Floral Visitors to *Helianthus verticillatus*, a Rare Sunflower Species in the Southern United States

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Abstract. *Helianthus verticillatus* Small (whorled sunflower) is a federally endangered plant species found only in the southeastern United States that has potential horticultural value. Evidence suggests that *H. verticillatus* is self-incompatible and reliant on insect pollination for seed production. However, the identity of probable pollinators is unknown. Floral visitors were collected and identified during Sept. 2017 and Sept. 2018. Thirty-six species of visitors, including 25 hymenopterans, 7 dipterans, 2 lepidopterans, and 2 other insect species, were captured during 7 collection days at a site in Georgia (1 day) and 2 locations in Tennessee (6 days). Within a collection day (0745–1815 hr), there were either five or six discrete half-hour collection periods when insects were captured. Insect visitor activity peaked during the 1145–1215 and 1345–1415 hr periods, and activity was least during the 0745–0845 and 0945–1015 hr periods at all three locations. Visitors were identified by genus and/or species with morphological keys and sequences of the *cox-1* mitochondrial gene. The most frequent visitors at all sites were *Bombus* spp. (bumblebees); *Ceratina calcarata* (a small carpenter bee species) and members of the halictid bee tribe Augochlorini were the second and third most common visitors at the two Tennessee locations. *Helianthus* pollen on visitors was identified by microscopic observations and via direct polymerase chain reaction of DNA using *Helianthus*-specific microsatellites primers. Pollen grains were collected from the most frequent visitors and *Apis mellifera* (honeybee) and counted using a hemocytometer. Based on the frequency of the insects collected across the three sites and on the mean number of pollen grains carried on the body of the insects, *Bombus* spp., *Halictus ligatus* (sweat bee), *Agapostemon* spp., and *Lasioglossum*/*Dialictus* spp., collectively, are the most probable primary pollinators of *H. verticillatus*.

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in Madison County; other populations were most likely extirpated due to habitat loss (Ellis et al., 2006; Matthews et al., 2002).

Helianthus verticillatus is a diploid ($2n = 2x = 34$) perennial species (Ellis et al., 2006). Plants can be propagated clonally via rhizomes either in the field or in containers (Edwards et al., 2017), or more efficiently by rooted cuttings until mid-to-late May or early June (Trigiano et al., 2018). *Helianthus verticillatus* are 2- to 4-m-tall plants that produce flowers from late August or early September to mid-October (Matthews et al., 2002) or until a killing frost. This sunflower is a self-incompatible and largely insect-pollinated species (R. Trigiano, unpublished data) that does not lend itself to wind pollination (Mandel, 2010). Pollinators of *H. verticillatus* and other self-incompatible plants are likely restricted to the flight range of locally available insect visitors (Ackerman, 2000; Faegri and Leendert, 1966). Plants and insects have co-evolved with tightening associations between energy expenditures in plants to produce pollen and with the demands of pollinators to obtain nectar and pollen for food (Kevan and Baker, 1983). Because insect pollination is of paramount importance to *H. verticillatus* propagation and crucial to understanding the biology and conservation of this rare plant, it is imperative to identify potentially important pollinator species (Kevan and Baker, 1983).

Flowers of species in the Asteraceae may be visited by only a single species or numerous species of insects (DeGrandi-Hoffman and Watkins, 2000; Horsburgh et al., 2011; Robertson, 1922). Species within the bee genera *Apis*, *Bombus*, *Halictus*, and *Melissodes* are among the common hymenopteran visitors to sunflowers (DeGrandi-Hoffman and Watkins, 2000; Robertson, 1922). Some common families of pollinators in Diptera, including Syrphidae and Bombyliidae, also have a significant role in the pollination of some *Helianthus* spp. (Robertson, 1922). Native bees are the most efficient pollinators of self-incompatible flowers (Free, 1970; Greenleaf and Kremen, 2006), and they have co-evolved with sunflowers, which are native to North America (Hurd, 1980). Despite this co-evolution, honeybees, which are not native to North America, have been reported to be the most efficient pollinators of commercial sunflowers (McGregor, 1976). In contrast, Parker (1981) claimed that two oligolectic native bees, *Andrena helianthi* and *Melissodes agilis*, were much more efficient pollinators. A combination of both native and domesticated bees provided efficient pollination of hybrid commercial sunflowers (DeGrandi-Hoffman and Watkins, 2000; Greenleaf and Kremen, 2006).

There has not been a study of floral visitors of *H. verticillatus*; therefore, an integral part of its reproductive biology is unknown. The goals of this study were as follows: to identify and catalog the diversity of potential pollinators visiting whorled sunflower inflorescences at diverse locations using morphological keys and sequences of

The whorled sunflower, *Helianthus verticillatus* Small, is a rare and federally endangered plant (U.S. Fish and Wildlife Service, 2014) found in only a few locations in the southern United States. This plant was described in 1892 by Samuel Bain (Ellis et al., 2006; Mandel, 2010; Matthews et al., 2002; Small, 1898) from collections obtained in Chester County, TN. The species was not collected again until 1993, when it was discovered in Floyd County, GA (Matthews et al., 2002). A regional census was conducted several years later, and an additional population was discovered in Cherokee County, AL (Matthews et al., 2002). In Tennessee, wild plants are now located only

the *cox-1* mitochondrial gene; to verify the pollen composition carried by captured insects via DNA amplification with *H. annuus* primers; and to determine loads of pollen grains carried on captured insects.

