8-2019

Floral visitors of Helianthus verticillatus, a rare sunflower species in the southeastern United States

Nicolas C. Strange
University of Tennessee

To the Graduate Council:

I am submitting herewith a thesis written by Nicolas C. Strange entitled "Floral visitors of Helianthus verticillatus, a rare sunflower species in the southeastern United States." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Robert N. Trigiano, Major Professor

We have read this thesis and recommend its acceptance:

William Klingeman, Ernest Bernard, Feng Chen

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Floral visitors of *Helianthus verticillatus*, a rare sunflower species in the southeastern United States

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Nicolas C. Strange

August 2019

DEDICATION

My thesis is dedicated to my parents, Mark and Andrea Strange, for their constant support and love. They never stopped believing in me and encouraging me, and I am eternally grateful for the sacrifices they have made to help give me this opportunity and help me succeed.

ACKNOWLEDGEMENTS

Special thanks to Dr. Robert Trigiano for giving me this opportunity to pursue my Master's degree. None of this would be possible without his belief in me as a student. I would also like to thank my committee members: Dr. Willian Klingeman, Dr. Ernest Bernard, and Dr. Feng Chen for their guidance and their assistance throughout my project. I would like to give a special thank you to colleagues and fellow scientists who helped me learn the skills necessary to complete this research: Dr. Kevin Moulton and Dr. Robert Pivar of the University of Tennessee and Dr. Blair Sampson of the USDA. Finally, I would like to thank the University of Tennessee Institute of Agriculture and the United States Department of Agriculture for their financial support.

ABSTRACT

Whorled sunflower (*Helianthus verticillatus*) is an endangered species of aster found exclusively in the southeastern United States. Evidence suggests that this species is self-incompatible and reliant on insect pollination for seed production. However, little is known about the general biology of this species, including the identity of probable pollinators. Floral visitors were collected and identified during September of 2017 and 2018. Forty-one species of visitor species, including 29 hymenopteran, 6 dipteran, 1 lepidopteran, and 5 other miscellaneous insects were trapped during seven collection days at one site in Georgia and two sites in Tennessee. Within a collection day (7:45 to 18:15), there were either 5 or 6 discrete half-hour time periods when insects were trapped. Insect visitor activity peaked during the 11:45-12:15 and 13:45-14:15 collection periods and was least during the 7:45- 8:45 and 9:45-10:15 periods at all three locations. Visitors were dentified to genus and species using morphological keys and some with sequences of the *COX-1* mitochondrial gene. A rarefaction analysis using the iNext Online package was used to assess species richness, while Simpson's Diversity Index was used to assess species diversity within and across each location. The most common visitors at all locations were *Bombus* spp. (bumblebees), while *Ceratina calcarata* (a carpenter bee) and members of the halictid bee tribe *Augochlorini* were second and third most common at the two Tennessee locations. Pollen on visitors was identified as belonging to the *Helianthus* genus via direct PCR of DNA using *Helianthus*-specific microsatellites. Pollen grains were obtained from the 10 most common visitors and *Apis mellifera* (honey bee) and counted using a hemocytometer. Of these visitors, *Bombus* spp., *Halictus ligatus* (a sweat bee), and *Melissodes* spp. (long-horned bees) carried the most *Helianthus* pollen grains. These visitors are the most likely candidates to be the primary pollinators of *H. verticillatus* flowers.

TABLE OF CONTENTS

Section 1 - INTRODUCTION.....1

Section 2 – MATERIALS AND METHODS.....5

Section 3 – RESULTS AND DISCUSSION.....13

Section 4 - CONCLUSION.....23

REFERENCES.....24

APPENDIX.....29

VITA.....43

LIST OF TABLES

Table 1… Numbers of all visitors captured at the three collection sites, listed taxonomically....31

Table 2… Primers developed for *cox-1* sequencing of floral visitors.....34

Table 3… Pollen counts from the ten most collected insects and *Apis mellifera* captured at *Helianthus verticillatus* collections sites 35

Table 4… Simpson's Diversity indices for the Maryville suburban site. Four collections were done: two in 2017 and two in 2018. In 2017, there was no 7:45-8:15 collection period.....36

Table 5… Simpson's Diversity indices shown for the Oak Ridge setting. The first collection was done in September and the second collection was done in October of 2018. No visitors were captured during the 7:45-8:15 collection periods at this site 37

LIST OF FIGURES

Figure 1. Floyd county, Georgia collection site.....37

Figure 2. Maryville, Tennessee home garden collection site.....38

Figure 3. *Helianthus verticillatus* plants established at Oak Ridge, Tennessee collection site one year before insects were collected 39

Figure 4. *Helianthus verticillatus* plants at the Oak Ridge, Tennessee collection site one year after the plot was planted. 40

Figure 5. Slide mounts of p collected fromMorphology of *Helianthus verticillatus*, suspended in Qiagen QX Wash Buffer.....41

Figure 6. Temperatures (°C) recorded for each collection site 42

Figure 7. *Stiria rugfrons* larvae feeding on *Helianthus verticillatus* 43

Figure 8. Species richness diversity plot. This plot signifies no significant difference in richness across all three sites. the point at which with providing an estimation of results expected had sampling been continued.....44

Figure 9. Species richness completeness plot. This plot indicates a successful coverage of visitors at each location. the point at which with providing an estimation of results expected had sampling been continued.....45

Section 1 - INTRODUCTION

Helianthus verticillatus Small (whorled sunflower) is a rare and endangered species found in only a few locations in the southern United States. It was described in 1892 by Samuel Bain (Small 1898, Matthews, Allison et al. 2002, Ellis, Pashley et al. 2006, Mandel 2010). Although originally described from collections from Chester County, Tennessee, this species was not found again until 1993 where it was rediscovered in Floyd County, Georgia (Matthews, Allison et al. 2002). A census was conducted several years later and an additional population was discovered in Cherokee County, Alabama (Matthews, Allison et al. 2002). In Tennessee, wild plants are now known only in Madison County only; other populations were lost likely due to habitat loss (Matthews, Allison et al. 2002, Ellis, Pashley et al. 2006).

Helianthus verticillatus is a diploid (2n=2x=34) perennial species (Ellis, Pashley et al. 2006). Plants can be propagated asexually via rhizomes either in the field or in containers (Edwards, Trigiano et al. 2017) or efficiently by rooted cuttings until mid-late May to early June (Trigiano pers. comm.). *Helianthus verticillatus* flowers on 2–4 m-tall plants from late August or early September to mid-October (Matthews, Allison et al. 2002). In Georgia and Alabama, the plant flourishes in wet, poorly drained soil, and in West Tennessee it grows in a silt loam from nearby alluvial deposits (Matthews, Allison et al. 2002, Ellis, Pashley et al. 2006). The plants grown in a home garden in Maryville, Tennessee for the purposes of this study are grown in a clay soil. *Helianthus verticillatus* is a self-incompatible species that does not lend itself to wind pollination (Mandel 2010). When floral visitors *H. verticillatus,* they compact pollen grains together on the flower, thus increasing the weight of the pollen grains and therefore making wind pollination less likely. Pollination partners for *H. verticillatus* and other self-incompatible plants are likely restricted to within thethese visitors' flight range (Faegri 1966, Ackerman 2000).

Limited flight distance for pollinators could have a negative effect on the genetic diversity of the whorled sunflower (Ghazoul 2005) as it would affect gene flow between individuals and subpopulations. The three major locations from which *H. verticillatus* is currently documented are separated by hundreds of kilometers, however, there are subpopulations within a kilometer of each other at the Cave Springs, Georgia location. The Alabama population is roughly one kilometer away from the Georgia location.

Matthews et al. (2002) used new population data (N = 60) from the newly found populations of *H. verticillatus* and described multiple morphological differences in these subpopulations from the original sample description by Small (1898) (N = 2). These differences include an increased height of the plant (from 0.9–1.80 m to 2–4 m), a wider range of the length of the leaves (from 8–12 cm to 7.5–18.5 cm), and a range of inflorescences per head (10–17) A description of the rhizomes and thick root system was also given.

Helianthus verticillatus exhibits unexpectedly high genetic diversity relative to common congeners such as *H. angustifolius* (Ellis, Pashley et al. 2006). *Helianthus verticillatus* was once thought to be a hybrid between either *H. angustifolius* and *H. eggertii* (Beatley 1963) or *H. angustifolius* and *H. grosseserratus* (Heiser, Smith et al. 1969), which could have accounted for the high genetic diversity (Rieseberg 1997, Soltis and Soltis 2009). However, at nuclear loci, *H. verticillatus* does not exhibit these parental alleles nor does it share chloroplast DNA haplotypes with these alleged parents (Ellis, Pashley et al. 2006). Therefore, when considering this and the morphological differences described by Matthews et al. (2002), *H. verticillatus* is a distinct, non-hybrid species.

Mandel (2010) found low genetic diversity in contrast to the results of Ellis et al. (2006). She postulated that this apparent discrepancy in genetic diversity may have indicated a decline in

individuals of the population due to compatible mate limitations and inbreeding within small populations. Mandel also argued that if individuals cannot be genetically characterized, counting distinct clusters of *H. verticillatus* plants would be a more accurate way of estimating genetically different individuals. Because *H. verticillatus* plants can reproduce via rhizomes, aggregates of individuals are likely genetically identical or clones (Mandel 2010).

