Error bars represent standard error.

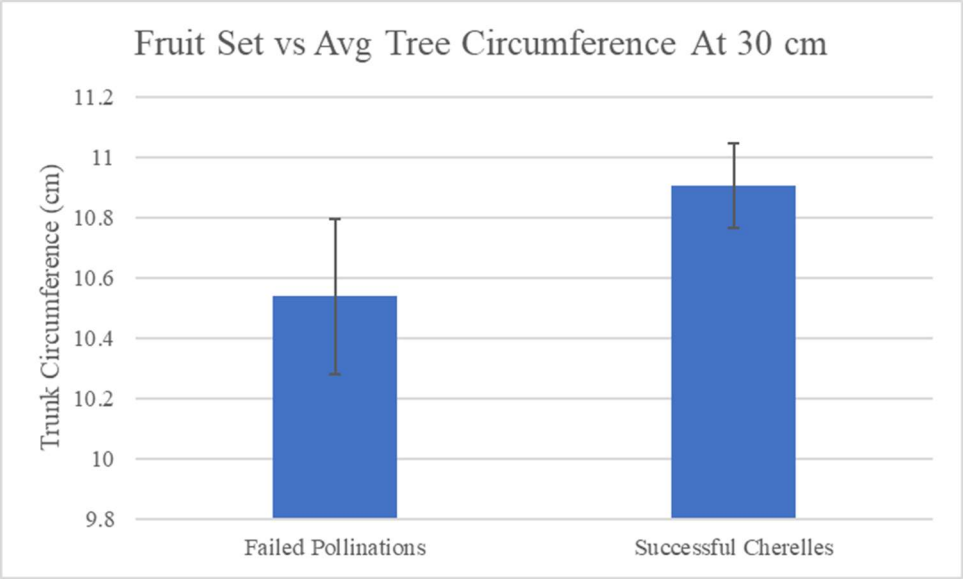

Figure 30. Bar graph showing the relationship between fruit set and the circumference of the cacao trunk or branch at 30 cm above soil level. Error bars represent standard error.

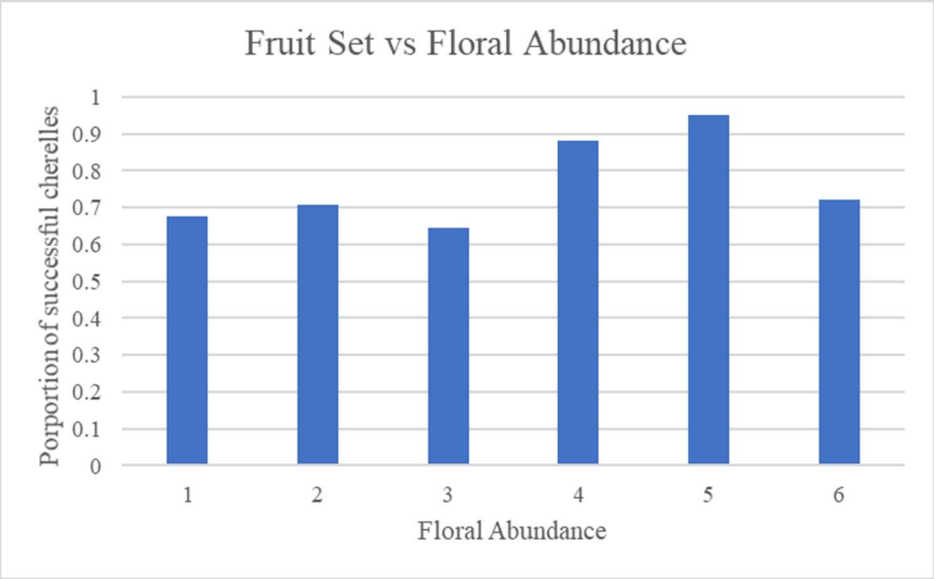

Figure 31. Bar graph comparing floral abundance and fruit set (proportion of successful cherelles) on the y-axis. Floral abundance was measured on a scale of 1 to 10, however trees did not flower above a level 6.

