DOES STYLE PERSISTENCE MEASURE POLLEN LIMITATION IN PERENNIAL HELIANTHUS SPECIES?

A THESIS SUBMITTED TO THE FACULTY OF THE PROGRAM IN PLANT BIOLOGY AND CONSERVATION

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PLANT BIOLOGY AND CONSERVATION FROM NORTHWESTERN UNIVERSITY AND THE CHICAGO BOTANIC GARDEN

15 OCTOBER 2013

COMMENTS ON THESIS DEFENSE FOR MASTER OF SCIENCE IN PLANT BIOLOGY AND CONSERVATION NORTHWESTERN UNIVERSITY AND THE CHICAGO BOTANIC GARDEN

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on the date of 29 October 2013. The committee members have rendered the following decision:

☐ Pass with no revisions ☒ Pass with minor revisions

☐ Pass with major revisions ☐ Fail to pass

Committee members:

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Stu W — 11 Dec 2013

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ABSTRACT

Pollination is a reproductive necessity in the majority of the world's flowering plants. Pollen limitation threatens plant reproduction, particularly in self-incompatible species. Although assessing pollen limitation usually requires pollen supplementation experiments, alternative methods support the findings of traditional pollen supplementation experiments. Style Persistence, is a measure of pollen limitation developed for the prairie forb *Echinacea angustifolia*, during flowering season. If effective in other species, this measure may be useful in predicting the reproductive fitness of individuals and populations. The genus *Helianthus* (Asteraceae) contains many perennial species common to prairies that are self-incompatible, making it a good candidate to test the effectiveness of Style Persistence. I conducted pollination experiments using four treatments (cross-pollination, pollen exclusion, open-pollination and self-pollination) in 236 inflorescences (heads) in remnant prairies in Illinois and Minnesota. Six native perennial *Helianthus* species were studied: *H. divaricatus, H. grosseserratus, H. hirsutus, H. maximilianii, H. pauciflorus,* and *H. strumosus.* I applied treatments and recorded style condition every day of flowering and assessed seed set. I quantified Style Persistence as a measure of pollen limitation in all six species ($p < 0.0001$). There was a significant additive effect of the row, with outer rows persisting longer than inner rows in each treatment. Styles that received compatible pollen persisted a mean 0.9 - 2.1 days (95% CI), while styles that did not receive compatible pollen persisted a mean 3.0 - 5.2 days (95% CI). I conclude that Style Persistence is an effective measure of pollen limitation and a good method to assess reproductive fitness in these native perennial *Helianthus* species.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following:

My advisor, Stuart Wagenius, for guidance in experimental design, data management, statistical analysis and especially for his infinite patience; my thesis committee members, Nyree Zerega and Krissa Skogen, for encouragement and support throughout; Lake County Forest Preserves, The Forest Preserve District of Cook County, and Minnesota Department of Natural Resources for use of their sites; Lauren Umek for help in obtaining permits and inclusion of my study in the Chicago Wilderness 100 Sites for 100 Years research program; Team Echinacea 2011 and 2012 for welcoming me to the field assistant residence in Minnesota; Susanne Masi, Josh Drizin and Rachel Goad for assistance in *Helianthus* species identification; Gretel Kiefer and Stuart Wagenius for collecting seed heads in Minnesota; Courtney King and Marissa Ruiz of Lake Forest College for cleaning seed heads and x-raying achenes; volunteers in the Population Biology Lab at the Chicago Botanic Garden for unyielding support; fellow PBC students for commiseration and support. I would especially like to thank Jeff Lomax for his unconditional support and willingness to periodically take over all things domestic while I pursue my dream, and Calvin and Dean Lomax for always being my biggest fans.

Without the help and support of all of you, this project would not have been successful. My sincerest thanks to you all.

My research was supported by a grant from the Plant Biology and Conservation program of Northwestern University, the Chicago Botanic Garden, and National Science Foundation awards 1052165, 10521791, and 0545072.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

An estimated 90 percent of flowering plant species worldwide rely on pollination (Menz et al. 2010). Pollen limitation occurs when an inadequate quantity of compatible pollen is deposited, resulting in low ovule fertilization. Many plants experience pollen limitation resulting in reduced reproduction (Burd 1994). This can have evolutionary consequences, such as selecting for traits that reduce pollen limitation or increase attraction for pollinators as well as ecological consequences like a decrease in seed production or seedling viability (Ashman et al. 2004).

Pollen limitation may result from lack of compatible mates or a lack of pollinators. Recent research has shown in some habitats, self-incompatible plants are not limited by pollinators, but rather by isolation from compatible mates (Campbell and Husband 2007, Wagenius and Lyon 2010). If pollinators do not limit reproduction but compatible pollen does, low fruit set is expected. In one study in which hand cross-pollination was performed using within-site donors, approximately half of the self-incompatible species studied showed no clear increase in the fruit set. This suggests that genetic load resulting from mates that are relatives can be an important cause of low fertilization and fruit set (Morales and Galetto 2003).

Pollen limitation in prairies is a great threat to plant reproduction with consequences for not only individual plants, but also for the ecology and evolution of populations and plant communities (Ashman et al. 2004). Remnant prairies in Wisconsin were found to have a rate of extinction in local plants of 0.5 - 1% per year (Leach & Givnish 1996). Self-incompatibility causes rejection of self pollen, resulting in an inability to set seeds (Morales and Galetto 2003). Pollination and reproduction decline in self-incompatible species more than self-compatible species, particularly

in combination with other stressors like habitat fragmentation (Aizen et al. 2002, Knight et al. 2005). Understanding the extent of pollen limitation is important for predicting reproductive success in prairies.

Assessing the extent of pollen limitation often requires researchers conduct pollination experiments or pollinator visit observations and compare effects on reproduction. This can be a time and resource intensive effort. Hand-pollination experiments, in which compatible pollen is applied by the researcher to a sample of the target species and excluded from others, are often an unreasonable choice for large-scale studies (Alonso et al. 2012), and may not account for the sudden increase in resources in the samples treated (Knight et al. 2006). Using pollinator visitation rates to determine pollination is also time consuming and has many challenges (Kearns and Inouye 1993). Measures of reproductive success that rely solely on measures of seed set at the end of season are confounded with occurrences such as resource reallocation and abortion of flowers (Burd 1994).

Recent studies suggest alternate methods of assessing pollen limitation can corroborate pollen supplementation experiment data, while providing information on possible causes (Knight et al. 2006). For example, pollinator visits can be assessed by visual examination of "tripped" flowers (Parker 1997), bumblebee claw marks (Washtani et al. 1995), and analysis of hydrocarbon residues, or "footprints," left by pollinators on a corolla (Witjes et al 2011). However, distinguishing between pollinator visitation and an actual pollination event is also a concern when assessing pollen limitation. Not all alternative methods designed to determine visitation rates are good proxies for identifying pollination and some have questioned their reliability

(Geerts and Pauw 2011). Many proxies are indirect estimates that determine that pollination has occurred as a result of visits rather than indication of pollination on its own. These methods rely on linking pollination to observed pollinator visits (Engel and Irwin 2003). Geerts and Pauw (2011) showed anther ring status could be used to indicate a sunbird visit in the genus *Erica*, and also indicate pollination has occurred.

Style Persistence (hereafter, SP), a measure of assessing pollen limitation in the field during flowering season (Wagenius 2004), was developed for the prairie plant *Echinacea angustifolia* (Asteraceae). The study examined the condition of the styles, persisting or shriveling, after receipt of compatible pollen or no pollen. The measure uses the natural progression of disk floret maturation (Figure 1a) to determine if pollination had occurred. Rows of anthers and styles progress from outer to inner. Anther are presented one day, styles the next. Styles shrivel within 24 hours of receiving compatible pollen, but persist up to 10 days after being restricted from receiving any pollen. As a result, an examination of *E. angustifolia* during flowering season indicates the reproductive fitness of individuals. SP reinforces results of pollen supplementation experiments by providing more information about the causes of pollen limitation (Knight et al. 2006). SP measures pollination, not just visitation, and is therefore a uniquely useful tool. If SP proves an effective measure of pollen limitation in other prairie species, it has the potential to reduce the amount of experimentation needed in predicting the reproductive fitness in a given population.

Alternative methods of measuring pollen limitation may have a practical application for restorationists and conservationists in the assessment of the long-term effectiveness of plant

management efforts. Invasive species removal, for example, has been shown to increase the pollinator community within the first year of restoration, becoming similar to uninvaded areas. Plant communities, however, may remain distinct from reference sites within the same timeframe (Feidler 2012), making long-term prediction of success difficult. In such an instance, SP may help determine if species are receiving compatible pollen during the flowering seasons after removal of invasives, giving some insight into the long-term reproductive fitness of those species. A common concern for restorationists is the possibility of Allee effects, reduced reproductive output in small populations. Pollen supplementation experiments are often used to measure such an effect (Menz 2011), but SP may provide information on pollen limitation and support data collected on seed production of the target species.