Reproductive fitness is considered when determining conservation practices. In a study by Ellis and McCauley (2009), several individuals of *H. verticillatus* were crossed to determine fitness. *Helianthus verticillatus* exhibited a low cumulative fitness, especially the Madison County, Tennessee population. This observation is contrary to past findings for other rare plants that indicate a positive correlation between genetic diversity and overall fitness (Newman and Pilson 1997, Leimu, Mutikainen et al. 2006). The likely reasons for low cumulative fitness could be extensive inbreeding within the populations, differing adaptations, or a limited number of compatible mates, which can be expected of rare species of plants (Ellstrand and Elam 1993, Ellis, Pashley et al. 2006, Ellis and McCauley 2009).

As plants and insects coevolved, energy expenditures in plants to produce pollen and the demands of pollinators, such as nectar rewards and pollen for food, have become closely associated (Kevan and Baker 1983). Because insect pollination is of paramount importance to *H. verticillatus*, it is imperative to identify the potential players. Both the pollination of the plant and its maintenance are linked (Kevan and Baker 1983). Therefore, knowing the potential primary pollinators of rare plants, such as *H. verticillatus*, is crucial to understanding its biology.

Species in the Asteraceae may be visited by a single species or many species (Robertson 1922, DeGrandi-Hoffman and Watkins 2000, Horsburgh, Semple et al. 2011). Members of the Hymenoptera, Diptera, Lepidoptera, and Coleoptera insect orders are pollinators of some

Helianthus species (Robertson 1922). Members of the bee genera *Apis, Bombus*, *Halictus*, and *Mellisodes* are among the common hymenopteran pollinators of sunflowers (Robertson 1922, DeGrandi-Hoffman and Watkins 2000), whereas common families of pollinators in Diptera include are Syrphidae and Bombyllidae also have a significant role in pollination of *Helianthus* spp. (Robertson 1922). Native bees are the most efficient pollinators of self-incompatible flowers (Free 1970, Greenleaf and Kremen 2006) and have coevolved with sunflowers in North America (Hurd 1980). Despite this coevolution, honeybees have been reported as the most efficient pollinators of sunflowers by McGregor (1976). However, Parker (1981) claimed that oligolectic native bees (*Andrena helianthi* and *Melissodes agilis*) were much more efficient pollinators. DeGrandi-Hoffman and Watkins (2000) and Greenleaf and Kremen (2006) stated that the presence of a combination of both native and domesticated bees provided efficient pollination of hybrid sunflower.

To date, there has not been a study to identify the floral visitors of *H. verticillatus*. Thus, an integral part of its biology is unknown. The goals of this study were to identify potential pollinators of *H. verticillatus*, determine which species was the most important to the reproductive biology of the whorled sunflower, and catalog the diversity of insect species visiting *H. verticllatus* flowers.

Section 2 - MATERIALS AND METHODS

Study Sites

Three sites with populations of *H. verticillatus* were used to assess potential insect pollinators: a native, rural setting in Cave Springs, Georgia; a suburban setting in Maryville, Tennessee; and a controlled field trial setting at the University of Tennessee Forest Resources Research and Education Center Arboretum in Oak Ridge, Tennessee.

The native setting (Figure 1, all tables and figures can be found in the appendix) is a forested site on a possible prairie remnant in Cave Springs, Georgia. All tables/figures can be found in the appendix. Weyerhauser Company granted permission to access this land which is used as a commercial slash pine plantation. There were several subpopulations of *H. verticillatus* scattered throughout the property however, for this project, insects were only collected from one. *Helianthus verticillatus* plants at this site were not numerous and were many clusters of a few plants separated from each other by various distances (estimate meters), which were occupied by a thick undergrowth of privet, honeysuckle and grasses. Most plants were growing in full sunlight, but some individuals were under trees and in partial shade. The center of the site was located at GPS coordinates (34.1375512N, -85.4042330W).

The suburban setting (Figure 2) was located in a private residential garden in Maryville, Tennessee. Plants from naturally occurring populations in West Tennessee and Alabama were collected in 2014 before *H. verticillatus* was declared endangered (US Fish and Wildlife Service, 2014) and transplanted to this East Tennessee location. In the study years 2017 and 2018, there were approximately 250 stems in a 10-m^2 area. The soil was heavy clay and the plants were exposed to filtered sunlight in the morning and direct sunlight in the afternoon. This site was

selected to examine potential pollinators that might be found in a home garden when *H. verticillatus* is present. The GPS coordinates of this site are (35.7196480N, -83.9848220W).

The Oak Ridge location (Figures 3 and 4) was at the University of Tennessee Arboretum (35.993936N, -84.221025W) in Oak Ridge, Tennessee, comprising 250-acres hosting many exotic and native species of plants. In October of 2017, 30 *H. verticillatus* plants collected from West Tennessee prior to *H. verticillatus* being listed endangered were arranged in two sections, with three groups in each section and five plants per group for uniformity. This site was considered intermediate between that of the other two sites, as it mimics a native setting while having suburban areas nearby.

Collection of Floral Visitors

Floral visitors were collected while flowering during September and October at the Cave Spring location and the Maryville location in 2017, and at the Maryville and Oak Ridge locations in 2018. The Cave Spring location was sampled on September 28, 2017 by one collector. The Maryville location was sampled September 16 and 30, 2017, and September 18 and 29, 2018, by two collectors at each collection date. The Oak Ridge location was sampled September 23, 2018 by three collectors and October 9, 2018 by one collector.

Floral visitors were trapped in FisherBrand (Waltham, Massachussetts) 27.25 × 70-mm vials that were held directly above insects on flowers. Care was taken to avoid contact of the flowers with the vial to prevent pollen transfer to the vial. The target insect typically flew upwards into the vial, and the vial was then capped. Captured insects were immediately placed on ice, transported to the laboratory, and stored at -20° C until processing for molecular and morphological identification, and enumeration of pollen carried on the body of specimens. At all

sites, specimens were collected in the following five, one-half hour intervals throughout the day: 9:45 to 10:15, 11:45 to 12:15, 13:45 to 14:15, 15:45 to 16:15, and 17:45 to 18:15 to assess species composition and abundance of visitors throughout the day. In 2018, an additonal interval from 7:45 to 8:15 was added to evaluate potential early morning visitors. During each period the temperature and weather conditions were recorded.

Pollen Identity Confirmation

Some visitors had pollen collecting modifications on their hind legs called scopae. For visitors with scopae, a hind leg with a pollen-bearing scopa was detached for pollen analysis. For visitors without scopae, or visitors with no visible pollen on their scopae, the entire body was processed to remove pollen. DNA from the pollen was extracted to specifically verify the presence of *Helianthus* pollen. Specimens were placed in 1.5-mL microcentrifuge tubes. Each tube was filled with 1 mL of Qiagen QX (Qiagen, Hilden, Germany) wash buffer and vigourously vortexed for 15 sec in order to dislodge pollen grains from the insects. Tubes were centrifuged at 10,000 × g for 5 min to sediment any pollen. Insects or body parts were removed and pollen samples stored at -20° C until they could be processed for molecular identification.

DNA extraction of the pollen pellet was completed via direct PCR using the Phire Direct Plant PCR Kit (ThermoFisher Scientific, Waltham, Massachussetts) and followed the manufacturer's instructions. Centrifuge tubes with pollen samples were placed in liquid nitrogen for 3 min, incubated at room temperature for 3 min, and both were repeated twice. PCR reaction mixtures contained 4 µL of GoTaq (nucleotides included), 0.5 µL of dimethyl sulfoxide (DMSO), 3.5 µL of sterile distilled water, 1 µL of forward primer, 1 µL of reverse primer, and 1 µL of DNA. The DNA concentration was not the same for all samples however, the Phire Direct Plant PCR Kit allows for this. A positive control of *H. verticillatus* DNA extracted from its

leaves and a negative control of water were used. Only the ten most commonly captured visitors (Table 1) had their pollen loads tested, with two pollen amplifications completed per visitor. Pollen was identified as *Helianthus* using EST-SSRs (expressed sequence tags – simple sequence repeats) primers from Ellis, Pashley et al. (2006). Locus HT1099 (forward GGCTTTCGTTTCTCGTTGTC and reverse CAGCTCACTCCTAATTGGTTCC) had an expected allele size of 302 bp, and locus HT1123 (forward GGGTTTGTACCAGGCACTTG and reverse TTCATAGAAATGAGGACCAAAGG) had an expected allele size of 322 bp. Both EST-SSRs were developed for *H. annuus,* but cross-amplified DNA of *H. verticillatus* (Ellis, Pashley et al. 2006, Edwards 2018). The thermocycler protocol was 95° C for 3 min, 10 cycles of 94° C for 30 secs, 65° C for 30 sec, 72° C for 45sec, 30 cycles of 94° C for 30 sec, 55° C for 30 sec, 72° C for 45 sec, and 72° C for 5 min and hold at 4° C. PCR products were separated by electrophoresis (100 volts/cm^2 for 1 h) on 2% low melting point agarose gels stained with ethidium bromide and visualized on an ultraviolet transilluminator. The detection of amplification products, visualized as discrete bands in the gels was considered a positive identification of *Helianthus* pollen.

