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Research Article

Adaptive floral traits that impact stigma pollen load and seed production in *Penstemon digitalis* (Plantaginaceae)

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Penstemon digitalis is an early spring-flowering species of the northwest Missouri prairie community, a period often characterized by low and unpredictable bee visitation. We conducted an experimental field study to evaluate how pollinator-mediated pollen transfer and floral longevity interact to influence pollen acquisition and seed production. The study involved the tagging and bagging of plants and flowers to record flower phenology, flower longevity, pollen tube growth to evaluate stigma receptivity, stigma pollen loads, and single visit deposition experiments to flowers by bees to evaluate pollinator effectiveness. Our results demonstrated that flowers were not autonomous but required bee visits for successful reproduction with fertilization dependent on the flowers remaining viable for three days. Single visit deposition experiments performed using *Anthophora terminalis*, *Bombus* sp., *Ceratina* sp. and *Hoplitis pilosifrons*, resulted in the deposition of relatively few pollen grains per visit indicating that multiple visits during the two-day period of stigma receptivity were likely necessary for successful reproduction. Pollen germinated on receptive stigmas on each of the two days of receptivity and flowers exhibited a fertilization interval of an estimated 48 hours, as evidenced by pollen tube growth through the style via fluorescence microscopy. Although pollen accumulated on stigmas over the two-day period of receptivity, the pattern of seeds produced per flower remained essentially the same for stigmas pollinated on day 2 of anthesis as the combined days of stigma receptivity, day 2 plus day 3. These results suggest that prolonged stigma receptivity functions as a mechanism that safeguards against temporally variable pollinator visitation typical of early spring, more so than increasing per-flower seed production.

Keywords: female fitness, flower longevity, *Penstemon digitalis*, pollen transfer dynamics, protandry, stigma pollen load, stigma receptivity

Introduction

Flower traits are frequently under selection from visiting pollinators and result in the evolution of floral adaptations that affect how pollinators influence pollen transfer,

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either through pollen dispensing or pollen load deposition on stigmas (Macior 1967, 1970, Bell 1985, Fenster et al. 2004, Armbruster 2014, Caruso et al. 2018, Ashman et al. 2020). Flower longevity and dichogamy represent two such floral traits that are typically intertwined and heavily influence pollen transfer and consequently, the amount of pollen ultimately deposited on floral stigmas and resultant female reproductive success (Primack 1985, Marshall et al. 2010, Losada et al. 2017, Dieringer and Cabrera R. 2024).

Flowers viable over several days are typically dichogamous where sexual function is separated into distinct male and female phases across anthesis. This separation of sexual function can occur within a day or across days in longer lived flowers. An advantage of dichogamy is reduced interference of sexual function (Loyd and Webb 1986) with pollen dispensing occurring before (protandrous) or after (protogynous) stigmas are receptive. This separation in time reduces intra-floral self-deposition of pollen. Most animal-pollinated flowers are protandrous (Bertin and Newman 1993).

Protandry influences the transfer of pollen within and among flowers, thereby shaping realized mating patterns (Bell 1985, Primack 1985). Mating systems vary considerably, ranging from predominantly selfing to predominatly outcrossing and mixed mating strategies**.** These patterns result in genetically different mixes of offspring, those with longer-lived flowers, exhibiting stigmas receptive over several days and thus having an opportunity to accumulate more pollen and more diverse pollen over time (Weber and Goodwillie 2013). Studies on self-incompatible *Passiflora incarnata* (Dai and Galloway 2011) demonstrated protandry enhanced pollen dispersal while studies on self-compatible *Silene acutifolia* indicated that protandry did not prevent self-deposition but did reduce its likelihood (Buide and Guitián 2002).

Low visitation rates or unpredictable visitation rates by pollinators to flowers might be expected to lead to the evolution of longer flower lifespans, such as those found in montane flora (Primack 1985, Stratton 1989) that display a mating system that can favor autogamy (automatic self-pollination) and self-compatibility to ensure adequate pollen deposition (Wheelwright et al. 2006). However, offspring from autogamous pollen transfer would be expected to have reduced genetic variability relative to offspring formed where xenogamy (cross-pollination) predominates (Lloyd 1992) ultimately affecting plant reproductive success. For example, studies by Larson and Barrett (2000) have demonstrated that plants with autogamous mating systems display a lesser degree of pollinator limitation than those with outcrossing systems due to differences in offspring quality.

One mechanism to enhance opportunities for cross-pollen deposition on stigmas is by extending flower longevity. Extended flower longevity can contribute to prolonged stigma receptivity thereby allowing more pollen and a more diverse pollen load to accrue over the flowering lifespan (Galen et al. 1986).

For example, in *Lupinus texensis* (Fabaceae), flowers persist for six days and exhibit a banner spot color change from white to red associated with flower age. Flowers are primarily out-crossing with flowers exhibiting a banner color spot change on day five. By day six, the spot is completely red and flowers are no longer receptive to incoming pollen but are retained to aid in bee attraction (Schaal 1980). Across the first five days of stigma receptivity, pollen loads increased cumulatively, likely reflecting contributions from multiple donors under variable visitation regimes (i.e. variation in both pollinator taxa and visitation frequency).

Penstemon digitalis (Plantaginaceae) is a noted component of the early spring flora in the tallgrass prairies of Missouri and is pollinated by a wide variety of bees, ranging from *Anthophora*, *Augochlorella, Bombus*, *Ceratina*, *Hoplitis*, *Megachile*, *Osmia*, to *Xylocopa* (Clinebell and Bernhart 1998) The anthesis of early spring-flowering taxa coincides with the post-winter emergence of bees active in spring, a period during which foraging dynamics are heavily constrained by stochastic meteorological conditions (Kehrberger and Holzschuh 2019). This temporal variation can result in unpredictable visitation and increased risk of pollen limitation. Studies by Kalisz and Vogler (2003) on *Collinsia verna* (Scrophulariaceae) suggest that increased autonomous fruit production is an adaptive response for plants that experience unpredictable pollinator conditions. The consequences of unpredictable visitation to pollen deposition and resultant female reproductive success remain poorly understood.