The genus *Helianthus* is in the Asteraceae, which, according to the USDA (NRCS2012), contains 478 genera and is the largest plant family. Perennial *Helianthus* are self-incompatible species (Free and Simpson 1964, Heiser et al.1969) found throughout North America in prairies and woodlands. According to recent taxonomy, there are 51 species of Helianthus, 37 of which are perennial (Vear 2011). The progression of disk floret development in perennial *Helianthus* species is similar to that of *Echinacea angustifolia* (Figure 1b), making it a good candidate to test the use of SP as a measure of pollen limitation. Flowering phenology of *Helianthus* is detailed in the methods section. If SP measures pollen limitation in native perennial *Helianthus*, it could help land managers assess pollen limitation in the flowering season. This is beneficial because the assessment of pollen limitation is done before predation of seeds, common in *Helianthus* (Heiser 1969), occurs. It may also direct further study, and serve as model for SP as a measure of pollen limitation in other Asteraceae species and even other families.

To quantify the extent to which SP measures pollen limitation in perennial *Helianthus* species, I conducted hand pollination and pollen exclusion experiments on six species in remnant prairie sites in Illinois and Minnesota in 2012. I applied four pollination treatments to inflorescences in each population: open, cross, self, and exclusion. I expected the persistence of styles in cross-pollination groups to be comparable to those of the open-pollination groups which represented the natural or control group, and style persistence in the self-pollination groups to be similar to the pollen exclusion group, verifying perennial *Helianthus* are self-incompatible (Free and Simpson 1964, Heiser 1969). I expected lower fruit set in inflorescences that did not receive compatible pollen, indicating that SP could also be a predictor for a decrease in reproductive success.

RESEARCH GOALS AND OBJECTIVES

Based on morphological and breeding system similarities to *Echinacea angustifolia*, I hypothesized that the styles of *Helianthus* species would: (1) respond to receipt of compatible pollen by shriveling and (2) persist if compatible pollen is not received, quantifying SP as a measure of pollen limitation in perennial *Helianthus* species. By comparing SP in six species, I quantify the effectiveness of SP among species as well as among treatments.

MATERIALS AND METHODS

Study Sites

I conducted my experiment in Illinois and Minnesota, USA. The species in Illinois began flowering July 8 and continued through August 10. The *Helianthus* species in Minnesota

flowered from August 14 to August 28 (Table 2). I chose remnant prairies in Cook and Lake Counties in northeast Illinois and Douglas County in western Minnesota. The sites I chose in Illinois had two or more *Helianthus* species and it was feasible to visit both sites and conduct experiments each day of the study. I obtained permits through the Chicago Wilderness 100 Sites for 100 Years research program. The Minnesota sites I chose are used by the Echinacea Project for ongoing study (*Our Study Site*). Hegg Lake Wildlife Management Area has more than two *Helianthus* species. I chose Riley because the *H. maximilianii* at this site would flower during the time of my study (Table 1).

Berkeley Prairie, Highland Park, IL, is a moderately degraded prairie and savanna owned and maintained by Lake County Forest Preserve District. It was acquired and designated as a preserve in 1968. Somme Prairie Grove, Northbrook, IL, is owned and maintained by the Forest Preserve District of Cook County. It includes remnant and restored prairie, woodland and savanna. The preserve has a 30-year restoration history. While some seeding has taken place, *Helianthus* species are reported to be native to the preserve. Hegg Lake Wildlife Management Area, Douglas County, MN, is a native prairie near Kensington, MN, owned and maintained by the Minnesota Department of Natural Resources. Riley, Douglas County, MN is so named by the Echinacea Project. It refers to a roadside remnant between two agricultural plots. It is in a Solem Township road right of way (Table 1).

Study Species

I chose native perennial *Helianthus* for this study due to similarities to *Echinacea angustifolia*, for which the SP measure was developed. *Helianthus* is an indigenous North American genus

(Heiser 1969) in the Asteraceae that has many species native to prairies (Swink and Wilhelm 1994).

I selected species for my experiment that had populations of 100 or more stems to avoid stressing the population and had disks large enough to easily view rows of anthers and styles. To complete the study in one growing season, I chose species that would begin and end flowering within the timeframe of the study (Seiler 1992). The species are*: H. divaricatus* L.*, H. grosseserratus* M. Martens*, H. hirsutus* Raf.*, H. maximilianii* Schrad*, H. pauciflorus* Nutt.*,* and *H. strumosus* L.(Gleason and Cronquist 1991, Swink and Wilhelm 1994, Mohlenbrock 2002) . Each population was assigned a unique identifying code. The identifier has an abbreviated species code followed by the state abbreviation and the first letter of the site name. For example, the *H. divaricatus* population at Berkeley Prairie is identified as HdivIL-B (Table 2). All species represent the *Helianthus* Section Divaricati, Series Corona-solis, except *H. pauciflorus*, which is Series Astrorubens as classified by Schilling and Heiser (1981). Recent phylogeny for *Helianthus* places all six in Section Divaricati and suggests independent hybrid speciation events have occurred within this section (Timme et al. 2007). I collected a representative individual from each population and deposited specimens for herbarium accession at the Chicago Botanic Garden (CHIC) (Table 2).

Phenology and structure

Perennial *Helianthus* species have composite inflorescences. The disk florets are hermaphroditic, protandrous, and open in a pattern progressing inwardly (Figure 1a and 1b). In their seven-year study of rewards and pollinator foraging behavior in the wild sunflower *Helianthus annuus,* Neff

and Simpson (1990) determined the flowering phenology of disk florets in that species. Staminate florets appeared one day and became pistillate the next, with the staminate phase lasting an average of 12 hours. The pistillate phase of the disk florets was observed to last 4 hours to 4 days, depending on the timing of pollination. They noted ray florets of open-pollinated heads persisted for 7-10 days and persistence could be prolonged up to 11 days if the pollination of the disk florets was prevented, but disk floret response to different pollination treatments was not noted.

I observed similarities in the pattern and timing of flowering among the *Helianthus* species in my study. The emergence of the ray florets preceded the emergence of anthers. The anthers emerged in rows and presented up to 24 hours before the emergence of styles. I assigned row numbers based on the emergence pattern, from the outer rows to the inner rows. Number of rows varied within populations, ranging from 5 to 10. In order to account for differing number of rows within populations I transformed the row numbers to scaled row numbers using statistical software R version 3.0.0. All inflorescence were analyzed on a scale of 1 to 10 rows, which ensures that the last row of any given inflorescence was comparable to the last row of each of the other inflorescences. This eliminated the possibility that differences among inflorescences and treatments were attributable to differences in disk size.

Perennial *Helianthus* species typically have more than one inflorescence, or head, per stem (Heiser 1969), and the number of heads varies within and among species (Swink and Wilhelm 1994). I attempted to include more than one head per stem in my study. However, the flowering

time of the heads varied and many did not flower within the time of the study. Of the 236 inflorescences in the study, only 16 represent multiple heads on single stems.

Treatments

I applied four treatments in each population: cross-pollination, self-pollination, pollen exclusion, and open-pollination. I chose individual inflorescences that appeared close to flowering, with ray flowers newly emerged or emerging and disk florets still immature. I assigned a unique identifying letter/number combination to each inflorescence, and wrote it in permanent ink on vinyl flag tape attached to the stem, or peduncle if multiple inflorescences per stem. I randomly assigned one treatment to each inflorescence. I bagged individual inflorescences in the cross, self, and exclusion treatments with pollinator exclusion bags made from nylon bridal veil material with 0.5mm holes and secured using plastic twist ties color coded for each treatment (Kearns and Inouye 1993). I bagged inflorescences once the ray florets were open, while disk florets were immature, July1-4 in Illinois and August 12-13 in Minnesota. I left the open-pollination inflorescences unbagged until flowering was completed and then bagged them until seedhead collection, which occurred September 4-20 in Illinois and October 8-10 in Minnesota. The number of inflorescences per population and treatment varied due to differences in flowering time (Table 2).

Pollen Collection and Application

The land managers of the Illinois sites requested that no pollen be brought from outside sources. Therefore, pollen donor plants were the same species as the recipients and were chosen from the same site. I used the same method in the Minnesota sites for consistency. Perennial *Helianthus*

species produce rhizomes and vegetative shoots (Rogers et al. 1982). Clonal growth has been researched in *Helianthus occidentalis*, another native perennial prairie species, and was found to have a mean distance ranging from 2.4 meters to 12 meters between clonal stems (Fore and Guttman 1999). Donors in my study were a minimum 20 meters from the outermost inflorescence in the treatment populations to avoid pollen from clones. Donors were usually 10 – 30 m from the closest other donor stem. I recorded GPS points for each population (Figure 3). I visually estimated the center of each population. Populations HpauMN-H and HstrMN-H each were more than were more than 50 meters wide and had two sections. I recorded two points in the center of each section. Populations HgroMN-H and HmaxMN-R had more than one donor population. Distances of donor populations from experimental populations were calculated using statistical software R 3.0.0 (Table 3).