Pollen Counts

Five specimens of each of the ten most commonly captured visitors and *Apis meliffera* were selected to determine the number of pollen grains carried on insect bodies. The visitors used for this experiment were selected at random from the entire collection regardless of location. Pollen was washed off with 1 mL of Qiagen QX wash buffer (Qiagen, Hilden, Germany). The following method was used to collect pollen from individual specimens, and is slightly modified from the methods reported by Jones (2012). Entire insects were placed in 1.5-mL microcentrifuge tubes with 1 mL of distilled water. Tubes were vortexed and centrifuged at

10,000 × g for 8 min to accumulate the pollen at the bottom of the tube, after which insects were removed. Tubes were vortexed to resuspend the pollen grains. The number of pollen grains per mL were estimated using a hemocytometer (Trigiano 2010). After agitation to ensure uniform suspension of the pollen sample, 10 µL of this suspension was drawn into a pipette tip and liquid delivered to the hemocytometer counting chamber by capillary action. Individual pollen grains lying on the top left, top right, middle, bottom left, and bottom right squares were counted. Some visitors carried pollen from plants other than *Helianthus*, but only those grains from *Helianthus* (Figure 5) were counted. The sum of pollen grains in the five counting squares was multiplied by 2000 to obtain the number of pollen grains/mL (Trigiano 2010). Each pollen sample was counted five times and the mean calculated. The lowest, highest, and means for pollen counts were recorded for each insect species.

Insect Taxonomic Identification - Morphology

Each specimen was examined using a stereo microscope and identified to the lowest taxonomic level described by available resources. For members of the Hymenoptera, Mitchell (1960) was used, whereas (McAlpine, Peterson et al. 1981) was employed for members of the Diptera. Assistance was also provided by entomologists at the University of Tennessee and the Thad Cochran Horticultural Research Center in Poplarville, Mississippi.

Insect Molecular Identification Using cox-1

Following morphological identification, representatives of each species after were selected for *cox*-1 gene sequencing. Primers were developed (Table 2) for this purpose and were mapped against the *A. mellifera* mitochondrial gene. Many potential primer combinations were developed for this task and the best matches for each visitor were determined with trial and error.

DNA was extracted from specimens using the Omega E.Z.N.A Insect DNA Kit (Omega Bio-Tek, Norcross, Georgia) with some modifications. One leg was used for large specimens (e.g., *Bombus* spp. and *Svastra* spp.), whereas three legs where removed for small insects (e.g., members of the tribe *Augochlorini*). For very small visitors, (e.g., *Lasiglossum* (*Dialictus*) spp.), the entire body was used. Samples from each visitor were pulverized with a pestle in a 1.5 mL Eppendorf tube filled with lysis buffer (CTL, kit provided) and Proteinase K solution. CTL buffer (300 µL), 10 µL of Proteinase K (this amount differs from the protocol), and 10 µL of RNase A solution were used. Samples were incubated overnight at 55° C in a Fisherbrand IsoTemp dry block.

Spin columns were filled with 150 µL of 1 N hydrochloric acid, incubated at room temperature for 5 min. and centrifuged at 13,000 × g for 2 min. The filtrate was decanted and 150 µL of CTL lysis buffer was added to the spin column. The columns were centrifuged immediately at 13,000 × g for 2 mins. DNA extraction followed the protocol provided in the Omega E.Z.N.A Insect DNA Kit. Extracted DNA was stored at -20°.

PCR Protocol

PCR reactions contained 36 µL of sterilized distilled water, 5 µL of 10x TaKaRa Taq buffer, 2.3 µL $MgCl_2$ (50 mM), 3.5 µL dNTP (10 mM) mixture, 0.2 µL TaKaRa hot start Taq, 3 µL of forward and reverse primers (Table 1), and 1 µL of DNA template (various concentrations). The PCR protocol was a touchdown method similar to that used by Senatore et al. (2014) with the following modifications: 95° C for 1 min, 10 cycles of 96° C for 15 sec, 58° C for 20 sec, 72° C for 1 min; 10 cycles of 96° C for 15 sec, 50° C for 20 sec, 72° C for 1 min; and 40 cycles of 96° C for 15 sec, 45° C for 20 sec, 72° C for 1 min, and 72° C for 5 min.

Purifing DNA Amplicons from Agarose Gel

PCR products were separated on a 1 % low melting point agarose gel (120 volts/cm^2 for 30 min) stained with ethidium bromide, and visualized with an ultraviolet transilluminator. Brightly fluorescing bands were excised from the gel using a sterile scalpel, placed into 1.5 mL Eppendorf microcentrifuge tubes, and placed in a FisherBrand IsoTemp dry block at 65 °C to melt the agarose before sequencing.

EconoSpin DNA Spin Columns were incubated with 250 µL of 1 N hydrochloric acid at room temperature for 5 min, and centrifuged at 13,000 × g for 30 sec. Afterwards, 100 µL of solubulization buffer (QG, kit provided) was added to each column and centrifuged at 13,000 x g for 30 sec. PCR products were extracted and purified using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, Norcross, Georgia). To assess the quality of the recovered amplicons, 2 µL of each sample was placed in 0.2 mL tubes, mixed with 3 µl of 6X dye (Ficoll blue + Orange G), separated on a 1% agarose gel (115 volts/cm^2 for 30 min) and visualized using an ultraviolet transilluminator.

Sequencing Preparation

Extracted DNA from gels were prepared for sequencing with the ABI Big Dye 3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, Massachussetts) and followed the manufacturer's protocol. Samples were placed into a thermalcycler for the Cycle Sequencing reaction. The protocol was a variation of the protocol used by Senatore et al. (2014) and was as follows: 95° C for 1 min, 15 cycles of 95°C for 15 sec, 47° C for 15 sec, 60° C for a min and 45 sec; 25 cycles of 95°C for 15 sec, 45° C for 15 sec, 60° C for 1 min and 45 sec, 30 cycles of 95°C for 15 sec, 43° C for 15 sec, 60° C for 1 min and 45 sec, and 60 °C for 3 min. .

Sephadex Tube Preparation

Bottom caps were placed on Sephadex tubes (Princeton Separations, Freehold, New Jersey), filled with Illustra Sephadex G-50 Fine DNA Grade (GE Healthcare, Chicago, Illinois) and 800 µL of sterile, distilled water added to each tube. The tubes were capped and incubated at room temperature. Both caps were removed from the tubes after 2 h and the tubes placed in 2 mL collection tubes to drain for 30 mins. Sephadex tubes were centrifuged at 900 × g for 2 min. Sephadex tubes were placed in 1.5-mL microcentrifuge tubes, PCR products were added, and the tubes were centrifuged at 750 × g for 3 min. Microcentrifuge tubes were placed in a vacuum concentrator (Labconco, Kansas City, Missouri), dried at 40° C for 30 min, and the products sequenced at the University of Tennesse Genomics Core Sanger Sequencing Laboratory.

Rarefaction Analysis and Diversity Indices

A rarefaction analysis (Chao, Gotelli et al. 2014) was used to assess the data. This was peformed with the iNext Online software (Chao, Ma et al. 2016) and the data was graphically illustrated using this same software. Simpson's Diversity index (Simpson 1949) was calculated for each location overall and for each time period at each location.

Section 3 - RESULTS AND DISCUSSION

Location Trends

Only 56 floral visitors were captured at the Cave Spring, Georgia location and was the least number of insects trapped at all sites (Table 1). The number of visitors captured steadily increased throughout the morning and mid-day collection periods (9:45-10:15, 11:45-12:15, 13:45-14:15), but activity decreased during the evening collection period (15:45-16:15). Individuals in the *B. bimaculatus/impatiens* complex were the most commonly captured visitor. *Apis mellifera* (honey bee) was collected here more often than at the other sites and is likely due to hobbyist colonies spotted within 5 miles of the collection site. Members of the *Megachilidae* were well represented at this site; *Megachile* spp. were more commonly collected here than at the other sites, and *Coelioxys* spp. was collected exclusively at this site (Table 4). Coincidentally, larvae of the noctuid moth *Stiria rugifrons* were feeding upon and destroying many of inflorescences (Figure 6). This pest of *H. verticillatus* could limit seed production and was observed only at the Cave Spring, Georgia site.

There were collection limitations associated with this location. The relatively few plants (compared to the other sites) were growing much further apart, which added the factors of time and distance among plants when collecting. Additionally, the space among the plants in this setting was covered with thick underbrush (Figure 1), making travel between individuals difficult. Overall, collection at this location was more difficult than at the other two sites, and the lower total number of visitors collected from this location may reflect these limitations.

At the Maryville location there were 776 visitors (Table 1) collected during four days of sampling over 2 years. The number of visitors trapped was lowest during the morning collection

periods (7:45-8:15 and 9:45-10:15), whereas the number of insects captured at the site increased during the mid-day collection periods (11:45-12:15, 13:45-14:15 and 15:45-16:15), and decreased during the evening collection period (17:45-18:15). The most commonly captured visitor at this location were members of the *B. bimaculatus/impatiens* species group. *Ceratina calcarata* was the second most numerous visitors collected during the entire study and was trapped almost exclusively at the Maryville garden site. Additionally, Halictidae were collected at this location in much greater numbers than at the other two study sites (Table 4).