This unpredictability in visitation may even be exacerbated by global warming causing a shift in flower phenology such as that observed for *Corydalis ambigua* (Papaveraceae) (Kudo and Cooper 2019). Studies by Kehrberger and Holzschuh (2019) on *Pulsatilla vulgaris* (Ranunculaceae) found that despite this unpredictability and low visitation by pollinators, there lies an advantage to early flowering plant species in that there is low competition for those available pollinators. Early-flowering plants still received higher pollinator visitation rates compared to later periods of time when pollinator abundance increases but more plant species are also in flower. Yet, few integrative field studies have examined the interplay between pollinator visitation, pollen transfer and consequent reproductive success (Clinebell and Bernhardt 1998, Dieringer and Cabrera R. 2002).

Theory predicts that longer lived flowers are favored when pollinator visitation is low or unpredictable thereby increasing the probability of flowers being visited (Bertin and Newman 1993, Ashman and Schoen 1996). Increased flower longevity is usually thought to favor dichogamy such as protandry and may include extended periods of stigma longevity. Protandry is therefore likely to lead to longer time periods of pollinator visitation that allow for greater quantities of pollen deposition by extending the duration of stigma receptivity while reducing interference between male and female function. This combination of protandry and extended periods of stigma receptivity may safeguard reproductive success under variable patterns of visitation by pollinator taxa and rates of visitation. Nevertheless, it remains unclear whether, in early-flowering spring systems, multi-day female function leads to cumulative pollen deposition across days; whether few pollinator visits are sufficient to saturate stigmas; and whether

variation in stigma pollen load translates directly into differences in seed production.

Penstemon digitalis is protandrous with a floral lifespan of approximately three days (Walker-Larsen and Harder 2001, Dieringer and Cabrera R. 2002), providing us an opportunity to evaluate how protandry and floral longevity interact under conditions of early spring flowering. How protandry influences the timing of pollen acquisition and subsequent pollen tube growth across flower lifespan is little studied. Pollen may accumulate early during the female-phase but pollen tube penetration and growth may be constrained over time by stigma receptivity.

We addressed four predictions derived from theory: 1) If early-spring flowering is characterized by low or unpredictable bee visitation, then flowers excluded from bee visits should display autonomously produced fruits as a mechanism of reproductive assurance. 2) If pollinator visits are infrequent in early spring, then selection should favor deposition of high pollen loads per visit sufficient to approach levels required for maximal seed production. 3) If flower longevity is a trait that buffers periods of low visitation, then flowers should continue to receive and accumulate pollen across the two days of the female phase. These two days should differ in pollen grains deposited, pollen germination, and pollen tubes growing through the style. 4) If prolonged stigma receptivity is a mechanism to allow for greater pollen accumulation and female reproductive success, then there should be a positive correspondence between stigma pollen loads and seed production.

To test these predictions, we designed a field study that included a combination of descriptive and experimental investigations. We began with documenting flower phenology, sexual phase progression of flowers, and visiting bee species, to characterize how male and female function varied over time. We then performed controlled pollination experiments to estimate the extent of autonomous versus pollinator-mediated fruit production. Single visit experiments (SVD) were used to measure pollen deposition by individual bees across 4 taxa. Experimental manipulation of days of the female phase was performed to quantify stigma pollen loads, pollen germination, and pollen tube growth across the two-day female phase (Table 1). Finally, over a three-year period, we evaluated the relationship between stigma pollen load and associated seed production (Table 1). This integrative approach allowed us to assess how floral longevity and protandry function together to influence pollen deposition and female reproductive success in early-spring conditions.

We acknowledge our study focuses on stigma pollen load and subsequent seed production and does not entail experimental study of pollen dispersal among flowers. Patterns of pollen dispersal in *Penstemon* flowers have previously been investigated in studies by Walker-Larsen and Harder (2001) and Dieringer and Cabrera R. (2002) allowing us to focus on consequences of stigmatic pollen load receipt as it relates to female reproductive success.

Material and methods

Study organism and site

Penstemon digitalis Small (Plantaginaceae), the foxglove beardtongue, is a short-lived perennial native prairie species found in the midwest and south-central United States, typically on sandy, well-drained soils. This species occurs in upland prairies, open woods, rocky cliffs, old fields, and along railways (Yatskievych 2013). Flowers are zygomorphic, pale-white, bearing a glandular pubescence. Corollas are trumpet-shaped with both the throat and petal lobes bearing faint purple lines as nectar guides. *Penstemon digitalis* does possess didynamous stamens and bears a prominent bristle staminode that lies on the lower (abaxial) side of the corolla throat. In addition, flowers bear a short, nectar producing, floral tube, and a capitate stigma.

The study took place at Nodaway County Community Lake owned by the Missouri Department of Conservation located near Maryville, MO (40°25′49.92″N, 94°51′24.54″W) over a period of years ranging from 2014 to 2020. The area is a mix of old fields, woodland, and cropland and is characterized by an abundance of *Sorghastrum nutans* (Poaceae), *Bidens aristosa* (Asteraceae), *Helianthus grosseseratus* (Asteraceae), *Chamaecrista fasciculata* (Fabaceae), *Fragaria virginiana* (Rosaceae), and *Rosa carolina* (Rosaceae)

Table 1. Set of pollination experiments for pollination study of *Penstemon digitalis* at Nodaway Lake from 2015 to 2020.

Experiment Day		Day 1	Day 2	Day 3
Pollination experiment		Flowers received natural pollination day 1 of anthesis only, bagged at end of day, 1700 hr	Flowers received natural pollination for days 1 and 2 of anthesis, bagged at end of day 2, 1700 hr	Flowers open days 1, 2, 3 of anthesis to natural pollination, never bagged
Year	Experiment	Variables measured		
2015	Stigma receptivity + Female reproductive success	Pollen deposited, pollen germinated, pollen tube growth through the style		
		NA	Pollen grains deposited on stigmas	
2017	Female reproductive success	NA	Seeds per flower	
2018 and 2020	Female reproductive success	NA	First set of plants: pollen grains deposited on stigmas Second set of plants: seeds per flower	

that also intermix with *Agalinis auriculata* (Orobanchaceae) (Dieringer and Cabrera R. 2024). Plant taxonomy follows Yatskievych (2013) and vouchers were deposited at the Missouri Botanical Garden (Dieringer and Cabrera R. 124-128; MO). Visiting bees were identified by either G. Dieringer, J. Neff, or M. Arduser; vouchers were retained in a reference collection. Statistical guidelines followed those outlined in Sokal and Rohlf (2012) and analyses performed using R ver. 4.5.2 (www.r-project.org).

