

**POLLINATION ECOLOGY IN *PENSTEMON*:
MECHANISMS OF REPRODUCTIVE ISOLATION IN
PENSTEMON VIRENS AND *PENSTEMON SECUNDIFLORUS***

**By Kelsey Beckrich
Department of Ecology and Evolutionary Biology, University of Colorado at
Boulder**

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**Thesis Advisor and Department: Dr. Erin Tripp
Ecology and Evolutionary Biology**

**Defense Committee Members and Departments:
Dr. Erin Tripp, Ecology and Evolutionary Biology;
Dr. Barbara Demmig-Adams, Ecology and Evolutionary Biology;
Dr. Joanna Lambert, Anthropology**

Abstract

Two distantly related species of *Penstemon*, *Penstemon virens* and *Penstemon secundiflorus* frequently flower contemporaneously throughout an overlapping range in the foothills and Front Range of Colorado. In cases where populations are sympatric (co-occurring) and hybrids do not form, non-geographic and non-temporal boundaries must be present that limit or prohibit gene flow between species. I examined two possible contributors to such boundaries, pollinator activity and floral traits, through a field pollination experiment in the foothills of central Colorado in the summer of 2016 and found that both species exhibit distinctive floral traits that may affect both pollinator behavior and mechanical compatibility. Field collection of floral visitors (totaling 68 individuals) indicated that only five bee species from two genera (*Lasioglossum* and *Hylaeus*) were shared between *P. virens* and *P. secundiflorus*, suggesting that pollinator behavior contributes to limiting gene flow between these two species. Thus, the limited overlap in pollinator species and floral morphology likely severely limit the capacity for hybridization between these two species of *Penstemon*. Finally, I found that floral traits could not be used to predict floral visitors in a way that is consistent with prior research on pollination syndromes in the genus of *Penstemon*. Further field research in pollination ecology is needed to assess pollinator behavior in response to the variety of floral traits exhibited in the genus and to assess the fitness and life history of these two iconic flowering-plant species of the Colorado foothills.

Introduction

Penstemon (Plantaginaceae) represents a diverse genus consisting of approximately 270 species whose distributions span much of North America and portions of Central America (Wolfe et al., 2006). Species of *Penstemon* are particularly abundant in, and sometimes endemic to, a diversity of habitats across the western United States (Wolfe et al., 2006; K. Beckrich, pers. obs.). Within Colorado, several species of *Penstemon* are sympatric throughout mixed grass prairies, foothills, and montane ecosystems and flower as early as May or as late as September, as shown from publicly available records at SEINET (<http://swbiodiversity.org/seinet>). Across this range, species of *Penstemon* may associate with hummingbirds or several different insect families and tend to flower during times when pollinators are most actively seeking food (Wilson et al. 2006; Wolfe et al., 2006). Thus, the combination of species range, species abundance, and diversity of floral visitors establishes *Penstemon* as a substantial food resource to pollen- and nectar-foraging animal visitors (Wilson et al., 2006).

Where species of *Penstemon* are sympatric, they have the potential to form hybrid zones wherein pollen is both transferred and compatible between co-flowering species and viable offspring are produced. Hybrid zones have been documented between closely related species (see Wilson and Valenzuela, 2002), and hybridization is suspected to have contributed speciation events both in the genus of *Penstemon* (Wolfe et al. 2006) as well as flowering plants on a broader scale. However, most species of *Penstemon* have never been studied from a perspective of potential hybridization (see Kimball, 2008; Lindgren and Schaaf, 2007; Wilson and Jordan, 2009; Wilson and Valenzuela, 2002). This absence of research is particularly glaring for naturally occurring populations (vs. those used for horticultural purposes) and for more distantly related species of *Penstemon*.

When species of *Penstemon* co-occur and do not form hybrid zones, isolating mechanisms must be present that prevent gene flow or the development of viable offspring and represent a case of reproductive isolation (RI). Reproductive isolation refers to genetic fidelity within a population or species and results in a distinct evolutionary trajectory. Reproductive isolation is a fundamental step in speciation events and can occur via geographical, temporal, ethological, or mechanical mechanisms (Grant, 1994). For sympatric populations, ethological (i.e. differences in pollen vectors determined by sensory and behavioral attributes among those animal species in response to floral traits) and mechanical (i.e. differences in flower size, shape, or pistil length; see Grant 1994) are among the most important mechanisms maintaining RI. However, there have been few studies exploring RI between sympatric species of *Penstemon* (see Chari and Wilson, 2001), although it has been speculated that pollinator-driven selection for distinct traits has promoted reproductive isolation and repeatedly contributed to speciation events in this genus (Wolfe et al, 2006).