There were some biases associated with this location. This location had the most flower heads of all collection sites, thus allowing more comprehensive sampling. Bees remember sources of pollen and nectar (Menzel and Erber 1978, Goulson 1999, Reinhard, Srinivasan et al. 2004) and thus, may be more attracted to *H. verticillatus* plants at this site than other nearby resources if the flowers had provided rewards in the past. These sunflowers were all planted closely to each other, included new plants growing from rhizomes, and were not surrounded by underbrush (Figure 2), thus limiting travel distance by collectors and floral visitors and making collection more continuous and efficient. This location had the most collection days, as well as two collectors each day. These biases may explain the significantly larger number of visitors captured at this setting.

At the University of Tennessee Arboretum in Oak Ridge, Tennessee, 191 visitors were collected (Table 1) on the two dates. At this site, the lowest number of captures was during the morning periods of 7:45-8:15, and 9:45-10:15, and capture of insects increased substantially during in the midday collection periods of 11:45-12:15, 13:45-14:15, and 15:45-16:15. The number of trapped insects decreased during the evening collection period of 17:45-18:15. The

most commonly collected visitors at this site were the species in *B. bimaculatus*/*impatiens* group. *Melissodes* spp., and *Svastra* spp. were also commonly trapped here (Table 4).

Like the Maryville location, this setting had individuals in a much more compact area and lacked the underbrush (Figure 3) present in the Cave Spring location, making travel among individuals much easier for collectors. A limitation of this setting was the height of the plants: At the Maryville location, tall plants were tied down to lower the flowerheads to aid in capturing visitors. In this location, no such preparations were made, although a few plants had lodged due to rain. Still, there were many flower heads above the collectors' reach and visitors to these flowers were not captured. Additionally, there were three collectors during the first collection day at this location and only one collector during the second, which may be one factor in the reduced numbers of captured visitors during the second collection.

Floral Visitor Trends

Insect activity around *H. verticillatus* was sparse during the morning collection periods of 7:45-8:15, with a slight increase at 9:45-10:15 at all three locations. Species of *Helianthus* secrete nectar at around 8:00 in the morning (Neff and Simpson 1990) and therefore, the sparse activity and low diversity of visitors present (only those foraging for nectar) was not unexpected. In regards to pollen, Neff and Simpson (1990) reported that *H. annuus* anthers dehisced in the morning and evening, and insect visitation coincided with these periods. The data from this study agree with this observation as insect visitation began to increase at 9:45-10:15, with a much larger increase in the late morning and early afternoon (11:45-12:15). However, the number of visitors captured peaked during late afternoon sampling time of 13:45-14:15. Activity stayed steady into the late afternoon (15:45-16:15), and then decreased into the evening sampling period (17:45-18:15). Despite this decrease in activity in the later collection periods, the numbers of

visitors captured were sometimes still greater than those of the morning collection periods. The observations of this study agree with Peat and Goulson (2005) who reported an increase in foraging behavior as temperature increased throughout the day, and decreased activity as the temperature decreased during the evening.

At all three sites, the *B. bimaculatus*/*impatiens* group was the most numerous visitor (Table 1). This species group was commonly captured throughout all time periods. Hoverflies in several genera (*Allograpta* spp., *Eristalis* spp., *Eupeodes* spp., and *Toxomerus* spp.) were not captured as frequently as *Bombus* spp. however, hoverflies were also found throughout most collection periods. The second most numerous visitor captured, *C. calcarata*, was exclusively captured at the suburban setting, but was only active during the 11:45-12:15 through the 15:45-16:15 collection period. All other visitors of note, such as *Augochlorini*, *H. ligatus*, *Melissodes* spp., and others were mostly active during the afternoon collection periods as well.

Limitations and Biases

Sampling with vials was used for collection instead of a sweep net for several reasons. First, the only desired pollen was that which would be collected by the visitors themselves. Using a sweep net over the flowerheads might dislodge pollen into the net and it would be impossible to tell if this pollen was being carried by the captured insects. Second, sweeping a net over the tops of the inflorescences would likely capture flying insects that may not have visited the flowers, thus possibly confusing them as potential pollinators. Third, *H. verticillatus* can grow to a height of 4 m, which is impractical for use of sweep nets unless the operation is done from a ladder. Fourth, sweeping a net over these flowers could destroy flower heads and thereby degrade the location and possibly influence pollinator numbers and species.

Potential biases for the vial method should also be considered. The first consideration is the ability to capture with this method. Most collections had more than one collector. One collector could be more skilled and efficient at collecting the insect visitors than other collectors, and if some visitors exclusively visit one side of the site that this collector collects from, this could skew the count. Second, some species, such as some *Bombus* spp. or lepidopterans, visiting *H. verticillatus* were too big to fit into the lumen of the vials. Third, some insects are more difficult than others to capture. There are limitations regarding each collection site, and these are individually detailed below.

Pollen Grain Counts

Abundance of a visitor does not guarantee pollination efficiency, but the amount of pollen they carry might be a better indication (Horsburgh, Semple et al. 2011). Five specimens for each species or species group were selected randomly from the collections to estimate pollen load carried (Table 3). *Apis mellifera* was also selected for this experiment because of its prevalent use as a pollinator in agricultural settings (Levin 1983). The estimated length of each visitor was used as a proxy variable to express how much pollen the visitor is carrying relative to its size. This was done to give a relative estimation of the pollination effort from each chosen visitor. Hypothetically, a large visitor such as *Bombus* (8.5-16 mm) could carry some 50,000 pollen grains at the time of washing. By contrast, if a smaller *Halictus* visitor (7-10 mm) carried about 40,000 pollen grains, then it could be inferred that the smaller visitor either spent more time at the flower or was more efficient in its method of pollen collection. Pollen counts were expressed as pollen grain/mL of water. The visitor with the highest mean number of pollen grains was *Melissodes* spp., with *H. ligatus* and members of *B. bimaculatus/impatiens* following (Table 3). These results are similar to those recorded for *H. annuus* by Parker (1981), who found

that female *Melissodes* spp. carried the most pollen grains, with female *Bombus* spp. also carrying a large number of pollen grains among the recorded visitors. Parker (1981) also found honeybees to carry fewer pollen grains than most of the native bees recorded in their study and mentioned their habit of grooming pollen off of their bodies, which likely affected the number of pollen grains found on this visitor.

Non-hymenopteran visitors, such as *Allograpta* spp. (Diptera: Syrphidae), *Atalopedes campestris* (Lepidoptera: Hesperiidae), and S*parnopolius* spp. (Diptera: Bombyliidae), carried relatively low amounts of pollen compared to hymenopteran visitors (Table 3). Members of Bombyliidae and Syrphidae seek both nectar and pollen rewards (Gilbert 1981, Kastinger and Weber 2001), but syrphid flies primarily seek pollen (Horsburgh, Semple et al. 2011). Horsburgh and Semple et al. (2011) also reported that smaller syrphid flies carried less pollen than other pollinators. Syrphid flies can also be seen cleaning pollen from their bodies (Gilbert 1981), which could affect the number of pollen grains observed by this method. Members of Hesperiidae do eat pollen, but their primary food is nectar (Gilbert and Singer 1975, Pivnick and McNeil 1985).

Pollen load evaluation using the wash method may have some bias. For example, a randomly selected visitor from the collection could have been captured before visiting many flowers and would yield low pollen counts and in contrast, an individual may have visited many flowers before capture. However, by calculating the mean of five samples, the numbers should be representative of actual pollen carrying ability.

Weather and Temperature

Weather conditions and temperature were recorded for each sampling period at each collection site to assess the effect(s) these factors may have had on insect visitation for *H. verticillatus* (Figure 6). Peat and Goulson (2005) stated that bees prefer to forage for pollen in warmer conditions with peak activity during the middle of the day. The data from this study support this conclusion, as there were more floral visitors captured during the afternoon collection periods when temperatures were warmer compared to the morning and evening collection intervals with lower temperatures.

Pollen vs. Nectar

Not all visitors seek the same rewards, and some visitors may seek different rewards depending on the time of day (Neff and Simpson 1990, Peat and Goulson 2005). *Bombus* spp. seek both pollen and nectar rewards (Cresswell 1999). Observations by Peat and Goulson (2005) concluded that *Bombus* visitors collected nectar in the mornings and evenings, whereas they collected pollen during the afternoon. This agrees with observations made during this study as *Bombus* spp., hesperiid, and syrphid visitors were observed collecting nectar during the 7:45-8:15 and 9:45-10:15 collection periods rather than pollen. During the mid-day collection periods the scopae on *Bombus* spp. were full, but contained less pollen during the evening collection periods.