Pollen Removal Method

I obtained pollen each day from a minimum of 6 donors. Donor plants were chosen based on which were presenting pollen that day. I collected pollen from each donor daily throughout flowering pushing from base to tip of the anther with a round wooden toothpick, and placing it into a 1.5 ml microtube (Kearns and Inouye 1993). Each day, outcross pollen was mixed in one microtube. Most pollen collected was used the same day, and any requiring storage was stored in a household refrigerator within 6 hours after collection.

Cross-Pollination

Each day, I loosened the twist tie and removed the bag completely. I removed pollen from presenting anthers as described above, applied pollen, and recorded style condition for each

inflorescence. I replaced and secured the bag when all data had been collected for the head before moving on to the next. Collected pollen was discarded. I applied outcross pollen to individual styles beginning the day they presented using a separate, clean, round toothpick for each inflorescence each day, and continued to apply pollen to every persisting style until styles were no longer observed. Donor pollen was stored a maximum of 48 hours, but usually was depleted on the day of collection. Sixty-one inflorescences received the cross-pollination treatment.

Self-Pollination

I removed and replaced the bag from each head as in the cross-pollination treatment. I wrote the unique identifier on the microtube and collected the pollen each day anthers presented. Pollen was removed as described in the pollen removal section above. Beginning on the first day of style presentation, I applied pollen to every persisting style and recorded style condition, using the same method as in the cross-pollination treatment until styles were no longer observed or pollen was depleted. Because pollen in this treatment had to be from a specific, limited source, pollen needed to be stored. Pollen was stored overnight to a maximum of 3 days. Sixty inflorescences received the self-pollination treatment.

Pollen Exclusion

I placed pollinator exclusion bags on each inflorescence before florets matured. I removed pollen each day from presenting anthers as in the self-pollination and cross-pollination treatments and discarded it. I recorded style condition each day. Anthers were not emasculated to avoid

disrupting style emergence. It is unlikely that all the pollen was removed. Fifty-five inflorescences received the pollination exclusion treatment.

Open-Pollination

Inflorescences in this treatment were left unbagged during flowering. I did not remove or add pollen. Each day, I recorded style condition for each inflorescence. At the end of flowering, I secured a nylon pollinator exclusion bag, using the same method as in the other treatments, over each inflorescence. Sixty inflorescences were in the open-pollination treatment.

Photographic data

I photographed each inflorescence in the study each day beginning when the first row of anthers emerged and ending when all styles had shriveled. The duration of flowering ranged from 4 to 10 days per inflorescence. I used a Nikon Coolpix P510 digital camera and uploaded images daily to an external hard drive.

Seed Collection

After flowering and treatments ended (Table 2), I left all inflorescences bagged until the seed heads ripened. I determined seed heads to be ripe if the inflorescence was dry and the peduncle was no longer green. We collected by clipping the stem 1-3 centimeters below the head. Heads, bags, twist ties, and identification tape were collected and placed in small brown paper bags marked with the unique identifier and placed in larger paper grocery bags. I dried seed heads from Illinois in the seed dryer at the Chicago Botanic Garden at standard settings, 15^{o} C and 15% relative humidity, for three weeks after harvesting. I did not dry the seed heads from the

Minnesota sites because they were very dry at time of harvest, I stored them in the Population Biology Lab at the Chicago Botanic Garden until cleaning began in November.

Assessing Fruit Set

We cleaned each seed head by removing all achenes and placing them in small coin envelopes with the corresponding identifier. We then placed all the achenes from each head in a Petri dish and x-rayed them using the Chicago Botanic Garden's Faxitron X-Ray Specimen Radiography System at 18kV for 20 seconds. Using this method, achenes appear as outlines and partially in shadow. I identified full achenes by a whitish embryo observed inside the outline of the achene (Figure 1). Total number of achenes and number of full achenes were counted from the resulting image using ImageTool software.

Statistical Analysis

I used the statistical software R version 3.0.0 for data transformation and analysis for the pollination experiment data. A significance level of $p \leq 0.05$ was used for all tests. I used backward selection and an ANOVA analysis of a generalized linear model with a Poisson error structure (Crawley 2005) to analyze the relationships between treatment, row number, and number of days styles persisted. I determined the proportion of fruits per seedhead by counting the number of full achenes and dividing by the total number of achenes.

RESULTS

Style Persistence as a measure of pollen limitation

Styles that did not receive compatible pollen persisted longer than those that received compatible pollen. Styles that received pollen exclusion treatment persisted an average 3.9 $\pm$ 0.06 days (1 s.e., n = 371) Styles that received self-pollination treatment persisted an average 4.1 $\pm$ 0.06 days (1 s.e., n= 404). Styles that received open-pollination treatment persisted an average 1.5 $\pm$ 0.05 days (1 s.e., n = 379). Styles that received cross-pollination treatment persisted an average 1.8 $\pm$ 0.05 days (1 s.e., n = 426) . Slight variations occurred in populations, but the results are consistent for each population and species (Figure 2). I recorded style persistence by row and the study resulted in data for 1580 rows. The number of days styles persisted differed by row, with inner 2 rows persisting a maximum of 5 days and outer 5 rows persisting a maximum of 9 days.

Anthers and styles emerged in complete rows, and styles persisted or shriveled in complete rows. The innermost rows were often single florets. I observed instances in every population in which two rows would present anthers on the same day. This occurred in 137 instances, about 8.6% of the total number of rows and the majority were within the innermost 2 rows.

For each population and species, treatment affected duration of SP. Style persistence is also affected by row and the effect is additive (p <0.0001, n=1578) rather than interactive (p = 0.28) according to a generalized linear model with a Poisson response. The effect is apparent in analyzing data from the entire study and individual populations (Table 3).

Photographic data

The photographic data recorded over 4 to 10 days of flowering of each inflorescence show that the appearance of disk florets differs depending on treatment. Styles that persist were clearly

distinguishable from styles that did not persist. A visual comparison of the disks of inflorescences in different treatments indicated a lack of compatible pollen (Figure 3).

Seed Set

We recovered 8,068 achenes, of which 1,547 were full, or 37% across the entire study. The number of total achenes ranged per head from 2 to 88 and the number of full achenes ranged from 0 to 66 assessed from x-ray images (Figure 1).

I determined the mean proportion of seeds produced by dividing the mean number of full achenes by the total number of achenes. I determined the mean proportion of full achenes per treatment per population (Figure 7) and per treatment for the entire study (Figure 8). The mean proportion of full achenes in open and cross-pollination was higher than in self and exclusion in every population (Figure 7).The mean proportions of full achenes were as follows: open-pollination treatment was 25.8 $\pm$ 3.3 (1 s.e., n = 58), cross-pollination treatment was 35.1 $\pm$ 3.6 (1 s.e., n = 61), self-pollination treatment was 5.2 $\pm$ 1.6 (1 s.e., n = 60), and pollen exclusion treatment was 4.8 $\pm$ 1.3 (1 s.e., n = 57).

DISCUSSION

Treatment and Row Affect Style Persistence in Helianthus

In all six species of *Helianthus* and in all eight populations, I quantified SP as a measure of pollen limitation by testing the hypothesis that the number of days styles persist is significantly affected by the receipt of compatible pollen (Table 4). The style persistence data, along with seed set assessment, support the long-standing assertion that perennial *Helianthus* species are

self-incompatible (Free and Simpson 1964, Heiser et al. 1969). In every population, styles that persist 3.5 days or more indicate that an inflorescence has not received compatible pollen. Styles that have received compatible pollen shrivel within 2.2 days (Figure 4).

The mean number of days of style persistence was predicted with a linear regression by days styles persist per treatment and scaled row number (Figure 6). Styles in the open and cross-pollination treatments will persist fewer days than styles in the self-pollination and exclusion treatments. The prediction indicates not only that pollen limitation affects style persistence, but also the number of days styles persist varies by row number. The innermost 2 rows of styles of an inflorescence persisted a maximum 5 days and can be expected to shrivel sooner than the outer 5 rows regardless of treatment or species. Using SP as a measure of pollen limitation in *Helianthus* may be less effective in the innermost 2 rows. The row effect may be due in part to the phenology of the ray florets. Ray florets function to attract pollinators to the center disk (Heiser 1969) and have been observed in annual species to persist 7-9 days before wilting (Neff and Simpson 1990). The flowering time of *Helianthus*, from first anther date to last anther date, ranged from 4 to 10 days in my study. The styles in the inner rows would emerge nearest the end of flowering time of the ray florets. Wilting of ray florets may signal the styles of disk florets to retract in order to use resources for seed production. In this study I did not record ray flowering duration.

In *Echinacea angustifolia*, there was no row effect, and the number of days styles persisted in the inner rows was comparable to the outer rows (Wagenius 2004). In another SP study of *Heliopsis helianthoides* and *Echinacea purpurea*, Lee Rodman (Grinnell College, unpublished data) found

that SP measured pollen limitation and there was a row effect. The row effect, as in my study, was present regardless of treatment.