Materials and Methods

Study sites. Three locations with populations of *H. verticillatus* were used to assess potential insect pollinators: a rural location where the sunflower naturally occurs (native) in Cave Spring, GA; plantings at a suburban garden site (suburban) in Maryville, TN; and a controlled field trial location at the University of Tennessee Forest Resources Research and Education Center Arboretum (semirural) in Oak Ridge, TN.

The rural location is a forested site on a prairie remnant near Cave Spring, GA. *Helianthus verticillatus* plants at this site were not numerous. Many clusters of a few plants were separated from each other either by 1 m or less or by more than 10 m and distributed among a thick undergrowth of privet, honeysuckle, and grasses. Most plants were growing in full sunlight, but some individuals were partially shaded under trees.

The suburban location is in a private garden in Maryville, TN. Plants from naturally occurring populations in West Tennessee and Alabama were collected in 2014 before *H. verticillatus* was declared federally endangered (United States Fish and Wildlife Service, 2014) and transplanted to this site. During the study years 2017 and 2018, there were ~250 stems of sunflower within a 10-m² area. Plants grew in filtered sunlight in the morning and direct sunlight in the afternoon. This site was selected to examine potential pollinators of the sunflower found in a residential garden.

The Oak Ridge location (semi-rural) at the University of Tennessee Arboretum in Oak Ridge, TN, has 250 acres of exotic and native plant species. Clones (Trigiano et al., 2018) of 30 *H. verticillatus* plants collected from West Tennessee were established in Oct. 2017 at the arboretum. Plant specimens were arranged in two sections with three groups in each section and five plants per group for uniformity. This location was intermediate for anthropogenic influence between the other two sites because it mimicked the native setting while having suburban areas nearby.

Collection and identification of floral visitors. Floral visitors were collected during September and October at the Cave Spring location and the Maryville location in 2017, and only at the Maryville and Oak Ridge locations in 2018. Floral visitors were caught in 27.25 × 70-mm vials (FisherBrand, Waltham, MA) that were held directly above insects on flowers. Contact of the collection vials with flowers was avoided to prevent pollen transfer to the vial. Individual insects were captured in vials rather than with sweep or aerial nets for several reasons. First, only pollen collected by individual insects would be present in comparison with catching many

insects concurrently in sweep nets. When sweep netting, specimens could become cross-contaminated with pollen from other plants, and pollen could potentially be collected from disturbed flower heads. Second, sweeping a net over the tops of the inflorescences would likely capture many flying insects, some of which that may not have visited flowers, thus possibly confusing them with potential pollinators. Third, *H. verticillatus* can grow to a height of 4 m, which is too tall for the practical use of standard sweep nets unless the operation is performed using a ladder. Fourth, moving a net over and around these plants could destroy flower heads and degrade the plants at the location. Potential biases using vials for capture were also introduced, including the following: capture skill among collectors could vary greatly; a few specimens, such as some large *Bombus* spp. and lepidopterans, were too large to fit into the lumen of the vials, but these were rare; some insects were more difficult than others to capture routinely; and visitors to flowers beyond the reach of collectors (more than ~2 m) were not collected.

Vials with captured insects were immediately placed on ice, transported to the laboratory, and stored at -20 °C until processing for morphological and molecular identification, enumeration of pollen grains, and molecular identification of pollen. At all sites, insects were collected during the following five 30-min intervals throughout the day: 0945–1015, 1145–1215, 1345–1415, 1545–1615, and 1745–1815 HR. In 2018, an additional interval from 0745 to 0815 HR was added to evaluate potential early morning visitors.

Insect morphological and molecular identification. All specimens were examined with a stereomicroscope and identified to the lowest taxonomic level described by available resources. For members of the Hymenoptera, the work of Mitchell (1960) was used; however, the work of McAlpine et al. (1981) was used for Diptera species.

Following morphological identification, representatives of each putative morphospecies were selected for *cox-1* gene sequencing. Several primers, in addition to the traditional barcoding primers, LepF and Lep R (Hebert et al., 2003), were designed using available GenBank *CoxI* sequences from related taxa to reliably amplify the gene from all captured taxa (Table 1). DNA was extracted from insect specimens with the Omega E.Z.N.A. Insect DNA Kit (Omega Bio-Tek, Norcross, GA). For large specimens (e.g., *Bombus* spp. and *Svastra* spp.), one leg was used; for smaller taxa (e.g., members of the tribe *Augochlorini*), the three left legs were used. For very small insects [e.g., *Lasiglossum* (*Dialictus*) spp.], the entire body was used with a nondestructive approach (i.e., proteinase K-mediated in situ dissolution of tissues). As a result, all voucher specimens were complete in a taxonomic sense. Extracted DNA was stored at -20 °C until use. Insect specimen vouchers were retained at the University of Tennessee-Knoxville Department

of Entomology and Plant Pathology Insect Museum.