All Visitors Collected

A total of 38 visitor species (25 Hymenoptera, 7 Diptera, 1 Lepidoptera, and 5 miscellaneous floral visitors) captured over the course of the two collection seasons (Table 4). Visitors in the *B. bimaculatus/impatiens* group were present at all locations and all collection

periods. *Ceratina calcarata* was the second most commonly captured visitor in this study, but was found almost entirely at the Maryville location. Because this site is in a home garden where the plants have been present since 2014, there are numerous dead old stems present, more so than at the Oak Ridge as it had just been planted. *Ceratina* species nest in dead stems (Rehan and Richards 2010); therefore; it is hypothesized that *Ceratina* visitors may be nesting in the old stems at this collection site, which could explain the higher number of *Ceratina* visitors captured there. Because of the prescribed burns that take place at the Cave Spring location, there are fewer dead stems surrounding *H. verticillatus*, which may explain the lack of *Ceratina* visitors captured there. The Oak Ridge location was planted much more recently than the Maryville location, so there has not been ample time to produce the same number of dead steams as the Maryville location. It would be interesting to see if numbers of *C. calcarata* or other *Ceratina* species rise after a few years at the Oak Ridge location. Dead stems of any plant host, not just *H. verticillatus*, are a sufficient nesting site for *Ceratina* visitors, but the hypothesis is that the abundance and close proximity of the dead *H. verticillatus* stems at the Maryville site may provide a suitable habitat for *Ceratina* visitors. *Melissodes* and *Halictus* species were reported as pollinators of *Helianthus* spp. (Robertson 1922, DeGrandi-Hoffman and Watkins 2000), as was *Agapostemon* spp. (Chandler and Heilman 1982, Posey, Katayama et al. 1986).

Molecular Identification Using cox-1

Representative visitors that were sequenced with *cox*-1 confirmed the morphological ID for most visitors (Table 5). Sequencing with *cox*-1 provided species identification for visitors not identified by use of dichotomous keys.

Rarefaction Analysis

The Cave Spring location had the highest overall diversity of captured visitors, but a lower sample size of captured insects compared to the other two collection sites. The Maryville location had the lowest diversity of captured visitors, but at this site, more insects were captured than at the other two collection sites. Despite having the greatest number of unique individuals captured, a few visitors were collected in greater numbers in the collection for this site, thus reducing the diversity of captured visitors. Overall, there was no significant difference in richness across the three locations (Figure 8).

The species accumulation curve (Figure 9) indicated the Cave Spring location had the least amount of species coverage, which was expected because of the low sample size and fewer collections. The Maryville location had the highest amount of coverage, which was to be expected when considering the large sample size and highest amount of collections. Overall, the data indicate a satisfactory coverage all potential visitors at all sites, as indicated by the plateaus of the lines (Chao and Jost 2012).

Simpson's Diversity Indices

Simpson's Diversity Index (Tables 6 and 7) ranges from 0 to 1. The closer to 0, the less diverse a collection is, and the closer to 1, the more diverse the collection (Simpson 1949).

In the Maryville location, the second collection in late September for the 2017 season was more diverse than the first collection in mid-September. However, in the first 2018 collection (mid-September) diversity was higher than the second collection (late September), despite having a lower overall number of visitors captured. This result can be explained by the larger proportion of *Agapostemon* spp. and *B. bimaculatus/impatiens*. *Bombus* species

experience population peaks in the fall, which could explain their overall abundance in this study (Neff and Simpson 1990). Other *Helianthus* pollination studies have shown an abundance of *Agapostemon* visitors as well (Chandler and Heilman 1982, Posey, Katayama et al. 1986).

At the Oak Ridge location, the first collection was in late September and had a much higher diversity than the second collection, which occurred in early October. The second collection day at this location was much later than other collection days in this study and was both late in the flowering period for *H. verticillatus* (Matthews, Allison et al. 2002) and late in the flight season for many of the visitors found in this study (Matthews, Allison et al. 2002). Additionally, temperatures on this day were lower and the weather was cloudier, which are less favorable for pollinator activity (Peat and Goulson 2005). *Bombus bimaculatus/impatiens* was the most frequently captured visitor on this collection day, which explains why the species richness was much lower for this collection day.

In the Maryville and Oak Ridge locations, the 11:45-12:15 and the 13:45-14:15 collection periods offered the most diversity, with the Maryville location showing high diversity during the 15:45-16:15 collection period as well. Morning collection periods had the least diversity at all sites.

Section 4 - CONCLUSION

The data collected in this study are in accordance with past *Helianthus* pollinator studies and found several Hymenoptera genera (*Bombus*, *Melissodes*) and Diptera families (Bombyllidae, Syrphidae) in common with these past studies. These visitors were the most abundant floral visitors of *H. verticillatus*. Some of these hymenopteran genera (*Bombus*, *Halictus*, and *Melissodes*) carried the highest amounts of *Helianthus* pollen grains, and likely represents pollination capabilities. Other genera in the Halictidae (*Agapostemon*, *Augochlora*, and *Augochlorella*) were also common visitors to *H. verticillatus*, however, they carried fewer *Helianthus* pollen grains than other hymenopteran genera. Previous studies concluded that native pollinators, rather than *A. mellifera*, are likely more efficient pollinators of *H. annuus*. Our findings agree that this is likely the case for *H. verticillatus* as well.

Despite some differences in species composition, there was no significant difference in the diversity of floral visitors across all three locations. *Helianthus verticillatus* is likely to attract a wide range of insect visitors regardless of its location, and species-specific composition is likely dependent on the location and its native species.

Temporal and spatial differences may influence the potential pollinators found at *H. verticillatus* (Herrera 1998). Therefore, it is recommended that this study be conducted again in the future. If one is to repeat this study, some modifications are recommended: If using the vial method to capture visitors, it is recommended that larger vials be used as not to miss larger visitors that could not fit into the vials used in this study. For each collection day, it is recommended to employ the same number of collectors each time. For estimating pollen grain counts on visitors, it is recommended to use a more intricate scale that has been published in similar pollination studies.

REFERENCES

Ackerman, J. D. 2000. Abiotic pollen and pollination: Ecological, functional, and evolutionary perspectives. Plant Systematics and Evolution 222:167-185.

Beatley, J. C. 1963. The sunflowers (genus *Helianthus*) in Tennessee. Journal of the Tennessee Academy of Science 38:135-154.

Chandler, L. D. and M. Heilman 1982. Hymenoptera associated with sunflower in the Lower Rio Grande Valley of Texas with notes on relative abundance, visitation times and foraging [*Apis mellifera* and native bees as pollinators, ecologic aspects, yields]. Southwest Entomology 7:170-173.

Chao, A., N.J. Gotelli, T.C. Hsieh, E.L. Sander, K.H. Ma, R.K. Colwell, and A.M. Ellison. 2014. Rarefaction and extrapolation with Hill numbers: a framework for sampling and estimation in species diversity studies. Ecological Monographs 84:45-67.

Chao, A. and L. Jost 2012. Coverage-based rarefaction and extrapolation: standardizing samples by completeness rather than size. Ecology 93:2533-2547.

Chao, A., K.H. Ma, and T.C. Hsieh. 2016. iNEXT (Interpolation and Extrapolation) Online: Software for interpolation and extrapolation of species diversity. Program and User's Guide published at http://chaohttp://chao. stat. nthu. edu. tw/wordpress/software_download.

Cresswell, J.E. 1999. The influence of nectar and pollen availability on pollen transfer by individual flowers of oil-seed rape (*Brassica napus*) when pollinated by bumblebees (*Bombus lapidarius*). Journal of Ecology 87:670-677.

DeGrandi-Hoffman, G. and J.C. Watkins. 2000. The foraging activity of honey bees *Apis mellifera* and non—*Apis* bees on hybrid sunflowers (*Helianthus annuus*) and its influence on cross—pollination and seed set. Journal of Apicultural Research 39:37-45.

Edwards, T.P. 2018. A Fine Scale Population Genetics Study of the Rare *Helianthus verticillatus*. Master's Thesis, University of Tennessee. https://trace.tennessee.edu/utk_gradthes/5395

Edwards, T.P., R.N. Trigiano, P.A. Wadl, B.H. Ownley, A.S. Windham, and D. Hadziabdic. 2017. First report of *Alternaria alternata* causing leaf spot on whorled sunflower (*Helianthus verticillatus*) in the southeast United States. Plant Disease 101:632-632.

Eickwort, G.C. 1981. Aspects of the nesting biology of five Nearctic species of *Agapostemon* (Hymenoptera: *Halictidae*). Journal of the Kansas Entomological Society 54:337-351.

Ellis, J.R., C.H. Pashley, J.M. Burke, and D.E. McCauley. 2006. High genetic diversity in a rare and endangered sunflower as compared to a common congener. Molecular Ecology 15:2345-2355.

Ellis, J.R. and D.E. McCauley. 2009. Phenotypic differentiation in fitness related traits between populations of an extremely rare sunflower: conservation management of isolated populations. Biological conservation 142:1836-1843.

Ellstrand, N.C. and D.R. Elam. 1993. Population Genetic Consequences of Small Population Size: Implications for Plant Conservation. Annual Review of Ecology and Systematics 24:217-242.

Faegri, K. and V.D.P. Leendert. 1966. Animals as Pollinators, 96-115. The principles of pollination ecology. Toronto, New York, Toronto, New York, Pergamon Press.

Free, J.B. 1970. Insect pollination of crops. London, New York, London, New York, Academic Press.

Ghazoul, J. 2005. Pollen and seed dispersal among dispersed plants. Biological Reviews 80:413-443.

Gilbert, F.S. 1981. Foraging ecology of hoverflies: morphology of the mouthparts in relation to feeding on nectar and pollen in some common urban species. Ecological Entomology 6:245-262.

Gilbert, L.E. and M.C. Singer. 1975. Butterfly ecology. Annual review of Ecology and Systematics 6:365-395.

Goulson, D. 1999. Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. Perspectives in plant ecology, evolution and systematics 2:185-209.

Greenleaf, S.S. and C. Kremen. 2006. Wild Bees Enhance Honey Bees' Pollination of Hybrid Sunflower. Proceedings of the National Academy of Sciences of the United States of America 103:13890-13895.