Style Persistence Varies Among Species

Although SP is a measure of pollen limitation in all six species, there are differences. A comparison of slopes (Figure 6) indicates that row has largest effect on SP in *H. divaricatus,* HdivIL-B. The number of days styles persist decreases significantly in the inner rows. Row has less of an effect in the *H. strumosus* species. For example, the predicted range for pollen exclusion in *H. strumosus*, HstrIL-S, is 3.3 to 3.7 days. The predicted range for range for pollen exclusion in *H. maximilianii* is 2.6 to 4.0 days. While each of these is a significant indication of pollen limitation, there is a greater difference in SP due to floret position in *H. maximilianii,* HmaxMN-R. Differences in species provide insight into how best SP can measure pollen limitation in a target species.

The *H. strumosus* species at Hegg Lake in Minnesota, HstrMN-H, had the least difference in days styles persisted across treatments (Figure 4), and produced fewest fruits across treatments in the study (Figure 7). This population had the highest p-value (Table 4) of the three *H. strumosus* populations and the predicted values (Figure 6) showed less differentiation between compatible and incompatible pollen. This population was difficult to key out in the field and I consulted with experts to identify the sample I collected. There were two areas of the population measured because the population was more than 50 meters (Figure 3) and donor pollen was taken from 276.1 and 233.5 meters away from the experimental population (Table 3). This was done to avoid collecting pollen from the same plant in the study. Eliminating the likelihood of pollen

from the same plant and considering the data for the other two populations of this species, the *H. strumosus* population at Hegg Lake may be less receptive to pollen, experiencing another stress, like inbreeding depression in which the populations are too genetically similar and the pollen is not compatible. The population produced seeds, although fewer per head than the others in the study (Table 4), and I have ruled out sterility as a cause for the SP results. *H. strumosus* is one of the most easily hybridized native perennial sunflowers (Rogers et al 1982) and it is possible that there were some hybridized specimens in the population. Hybridization in annual *Helianthus* was studied and theorized by Heiser (1969, 1976) and verified in perennial species by Seiler (1982). Hybrid speciation occurs when genotypes are generated by hybridization and become genetically stable and independent from the parent genotypes. Rieseberg observed rapid hybrid speciation in annual sunflowers species that established distinct reproductively fit populations within four generations (2006). A hybridized population may be less receptive to donor pollen. However, a cause such as inbreeding depression or hybridization could not be verified without further study of the population.

Style Persistence Predicts Seed Set

The proportion of fruits per treatment provides evidence that SP indicates low fruit set. The number of achenes produced ranged among populations from 22-35, and the proportion of full achenes ranged from 5.0 – 40% (Figure 7). There was a clear difference across all populations in the number of achenes produced per treatment (Figure 8). This is evidence that pollen limitation results in low seed set and that SP is a good predictor (Figure 8). In every population, self-pollination and pollen exclusion resulted in lower fruit set than open-pollination and cross-pollination (Figure 7).

In addition to examining the x-ray image, the appearance of the achenes indicates which are full and which are empty. Empty achenes are often flat and full achenes are rounded. A visual comparison of achene shape and size and the x-ray image demonstrates this (Figure 2). In assessing seed set of these six species, the outward appearance may be sufficient in determining seed set.

I observed a smaller proportion of full achenes than I expected in the open-pollination groups. The mean number of achenes recovered in the open-pollination samples was lower than the cross-pollination treatment (Figure 8). One possibility is predation of seeds may have occurred, resulting in proportionally lower seed sets at the time of collection. During seed head cleaning, we observed insect larvae, dead insects, or chewed achenes. We recorded these in 26 seeds heads in six of the eight populations, or 11% of the heads collected. Of those 26, 10 were in open treatments, 5 in cross treatments, 6 in self-treatments and 5 in exclusion treatments, and 61% of the affected inflorescences were from the Minnesota sites. Inflorescences in the cross-pollination treatment were bagged before disk florets began flowering and remain bagged until the seed head was collected. Inflorescences in the open-pollination treatments were only bagged after flowering was complete. This may indicate that the timing of bagging of inflorescences affect predation rates. The two species for which no evidence of predation was noted were in Somme Prairie Grove. It is possible that this is due to the difference in type, location, size, or management practices at the sites.

Differences in quality of pollen received may also have affected seed set. Inflorescences in cross-pollination received pure outcross pollen collected from the target species, whereas the open-pollination inflorescences likely received a blend of compatible and incompatible pollen. In addition, excluding natural pollinators by bagging the cross-pollinated inflorescences likely increased the difference in pollen quality (Ashman et al. 2004). In their assessment of experiments to determine causes of pollen limitation, Aizen and Harder (2007) concluded that increased seed production observed by pollen supplementation methods are due to plants receiving higher-quality pollen than is received in nature. Low seed set in the open-pollination treatments may also be due to interference from incompatible pollen. Stigma or stylar "clogging" may occur, in which non-compatible pollen mechanically or chemically inhibits fertilization by compatible pollen (Brown and Mitchell 2000). Still another explanation is the rate at which compatible pollen is deposited affected fertilization. Open-pollination is gradual addition of pollen, while pollen applied by hand may represent a sudden increase in compatible pollen resulting in increased fertilization (Ashman et al. 2004).

The seed yield for Illinois sites were lower than the Minnesota sites overall, less than 35% regardless of treatment (Figure 7). One possible explanation is weather. In 2012, Illinois experienced severe drought conditions with record high temperatures. Douglas County, Minnesota did not experience drought or record high temperatures (noaa.gov). Disruption in water availability during flowering prevents fertilization and decreases seed yield in *Helianthus annuus* (Mehrpouyan et al. 2010). The drought in Illinois may have reduced the ability to set seed. Because my study was conducted only one growing season, it is difficult to conclude the role of the drought in seed production.

Style Persistence in Asteraceae

Quantifying SP as a measure of pollen limitation in *Echinacea angustifolia* (Wagenius 2004), *E. purpurea*, *Heliopsis helianthoides* (*Lee Rodman*), and in six *Helianthus* species is a good indication that SP may prove a good measure of pollen limitation in other self-incompatible Asteraceae genera. Style Persistence has already proven useful in assessing pollination events. Wist and Davis (2013) used the SP measure to test the reliability of style retraction as indicator that pollination had occurred in their study of pollinators in *Echinacea angustifolia*. If SP is an effective measure of pollen limitation in other Asteraceae species, it has the potential to inform conservation efforts. I would expect SP to be an effective measure in other Asteraceae as well. Phylogenetic research may give insight into which genera and species would be good candidates for using SP to measure pollen limitation. *Helianthus*, *Echinacea*, and *Heliopsis* are in the Heliantheae tribe. Helianthus is in the Helianthinae subtribe and *Echinacea* and *Heliopsis* are in the Zinniinae subtribe (Urbatsch et al. 2000). Other species in the Heliantheae tribe might prove good species for using SP, even if they are in different subtribes. Some Asteraceae genera that are common to prairies include *Rudbeckia*, *Coreopsis*, *Silphium,* and *Ratibida* (Swink and Wilhelm 1994). Using SP might prove useful in other families with species in which styles are known to retract after successful pollination.

Practical Applications and Limitations of SP

Assessing pollen limitation is important for testing the effectiveness of conservation efforts. Restorationists and conservationists would benefit from having a variety of methods to predict the reproductive success of species of concern. SP is an effective measure of pollen limitation for

the six *Helianthus* species in my experiment. Wild *Helianthus* species can been used as sources of genetic material, and crossed with crop species for disease and insect resistance, and to increase oil content, drought resistance and salt tolerance. All the species I studied have shown potential in providing genetic material for these purposes (Seiler 1992, Seiler 1994). *H. strumosus* has successfully been crossed with sunflower crop species to restore fertility, and provide genetic diversity (Jan et al. 2002, Vear 2011). There are not sufficient resources to preserve all wild and locally adapted Helianthus species in seed banks, making the preservation of wild populations more critical to the survival of the genus (Vear 2011).

I recommend two practical methods that a land manager can follow using SP to predict reproductive fitness of *Helianthus* species. First, one can chose individual plants before flowering and observe them over the entire flowering period of the chosen inflorescence(s). If styles in the outer 5 rows persist for 3 days or more, compatible pollen has not been received. Second, an individual inflorescence can be selected during flowering and examined on that day. If more than three rows of styles are present, that inflorescence may not be receiving compatible pollen. In each case I would recommend assessing seed set to verify the effect on reproductive fitness. I provided a suggested protocol for using SP in the field as a measure of pollen limitation in native perennial *Helianthus* species (Appendix 1) and sample data sheet (Appendix 2).

Using SP to measure pollen limitation has been quantified in three genera *Echinacea*, *Helianthus* and *Heliopsis*. The non-manipulative approach may prove useful in using SP to measure pollen limitation in threatened or endangered species. While it may prove a useful tool in families other than Asteraceae, there are some limitations. First, SP indicates that a style has not received

compatible pollen, but shriveling may not always indicate receipt of compatible pollen. Styles may retract or shrivel in response to other factors like drought (Mehrpouyan et al. 2010), or predation of styles (Wagenius 2004). Second, in order for SP to be an effective measure, the style emergence pattern and response to pollination of the target species should be documented, either before or during the experiment. Third, In order to be certain that SP is measuring a response to pollination other data may need to be collected by, for example, conducting a pollen addition/exclusion experiment, assessing seed set, or analyzing pollen tube growth (Wist and Davis 2013).