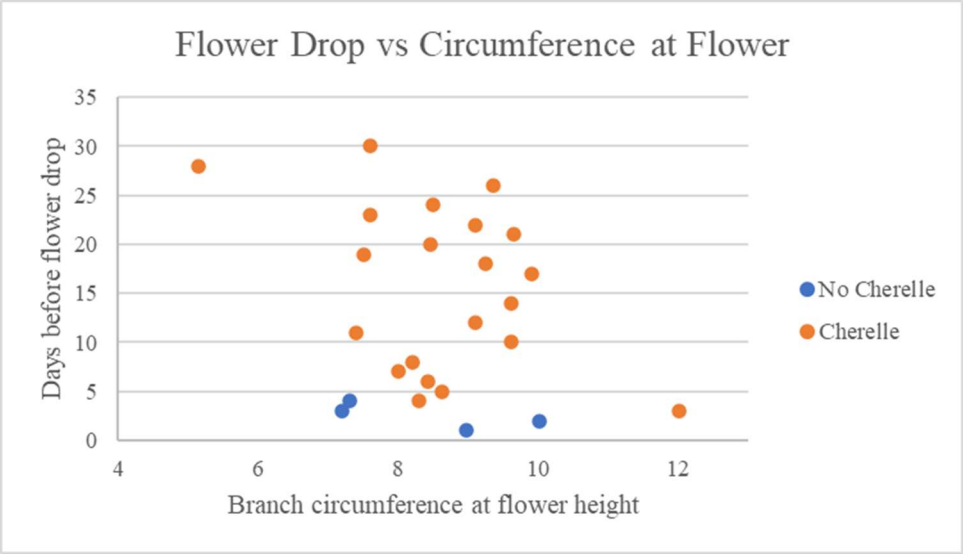

Figure 32. The circumference of the branch at flower location (cm) on the x-axis with the number days before the flower or cherelle fell off the tree. The orange points represent successful fruit set (cherelles) and the blue points represent failed fruit set (no cherelle).

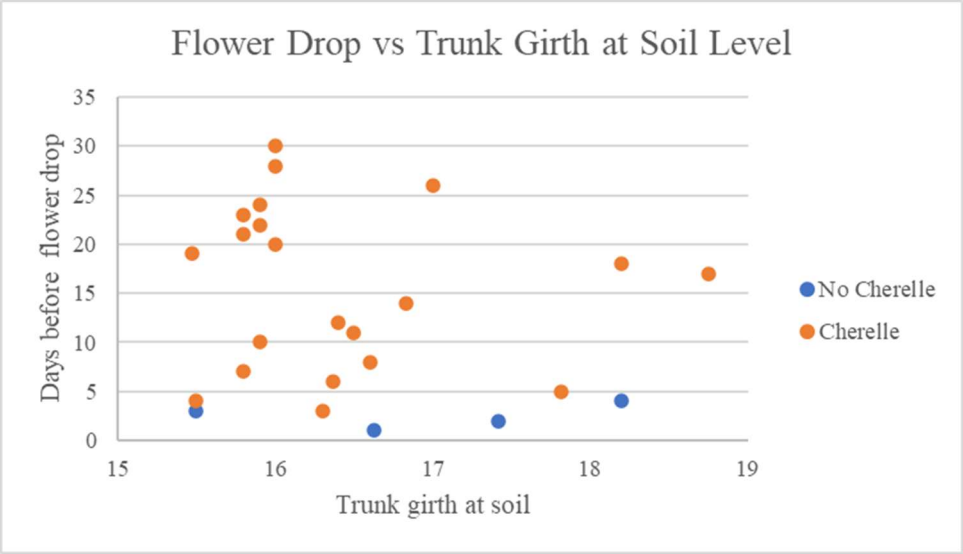

Figure 33. The circumference of the trunk at soil level (cm) on the x-axis with the number days before the flower or cherelle fell off the tree. The orange points represent successful fruit set (cherelles) and the blue points represent failed fruit set (no cherelle).

Table 4. Greenhouse pollination treatments. CCN51 F1 self trees were labelled A through W and the mother-father pairings are shown on the left. Unsuccessful pollinations represent the flowers that did not set fruit. The Total column shows how many pollinations were made per pairing.

Tree Pairings	Unsuccessful Pollination	Cherelle Formed	Total Pollinations
B-I	1	4	5
B-L	3	2	5
C-J	0	5	5
C-U	0	5	5
D-I	5	0	5
D-W	2	3	5
E-D	0	5	5
E-L	0	5	5
F-I	1	4	5
F-O	5	0	5
F-P	1	1	2
G-D	1	4	5
G-O	1	4	5
H-I	3	2	5
H-R	1	4	5
I-O	3	2	5
I-P	0	5	5
I-W	5	0	5
J-W	0	5	5
K-B	4	1	5
L-O	0	5	5
L-Q	1	4	5
L-R	0	5	5
L-W	2	3	5
N-P	1	2	3
N-Q	1	5	6
O-B	0	5	5
O-L	0	10	10
P-H	5	0	5
P-I	1	4	5
P-W	1	4	5
Q-G	2	3	5
Q-P	0	5	5
R-G	3	2	5
R-Q	3	2	5
T-F	2	3	5
T-L	1	4	5
T-O	2	3	5
T-R	2	3	5
U-D	0	5	5
U-Q	3	2	5
W-Q	2	3	5
W-R	0	5	5
Totals:	68	148	216

CONCLUSION

In the landscape survey (Chapter 1), we collected or observed 3,350 flower-visiting insects during 16.67 hours of sampling and completed five full rotations of sampling at five different sites. We collected 2,914 (88%) bees of 62 species. In addition, we recorded two bee species new to the state of Tennessee, *Lasioglossum fattigi* and *Lasioglossum simplex*. We compared our landscape collections to flower-visiting insects collected from experimental garden plots. We determined that the flower-visiting insects collected from within the research plots were a subset of the surrounding landscape diversity. We did not see any effect of land-use at a radius of 2 km on the overlap of flower-visiting insects between the plots and the landscape. Thus, we failed to detect any signal of land-use on homogenization of the pollinator communities in eastern Tennessee.