Heiser, C.B., D.M. Smith, S.B. Clevenger, and W.C. Martin. 1969. The north american sunflowers (*Helianthus*). Memoirs of the Torrey Botanical Club 22:1-218.

Horsburgh, M., J.C. Semple, and P.G. Kevan. 2011. Relative pollinator effectiveness of insect floral visitors to two sympatric species of wild aster: *Symphyotrichum lanceolatum* (Willd.) Nesom and *S. lateriflorum* (L.) Löve & Löve (Asteraceae: *Astereae*). Rhodora 113:64-86.

Hurd, P.D. 1980. Principal sunflower bees of North America with emphasis on the Southwestern United States (Hymenoptera, *Apoidea*). Smithsonian contributions to zoology. Smithsonian Institution, Washington, D.C.

Jones, G.D. 2012. Pollen extraction from insects. Palynology 36:86-109.

Kastinger, C. and A. Weber. 2001. Bee-flies (*Bombylius* spp., Bombyliidae, Diptera) and the pollination of flowers. Flora 196:3-25.

Kevan, P.G. and H.G. Baker. 1983. Insects as flower vectors and pollinators [Foraging behavior, nectar secretion, pollination ecology]. Annual Review of Entomology 28:407-453.

Leimu, R., Mutikainen, P.I.A., Koricheva, J., and Fischer, M. 2006. How general are positive relationships between plant population size, fitness and genetic variation? Journal of Ecology 94:942-952.

Levin, M. 1983. Value of bee pollination to US agriculture. American Entomologist 29:50-51.

Mandel, J.R. 2010. Clonal diversity, spatial dynamics, and small genetic population size in the rare sunflower, *Helianthus verticillatus*. Conservation Genetics 11:2055-2059.

Matthews, J.F., J.R. Allison, R.T. Ware Sr., and C. Nordman. 2002. *Helianthus verticillatus* Small (Asteraceae) Rediscovered and Redescribed. Castanea 67:13-24

McGregor, S.E. 1976. Insect pollination of cultivated crop plants. U.S. Department of Agriculture, Agriculture Research Service, Washington, D.C.

Menzel, R. and J. Erber. 1978. Learning and memory in bees. Scientific American 239:102-111.

Mitchell, T.B. 1960. Bees of the eastern United States. 2nd ed. North Carolina Agricultural Experiment Station. Salisbury, N.C.

Neff, J.L. and B.B. Simpson. 1990. "The roles of phenology and reward structure in the pollination biology of wild sunflower (*Helianthus Annuus* L., Asteraceae). Israel Journal of Botany 39:197.

Newman, D. and D. Pilson. 1997. Increased probability of extinction due to decreased genetic effective population size: experimental populations of *Clarkia pulchella*. Evolution 51:354-362.

Parker, F.D. 1981. How efficient are bees in pollinating sunflowers? Journal of the Kansas Entomological Society: 54:61-67.

Peat, J. and D. Goulson. 2005. Effects of experience and weather on foraging rate and pollen versus nectar collection in the bumblebee, *Bombus terrestris*. Behavioral Ecology and Sociobiology 58:152-156.

Pedersen, J.F., K.P. Vogel, and D.L. Funnell. 2005. Impact of reduced lignin on plant fitness. Crop Science 45:812-819.

Peterson, B.V., G.E. Shewell, H.J. Teskey, J.R. Vockeroth, and D.M. Wood. 1981. Manual of Nearctic Diptera ,Vol. 2. Pp. 675-1332 (J. F. McAlpine et al. Eds.). Biosystematics Research Centre, Monograph 28. Agriculture Canada, Ottawa, CAN.

Pivnick, K.A. and J.N. McNeil. 1985. Effects of nectar concentration on butterfly feeding: measured feeding rates for *Thymelicus lineola* (Lepidoptera: Hesperiidae) and a general feeding model for adult Lepidoptera. Oecologia 66:226-237.

Posey, A.F., R.W. Katayama, and J.G. Burleigh. 1986. The abundance and daily visitation patterns of bees (Hymenoptera: *Apoidea*) on oilseed sunflower, *Helianthus annuus* L., in southeastern Arkansas. Journal of the Kansas Entomological Society 59:494-499.

Rehan, S.M. and M.H. Richards. 2010. Nesting biology and subsociality in *Ceratina calcarata* (Hymenoptera: Apidae). The Canadian Entomologist 142:65-74.

Reinhard, J., M.V. Srinivasan, D. Guez, and S.W. Zhang. 2004. Floral scents induce recall of navigational and visual memories in honeybees. Journal of Experimental Biology 207:4371-4381.

Rhoades, P.R., W.E. Kingeman, R.N. Trigiano, and J.A. Skinner. 2011. Evaluating Pollination Biology of *Cornus florida* L. and *C. kousa* (Buerger ex. Miq.) Hance (Cornaceae: *Cornales*). Journal of the Kansas Entomological Society 84:285-297.

Rieseberg, L.H. 1997. Hybrid Origins of Plant Species. Annual Review of Ecology and Systematics 28:359-389.

Robertson, C. 1922. The sunflower and its insect visitors. Ecology 3:17-21.

Schlindwein, B. 2002. A hypermedia glossary of genetic terms, Technische Universität München-Weihenstephan. Library Weihenstephan.

Senatore, G.L., E.A. Alexander, P.H. Adler, and J.K. Moulton. 2014. Molecular systematics of the *Simulium jenningsi* species group (Diptera: *Simuliidae*), with three new fast-evolving nuclear genes for phylogenetic inference. Molecular phylogenetics and evolution 75:138-148.

Simpson, E.H. 1949. Measurement of diversity. Nature 163:688.

Small, J.K. 1898. Studies in the Botany of the Southeastern United States.-XIV. Bulletin of the Torrey Botanical Club 25:465-484.

Soltis, P.S. and D.E. Soltis. 2009. The Role of Hybridization in Plant Speciation. Annual Review of Plant Biology 60:561-588.

Trigiano, R.N. 2010. p. 365-371. Plant Tissue Culture, Development, and Biotechnology. In: Trigiano, R.N. and D.J. Gray (eds.) Demonstration of principles of protoplast isolation using chrysanthemum and orchardgrass leaves. CRC Press, Boca Raton, FL.

US Fish and Wildlife Service. 2014. Endangered Status for *Physaria globosa* (Short's Bladderpod), *Helianthus verticillatus* (Whorled sunflower), and *Leavenworthia crassa* (Fleshy-Fruit Gladecress). 79:44712-44718.

APPENDIX

Tables and Figures

Table 1. Numbers of all visitors captured at the three collection sites, listed taxonomically

Order	Family	Visitor	Native - GA	Suburban - TN	Arboretum - TN	Total #
Hymenoptera	Apidae	*Bombus bimaculatus/impatiens* Cresson	11	210	90	311
Hymenoptera	Apidae	*Ceratina calcarata* Robertson	0	124	1	125
Hymenoptera	Halictidae	*Augochlorella aurata* Smith	0	81	5	86
Hymenoptera	Halictidae	*Halictus ligatus* Say	0	63	4	67
Hymenoptera	Halictidae	*Agapostemon virescens* Fabricius	0	52	0	52
Hymenoptera	Apidae	*Melissodes agilis* Laberge	2	15	33	50
Hymenoptera	Halictidae	*Agapostemon sericeus* Lepeletier	0	46	0	46
Hymenoptera	Halictidae	*Lasioglossum (Dialictus)* spp.	0	40	3	43
Hymenoptera	Halictidae	*Augochlora pura* Say	0	22	2	24
Hymenoptera	Apidae	*Apis meliffera* Linnaeus	8	2	7	17
Hymenoptera	Megachilidae	*Megachile* spp.	12	2	0	14
Hymenoptera	Ichneumonidae		0	1	7	8
Hymenoptera	Apidae	*Svastra aegis* Laberge	6	0	1	7
Hymenoptera	Apidae	*Melissodes dentiventris* Smith	0	1	5	6
Hymenoptera	Apidae	*Xylocopa virignica* Lepeletier	1	5	0	6
Hymenoptera	Apidae	*Svastra obliqua Say*	0	1	4	5
Hymenoptera	Andrenidae	*Andrena helianthi* Robertson	0	0	5	5
Hymenoptera	Andrenidae	*Andrena asteroides* Laberge	4	0	0	4