Floral biology

Flowering phenology was estimated in May–June 2014, 2017, 2018, and 2020 using tagged, randomly chosen plants during May, right before flowering, to monitor number of plants in flower and open flowers over time (n = 30–35 plants). Number of plants in flower was recorded every 2–7 d (Supporting information). Once flowering started, the appearance of bees was recorded every 2–3 d over the four years of phenological data presented (Supporting information).

In 2018, we evaluated floral sexual development. Following Straw (1956), Dieringer and Cabrera R. (2002), and previously collected field data, flowers were known to be protandrous with anthers dehiscing on the first day of anthesis (day 1; male-phase) and with stigmas recurved and receptive on the second and presumably the third day of anthesis (days 2 and 3; female-phase). To estimate the duration of floral anthesis, a sample of 57 plants were randomly selected with one to three flowers per plant being tagged depending on availability (n = 101 flowers). The proportion of flowers opening in morning versus afternoon (08:00–10:00 h versus 16:00–18:00 h) were assessed for differences in frequency of morning versus afternoon flower opening categories. Studies by Straw (1956) and Dieringer and Cabrera R. (2002) have indicated that stigmas are receptive on day 2 of anthesis with stigmas recurved but do not mention specifics regarding stigma receptivity or style morphology on day 3 of anthesis. Therefore, additional characteristics noted for tagged flowers included the shape of the style-stigma (straight, curved downward, or slightly bent), and time of corolla drop. Photographs of style-stigma shapes across the lifespan of the flowers were taken using a Keyence VHX-600 digital stereoscope. Observations were carried out twice a day (morning and afternoon) or every half-day, over an eight-day period in May and June.

In 2014, hand-pollinations were performed to assess 1) the role of bee visitation in pollen transfer under natural conditions to estimate the compatibility relationships of flowers with self- and cross-pollen (breeding system). Flower buds were bagged with bridal veil and then unbagged on day 2 (first day of stigma receptivity) for hand-pollinations. Three manipulations were performed. 1) To estimate self-compatibility, pollinations were performed using pollen from flowers of the same plant, geitonogamy (n = 20 plants; n = 30 flowers). 2) To estimate cross-compatibility, pollinations were performed using pollen from male-phase flowers from different individuals at least one meter away, xenogamy (n = 23 plants; n = 31 flowers). 3) To estimate the possibility of autogamy (automatic self-deposition, assuming flowers are self-compatible), flowers were left in the mesh bag with no further manipulation (n = 21 plants; n = 21 flowers). Following each set of manipulations, flowers were rebagged until corolla drop and fruits collected later in July.

For the purpose of comparison, fruit production was estimated from flowers exposed to open-pollination or natural pollen transfer (n = 20 plants; n = 422 flowers). Resultant fruit production was analyzed using a global Fisher's exact test to determine whether differences occurred among crossing treatments. Following a significant global effect, planned pairwise comparisons among treatments were conducted.

Pollinator effectiveness experiment

Pollinator effectiveness was measured for the four most common bee visitors in 2014 and estimated as SVD. We asked: do different bee taxa deposit differing amounts of pollen on stigmas and what is the relationship between pollen deposited and resultant fruit and seed production? Flowers utilized were tagged and bagged across 11 days of the 22-day phenology and involved a total of 47 person hours of observation. Each day, SVD experiments were performed on day 2 flowers (first day female) having been previously bagged in bridal vail as buds; only one flower per plant was manipulated. Times of observation, waiting for bees to visit flowers, ranged from 08:00 to 14:00 h. Following a SVD, flowers were then re-bagged until corolla drop to prevent any further visitation, leaving styles-stigmas intact. A total of 126 flowers were manipulated with 80 ultimately receiving a bee visit (Supporting information).

Bee species included *Anthophora terminalis* (n = 24), *Bombus* sp. (n = 32), *Ceratina* sp. (n = 14), and *Hoplitis psilifrons* (n = 10). Pollen from SVD experiments was then contrasted with the total pollen accumulated on stigmas available to open-pollination during that same time period (n = 19).

To count pollen deposited on stigmas from SVD experiments, styles-stigmas were removed the following day (day 3) after corollas had dropped around 17:00 h and fixed in FAA (90: 5: 5, (v/v/v) of 70% ethanol: glacial acetic acid: 10% formalin) for one hour and then stored in 70% ethanol. Fixed stigmas were then washed in deionized water, cleared overnight in 8N NaOH, and stained with a modified Alexander's stain (Peterson et al. 2010). Styles-stigmas were split lengthwise and the number of pollen grains deposited on stigmas was counted using a compound microscope at 40×. Number of fruits produced were noted later in July for the tagged, visited flowers.

Number of pollen grains deposited by SVD was analyzed to test if SVD differed among pollinator taxa (four levels). The number of '0' counts for pollen on stigmas ranged from 16 for *Bombus* to 40% for *Hoplitis* (Supporting information) suggesting data were overdispersed.

Protandry and stigma receptivity experiment

In 2015, we designed an experiment to examine stigma pollen load for each of the three days of flower longevity and

any pollen tube growth occurring through the stigma and style. For each day of flower lifespan (days 1–3), 20 plants were randomly chosen (Table 1). Each day had a different treatment applied. Day 1 flowers received natural bee visitation before removing the styles-stigmas (n = 20 plants/one flower per plant). Day 2 flowers received natural bee visitation over days 1 and 2 before removing the styles-stigmas (n = 20 plants/one flower per plant). Day 3 flowers represent the normal floral lifespan and also received natural bee visitation (n = 20 plants/one flower per plant). Each treatment had styles-stigmas collected at 17:00 h of that day to observe pollen deposition and pollen tube growth occurring up through that day of anthesis. Styles-stigmas were fixed in a 3:1 (v/v) preservative (70% ethanol: glacial acetic acid) for one hour and then stored in 70% ethanol.