Though we did not see an influence of land use on flower-visiting insects, it is possible that we did not include a wide enough range of land-use types in our survey. For example, our sites mostly included research farms (UTK Organic Crops Unit, East Tennessee AgResearch and Education Center, and Plateau AgResearch and Education Center) and these do not necessarily represent typical agricultural practices. Research farms tend to be more diverse with plots containing a different variety of crops, per the research questions currently being explored. Our findings may not be consistent in more conventional agricultural settings. Our sites also had very patchy landscapes and native bees have been shown to maintain large populations under patchy conditions (Winfree et al. 2009; Ruiz-Toledo et al. 2020). The next steps for further research may include comparing the heterogeneity of land-use at these locations.

During the three-week study in Belize at BFREE, we hand-pollinated two types of cacao varieties, Trinitario and the heirloom Criollo variety. We used five treatments to test if each variety could self-pollinate, receive self-pollen (as if from a pollinator), cross-pollinate between the two varieties, or if the cacao was pollen-limited. We have shown that Criollo cacao at BFREE is self-compatible and potentially able to self-pollinate. Fruit set occurred successfully on both Trinitario and Criollo when they received pollen from the opposite variety. Using the data from these hand-pollination experiments, we also found no evidence that the cacao trees growing in an agroforestry setting were pollen-limited. Ambient pollination and hand-pollinated flowers formed similar numbers of cherelles. This shows that agroforestry does not necessarily limit pollination services to cacao. Cacao's most important pollinators, very small biting midges of the genus *Forcipomyia* in Ceratopogonidae (Toledo-Hernández et al. 2017), are found in shady forest near moist habitats such as rotting logs and cacao husks (Kaufmann 1975). An agroforestry setting for growing cacao would provide a similar environment and may lead to large populations of the pollinators.

Overall, we found that the pollinators and pollination services in these systems are resilient to land-use. We saw no effect of land-use on the homogenization of pollinator communities in eastern Tennessee, and we saw that pollination systems in cacao

agroforestry were successful in promoting pollinators while providing a more sustainable way to grow cacao.

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VITA

Devon Eldridge attended Texas A&M University where she graduated in 2018 with her Bachelor's in Science in Wildlife & Fisheries Sciences with a concentration in Ecology & Conservation and a double major in Entomology.

While at Texas A&M University, she held multiple internships and volunteered on various research projects. Beginning in August 2017, she interned at the Biodiversity Research and Teaching Collections and later became a Curatorial Assistant. As a Curatorial Assistant, Devon managed the dermestid colony and processed vertebrate specimens into the collection. She also completed other various projects around the collections such as the cleanup and organization of the vertebrate wet collection and reorganization of the herpetological genomic resources collection. Also in the fall of 2017, Devon completed an Apprenticeship under Dr. Adrianne Brundage. She assisted in providing supporting research for two forensic cases including practicing correct handling, unpackaging, and documentation of evidence. During the Apprenticeship, she learned to correctly identify fly larva to genus and family using a dichotomous key. In the summer of 2018, Devon interned at the Trinity River National Wildlife Refuge as an Invasive Species Specialist. She actively conducted herbicide applications on invasive plant species (Chinese tallow, trifoliate orange, and McCartney rose) and built, baited, and trapped invasive feral hogs. Devon also performed a variety of wildlife surveys such as bat acoustic surveys, giant salvinia weevil counts, mosquito surveillance surveys using BG-Sentinel traps, alligator gar with seine, plant collections, and zoonotic disease surveys by collecting blood and lymph node samples from baited and trapped feral hogs. Finally, Devon worked with Dr. Jim Woolley in his Parasitic Hymenoptera Lab in Texas A&M University's Department of Entomology for two years between 2017 and 2019. There she continued developing her passion for collections and she performed collections improvement of the Chalcidoidea collection of Texas A&M University. She identified the tiny wasp specimens to family and subfamily level with a dichotomous key, then prepared and organized the specimens into the collection. She gained valuable experience with pinning, point mounting, labelling, curatorial organization, and insect photography using Leica & Keyence microscopes and a macropod.

In fall of 2019, Devon joined the Russo Lab at the University of Tennessee in Knoxville where she is currently completing her Master's Degree in Ecology & Evolutionary Biology with a minor in Entomology. Her thesis focuses on pollination ecology in agroecosystems. Not only did she enjoy romping around the Eastern Tennessee landscape with an insect net, but she also experienced first-hand what it was like to work within a rainforest while hand-pollinating cacao in Belize. Towards the end of Devon's Master's degree, she joined the Children's Museum Houston's team as a Science Educator where she teaches kids all about the wonders of science at the museum's Science Station!