As pollen limitation continues to threaten plant reproduction, assessing its extent becomes more urgent. The non-manipulative SP measure is an efficient and cost-effective measure of pollen limitation. Due to the success of SP as in native perennial *Helianthus* species, further study in the Asteraceae and other families could prove very beneficial.

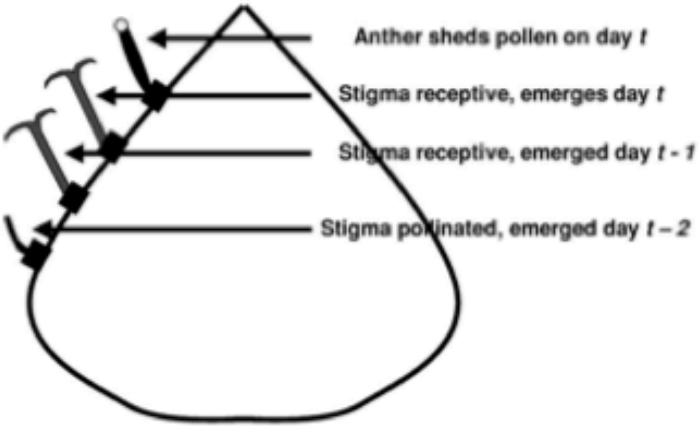


Figure 1a: Schematic section of *Echinacea* head with detail of four florets, representing a snapshot in time on day *t*. Each floret represents a row of florets. Floret rows emerge sequentially from bottom to top. The florets are protandrous, presenting anthers and pollen one day, and styles the next. A florets position relative to the pollen-shedding row is revealed when the style emerges. A style's shape indicates receptivity because it shrivels within 24 h after compatible pollen lands on the surface of the style branches. The progression of florets from outer row to inner is the same with *Helianthus* species. From Wagenius 2004.

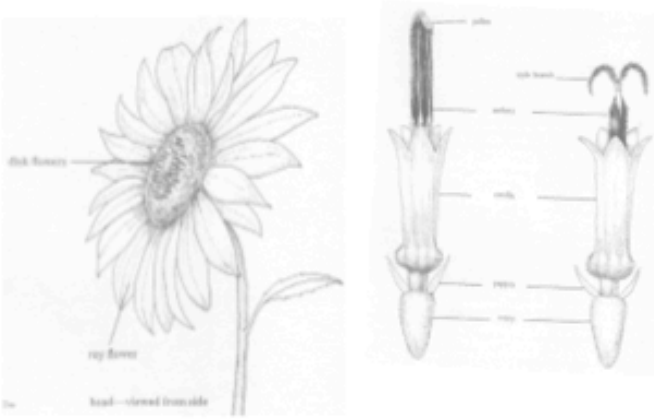
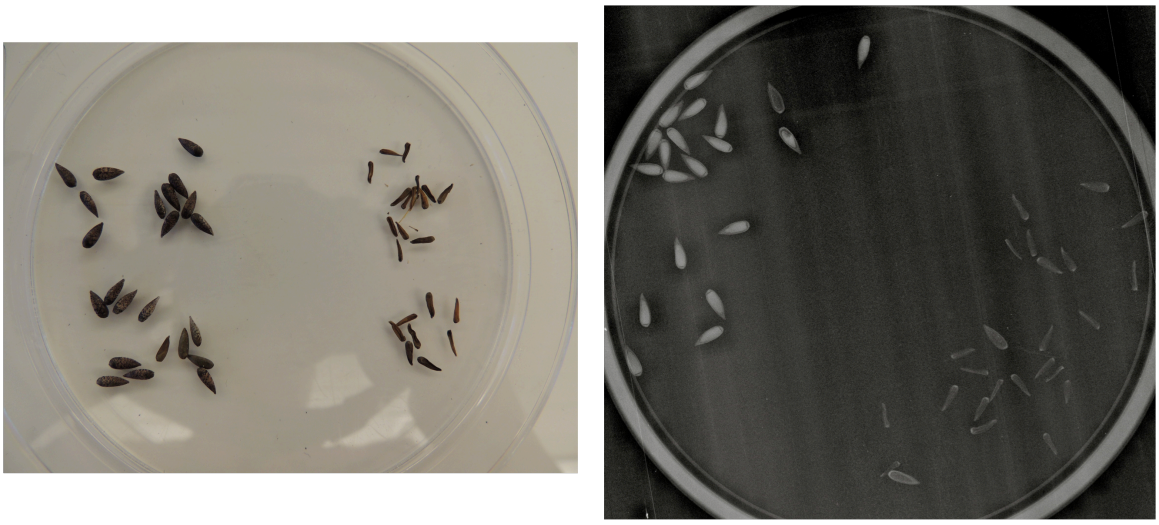


Figure 1b: Sterile ray and monoecious disk florets in *Helianthus*. The outer styles have shriveled and the inner rows are presenting anthers and styles. A close up shows the anther with pollen and the style that emerges within 24 hrs. after the anther. Drawing by Joan Wood. From Heiser 1976.



A B

Figure 2. Photograph (A) and X-ray (B) images of *H. maximilianii* achenes from cross-pollination treatment. A. Some achenes appear round and others flat. The round achenes are often larger. B. Achenes that appear round show a white embryo inside the achene outline, while those that appear flat are shadow shapes in the x-ray. The relationship of achene appearance and fullness is consistent in all species in the study. The x-ray image of each inflorescence was used to count total number of achenes, all that appear in the Petri dish and number of full achenes, those that show the embryo. Proportion seed set in each inflorescence is determined by dividing the number of full achenes by the number of total achenes.

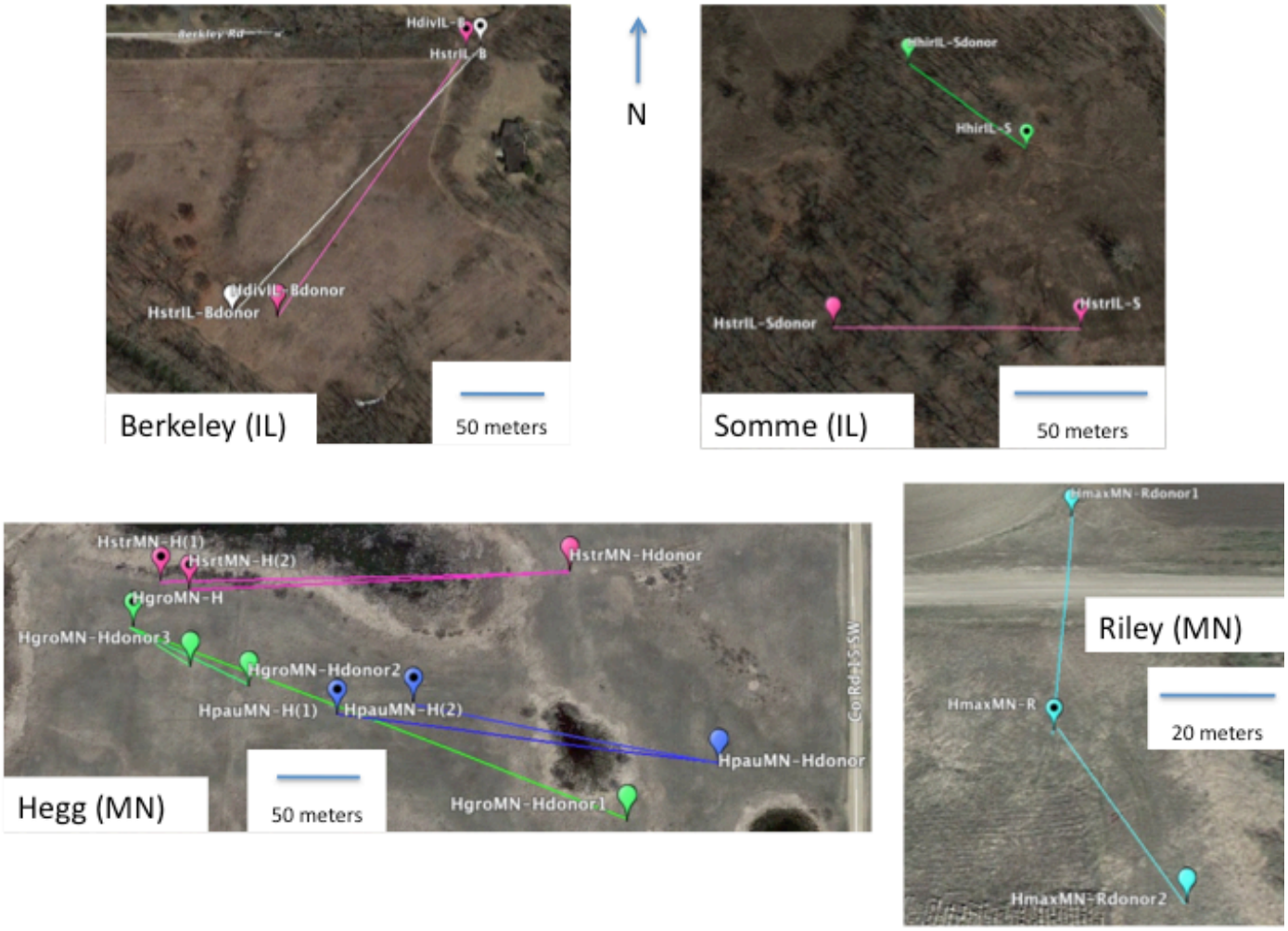


Figure 3. Maps of each site with experimental and donor populations. Maps were made using Google Earth software. Experimental populations are indicated by a dot in the marker. A marker in a corresponding color with no dot indicates donor populations. Due to the variety of site sizes and donor distances, the maps are not on the same scale. All map scale legends are 50m except Riley (MN), which is 20m. At Hegg (MN), HpauMN-H and HstrMN-H were each more than 50m wide and had 2 sections. Two GPS points were recorded in the estimated center of each section. HgroMN-H. At Riley (MN), HmaxMN-R had two donors. Donor distances from experimental populations are listed in Table 3.