Table 1 Continued

Order	Family	Visitor	Native - GA	Suburban - TN	Arboretum - TN	Total #
Hymenoptera	Halictidae	*Augochloropsis sumptuosa* Smith	0	1	0	1
Hymenoptera	Apidae	*Bombus fervidus* Fabricius	0	1	0	1
Hymenoptera	Bracronidae		1	0	0	1
Hymenoptera	Megachilidae	*Coelioxys asteris* Crawford	1	0	0	1
Hymenoptera	Megachilidae	*Coelioxys sayi* Say	1	0	0	1
Hymenoptera	Scoliidae	*Scolia dubia* Say	0	1	0	1
Hymenoptera	Apidae	*Xylocopa micans* Lepeletier	1	0	0	1
Hymenoptera	Andrenidae	*Andrena asteroides* Laberge	4	0	0	4
Hymenoptera	Andrenidae	*Andrena asteroides* Laberge	4	0	0	4
Hymenoptera	Halictidae	*Augochloropsis sumptuosa* Smith	0	1	0	1
Hymenoptera	Apidae	*Bombus fervidus* Fabricius	0	1	0	1
Hymenoptera	Bracronidae		1	0	0	1
Hymenoptera	Megachilidae	*Coelioxys asteris* Crawford	1	0	0	1
Hymenoptera	Megachilidae	*Coelioxys sayi* Say	1	0	0	1
Hymenoptera	Scoliidae	*Scolia dubia* Say	0	1	0	1
Hymenoptera	Apidae	*Xylocopa micans* Lepeletier	1	0	0	1
Diptera	Syrphidae	*Allograpta* spp.	3	23	5	31
Diptera	Syrphidae	*Eupeodes* spp.	2	20	1	23
Diptera	Bombyliidae	*Sparnopolius* spp.	0	13	5	18
Diptera	Syrphidae	*Toxomerus* spp.	0	4	4	8

Table 1 Continued

Order	Family	Visitor	Native - GA	Suburban - TN	Arboretum - TN	Total #
Diptera	Syrphidae	*Eristalis* spp.	0	3	0	3
Diptera	Dolichopodidae		0	1	0	1
Diptera	Syrphidae	*Lepidophora* spp.	1	0	0	1
Lepidoptera	Hesperiidae	*Atalopedes campestris* Boisduval	0	33	2	35
Lepidoptera	Attevidae	*Atteva aurea* Cramer	1	0	2	3
Coleoptera	Cantharidae	*Chauliognathus pennsylvanicus* DeGeer	0	7	1	8
Coleoptera	Chrysomelidae	*Diabrotica undecimpunctata* Mannerheim	1	0	2	3
Hemiptera	Reduviidae		0	3	2	5
Dermaptera			0	1	0	1

Table 2. Primers developed for *cox*-1 sequencing of floral visitors

Name	Sequence (5' → 3')	Position*
Lep FWD	ATAATYGGRGGATTTGGWAAYTG	2000
Beetle FWD	ATRGTNATRCCNATYATRATYGG	1985
Hym FWD1	ATRATTTTYTTYATRGTWATRCC	1973
Hym FWD2	CAYGCHTTYMTWATRATTTTYTTYAT	1961
Lep REV	GTTARTCCNCCYAGWGTRAA	2841
Beetle/Fly REV	ACNACATARTAWGTRTCRTG	2901
Hym REV1	ATNGANARWACRTARTGRAARTG	2928
Hym REV2	ATRATTGMRAAWACWGCYCCYAT	2949
Hym REV3	CCTARRAARTGTTGNGGRAARAA	3075

* Positions mapped against *Apis mellifera* mitochondrial genome. Degeneracies are as follows: **Y** = C or T, **R** = A or G, **W** = A or T, **N** = any base, **H** = A or C or T, **M** = A or C

Table 3. Pollen counts from the ten most collected insects and *Apis mellifera* captured at *Helianthus verticillatus* collection sites.

Insect Visitor	**Long Dimension of the Insect Visitor (mm)**	**Mean and (Range) of Pollen Counts x 10^4 per Insect Visitor**[1]	**Mean Pollen Counts per mm visitor length**
Agapostemon spp.	10-11	(0.2) – 4.7 – (11.8)	0.44
Allograpta spp.	6.5 - 8.0	(0.2) – 0.3 – (0.4)	0.041
Apis mellifera	12	(0.6) – 1.3 – (2)	0.11
Augochlorini tribe	5.5-8	(0.4) – 0.74 – (1.8)	0.11
Bombus spp.	8.5-16	(1.2) – 11.0 – (44.6)	0.90
Ceratina calcarata	6.5-8	(0.4) – 1 – (1.6)	0.14
Halictus ligatus	7-10	(4.6) – 15.9 – (25.8)	1.90
Hesperiidae	12-15	(0) – 0.12 – (2.0)	0.011
Lasioglossum	<5	(0.2) – 1.4 – (2.4)	0.48
(Dialictus) spp.	9-12	(1.8) – 18.1 – (50.0)	1.73
Melissodes spp.	6-9	(0) – (0.4) – (1.0)	0.051
Sparnopolius spp.			

[1] Mean of five counts

Table 4. Simpson's Diversity indices for the Maryville suburban site. Four collections were undertaken: two in 2017 and two in 2018. In 2017, there was no 7:45-8:15 collection period.

Period	Collection 1 - 2017	Collection 2 - 2017	Collection 1 - 2018	Collection 2 - 2018
7:45-8:15	N/A	N/A	0.560	0.370
9:45-10:15	0.340	0.630	0.753	0.291
11:45-12:15	0.527	0.859	0.815	0.855
13:45-14:15	0.446	0.816	0.827	0.839
15:45-16:15	0.735	0.790	0.842	0.805
17:45-18:15	0.750	N/A	0.776	0.586

Table 5. Simpson's Diversity indices shown for the Oak Ridge setting. The first collection was undertaken in September and the second collection was undertaken in October of 2018. No visitors were captured during the 7:45-8:15 collection periods at this site.

Period	Collection 1 - 2018	Collection 2 - 2018
7:45-8:15	N/A	N/A
9:45-10:15	0.775	0.194
11:45-12:15	0.789	0.609
13:45-14:15	0.780	0.711
15:45-16:15	0.759	0.219
17:45-18:15	0.571	0.560

Figure 1. Floyd county, Georgia collection site

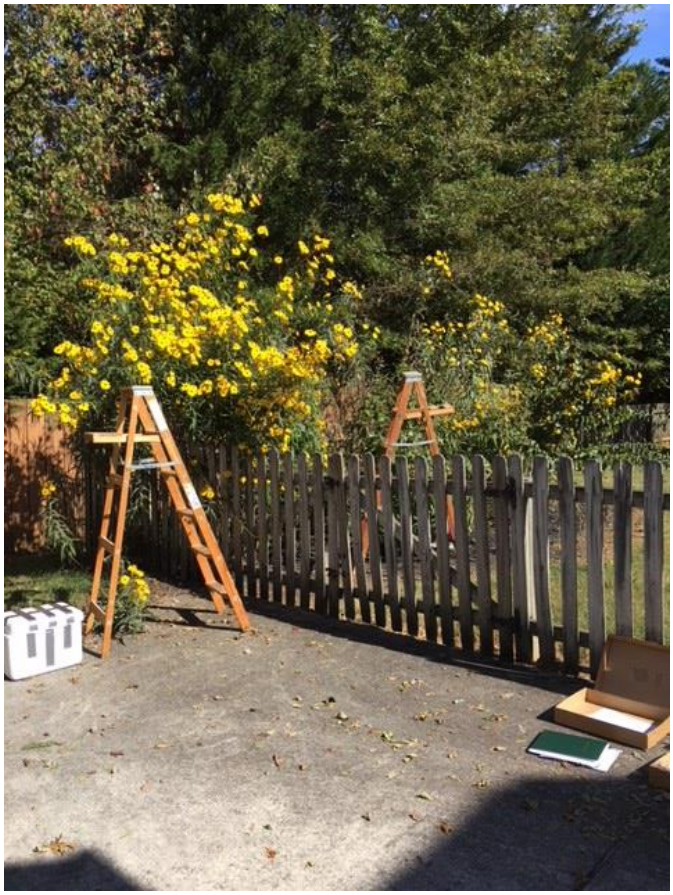

Figure 2. Maryville, Tennessee home garden collection site

Figure 3. *Helianthus verticillatus* plants established at Oak Ridge, Tennessee collection site one year before insects were collected

Figure 4. *Helianthus verticillatus* plants at the Oak Ridge, Tennessee collection site one year after establishment

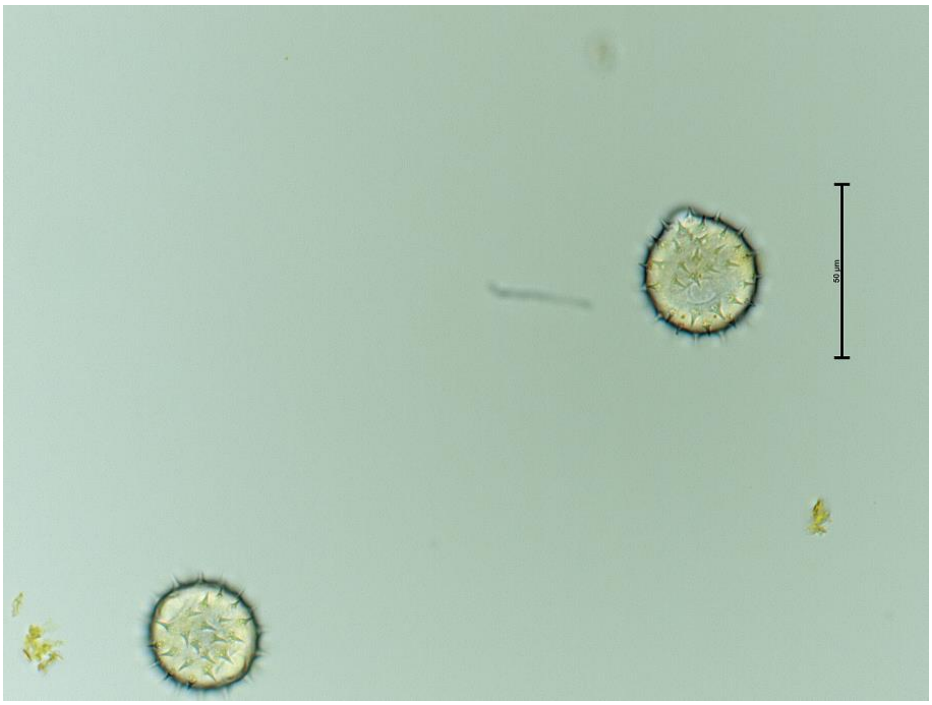

Figure 5. Slide mounts of pollen grains collected from *Helianthus verticillatus*, suspended in Qiagen QX Wash Buffer