Pollinators drive selection for a variety of floral traits, such as color, shape, size, inclination, reproductive organ length, nectar production, and nectar replenishment (Wessinger and Hileman, 2016). It has been shown that flowers evolve deterministically in response to specific pollinator taxa (such as birds or bees) or functional groups (i.e. similarly functioning species such as long-tongued or large-bodied insects; see Fenster et al., 2004; Wilson et al. 2004). The deterministic nature of these selective pressures leads to plants producing an array of traits corresponding to their pollinators in a process known as pollinator specialization. This results in predictable pollination syndromes wherein a suite of floral traits can be used to predict pollinator taxa or functional group. The relationship between floral traits and pollinator behavior has been the subject of extensive research for some species of *Penstemon*, particularly those that

are bird-pollinated or bee-pollinated (Castellanos et al., 2004; Wessinger and Hileman, 2016; Wilson et al. 2004). Species of *Penstemon* pollinated by birds typically produce abundant nectar; have long, tubular corollas; and exhibit colors that deter bee-visitation while bee-pollinated species tend to have small, blue or violet flowers, stark color contrasts around the floral throat known as nectar guide, and produce either infrequent of low volumes of nectar (Figure 1; see Bergamo et al., 2016; Castellanos et al., 2004; Wilson et al., 2006).

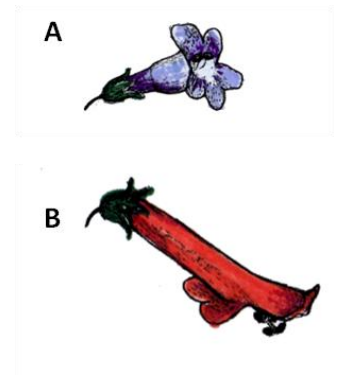


Fig.1. Illustrations of the two most common pollination syndromes in the genus of *Penstemon*. A: A characteristic bee-syndrome phenotype. B: A characteristic bird-syndrome phenotype.

However, only a few of the approximately 270 species of *Penstemon* have been targeted for research on pollination syndromes, and these macroevolutionary studies focusing on pollination syndromes often overlook population-level dynamics in the field, such as species of pollinators, pollination frequencies, and other behavioral movements. The present study addresses these oversights by exploring mechanisms of RI in two distantly related (see Wolfe et al., 2006) but frequently sympatric species of *Penstemon*, *P. virens* and *P. secundiflorus*, through a field-oriented study in pollination ecology. Despite extensive field observations and a broad zone of species co-occurrence, very few putative hybrids have been seen in nature, particularly at the present study site (K. Beckrich, pers. obs.). Consequently, the present study focuses on ethological and mechanical mechanisms of isolation potentially limiting or preventing hybridization events. The hypotheses were as follows: (1) that differences between flower morphologies of *P. virens* and *P. secundiflorus* limit transfer of pollen across species in a manner that results in mechanical isolation, (2) that either *P. secundiflorus* or *P. virens* has evolved in response to a specific group or groups of pollinators in a manner that results in ethological

isolation, (3) and that if mechanical and ethological barriers are overcome, one or both species will produce seeds from an interspecific cross. This species pair represents an excellent natural study system to investigate the accuracy of pollination syndromes based on floral traits and broader evolutionary questions regarding the significance of hybridization or pollinator specialization in the evolutionary history of the genus.

Materials & Methods

Study Site: Field work occurred between June 14-20, 2016 on 40 acres of privately owned farmland that had been unaltered by agriculture for a minimum of five years (elevation: 2160 meters, coordinates: 39°9'38.568"N, 104°33'27.521"W). *Penstemon virens* and *Penstemon secundiflorus* were identified by Dr. Erin Tripp and individuals from each species were pressed and submitted as permanent voucher specimens to the University of Colorado Herbarium. Populations of *P. virens* and *P. secundiflorus* exhibited noticeably uneven abundances; *P. virens* were frequent across the landscape and plants were dense within each population. Populations of *P. secundiflorus* were comparatively sparse, with clusters of two or three individuals each scattered intermittently across the landscape. Consequently, field ecological study sites were structured to include *P. secundiflorus*, which almost always grew within a meter or less of *P. virens*. Three different study sites for the observation and collection of floral visitors were sectioned off in 3 x 3 meter plots, each of which consisted of > 50 *P. virens* individuals and < 5 *P. secundiflorus* individuals. Strategies for data collection to address the research question are organized into three subsections, *Floral Traits*, *Floral Visitors*, and *Bagged Experiments*.