For each day of flower anthesis, we quantified the number of pollen grains deposited on stigmas, the number of pollen tubes that had penetrated the stigma and traversed approximately a quarter of the style length, and number of pollen tubes reaching the base of the style which presumably entered the ovary. Counting of pollen and pollen tubes for all three treatment groups was performed using fluorescence microscopy.

For fluorescence microscopy, styles-stigmas were fixed, washed in deionized water, cleared in 8N NaOH overnight, rinsed in deionized water, placed in a glycerin:Tris basic buffer, pH 8.4 for 2 h, counterstained with 0.05% aqueous toluidine blue for one hour, stained for 36 h in decolorized aniline blue (DAB); a 0.01% aqueous aniline blue prepared in 0.1M potassium phosphate buffer, and then mounted on a slide in 50:50 DAB: glycerin (Martin 1959, Sakai 1973, Mori et al. 2006). Styles-stigmas were imaged using a DAPI filter and photographed with an i Habanero tm-DSU Decon Spinning Disk Confocal/Deconvolution engineered on an Olympus IX81 Inverted Scope.

Experiment on reproductive success across flower lifespan

Results from the 2015 experiments on stigma receptivity then led us to conduct a series of field experiments pertaining to pollen accumulated on stigmas and seeds/flower produced over a flower's female phase (Table 1). Here we asked: how does stigma pollen load vary over the female-phase of floral lifespan and, is there consistency in pattern over years? Additionally, we asked: how does seeds per flower vary over the female-phase of floral lifespan and is there consistency in pattern over years? Data were collected over a three-year period, and different sets of plants were utilized to quantify pollen deposition and seed production across floral lifespan. We used separate sets of plants to quantify these two variables since removing styles to count pollen would interfere with pollen tube growth and subsequent seed production (Results for stigma receptivity experiments below). Unfortunately, only two of the three years of data overlap for each response variable. Adding the third, non-overlapping year of data to the analyses provided valuable insight into pattern.

We compared flowers open to natural bee pollination for day 2 flowers with those open to natural bee pollination for day 3 flowers. For pollen accumulated on stigmas, for each set of flowers, styles-stigmas were removed and fixed in FAA around 17:00 h of that day (day 2 or 3). Pollen deposited on stigmas was counted as previously described above.

For seeds produced, the experiment had flowers prepared as described above for pollen accumulated on stigmas. For each set of flowers (day 2 and 3), flowers were left intact so they could produce fruits and seeds. Day 2 flowers were bagged with bridal veil around 17:00 h to exclude further bee visits but allow time for pollen tubes to grow through styles and fertilization of ovules.

Stigma pollen load data were collected for years 2015, 2018, and 2020 as described above, while seeds produced measured as seeds/flower were collected in years 2017, 2018 and 2020. For consistency over time, experiments were conducted during periods of peak flowering for the population, typically during the first week of June.

Data analysis

To evaluate the proportional differences of fruit production between crossing treatments and open-pollinated flowers, we initially analyzed the data using generalized linear (mixed) models with both binomial and betabinomial error structures. However, these models exhibited complete (or near-complete) separation, because autogamous crosses produced no fruits while open-pollinated flowers produced a high proportion of fruits, preventing reliable estimation of model parameters. Consequently, differences among treatments were evaluated using Fisher's exact tests on aggregated counts of flowers fruiting or not fruiting. We applied a global test across all crossing categories followed by pairwise comparisons using Holm adjusted p-values which controls family-wise error rate. While this approach does not explicitly model plant-level non-independence, the strong treatment effects and limited replication per plant make it a robust and appropriate alternative for testing differences in proportional fruit production.

SVD experiments consisted of overdispersed count data ($\theta = 0.48$). We then fit a negative binomial (GLM) (log link) using the R package 'MASS' with pollinator taxon included as a fixed effect.

To evaluate differences among pollinator taxa in SVD we estimated marginal means using the 'emmeans' package. Pairwise comparisons among taxa were conducted using Tukey-adjusted p-values. Means (± SE) were then back-transformed to the response scale to facilitate interpretation and to provide effect size estimates. This test quantifies differences among pollinator taxa in number of pollen grains deposited by SVD experiments. A general inference regarding number of visits required for observed stigma pollen load from natural bee visitation was then inferred separately by simple observation of the relationship between pollen deposition from SVDs.

For stigma receptivity data, number of pollen grains deposited on stigmas and pollen tubes growing through

the style were analyzed using a negative binomial GLM as described above for the SVD experiment since both variables were overdispersed; for pollen on stigma ($\theta = 0.45$) and for pollen tube growth through the top quarter of the style ($\theta = 0.45$). We used the R package 'MASS' to estimate fit of the model and evaluated differences among days of floral lifespan for pollen deposited on day 1, day 2, and day 3 whereas difference in pollen tubes growing through the top quarter of the style was evaluated for only day 2 and day 3 as no pollen tubes penetrated into the style on day 1. Pollen grains deposited and pollen tube growth were response variables with days across anthesis as a fixed effect. Estimated marginal means were calculated using the 'emmeans' package (R ver. 4.5.2; www.r-project.org). Pairwise comparisons among taxa were conducted using Tukey-adjusted p-values for pollen deposited on stigmas across the three days but no Tukey-adjustment was needed for comparing pollen tube growth across just two days.

Stigma pollen load and seeds/flower were analyzed separately because they were measured on different sets of plants and not all years were shared between datasets. For each response variable, we fit a generalized linear mixed-effects model with day of anthesis (day 2 versus day 3) as a fixed effect and Year as a random effect to account for interannual environmental variation. The fixed effect of day was evaluated using likelihood-ratio tests comparing models with and without the day term. For models with two levels of day, no multiple-comparison adjustment was required. Analyses were performed in R (ver. 4.5.2; www.r-project.org) using the 'glmmTMB' and 'emmeans' packages allowing us to incorporate random effects of year.