Polymerase chain reactions (PCRs) for the *cox-1* mitochondrial gene contained 36 µL of sterile distilled water, 5 µL of 10× TaKaRa Taq buffer, 2.3 µL MgCl₂ (50 mM), 3.5 µL dNTPs (10 mM), 0.2 µL TaKaRa hot start Ex Taq, 3 µL of 10 mM aliquots of forward and reverse primers (Table 1), and 1 µL of DNA template (various concentrations). A touchdown amplification program (Senatore et al., 2014) was used with the following modifications: 95 °C for 1 min, 10 cycles of 96 °C for 15 s, 58 °C for 20 s, 72 °C for 1 min; 10 cycles of 96 °C for 15 s, 50 °C for 20 s, 72 °C for 1 min; and 40 cycles of 96 °C for 15 s, 45 °C for 20 s, 72 °C for 1 min, and 72 °C for 5 min. PCR products were separated on a 1% agarose gel (120 V/cm² for 30 min), stained with ethidium bromide, and visualized with an ultraviolet transilluminator. PCR products were freed from gel pieces and bound to EconoSpin silica columns (Epoch Life Science, Sugar Land, TX) using 5× volume of 5 M guanidium thiocyanate (pH = 5), washed, and eluted in 10 mM Tris (pH = 8.5). Purified PCR amplicons were cycle-sequenced using 1/16 diluted Big Dye v3.1 terminators (Applied Biosystems, Waltham, MA) and cleaned using Sephadex-50 spin columns (Princeton Separations, Adelphia, NJ) before being dried and sent to the University of Tennessee Genomics Core Sequencing Facility for analysis using an ABI 3730 Genetic Analyzer. Both directions were sequenced using amplification primers. Resulting chromatograms were reconciled using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI) to generate a consensus sequence. Resulting sequences were compared with entries in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and the Barcode of Life (<http://www.barcodinglife.org/>) databases.

Molecular confirmation of pollen identity. For some visitors, a hind leg with a pollen-bearing scopula was detached for pollen analyses. For visitors without scopulae or visitors without visible pollen on their scopulae, the entire body was processed to remove pollen. For these individuals, specimens were placed in 1.5-mL microcentrifuge tubes with 1 mL of Qiagen QX wash buffer (Qiagen, Hilden, Germany) added; samples were vigorously vortexed for 15 s to dislodge pollen grains from the insects. Tubes were centrifuged at 10,000 g_n for 5 min to obtain a pollen pellet. Insects or body parts were removed, and pollen samples were stored at -20 °C until being processed for molecular identification.

DNA extraction from the pollen pellet was completed using the Phire Direct Plant PCR Kit (ThermoFisher Scientific, Waltham, MA) and following the manufacturer's instructions. PCR mixtures contained 4 µL GoTaq (nucleotides included), 0.5 µL dimethyl sulfoxide (DMSO), 3.5 µL sterile distilled water, 1 µL forward primer, 1 µL reverse primer, and 1 µL DNA. The DNA concentration was not the same for all samples; however, the Phire Direct Plant PCR Kit

Table 1. Degenerate primers developed for *cox-1* sequencing of floral visitors to *Helianthus verticillatus* flowers.

Locus	Sequence (5' → 3') ^z	Position ^y
Lep FWD	ATAATYGGRGGATTTGGWAAATG	2000
Beetle FWD	ATRGTNATRCNATYATRAYGG	1985
Hym FWD1	ATRATTTTYYATRGTWATRC	1973
Hym FWD2	CAYGCHTTYMTWATRATTTTYYAT	1961
Lep REV	GTTARTCCNCCYAGWGTAA	2841
Beetle/Fly REV	ACNACATARTAWGTRTCRTG	2901
Hym REV1	ATNGANARWACRTARTGRAARTG	2928
Hym REV2	ATRATTGMRAAWACWGCYCCYAT	2949
Hym REV3	CCTARRAARTGTTGNGGRAARAA	3075

^zDegeneracies 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.

^yPositions mapped against the *Apis mellifera* (honeybee) mitochondrial genome (Crozier and Crozier, 1993; GenBank accession number L06178).

allows for this. A positive control consisted of *H. verticillatus* DNA extracted from leaves; DNA from *Cornus florida* and distilled water were used as negative amplification controls. Pollen pellets from the 10 most frequently captured visitors were tested, and two amplifications were performed using two expressed sequence tag–simple sequence repeat (EST-SSR) primers from Ellis et al. (2006): locus HT1099 (forward 5' GGCTTTCGTTTCTCGTTGTC and reverse CAGCTCACTCCTAATTGGTTCC) had an expected allele size of 302 bp, and locus HT1123 (forward 5'-3' GGGTTTGTACAGGCACTTG and reverse TTCATAGAAATGAGGACCAAAGG) had an expected allele size of 322 bp. Both EST-SSRs were developed for *H. annuus* (Ellis et al., 2006) but cross-amplified DNA of *H. verticillatus* (Edwards et al., 2020). The thermocycler protocol comprised the following: 95 °C for 3 min; 10 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 45 s; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and 72 °C for 5 min; and hold at 4 °C. PCR products were separated by electrophoresis (100 V/cm² 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 positive identification of *Helianthus* pollen.