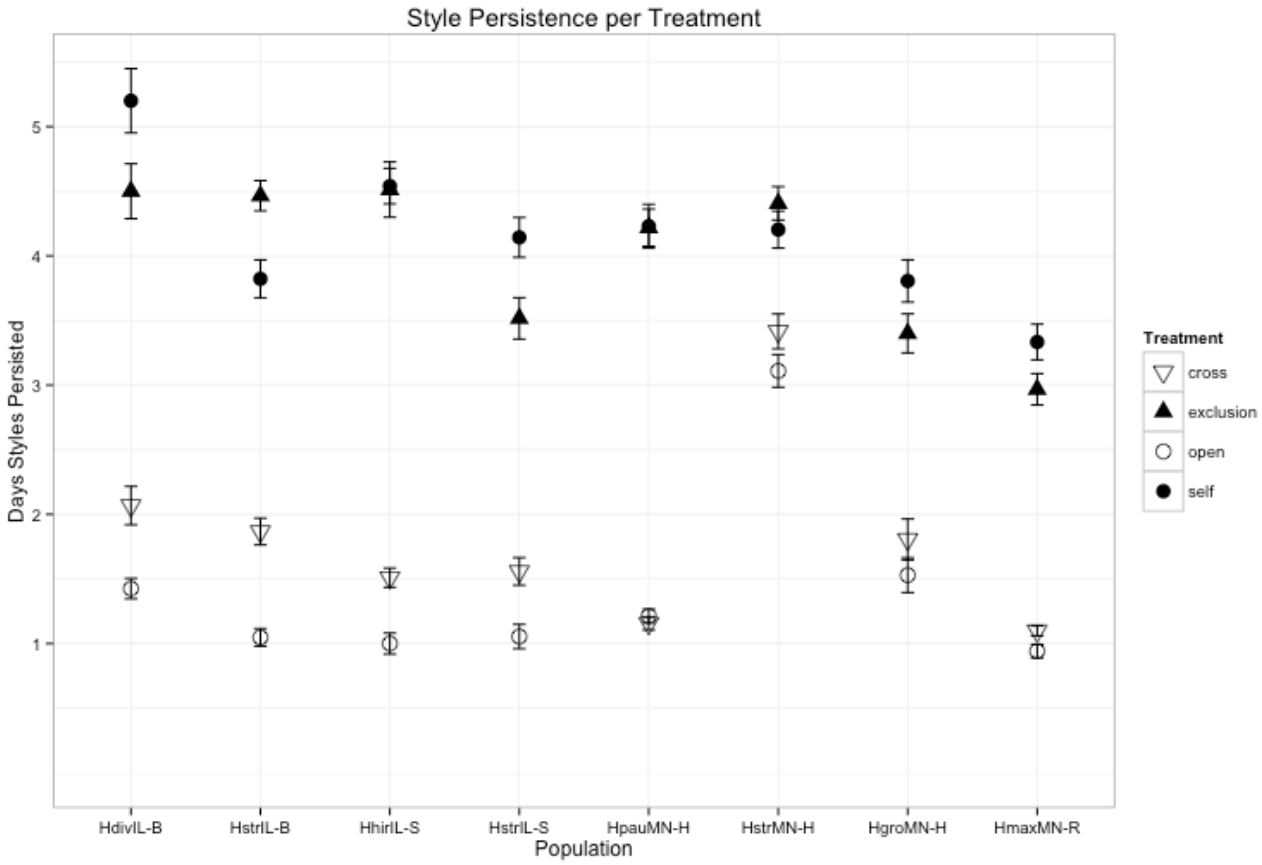


Figure 4. Mean number of days styles persisted per treatment in each population. Number of days styles persisted is counted by row for cross-pollination (n = 426), pollen exclusion (n = 371), open-pollination (n = 379), and self-pollination (n = 404). Each symbol represents of different treatment, and data are means $\pm$ S.E based on a generalized linear model. In all populations, styles in open-pollination treatments had the lowest mean number of days styles persisted. Mean number of days persisted in cross-pollination treatments was the second to lowest in all populations. The mean number of days styles persisted was highest in self-pollination and pollen exclusion treatments, with all mean values 3 days or more. Population HstrMN-H had the highest mean for open and cross-pollination treatments and had the least difference in mean days of persistence among treatments.

Figure 5. *H. pauciflorus* inflorescences from cross-pollination treatment (A), open-pollination treatment (B), pollen exclusion treatment (C), and self-pollination treatment (D) on day 4 of flowering. A comparison of the disks of inflorescences in the different treatments illustrates the use of SP as a measure of pollen limitation. Styles in the cross and open-pollination treatments (A and B), received compatible pollen and shriveled within 1-2 days. On day 4, styles in the outer two rows have shriveled, in the third row styles have emerged, and anthers are presenting in fourth row. Styles in the pollen exclusion and self-pollination treatments (C and D), did rot received compatible pollen. On day 4, styles in the outer two rows persist. Styles in the third row have emerged and anthers are presenting in the fourth row. In the self-pollination treatment (D), dead and dying pollen remains on the styles.