Results

Floral biology

The flowering phenology of *P. digitalis* at Nodaway Lake showed that, while varying over years, the plants typically initiated flowering in mid-May and continued into mid- to late-June (Fig. 1). The earliest date to initiate flowering was 15 May 2018 and the latest date to end flowering was 30 June 2020. Peak flowering typically occurred in early June with fruits maturing in mid- to late-July.

Bees typically emerge first in wooded areas which have earlier flowering plants than local meadows as previously documented by Mola et al. (2021). Locally, bumble bees emerge from overwintering and begin to forage in mid- to late-April with early favorites being *Ribes missouriense* and *Dicentra cucularia* found in wooded areas (G. Dieringer unpubl.). Bees began foraging on *P. digitalis* as soon as plants initiated flowering (Fig. 2) with bumble bees typically exhibiting a low visitation frequency at first but increasing substantially by peak flowering. For *P. digitalis*, a total of 22 bee species were recorded visiting flowers. Their earliest noted date of appearance is presented in supporting information.

Flower anthesis data on sexual development indicated that flowers opened progressively over the day. Our data indicated

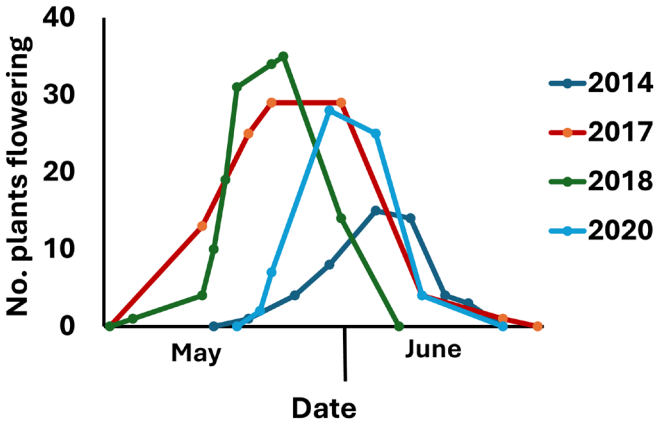


Figure 1. Flowering phenology of *Penstemon digitalis* at Nodaway Lake documented over a period of years including 2014, 2017, 2018, and 2020. Flowering started in mid- to late-May and continued into mid- to late-June.

plants differed little in the proportion of flowers opening in the morning versus afternoon (08:00–10:00 h versus 16:00–18:00 h) with approximately 46 of the flowers open in the morning and 54% open in the afternoon. For consistency we only worked with flowers opening in the morning (07:00 – 08:00 h).

While it was not uncommon for corollas to drop before 17:00 h on the third day, the styles-stigmas tended to remain white and turgid through 17:00–18:00 h. There was, however, a notable change in the shape of the stigma-style across the three-day lifespan (Fig. 3). On day 1, male-phase, the style-stigma was very straight and forward projecting (Fig. 3A). On day 2, first day female, the style-stigma exhibited a strong downward curvature as previously noted by Straw (1956) (Fig. 3B). On day 3, second day female, the style-stigma had straightened to a large extent but still exhibited a noticeable bend or nod (Fig. 3C).

A global test for differences in proportional fruit production from hand-pollination crosses in 2014 indicated a significant difference across treatments ($p < 0.0001$; Fisher's exact test, Table 2). Flowers did not experience autonomous self-pollination. Fruit production did not differ between self- and cross-pollinated flowers ($p = 0.46$; Fisher's exact test) with flowers expressing a high degree of self-compatibility (70%) and most open-pollinated flowers producing fruit (85.1%).

Pollinator effectiveness experiment

Bee taxon significantly affected pollen deposition overall ($\chi^2 = 8.22$, $df = 3$, $p = 0.042$), but Tukey-adjusted pairwise comparisons did not detect significant differences between individual taxa ($p > 0.08$ or greater). Estimated mean deposition varied between 4 to 13 pollen grains deposited for a single bee visit to a flower (Table 3). Although no specific pairwise differences were statistically supported after Tukey p-adjustment, effect sizes suggest that *Bombus* tended to deposit more pollen per visit than other taxa.

Of particular note, flowers utilized in SVD experiments did not produce any fruit, indicating that excising stigmas

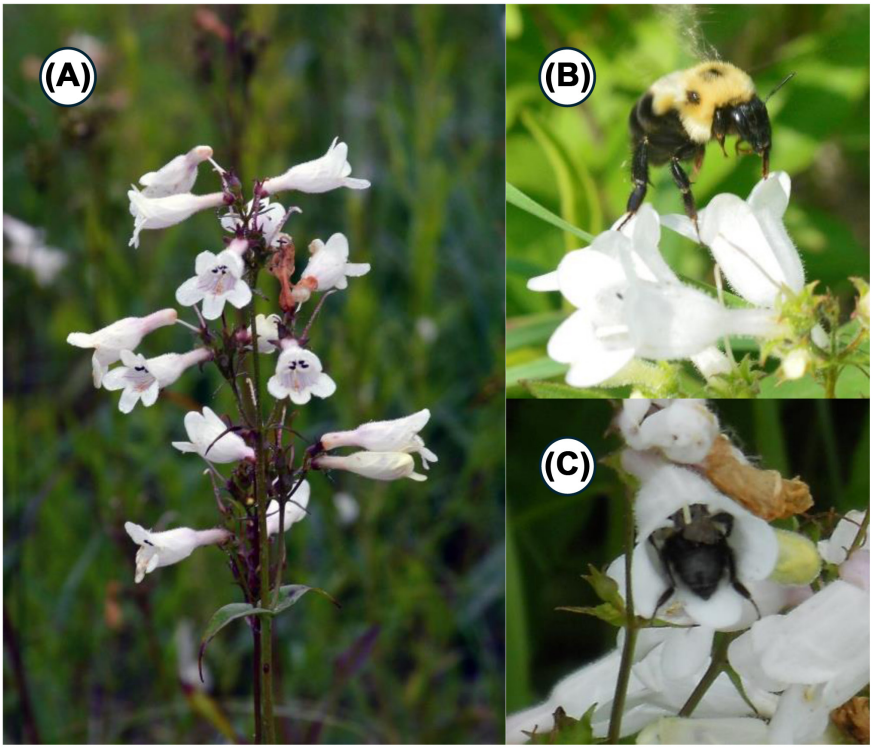


Figure 2. *Penstemon digitalis* flowers and visiting bees. (A) Inflorescence of *P. digitalis,* (B) *Bombus bimaculatus* visiting *P. digitalis* flower, (C) *Bombus impatiens* visiting *P. digitalis* flower showing the position of a day 2 receptive style-stigma. Photos taken using a Nikon 3200 DSLR camera.

at 17:00 h on day 3 of anthesis, after corolla drop, interrupts fertilization, or that pollen deposited by SVDs were below the minimum threshold to stimulate fruit development. This indicates that pollen deposition could not reliably be associated with fruit or seed production for the same manipulated flower. Hence, later experiments on stigma pollen load and seed production would require separate sets of plants (presented below).