Pollen counts. Five specimens of each of the nine most commonly captured visitors, plus *Apis mellifera*, were selected to determine the number of pollen grains carried on insect bodies. Individuals used for this experiment were selected at random from the entire collection of that taxon regardless of location. The following method was used to collect pollen from individual specimens and was slightly modified from the methods reported by Jones (2012). Then, 1 mL distilled water was added to the collection vial, and entire insects were placed in 1.5-mL microcentrifuge tubes, vortexed, and centrifuged at 10,000 *g*_n for 8 min; after which, the insects were removed. Pollen grains were resuspended with agitation, and the number of pollen grains per milliliter was estimated using a hemocytometer (Trigiano, 2010). Each pollen sample was counted five times, and the mean was calculated from these results. Because insect total surface area and surface composition (e.g., hairs) varied

greatly, an attempt to normalize counts was completed by dividing the pollen counts by the mean length of the insect, which served as a proxy for the specimen size. Finally, the mean number of pollen grains carried on the bodies of insects was multiplied by the number of collected specimens from all sites to gain an estimate of which insects were potentially moving the greatest amount of pollen.

Results and Discussion

Collection sites. Fifty-six floral visitors were captured at the location in rural Cave Spring, GA (Table 2). The number of visitors captured increased throughout the morning and mid-day collection periods (0945–1015, 1145–1215, and 1345–1415 HR), but activity was lower during the late afternoon period (1545–1615 HR). The most commonly captured visitors were individuals of the *B. bimaculatus/impatiens* complex. *Apis mellifera* was collected more often at the rural location than at any of the other study sites, likely due to hobbyist hives observed close to the site. *Megachile* spp. were collected more there than at the sites in Tennessee, and *Coelioxys* spp. were collected only at the Cave Spring site (Table 2).

Collection limitations were associated with the rural location. The density and distance among the plants affected collection efficiency. Additionally, thick underbrush surrounding individual and clustered plants made travel between them slow and difficult. Overall, collection of floral visitors at this location was more challenging than that at the other two sites, and the lower total number of visitors captured may reflect these limitations.

There were 776 floral visitors captured during 4 d of sampling over 2 years at the suburban Maryville, TN location (Table 2). The number of visitors captured was lowest during the morning collection periods (0745–0815 and 0945–1015 HR); however, the number of insects captured at the site increased during the midday collection periods (1145–1215, 1345–1415, and 1545–1615 HR) and decreased during the late afternoon period (1745–1815 HR). The most commonly captured visitors at this location were members of the *B. bimaculatus/impatiens* species group and *Ceratina calcarata* (a carpenter bee), which was found almost exclusively at

the Maryville garden site. Additionally, species in the Halictidae family were captured in much higher numbers at this location than at the other two study sites (Table 2).

There were some collection biases associated with the Maryville location. Plants at this location had many more flower heads compared with the other two sites, thus allowing for more comprehensive and exhaustive sampling. Insects, especially the various bee species, remember sources of pollen and nectar (Goulson, 1999; Menzel and Erber, 1978; Reinhard et al., 2004) and may have been more attracted to the more abundant *H. verticillatus* flowers at this site compared to other nearby resources. Individual stems were growing close to each other, and sunflowers were not surrounded by underbrush as they were at the Cave Spring, GA site. These biases explain the significantly greater number of visitors captured in the garden setting.

At the University of Tennessee Arboretum in Oak Ridge, TN (semirural), 191 floral visitors were collected (Table 2) on two dates. At this site, the lowest number of captures occurred during the morning periods of 0745–0815 and 0945–1015 HR, and increased during the midday and afternoon collection periods of 1145–1215, 1345–1415, and 1545–1615 HR. The number of captured insects decreased during the evening period of 1745–1815 HR. The most commonly collected visitors at this site belonged to the *B. bimaculatus/impatiens* group. *Melissodes* spp. and *Svastra* spp. were also commonly caught (Table 2).

Insect activity around *H. verticillatus* was sparse during the morning collection period of 0745–0815 HR, when the temperature was cooler, and slightly increased during the 0945–1015 HR period at all three locations. *Helianthus* species secrete nectar early in the morning (Neff and Simpson, 1990), and the sparse activity and low diversity of visitors present (only those foraging for nectar, such as some *Bombus* species and syrphid flies) were not unexpected. Neff and Simpson (1990) also determined that *H. annuus* anthers dehiscence in the morning and evening, and that insect visitation coincided with these periods. The data from this study agree with this observation because insect visitation increased during the 0945–1015 HR period, with much greater activity in the late morning and

Table 2. Insect visitors to *Helianthus verticillatus* flowers at three collection sites during 2017 and 2018.