Floral Traits. To assess the potential for mechanical barriers in driving RI, floral traits associated with reproductive isolation and pollination syndromes (i.e., floral color, nectar

production/nectar volume, and floral morphology) were recorded and compared between both focal species. Floral color, indicative of pollination syndrome, was not quantified via spectroscopy, but the relative coloration of the focal species was observed and compared to species of *Penstemon* known to exhibit either a bee-syndrome or a bird-syndrome.

Nectar volume, also indicative of pollinator specialization, was surveyed daily between 07:00 hours and 09:00 hours from June 15-19, 2016 (note: nectar surveys were conducted prior to insect activity to reduce the chance of sampling depleted flowers). Nectar volume was measured by inserting 5 μ l pipettes into the corolla until they reached the nectary, whereupon any present nectar was withdrawn and the volume recorded. Multiple corollas from several inflorescences were sampled per individual per species per day.

As floral morphology yields insights into pollinator specialization and comparison of floral morphologies can reveal existing mechanical RI, floral morphologies were quantified through length of longest stamen, length of pistil, length of corolla tube, and area of corolla (Figure 2). These traits were measured by removing and dissecting flowers from randomly selected plants to expose the pistil (Figure 2C) and stamen (Figure 2D). Dissected flowers were photographed adjacent to a ruler while fresh and subsequently analyzed using ImageJ (National Institute of Health, 2016). The frequency of nectar production, volume of nectar production, and floral morphological data were analyzed in R Studio.

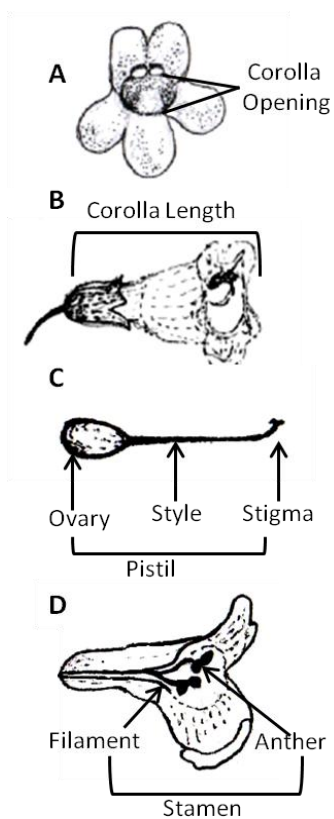


Fig. 2. Illustration showing floral anatomy and dissection techniques to measure focal traits. **A:** The corolla opening. **B:** The length of the corolla. **C:** The pistil after removal from the corolla. **D:** The dissected corolla exposing stamen for measurement.

Floral Visitors. To evaluate the role of ethological barriers in promoting RI, any insect seen entering the corolla (thus likely contacting the pollen-producing anthers) of either species within a study plot was collected via a net or directly from the flower using a centrifuge tube when floral visitors were most active, between 08:00 and 13:00 hours. Insects were then frozen for a minimum of three days before being thawed, pinned, and labeled with the date, location, source flower, and taxonomic name. Where possible, specimens were identified to species level by Virginia Scott based on morphological traits. If the species could not be determined through current taxonomic keys, they were identified at the morphospecies level, the level at which individuals with sufficient morphological differences are assigned to different species types. Insects collected for this study were donated to University of Colorado Museum entomology collection.

Bagged Experiments. Manual crosses were carried out to determine if the focal species were capable of hybridizing even when mechanical and ethological barriers were circumvented. To test this, five *Penstemon virens* and two *P. secundiflorus* individuals were bagged prior to flowering to prevent pollen transmission. When the flowers opened, the anthers were removed to eliminate the possibility of self-fertilization and the stigma was inserted into a 5 μ l pipette containing hydrogen peroxidase (see Kearns, 1993). If the stigma produced peroxidase which indicates receptivity, the peroxidase would break down the hydrogen peroxide into liquid water and oxygen gas. Thus, the presence of bubbles within the pipette confirmed receptivity. If the peroxidase test was positive, manual crosses were conducted to evaluate the potential for either *P. virens* to fertilize *P. secundiflorus* or vice versa. Each manually crossed flower was marked with colored thread, recorded, and monitored daily (Tripp et al., in review).