Figure 3. Shape of style-stigmas of *Penstemon digitalis* across the floral lifespan. (A) Style-stigma of day 1 flowers or male-phase flowers when anthers dehisce. Style-stigma is straight; (B) style-stigma of day 2 flowers or first day female-phase flowers when stigmas first become receptive to pollen. Style-stigma is strongly recurved; (C) style-stigma of day 3 flowers or second day female-phase flowers when stigmas remain receptive. Style-stigma is only slightly recurved or bent downward. Image was taken with a Keyence VHX-600 digital stereoscope.

Table 2. Hand-pollination experiments to estimate the compatibility system and the likelihood of autonomous pollination for *Penstemon digitalis* compared with proportional fruit production from open-pollinated flowers at Nodaway Lake in 2014. Pairwise contrasts were evaluated using Fisher's exact tests combined with Holm adjusted p-values; all significant comparisons were $p < 0.02$ or less. Proportions sharing the same letter were not significantly different. A global test for differences between all crossing categories was significant ($p < 0.0001$; Fisher's exact test).

Cross	N_{plants}	$N_{flowers}$	N_{fruits}	% fruit
Autogamy	21	21	0	0^{A}
Hand-selfed	20	30	21	$70^{B,C}$
Hand-outcrossed	23	32	20	62.5^{B}
Open-pollination	20	422	359	85.1^{C}

Protandry and stigma receptivity experiment

Pollen deposition on stigmas varied significantly across floral lifespan ($\chi^2 = 44.92$, $p < 0.001$; likelihood-ratio test; Table 4, Fig. 4A). Pollen deposited on day 1 did not germinate or produce pollen tubes. Model-based estimated means for pollen increased from day 1 to day 2 and day 3. Tukey-adjusted contrasts indicated that pollen deposition for both female-phase days exceeded day 1 ($p < 0.001$). Although mean deposition on day 3 was more than twice that of day 2, this difference was not significant after Tukey adjustment ($p = 0.192$) due to substantial among-plant variation in pollen deposition. Therefore, we lack sufficient evidence to conclude they differ.

Pollen tube growth through the top quarter of the style also increased across days of female-phase ($\chi^2 = 3.93$, $p = 0.047$; likelihood-ratio test; Table 4). Day 3 flowers exhibited 2.3 times more tubes than day 2 flowers ($p = 0.044$).

It is noteworthy that while pollen continued to accumulate over the two days of the stigma receptivity, pollen tubes did not grow completely through the style on day 2 (Fig. 4B–C) but required the two days of the female-phase to reach the base of the style and presumably enter the ovary (Fig. 4D).

Table 3. Model-based predicted means (± SE) for pollen grain deposition on stigmas and resultant percent fruit production from SVD experiments to flowers of *Penstemon digitalis* at Nodaway Lake in 2014. No SVD cross resulted in fruit production. Effect size for each bee taxon relative to *Anthophora* is presented. Pollen deposition and percent fruit production from open-pollination by bees are presented for comparison but not included in any statistical comparison. $\chi2 = 8.22$, $df = 3$, $p = 0.042$ for comparison of differences in pollen deposition from SVDs among bee taxa. Post hoc comparisons: Tukey-adjusted pairwise contrasts showed no significant differences among taxa ($p > 0.08$ or greater). *Bombus* species included *B. bimaculatus*, *B. griseocollis*, and *B. impatiens*. *Ceratina* species included were *C. calcarata*, *C. milkmaqi*, and *C. strenua*.

Bee taxa	n	Pollen deposited	Effect size	% Fruit
Anthophora terminalis	24	4.92 (±1.52)	1.0 (reference)	0
Bombus sp.	32	12.97 (±3.37)	2.64	0
Ceratina sp.	14	7.57 (±3.01)	1.54	0
Hoplites pilosifrons	10	3.70 (±1.79)	0.75	0
Open-pollination	19	119.2 (±21.93)	–	52.6

Experiment on reproductive success across flower lifespan

The previous experiment evidenced a two-day delay from pollen deposition to pollen tubes reaching the base of the style, hence, separate sets of plants were used for this set of experiments (Fig. 5, Supporting information). This reality imposed a constraint on the design of our experiments and no direct relationship between pollen deposition and seeds per flower can be inferred. Instead, we compare patterns of similarity across floral lifespan.

Pollen deposition on stigmas increased significantly between the two days of stigma receptivity. A negative binomial GLM test indicated a significant effect of day on stigma pollen load ($\chi^2 = 12.86$, $p = 0.0004$; likelihood-ratio test). Across the three years of study, the comparison of pollen deposition between day 2 and day 3 revealed substantial differences (rate ratio = 0.49; $z = -3.71$, $p = 0.0002$) with an estimated mean (± SE) pollen deposition of 86.8 (± 18.0) and 175.6 (± 36.4) respectively. Fig. 5A). These results suggests a continuous pollen accumulation across the flower lifespan.

Seed production, in contrast, did not vary across the two days of the female-phase portion of the flower lifespan (rate ratio = 0.99, $z = -0.08$, $p = 0.94$). Across the three years of study, model-based estimated means (± SE) for seed production were similar between days of stigma receptivity with 86.2 (± 12.4) seeds per flower produced for day 2 and 87.6 (± 12.5) seeds per flower produced for day 3 (Fig. 5B). This pattern contrasts with that of stigma pollen loads across the two days of the female-phase portion of the flower anthesis.