Taxonomic designation			Collection site			
Order ^z	Family	Species	Cave Spring, GA	Maryville, TN	Oak Ridge, TN	Total
Hymenoptera	Apidae	<i>Bombus bimaculatus/impatiens</i> Cresson	11	210	90	311
	Apidae	<i>Ceratina calcarata</i> Robertson	0	124	1	125
	Halictidae	<i>Augochlorella aurata</i> Smith	0	81	5	86
	Halictidae	<i>Halictus ligatus</i> Say	0	63	4	67
	Halictidae	<i>Agapostemon virescens</i> Fabr.	0	52	0	52
	Apidae	<i>Melissodes agilis</i> Laberge	2	15	33	50
	Halictidae	<i>Agapostemon sericeus</i> Lepeletier	0	46	0	46
	Halictidae	<i>Lasioglossum/Dialictus</i> spp. ^y	0	40	3	43
	Halictidae	<i>Augochlora pura</i> Say	0	22	2	24
	Apidae	<i>Apis mellifera</i> L.	8	2	7	17
	Megachilidae	<i>Megachile</i> spp.	12	2	0	14
	Ichneumonidae		0	1	7	8
	Apidae	<i>Svastra aegis</i> Laberge	6	0	1	7
	Apidae	<i>Melissodes dentiventris</i> Smith	0	1	5	6
	Apidae	<i>Xylocopa virignica</i> Lepeletier	1	5	0	6
	Apidae	<i>Svastra obliqua</i> Say	0	1	4	5
	Andrenidae	<i>Andrena helianthi</i> Robertson	0	0	5	5
	Andrenidae	<i>Andrena asteroides</i> Laberge	4	0	0	4
	Halictidae	<i>Augochloropsis sumptuosa</i> Smith	0	1	0	1
	Apidae	<i>Bombus fervidus</i> Fabr.	0	1	0	1
	Braconidae		1	0	0	1
	Megachilidae	<i>Coelioxys asteris</i> Crawford	1	0	0	1
	Megachilidae	<i>Coelioxys sayi</i> Say	1	0	0	1
	Scoliidae	<i>Scolia dubia</i> Say	0	1	0	1
	Apidae	<i>Xylocopa micans</i> Lepeletier	1	0	0	1
Diptera	Syrphidae	<i>Allograpta</i> spp.	3	23	5	31
	Syrphidae	<i>Eupeodes</i> spp.	2	20	1	23
	Bombyliidae	<i>Sparnopolius</i> spp.	0	13	5	18
	Syrphidae	<i>Toxomerus</i> spp.	0	4	4	8
	Syrphidae	<i>Eristalis</i> spp.	0	3	0	3
	Dolichopodidae		0	1	0	1
Lepidoptera	Syrphidae	<i>Lepidophora</i> spp.	1	0	0	1
	Hesperiidae	<i>Atalopedes campestris</i> Boisduval	0	33	2	35
	Attevidae	<i>Atteva aurea</i> Cramer	1	0	2	3
Coleoptera	Cantharidae	<i>Chauliognathus pennsylvanicus</i> DeGeer	0	7	1	8
	Chrysomelidae	<i>Diabrotica undecimpunctata</i> Mannerheim	1	0	2	3
Hemiptera	Reduviidae	Undetermined	0	3	2	5
Dermaptera	Undetermined	Undetermined	0	1	0	1

^zList of visitors is arranged in order of the most frequently collected and abundance.^y*Lasioglossum* and *Dialictus* species are difficult to differentiate (Gibbs, 2012); therefore, they were considered one group for this study.

at approximately noon (1145–1215 HR). Furthermore, the number of visitors that were captured peaked during the early afternoon sampling time of 1345–1415 HR. Activity was steady into the late afternoon (1545–1615 HR), and then it decreased into the evening (1745–1815 HR). Despite this decrease in

activity during the later collection periods, the number of visitors captured was sometimes still greater than those of the morning collection periods. The observations of our study agree with those of Peat and Goulson (2005), who reported an increase in bumblebee foraging behavior as temperature in-

creased throughout the day and decreased activity as the temperature decreased during the evening.

The *B. bimaculatus/impatiens* group was the most numerous visitor type captured at all three sites (Table 2). This species group was present during all collection periods.

Hoverflies (*Allograpta* spp., *Eristalis* spp., *Eupeodes* spp., and *Toxomerus* spp.) were not captured as frequently as *Bombus* spp.; however, they were collected throughout most of the collection periods. The second most abundant visitor, *C. calcarata*, was captured almost exclusively at the suburban setting, but only during the 1145–1215 and 1545–1615 HR periods. *Augochlorini* spp., *H. ligatus*, *Melissodes* spp., and others were active during the afternoon collection periods at all locations as well.

Visitor abundance does not guarantee pollination efficiency (Horsburgh et al., 2011); however, together with the amount of pollen carried by individuals, this may be a better indicator of pollination potential. Five specimens of each of the nine most commonly collected species or species groups were selected randomly to estimate the pollen load carried, which was expressed as pollen grains per millimeter of length for the specimen (Table 3). Including the length to express the pollen load was an attempt to standardize the data presentation. Additionally, *A. mellifera* was included because of its prevalent use as a pollinator of sunflowers in agricultural settings (Levin, 1983). *Dialictus* spp. had the greatest mean number of pollen grains per millimeter of specimen, followed by *H. ligatus* and members of the *B. bimaculatus/impatiens* group (Table 3). Of the insects observed visiting *H. annuus*, female *Melissodes* spp. and *Bombus* spp. were reported to carry the most pollen grains (Parker 1981). *Melissodes* and *Halicus* species were reported as pollinators of other *Helianthus* spp. (DeGrandi-Hoffman and Watkins, 2000; Robertson, 1922), as was *Agapostemon* spp. (Chandler and Heilman, 1982; Posey et al., 1986). Honeybees carried fewer pollen grains than most of the native bees recorded by previously mentioned sunflower studies. In our study, when a measure of pollen-carrying ability was expressed as insect abundance (collected across the three study sites) $\times$ mean number of pollen grains/length of the insect (Table 3), *B. bimaculatus/impatiens*, followed by *H. ligatus*, *Agapostemon* spp., and *Lasioglossum/Dialictus* spp., had the potential to disperse more pollen than the other collected species. Our results are similar to and

support the findings of other *Helianthus* species pollinator studies (Chandler and Heilman, 1982; DeGrandi-Hoffmann and Watkins, 2000; Parker, 1981; Posey et al., 1986; Robertson, 1922).