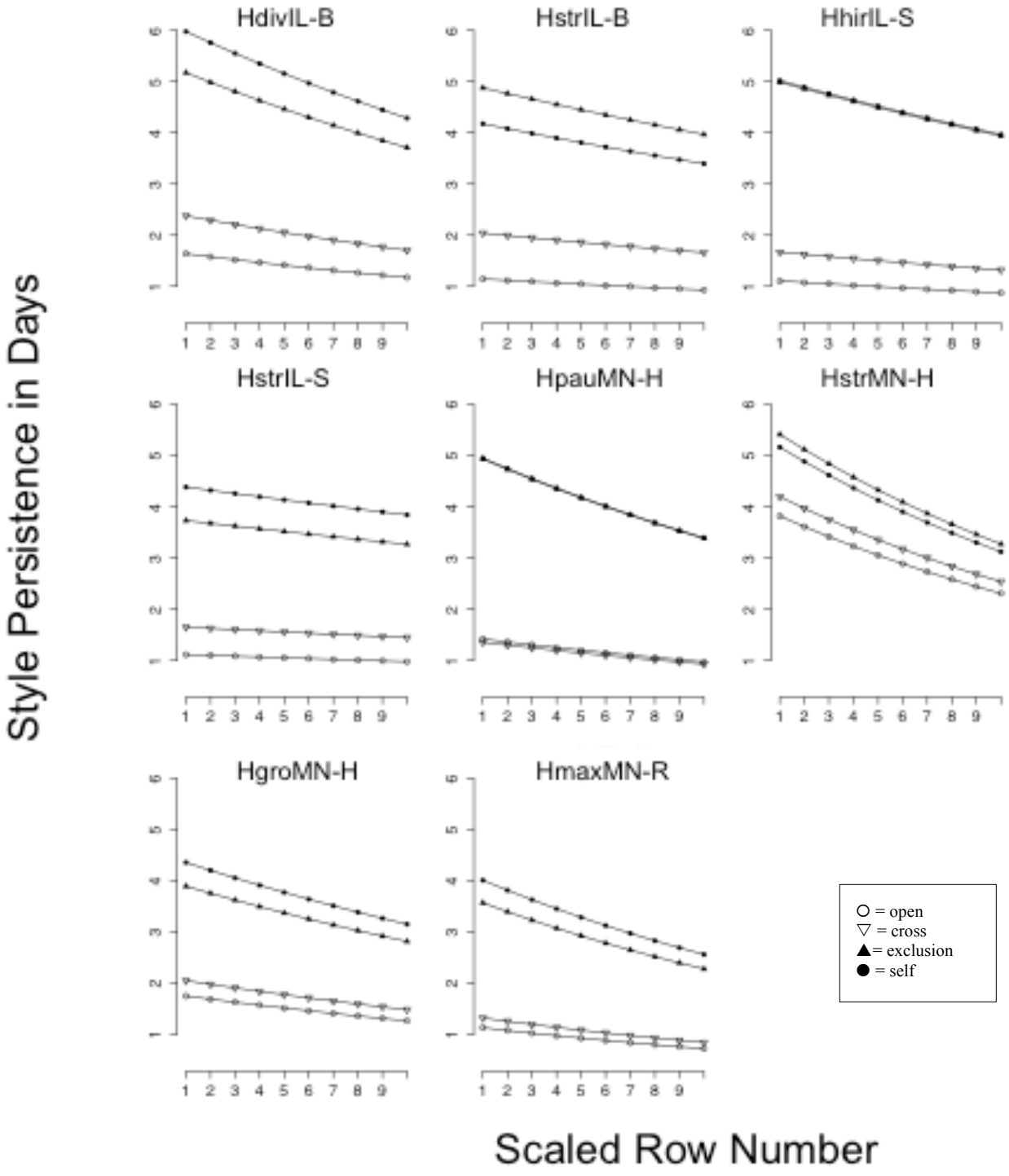


Figure 6. Style persistence for each population as predicted by the Style Persistence generalized linear model. The generalized linear model relates responses to linear combinations of continuous dependent variables. Styles will persist up to 6 days if they receive incompatible pollen or no pollen. Styles will persist up to 2.4 days if they receive compatible pollen. A row effect exists (P-value <0.0001 for all populations, except HstrMN-H which has a P-value = 0.0002; n=1580 rows); the number of days styles will persist decreases from the outer to the inner rows regardless of treatment. The outer rows may be more reliable in predicting pollen limitation. Species HstrIL-S, HstrIL-R and HhirIL-S populations show less of a row effect than the other populations.

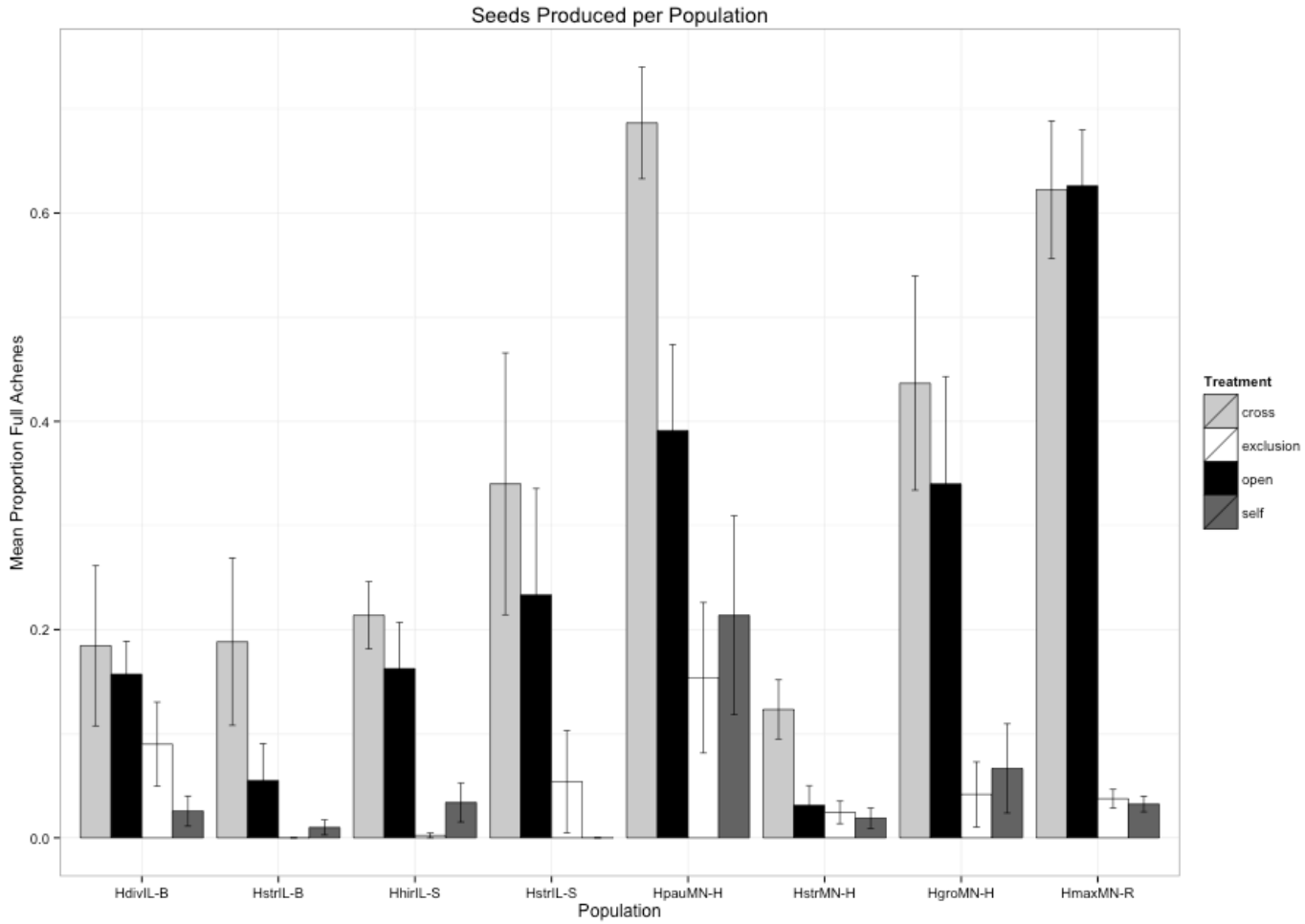


Figure 7. Mean proportion of full achenes per head in each population for each treatment. All data are means ± S. E. Cross-pollination and open-pollination treatments yielded the highest proportions in all populations. Pollen exclusion and self-pollination yielded the lowest proportions in all populations. Population 6 has the lowest proportion of full achenes in every treatment.

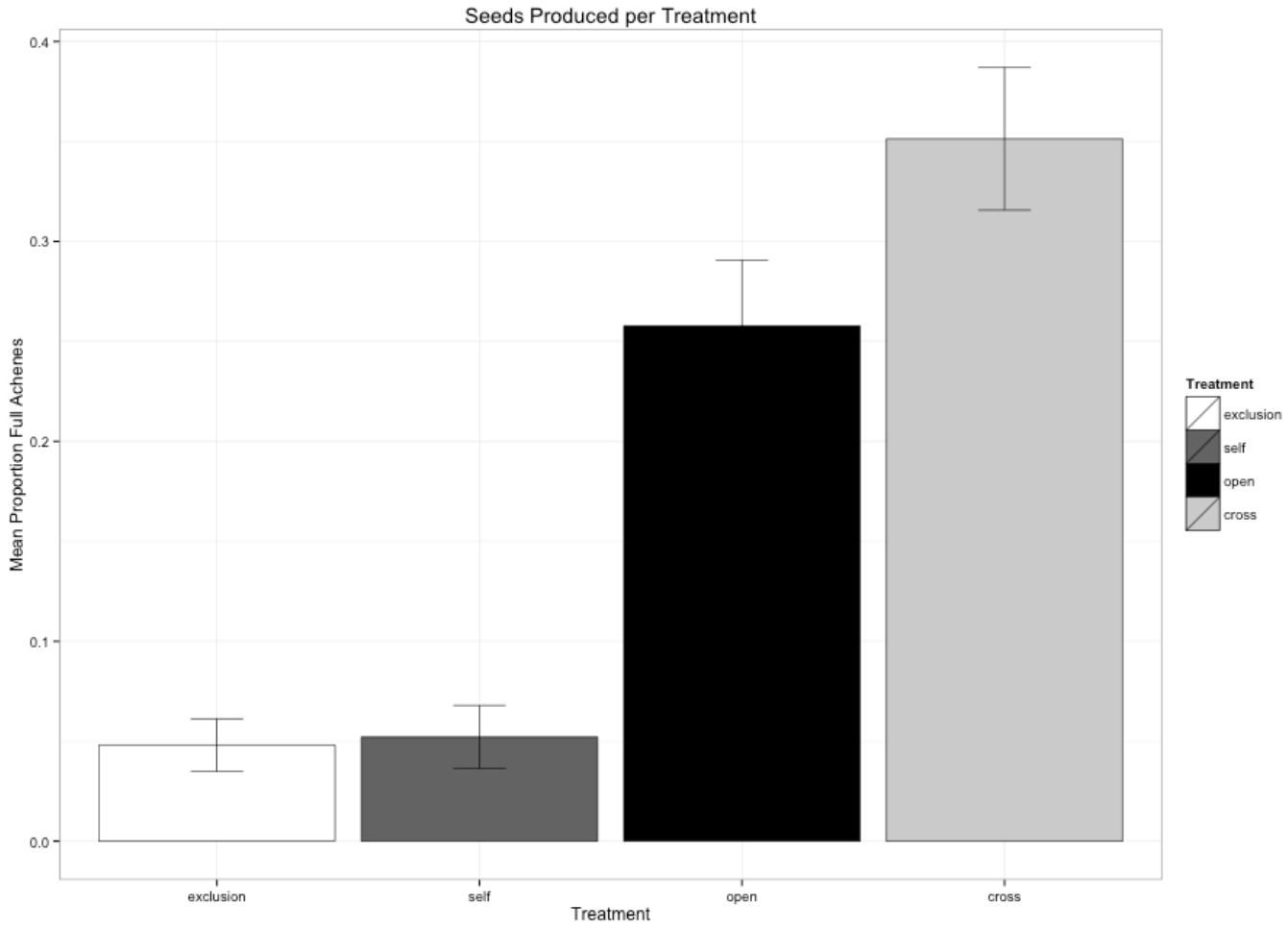


Figure 8. Mean proportion of full achenes per treatment. All data are means ± S. E. Cross-pollination and open-pollination treatments yielded the highest proportions in all populations. Pollen exclusion and self-pollination yielded the lowest proportions in all populations.