Discussion

Penstemon digitalis is a member of the early spring-flowering, tall-grass prairie in northwest Missouri (Clinebell and Bernhardt 1998). Flowers were short-lived, lasting approximately three days and were protandrous. On the first day of anthesis, anthers dehisced presenting pollen to visiting bee while stigmas were not yet receptive. The following two days represent the female-phase of anthesis with stigmas receptive and accumulating pollen across both days.

Flower longevity favors dichogamy (Schoen and Ashman 1995) where flowers have male and female function separated in time. The separation of sexual phases in time thus aids in minimizing interference of pollen transfer dynamics associated with pollen dispersal and stigma deposition within the same flower. Contrary to our expectation, bagged, unmanipulated flowers produced no fruit, indicating that seed production depends on pollen transfer by floral visitors. Combined with the absence of autonomous self-pollination and consequent requirement for bee visitation, a high proportion of outcrossed offspring would be expected. This would allow for

Table 4. Model-based means (± SE) for pollen grains and pollen tube growth through the style for *Penstemon digitalis* at Nodaway Lake across three days of flower viability in 2015; n=20 for days 1, 2, and 3, respectively (one flower per plant). Means (± SE) for pollen tubes reaching the base of the style represent actual counts and were not included in any statistical analysis as no pollen tubes reached the style base for day 1 and day 2. Means sharing the same letter were not significantly different. For pollen deposition, rate ratio contrasts were day 2/day 1=20.8, z=−6.33, p < 0.0001; day 3/day 1=47.2, z=−8.03, p < 0.001; day 3/day 2=2.26, z=−1.74, p=0.19. For pollen tubes reaching through the top quarter of the style, the rate ratio contrast was day 3/day 2=2.30, z=2.10, p=0.04

	Day 1		Day 2		Day 3	
Location	No. grains or pollen tubes	SE	No. grains or pollen tubes	SE	No. grains or pollen tubes	SE
Stigma (pollen deposition)	5.45[A]	1.88	113.50[B]	37.80	256.70[B]	85.30
Upper style (pollen tubes)	0	0	68.0[A]	20.0	157.0[B]	45.90
Base of style (pollen tubes)	0	0	0	0	96.1	14.10

variation in quantity and quality of seeds on an individual plant (Bertin and Newman 1993).

Flowers were visited by a diverse assemblage of bee taxa with only small, non-significant differences in SVD detected among bee taxa (Table 3). Contrary to our expectation, pollen from SVD was notably lower in quantity than pollen accumulated on stigmas from open-pollination during the female-phase portion of anthesis. SVD values ranged from 3.1 to 10.9% of the mean stigma pollen load across the female-phase portion of floral anthesis. Although these results suggest that repeated visitation contributes to pollen accumulation, our data did not allow direct estimation of the number of visits required to achieve full seed production because pollen-to-seed conversion was not explicitly evaluated. At first, these data suggest the two-day period of stigma receptivity might seem to be a response to low pollen deposition by bee visits with longer periods of stigma receptivity to allow for stigma saturation (below).

Flower longevity is typically considered an adaptation to allow for pollen accumulation during times of low or inadequate pollinator visitation (Primack 1985). Experiments on stigma receptivity supported the progressive accumulation of pollen on stigmas over the two-day period of stigma receptivity, but this duration appears necessary for more than just pollen collection. It also allows time for pollen tubes to grow through the style, meaning fertilization likely requires two days. Extended receptivity for pollen accumulation aligns with Castro et al. (2008) work on *Polygala vayredae* (Polygalaceae), however, their study did not consider the additional time required for pollen tube growth.

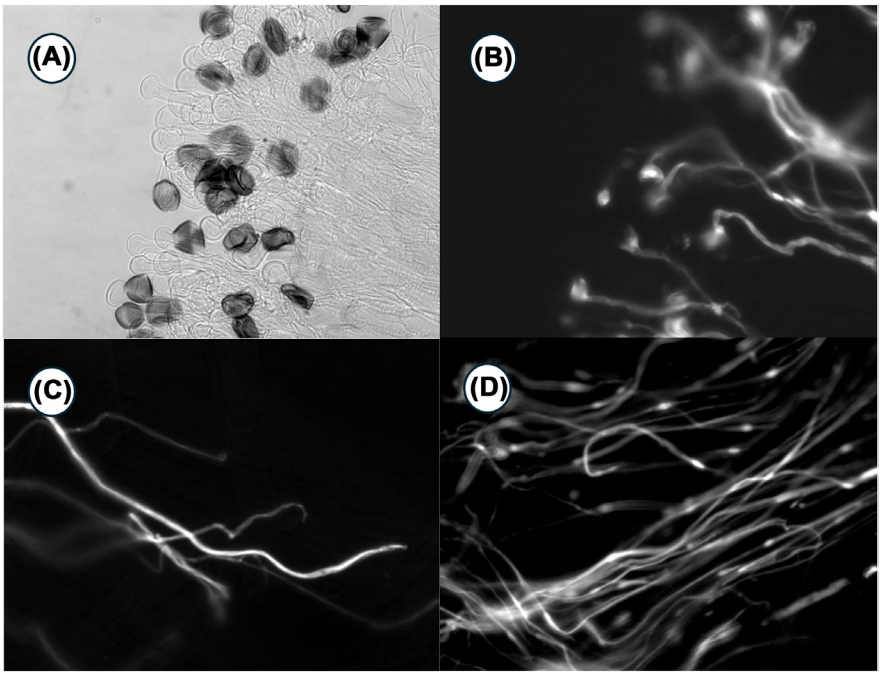


Figure 4. Pollen and pollen tube growth through stigmas and styles of *Penstemon digitalis* documented across floral lifespan. (A) Ungerminated pollen on the stigma of day 1 flowers; male-phase flowers; (B) germinating pollen grains and pollen tubes growing through the style of day 2 flowers; first day female-phase flowers; (C) pollen tubes growing through styles of day 2 flowers illustrating pollen tubes not yet having traversed the entire style; (D) pollen tubes of day 3 flowers; second day female-phase flowers, showing pollen tubes at the terminus of the style and presumably penetrating the ovary.