Nonhymenopteran visitors, such as *Allograpta* spp. (Diptera: Syrphidae), *Atalopedes campestris* (Lepidoptera: Hesperidae), and *Sparnopolius* spp. (Diptera: Bombyliidae), carried a relatively low number of pollen grains compared with bees (Table 3). Members of Apidae (Cresswell, 1999) and Syrphidae seek both nectar and pollen rewards (Gilbert, 1981; Kasting and Weber, 2001). However, syrphid flies primarily use pollen as a food source (Horsburgh et al., 2011). In another study (Gilbert 1981), it was reported that smaller syrphid flies carried less pollen than other pollinators, and that syrphid flies often cleaned pollen from their bodies. Members of Hesperidae do consume pollen, but their primary food is nectar (Gilbert and Singer, 1975; Pivnick and McNeil, 1985).

Helianthus pollen was morphologically identified when counting pollen grains. Sunflower pollen was overwhelmingly present in all samples, and some insects contained pollen from other plant species (*Echinacea* species and other composite species were often nearby, but they were not identified). *Helianthus* pollen was also detected using *H. annuus* SSRs, and it is possible that DNA of pollen from other local asteraceous plants, such as *H. tuberosum* and *E. purpurea*, was weakly amplified by *H. annuus* primers (R.N. Trigiano, unpublished data).

Thirty-six insect visitors (25 Hymenoptera, 7 Diptera, 2 Lepidoptera, and 2 other orders) were captured during the two collection seasons across all locations (Table 2). Visitors in the *B. bimaculatus/impatiens* group were present at all locations and during all collection periods (data not shown). *Ceratina calcarata* was the second most commonly captured visitor in this study, but it was found almost entirely at the Maryville or suburban location. Because this site is in a residential garden where the plants have been established since 2014, there are numerous dead stems, which *Ceratina* species use for nesting; however, they were absent at the Oak Ridge and Cave Spring sites (Rehan and

Richards, 2010). The greater availability of dead pithy stems could explain the higher abundance of *Ceratina* visitors at the longer-established suburban sunflower plot. Because of the prescribed burns that take place at the Cave Spring location, there are fewer dead stems, which explains the lack of *Ceratina* visitors at this site. The Oak Ridge location, established in late 2017, was planted much more recently than the Maryville location, and there were very few closely adjacent potential nesting sites for *Ceratina* spp.

The Cave Spring location had the highest overall diversity of captured visitors but a smaller sample size compared with the other two sites. The Maryville location had the lowest diversity of captured visitors; however, more insects were collected at this site than at the other two collection sites. Despite having the highest number of unique individuals captured, a few visitors were collected in greater numbers during the collection at Maryville, thus reducing the relative diversity of captured visitors.

At the Maryville location, the second collection during late September was more diverse than the first collection during mid-September. This result can be explained by the larger proportion of *Agapostemon* spp. and *B. bimaculatus/impatiens* group members that were present. *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 reported that *Agapostemon* spp. were frequent visitors (Chandler and Heilman, 1982; Posey et al., 1986), and this was supported by our study. At the Oak Ridge location, the first collection occurred in late September, and that timing yielded a much higher floral visitor diversity than that observed during the second collection period, which occurred in early October. Both collection dates were late in the flowering period for *H. verticillatus* (Matthews et al., 2002). Furthermore, temperatures on both collection dates were cool and overcast (data not shown), which were less favorable for pollinator activity (Peat and Goulson, 2005).

Hymenoptera genera (*Bombus* and *Melissodes*) and Diptera families (Bombyliidae,

Table 3. Counts of *Helianthus verticillatus* pollen recovered from the taxa representing the most collected insect visitors and *Apis mellifera* at the three collection sites.

Insect visitor ^a	Length of the visitor (mm)	Mean (range) of pollen counts $\times 10^4$ ^b	Mean pollen count per mm insect visitor $\times 10^4$	No. of visitors collected at all sites (Table 2)	No. of visitors $\times$ mean pollen count per mm of insect
<i>Agapostemon</i> spp. (2)	10–11	4.7 (0.2–11.8)	0.44	98	43.1
<i>Allograpta</i> spp.	7–8	0.3 (0.2–0.4)	0.04	31	1.2
<i>Apis mellifera</i>	12	1.3 (0.6–2)	0.11	17	1.9
<i>Augochlorini</i> spp. (3)	6–8	0.74 (0.4–1.8)	0.11	111	12.2
<i>Bombus bimaculatus/impatiens</i>	9–16	11.0 (1.2–44.6)	0.90	311	279.9
<i>Ceratina calcarata</i>	6.5–8	1.0 (0.4–1.6)	0.14	125	17.5
<i>Halicus ligatus</i>	7–10	15.9 (4.6–25.8)	1.90	67	127.3
Hesperidae	12–15	0.12 (0–2.0)	0.01	35	0.35
<i>Lasioglossum/Dialictus</i> spp.	<5–10	1.4 (0.2–2.4)	0.48	43	20.6
<i>Melissodes</i> spp. (2)	6–9	0.4 (0–1.0)	0.05	56	2.8

^aNumber in parentheses indicates the number of species.

^bFrom five randomly selected specimens.