Results

Floral Traits. The floral traits recorded from the focal species differed significantly and suggest both mechanical isolation and some degree of pollinator specialization. Observations of floral color throughout the study site revealed that the corollas of *P. virens* were consistently medium violet externally while the inside of the throat was pale violet with subtle dark lines and contrasting dark purple anthers, which likely provide nectar guides for pollinators. In contrast, *P. secundiflorus* individuals at the study site had pale pink corollas and no detectable nectar guides.

P. secundiflorus produced nectar across significantly more of the individuals sampled (*P. secundiflorus*: $n = 93$; *P. virens*: $n = 96$; $X^2 = 15.4$, d.f. = 1, $p < 0.001$, see Figure 3). However, nectar volume was often too small to be quantified, so these negligible volumes (less than $0.5 \mu\text{l}$) were not included for comparison of mean nectar volume. A subsequent t-test revealed that nectar volume, when quantifiable, did not significantly differ between species (*P.*

secundiflorus $n = 11$, $\bar{x} = 0.909$, $\text{SE} = 0.222$; *P. virens* $n = 3$, $\bar{x} = 0.833$, $\text{SE} = 0.167$; t-test: $t = 0.273$, d.f. = 9.44, $p\text{-value} = 0.791$). The nectar surveys of bagged flowers were consistent with uncovered flowers, which indicates that nectar was likely not depleted by floral visitors prior to sampling.

The two species differed significantly in all measured floral traits: the area of the opening to the corolla (*P. secundiflorus* $n = 20$, $\bar{x} = 33.0\text{mm}^2$, $\text{SE} = 1.60 \text{mm}^2$; *P. virens* $n = 27$, $\bar{x} = 11.3\text{mm}^2$, $\text{SE} = 0.634 \text{mm}^2$; t-test: $t = 12.6$, d.f. = 25.0, and $p < 0.001$), corolla length (*P. secundiflorus* $n = 20$, $\bar{x} = 18.9\text{mm}$, $\text{SE} = 0.241\text{mm}$; *P. virens* $n = 30$, $\bar{x} = 11.3\text{mm}$, $\text{SE} = 0.213$; t-

Proportion of Flowers with Nectar

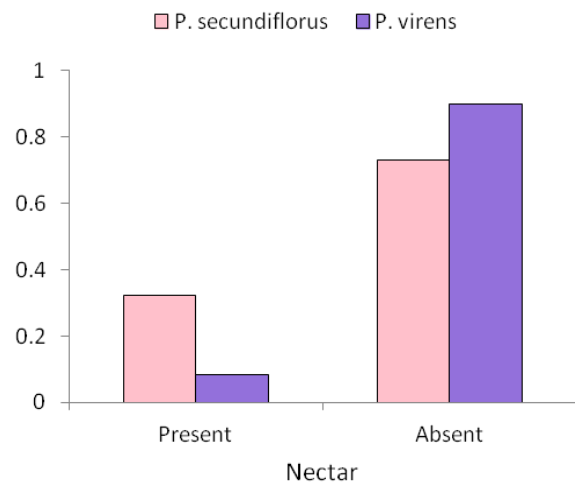


Fig. 3. Proportion of the focal species producing nectar June 15th-19th in Elbert, CO, USA. *P. secundiflorus*: $n = 93$; *P. virens*: $n = 96$; $X^2 = 15.4$, d.f. = 1, $p < 0.001$.

test: $t = 23.7$, $d.f = 43.0$, and $p < 0.001$), pistil length (*P. secundiflorus* $n = 20$, $\bar{x} = 15.6$, $SE = 0.291\text{mm}$; *P. virens* $n = 30$, $\bar{x} = 10.9\text{mm}$, $SE = 0.189\text{mm}$; t-test: $t = 14.0$, $d.f. = 34.4$ and $p < 0.001$), and stamen length (*P. secundiflorus* $n = 19$, $\bar{x} = 17.2\text{mm}$, $SE = 0.197\text{mm}$; *P. virens* $n = 30$, $\bar{x} = 11.2\text{mm}$, $SE = 0.197\text{mm}$; t-test: $t = 21.6$, $d.f = 44.6$, and $p < 0.001$, Figure 4). The corollas, stamen, and pistil of *P. secundiflorus* are significantly longer than those of *P. virens*, and the floral tube, as indicated by the area of corolla opening, is proportionally larger in *P. secundiflorus* as well.