Table 1. Description of site location, size, owner and species present. Species codes in parentheses correspond to assigned identifying code for each population and indicate which were used in the study.

Site Name	Size	Latitude	Longitude	Owner	All Species Present
Berkeley Prairie	18.8 acres (7.6 ha)	42.181711	-87.831465	Lake County Forest Preserve District	*H. divaricatus* (HdivIL-B) *H. strumosus* (HstrIL-B) *H. grosseserratus* *H. x laetiflorus*
Somme Prairie Grove	85 acres (34.4 ha)	42.1405559	-87.8316429	Forest Preserve of Cook County	*H. strumosus* (HstrIL-S) *H. hirsutus* (HhirIL-S) *H. grosseserratus* *H. divaricatus* *H. pauciflorus*
Hegg Lake Wildlife Management Area	345 acres (139.6 ha)	45.782500	-95.658389	Minnesota Department of Natural Resources	*H. strumosus* (HstrMN-H) *H. pauciflorus* (HpauMN-H) *H. grosseserratus* (HgroMN-H) *H. giganteus*
Riley	< 1 acre (<0.40 ha)	45.788625	-95.7474807	Solem Township	*H. grosseserratus* *H. maximilianii* (HmaxMN-R)

Table 2. Comparison of number of inflorescences per treatment for each population with flowering duration and seed collection dates.

Population	Species	Number Open	Number Cross	Number Self	Number Exclusion	Begin Date	End Date	Harvest Date	Herbarium Accession ID
HdivIL-B	*H. divaricatus*	8	9	7	6	8 July	4 Aug	10 Sept	18168
HstrIL-B	*H. strumosus*	7	6	7	8	7 July	8 Aug	4 Sept	18169
HhirIL-S	*H. hirsutus*	7	8	9	7	22 July	10 Aug	20 Sept	18167
HstrIL-S	*H. strumosus*	6	6	5	4	24 July	4 Aug	20 Sept	18166
HpauMN-H	*H. pauciflorus*	9	9	9	8	14 Aug	27 Aug	10 Oct	18172
HstrMN-H	*H. strumosus*	9	9	9	9	15 Aug	28 Aug	8 Oct	18170
HgroMN-H	*H. grosseserratus*	6	6	6	6	14 Aug	24 Aug	10 Oct	18171
HmaxMN-R	*H. maximilianii*	8	8	8	8	15 Aug	26 Aug	10 Oct	18173

Table 3. Distances of donor populations from experimental populations. Site names and population identifiers correspond to those in Figure 3.

Population	Donor Population	Distance (meters)	Site in Figure 3
HdivIL-B	HdivIL-Bdonor	198.6	Berkeley (IL)
HstrIL-B	HstrIL-Bdonor	179.8	Berkeley (IL)
HhirIL-S	HhirIL-Sdonor	93.4	Somme (IL)
HstrIL-S	HstrIL-Sdonor	66.4	Somme (IL)
HpauMN-H(1)	HpauMN-Hdonor	252.9	Hegg (MN)
HpauMN-H(2)	HpauMN-Hdonor	207.2	Hegg (MN)
HstrMN-H(1)	HstrMN-Hdonor	276.1	Hegg (MN)
HstrMN-H(2)	Hstr-MN-Hdonor	233.5	Hegg (MN)
HgroMN-H	HgroMN-Hdonor1	344.6	Hegg (MN)
HgroMN-H	HgroMN-Hdonor2	89.9	Hegg (MN)
HgroMN-H	HgroMN-Hdonor3	55.6	Hegg (MN)
HmaxMN-R	HmaxMN-Rdonor1	45.9	Riley (MN)
HmaxMN-R	HmaxMN-Rdonor2	38.6	Riley (MN)

Table 4. ANOVA summary of the generalized linear model showing treatment affects style persistence with an additive row effect.

Population	Species	N Rows	P-value
HdivIL-B	*H. divaricatus*	184	< 0.0001
HstrIL-B	*H. strumosus*	175	< 0.0001
HhirIL-S	*H. hirsutus*	198	< 0.0001
HstrIL-S	*H. strumosus*	146	< 0.0001
HpauMN-H	*H. pauciflorus*	241	< 0.0001
HstrMN-H	*H. strumosus*	257	0.0002
HgroMN-H	*H. grosseserratus*	146	< 0.0001
HmaxMN-R	*H. maximilianii*	233	< 0.0001
All	All	1580	< 0.0001

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Appendix 1
Protocol for using Style Persistence to measure pollen limitation in perennial *Helianthus* species

Method 1: Tagging stems and heads before the disk flowers and visiting each head over multiple days

Materials:
Flag tape in different colors for different species
Permanent marker
Data sheet
Pen/pencil
Camera (optional)

In this method, you will visit each inflorescence over multiple days. Due to the rapid progression of anther and style emergence, visiting the site the site every day is recommended.

- Select individuals that have ray florets flowering or beginning to flower but immature disk florets (no anthers presenting).
- Create a unique identification code (id) for each individual stem.
    - If you have only one site, you may choose to use only numbers. Choose numbers unique to each species. Example: 1001-1999 for one species, 2001-2999 for another, etc.
    - If you have multiple sites, you may choose an identifier for the site, species and head. Example: *Helianthus divaricatus* at Berkeley Prairie could be HdivB1001-HdivB1999.
    - If you have multiple heads on one stem, use one identifier for the stem and add A, B, C, etc. per head. Example: HdivB1001A, HdivB1001B.
- Cut a length of flag tape 20-25cm and write the id code on one end
- Attach the flag tape
    - Tie one end of the flag tape to the main stem, leaving the end with the id clearly visible. Take care to avoid tying the tape too tightly around the stem. Tape should be secure but able to move slightly up and down on the stem without damaging it.
    - If you have more than one head per stem, make one flag tape id for each head. Tie the tape on the peduncle of each head.

***For each head, each day*:**
- Record the date ray flowers open. The green phyllaries that enclosed the head will no longer appear closed. The disk will be visible and the yellow rays will emerge around the disk. The rays may not be flat and spread out on the first day of flowering.
- Record the date each row of anthers emerges. The disk floret corolla will be open. Anthers will appear erect and have pollen at the top.
- Record the date styles emerge in each row. As styles emerge, they appear split into two style branches that curve away from the center.
- Record the date styles are no longer erect past the end of the anther corolla (shriveled)
- If photographing, photograph the entire disk each day. It is helpful to place a small dot on one ray flower near the disk with a permanent marker. When photographing, make sure the dot is in each photo. This can be a useful reference point when analyzing the images.

- Record the date ray flowers wilt. Ray flowers may turn brown or become curled and dry. Some fall off of the head. (The data may help in comparing SP and the timing of shriveling in the innermost rows)

At the end of fieldwork

Dispose of all flag material properly.

Assessing SP:

Styles that persist 3 days or more in the outermost rows indicate the styles have not received compatible pollen. Shriveled styles may not always indicate successful pollination. Styles may shrivel due to weather or predation, for example.

Method 2: Observing heads on one day after flowering has begun

Materials:

Data sheet
Pen/pencil
Camera (optional)

Because this is a visit on only one day, this method only relies on SP as an estimation of how long styles persist. It is potentially less conclusive than Method 1.

- Choose a head in which at least the first three rows of florets have begun to present styles. The number of heads you choose depends on the site and the size of the population. I would recommend at least choosing a sample that is at least 10% of the population.
- Record the date of observation.
- Count or estimate the number of rows of disk florets for the entire head and record.
- From outer to inner, determine and record whether rows have shriveled, are presenting styles, or presenting anthers. Assess as described in Method 1 above.
- If photographing, photograph the entire disk

- ***Assessing SP:*** If there are three or more rows of styles presenting, compatible pollen has not been received. If rows of styles are shriveled, pollen limitation cannot be concluded. Shriveled styles may not always indicate successful pollination.

Appendix 2
Sample data sheet for using Style Persistence to measure pollen limitation in perennial *Helianthus* species

Sample data sheet for using SP in *Helianthus*

Species	id	row	rayDate	antherDate	styleDate	shrivelDate	daysPersist	notes