Syrphidae) were the most abundant floral visitors to *H. verticillatus*. *Bombus*, *Halictus*, and *Lasioglossum* species carried the highest number of *Helianthus* pollen grains and most likely represent important pollinators of this sunflower species. Other genera in the Halictidae family (*Agapostemon* spp., *Augochlora* spp., and *Augochlorella* spp.) were also frequent visitors to *H. verticillatus* inflorescences; however, these visitors carried only limited numbers of pollen grains. Native bee pollinators (DeGrandi-Hoffman and Watkins, 2000; Robertson 1922), rather than the exotic *A. mellifera*, are likely more efficient pollinators of *H. annuus* (Parker, 1981), and our findings indicate that this is probably also true for *H. verticillatus*.

Despite some differences in species composition, the relative diversity of floral visitors from all three locations was similar. *Helianthus verticillatus* attracts a wide range of insect visitors regardless of its location, and species-specific composition appears to be dependent on the location. Furthermore, temporal and spatial differences at specific locations may influence the potential pollinators of *H. verticillatus* (Herrera, 1988). Our conclusion from this study is that *Bombus* spp., *H. ligatus* (a sweat bee), *Agapostemon* spp., and *Lasioglossum/Dialictus* spp. are collectively the most probable primary pollinators of *H. verticillatus*, which is similar to the conclusions of other pollinator studies involving *Helianthus* species.