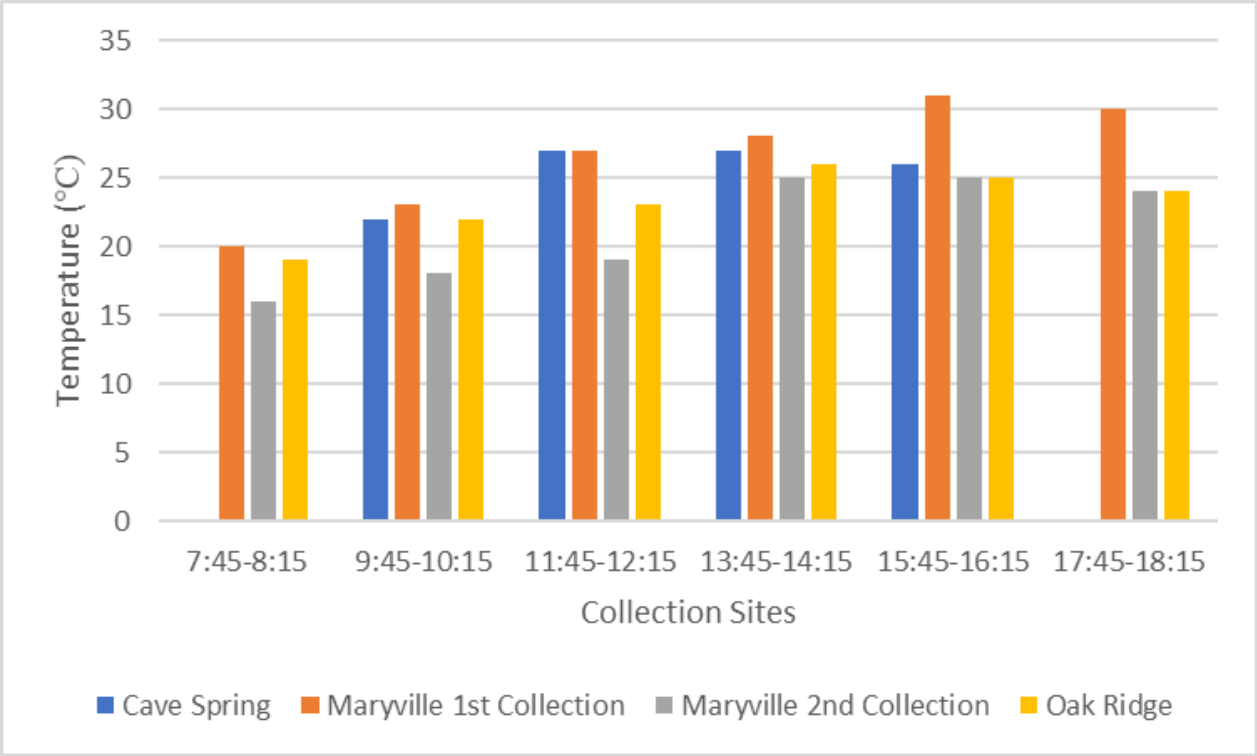

Figure 6. Temperatures (°C) recorded for each collection site

Figure 7. *Stiria rugfrons* larvae feeding on *Helianthus verticillatus* infloresences

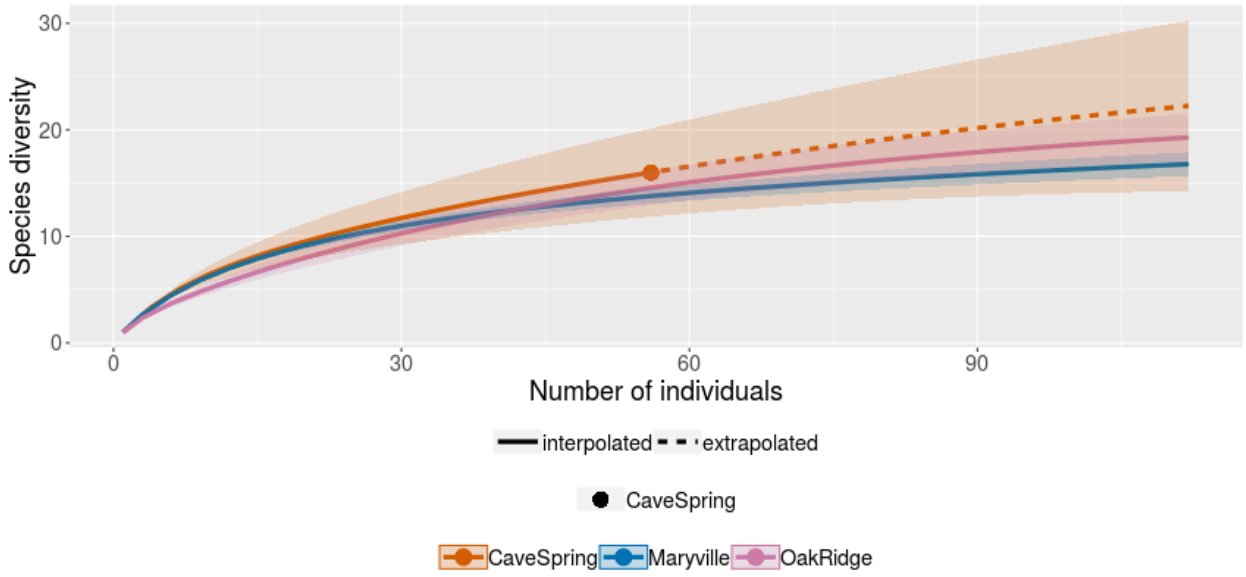

Figure 8. Species richness diversity plot. This plot signifies no significant difference in richness across all three sites. The dot on the Cave Spring line indicates the point at which sampling stopped (56 visitors) with the extrapolation line providing an estimation of results expected had sampling been continued

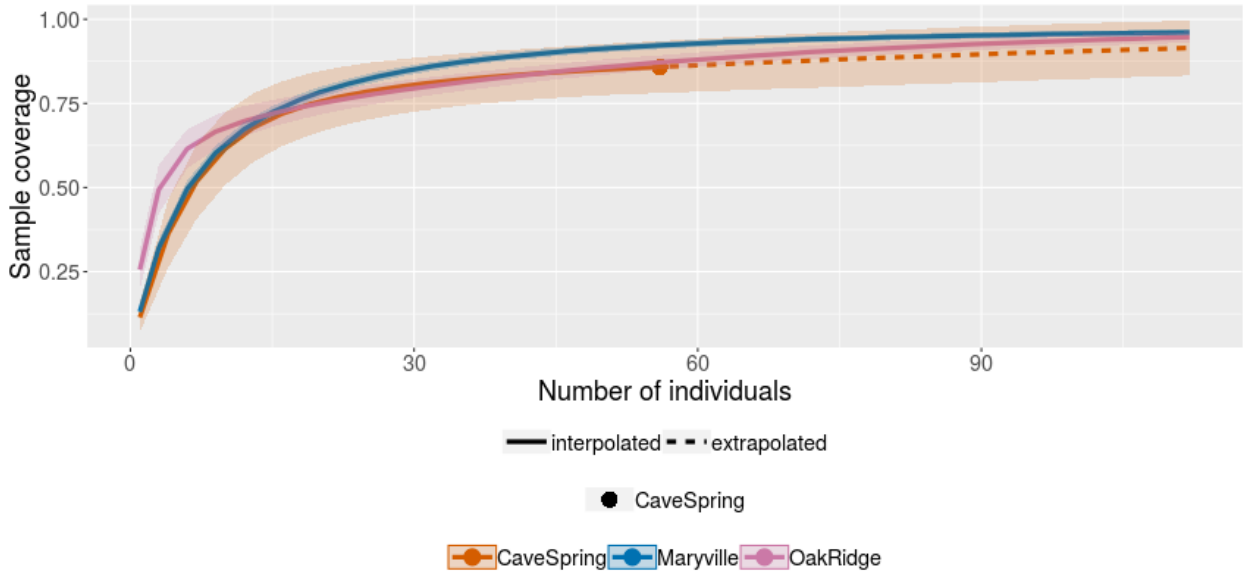

Figure 9. Species richness completeness plot. This plot indicates a successful coverage of visitors at each location. The dot on the Cave Spring line indicates the point at which sampling stopped (56 visitors) with the extrapolation line providing an estimation of results expected had sampling been continued

VITA

Nicolas Connor Strange received his bachelor's degree in agriculture from Austin Peay State University in Clarksville, Tennessee. He graduated in 2017 and began his research in the Entomology and Plant Pathology department at the University of Tennessee the same year. He is expected to graduate with a Master's degree in plant pathology in August of 2019.