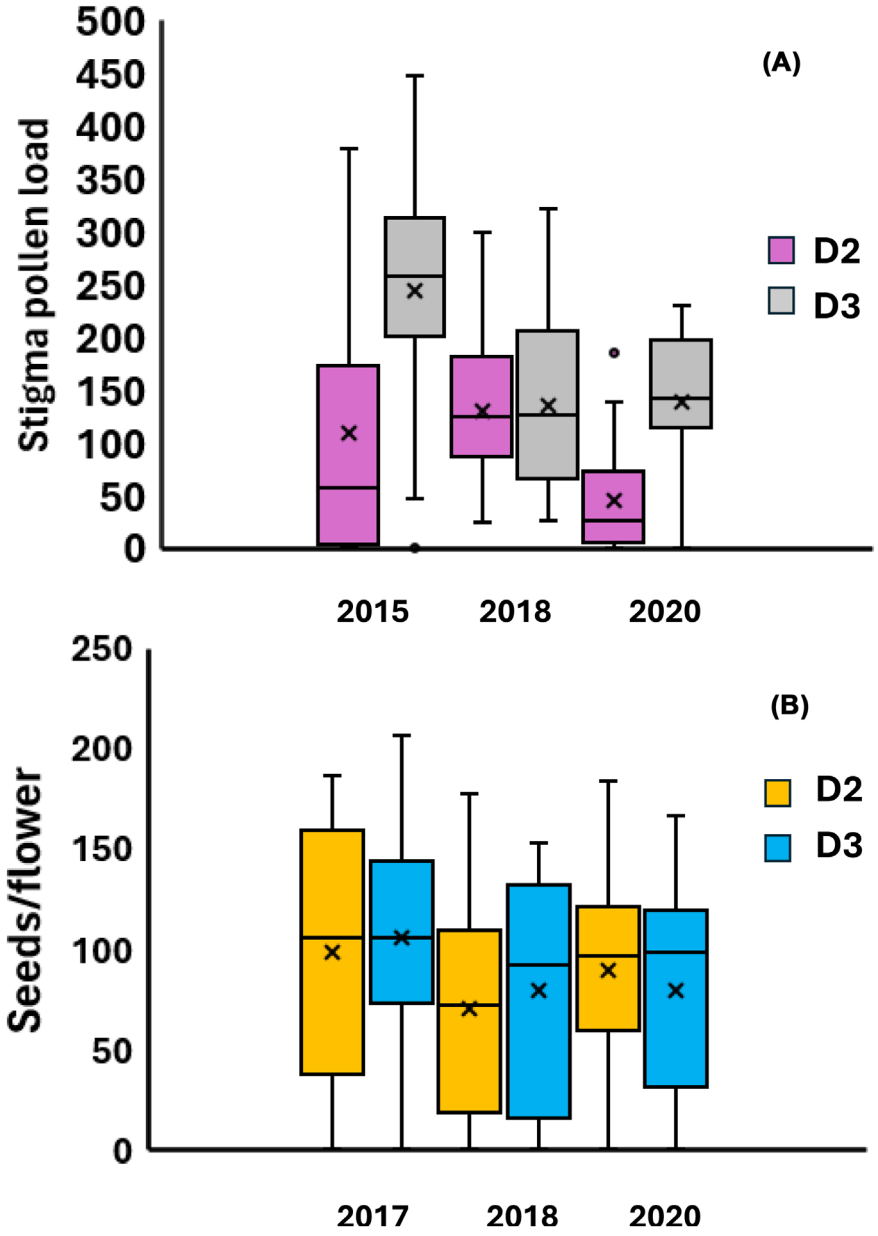


Figure 5. Boxplots for response counts of pollen stigma load and seeds per flower produced by flowers exposed to open-pollination by bees. Day 2 flowers were exposed to open-pollination only on day 1 and 2 of anthesis whereas day 3 flowers were exposed to open-pollination over the three consecutive days of anthesis. (A) Pollen stigma load on day 2 and 3 flowers of anthesis; (B) seeds per flower produced by day 2 and 3 flowers of anthesis. D2 represents day 2 flowers and D3 represents day 3 flowers.

A two-day period of stigma receptivity might be expected given early spring flowering and unpredictability of bee visitation to flowers, the second day being necessary for sufficient stigma pollen saturation leading to a full complement of seeds. Our results across three years demonstrate that day of stigma receptivity had an effect on pollen accumulation, with day 3 accumulating significantly more pollen than day 2 flowers (Fig. 5A, Supporting information) which met our expectations.

Unexpectedly, seed production, measured as seeds per flower did not differ between day 2 and day 3 of flower anthesis in contrast to the pattern observed by stigma pollen load accumulation. We consider this our most interesting finding of the study. It would appear stigmas were pollen-saturated after day 2 with exception of year 2018 when little difference in pollen deposited on stigmas across the two days was recorded.

We suggest future research focus on offspring quality for seeds produced on day 2 versus day 3 of the female-phase portion of anthesis. Variation in offspring quality has been suggested by Christopher et al. (2020) when plants are not pollen limited. Male reproductive success should then be

reflected by pollen abundance on stigmas and differential siring success evident among seeds within a fruit.

In early-spring flower plants such as *Penstemon digitalis*, protandry and extended longevity and associated stigma receptivity may function primarily to increase the probability of successful pollination under variable visitation conditions, rather than increase seed production once stigmas have received adequate pollen deposition. By linking patterns of pollen deposition, pollen tube growth, and seed production across anthesis, this study provides a mechanistic perspective on how protandry and floral longevity interact to influence fertilization timing and reproductive success.

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Author contributions

Gregg Dieringer: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (lead); Resources (equal); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). **Leticia Cabrera R.:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Funding acquisition (equal); Investigation (equal); Methodology (equal); Resources (supporting); Visualization (supporting); Writing – review and editing (equal).

Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.xpnvx0kwt (Dieringer and Cabrera R. 2026).

Supporting information

The Supporting information associated with this article is available with the online version.

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