Literature Cited

- Ackerman, J.D. 2000. Abiotic pollen and pollination: Ecological, functional, and evolutionary perspectives. *Plant Syst. Evol.* 222(1):167–185, doi: 10.1007/BF00984101.
- 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, ecology aspects, yields]. *Southwest. Entomol.* 7:170–173, https://digitalcommons.usu.edu/bee_lab_ca/72/.
- 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*). *J. Ecol.* 87:670–677, doi: 10.1046/j.1365-2745.1999.00385.
- Crozier, R.H. and Y.C. Crozier. 1993. The mitochondrial genome of the honeybee *Apis mellifera*: Complete sequence and genome organization. *Genetics* 133(1):97–117, <https://www.genetics.org/content/133/1/97.short>.
- 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. *J. Apic. Res.* 39:37–45, doi: 10.1080/00218839.2000.11101019.
- Edwards, T.P., R.N. Trigiano, B.H. Ownley, A.S. Windham, C.R. Wyman, P.A. Wadl, and D. Hadziabdic. 2020. Genetic diversity and conservation status of *Helianthus verticillatus*, an endangered sunflower of the southern United States. *Front. Genet.* 11, doi: 10.3389/fgene.2020.
- 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 Dis.* 101:632, doi: 10.1094/PDIS-08-16-1216-PDN.
- 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. *Molec. Ecol.* 15: 2345–2355, doi: 10.1111/j.1365-294X.2006.02937.x.
- Faegri, K. and V.D.P. Leendert. 1966. The principles of pollination ecology. Toronto, New York, Toronto, New York, Pergamon Press. <https://books.google.com/books?hl=en&lr=&id=3zfLBAAQBAJ&oi=fnd&pg=PP1&ots=3yLhBzGv&sig=42cWITbKrv5USiOD5H7OT-itok#v=onepage&q&f=false>.
- Free, J.B. 1970. Insect pollination of crops. Academic Press, London, New York. <https://www.cabdirect.org/cabdirect/abstract/19710305447>.
- Gibbs, J. 2012. Revision of the metallic *Lasioglossum* (*Dialictus*) of eastern North America (Hymenoptera: Halictidae: Halictini). *Zootaxa* 3076:1–216, doi: 10.11646/zootaxa.3073.1.1.
- 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. *Ecol. Entomol.* 6: 245–262, doi: 10.1111/j.1365-2311.1981.tb00612.x.
- Gilbert, L.E. and M.C. Singer. 1975. Butterfly ecology. *Annu. Rev. Ecol. Syst.* 6:365–395, doi: 10.1146/annurev.es.06.110175.002053.
- Goulson, D. 1999. Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. *Perspect. Plant Ecol. Evol. Syst.* 2:185–209. <https://www.urbanfischer.de/journals/ppees>.
- Greenleaf, S.S. and C. Kremen. 2006. Wild bees enhance honey bees' pollination of hybrid sunflower. *Proc. Natl. Acad. Sci. USA* 103:13890–13895, doi: 10.1073/pnas.0600929103.
- Hebert, P.D.N., A. Cywinska, S.L. Ball, and J.R. deWaard. 2003. Biological identifications through DNA barcodes. *Proc. Biol. Sci.* 270:313–322, doi: 10.1371/journal.pbio.0020312.
- Herrera, C.M. 1988. Variation in mutualisms: The spatiotemporal mosaic of a pollinator assemblage. doi: 10.1111/j.1095-8812.1988.tb00461.x.
- Horsburgh, M., J.C. Semple, and P.G. Kevan. 2011. Relative pollinator effectiveness of insect floral visitors to two sympatric species of wild aster: *Symphotrichum lanceolatum* (Willd.) Nesom and *S. lateriflorum* (L.) Löve & Löve (Asteraceae: Astereae). *Rhodora* 113:64–86, doi: 10.3119/08-09.1.
- Hurd, P.D. 1980. Principal sunflower bees of North America with emphasis on the southwestern United States (Hymenoptera, Apoidea). *Smithson. Contrib. Zool.* 310:1–158, doi: 10.5479/si.00810282.310.
- Jones, G.D. 2012. Pollen extraction from insects. *Palynology* 36:86–109, doi: 10.1080/01916122.2011.629523.
- Kastinger, C. and A. Weber. 2001. Bee-flies (*Bombylius* spp., Bombyliidae, Diptera) and the pollination of flowers. *Flora* 196:3–25, doi: 10.1016/S0367-2530(17)30015-4.
- Kevan, P.G. and H.G. Baker. 1983. Insects as flower vectors and pollinators [Foraging behavior, nectar secretion, pollination ecology]. *Annu. Rev. Entomol.* 28:407–453, doi: 10.1146/annurev.en.28.010183.002203.
- Levin, M. 1983. Value of bee pollination to US agriculture. *Amer. Entomol.* 29:50–51, doi: 10.1093/besa/29.4.50.
- Mandel, J.R. 2010. Clonal diversity, spatial dynamics, and small genetic population size in the rare sunflower, *Helianthus verticillatus*. *Conserv. Genet.* 11:2055–2059, doi: 10.1007/s10592-010-0062-3.
- 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, <https://www.jstor.org/stable/4034312>.
- McAlpine, J.F., B.V. Peterson, G.E. Shewell, H.J. Teskey, J.R. Vockeroth, and D.M. Wood. 1981. Manual of nearctic diptera. vol. 1. <https://www.cabdirect.org/cabdirect/abstract/19810582668>.
- McGregor, S.E. 1976. Insect pollination of cultivated crop plants. *Agricultural handbook no. 496*, Agricultural Research Service, U.S. Department of Agriculture. <<http://citeserx.ist.psu.edu/viewdoc/download?doi=10.1.1.463.3378&rep=rep1&type=pdf>>.
- Menzel, R. and J. Erber. 1978. Learning and memory in bees. *Sci. Amer.* 239:102–110, doi: 10.1038/scientificamerican0778-102.
- Mitchell, T.B. 1960. Bees of the eastern United States. vol. 1. North Carolina Tech. Bul. 141:1–538.
- 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). *Isr. J. Bot.* 39:197–204, doi: 10.1080/0021213X.1990.10677144.
- Parker, F.D. 1981. How efficient are bees in pollinating sunflowers? *J. Kansas Entomol. Soc.* 54:61–67, doi: 10.1007/978-3-7091-6306-1.
- Peat, J. and D. Goulson. 2005. Effects of experience and weather on foraging rate and pollen versus nectar collection in the bumblebee, *Bombus terrestris*. *Behav. Ecol. Sociobiol.* 58(2):152–156, doi: 10.1007/s00265-005-0916-8.
- Pivnick, K.A. and J.N. McNeil. 1985. Effects of nectar concentration on butterfly feeding: Measured feeding rates for *Thymelicus lineola* (Lepidoptera: Hesperidae) and a general feeding model for adult Lepidoptera. *Oecologia* 66:226–237, doi: 10.1007/BF00379859.
- Posey, A.F., R.W. Katayama, and J.G. Burleigh. 1986. The abundance and daily visitation patterns of bees (Hymenoptera: Apoidea) on oil-seed sunflower, *Helianthus annuus* L., in southeastern Arkansas. *J. Kansas Entomol. Soc.* 59:494–499, doi: 10.1007/s11829-009-9062-y.
- Rehan, S.M. and M.H. Richards. 2010. Nesting biology and subsociality in *Ceratina calcarata* (Hymenoptera: Apidae). *Can. Entomol.* 142:65–74, doi: 10.4039/n09-056.
- Reinhard, J., M.V. Srinivasan, D. Guez, and S.W. Zhang. 2004. Floral scents induce recall of navigational and visual memories in honeybees. *J. Exp. Biol.* 207:4371–4381, doi: 10.1242/jeb.01306.
- Robertson, C. 1922. The sunflower and its insect visitors. *Ecology* 3:17–21, doi: 10.2307/1929086.
- 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. *Molec. Phylogenet. Evol.* 75:138–148, doi: 10.1016/j.ympev.2014.02.018.
- Small, J.K. 1898. Studies in the botany of the southeastern United States. XIV. *Bul. Torrey Bot. Club* 25:465–484, doi: 10.2307/2477834.
- Trigiano, R.N. 2010. Demonstration of principles of protoplast isolation using chrysanthemum and orchardgrass leaves, p. 365–371. In: R.N. Trigiano and D.J. Gray (eds.). *Plant tissue culture, development, and biotechnology*. CRC Press, Boca Raton, FL.
- Trigiano, R.N., S.B. Wilson, and C.N. Steppe. 2018. Whorled sunflower (*Helianthus verticillatus*) – A potential landscape plant. *Comb. Proc. Intl. Plant Prop. Soc.* 68:1–13.
- U.S. Fish and Wildlife Service. 2014. Endangered status for *Physaria globosa* (Short’s Bladder-pod), *Helianthus verticillatus* (whorled sunflower), and *Leavenworthia crassa* (Fleshy-Fruit Gladecress). 79:44712–44718. <<https://www.federalregister.gov/documents/2014/08/01/2014-18103/endangered-and-threatened-wildlife-and-plants-endangered-status-for-physaria-globosa-shorts>>.