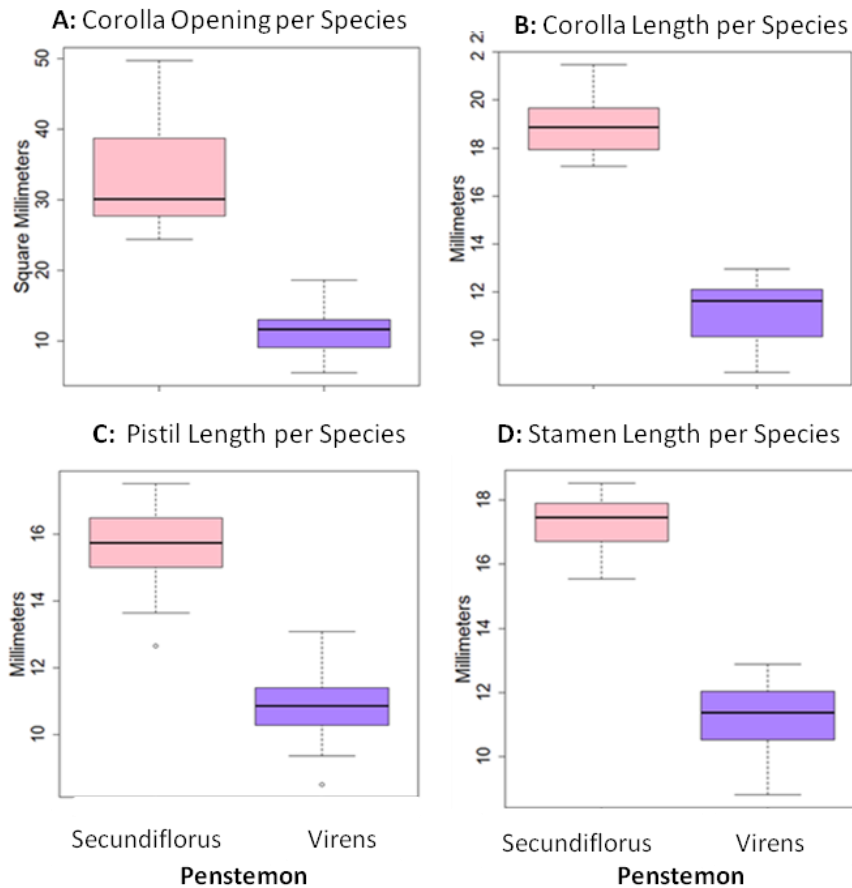


Fig.4. Comparison of floral morphologies for *P. secundiflorus* and *P. virens*. Flowers of *P. secundiflorus* were significantly larger. **A:** *P. secundiflorus* $n=20$, $\bar{x}=33.0\text{mm}^2$, $SE=1.60 \text{mm}^2$; *P. virens* $n=27$, $\bar{x}=11.3\text{mm}^2$, $SE=0.634 \text{mm}^2$; t-test: $t=12.6$, $d.f.=25.0$, and $p<0.001$. **B:** *P. secundiflorus* $n=20$, $\bar{x}=18.9\text{mm}$, $SE=0.241\text{mm}$; *P. virens* $n=30$, $\bar{x}=11.3\text{mm}$, $SE=0.213$; t-test: $t=23.7$, $d.f=43.0$, and $p<0.001$. **C:** *P. secundiflorus* $n=20$, $\bar{x}=15.6$, $SE=0.291\text{mm}$; *P. virens* $n=30$, $\bar{x}=10.9\text{mm}$, $SE=0.189\text{mm}$; t-test: $t=14.0$, $d.f.=34.4$ and $p<0.001$. **D:** *P. secundiflorus* $n=19$, $\bar{x}=17.2\text{mm}$, $SE=0.197\text{mm}$; *P. virens* $n=30$, $\bar{x}=11.2\text{mm}$, $SE=.197\text{mm}$; t-test: $t=21.6$, $d.f=44.6$, and $p<0.001$. All samples taken from, Elbert, CO, USA and analyzed in ImageJ from photographs of fresh flowers.

Floral Visitors. The floral visitors ($n = 68$) also differed to some extent between the focal species, demonstrating partial, but not complete, pollinator specialization. Most floral visitors were bees (super family Apoidea). A total of seven bee morphospecies were collected visiting *P. secundiflorus* (Table 1, Figure 5) and 20 from *P. virens* (see Table 2, Figures 6-7). Five of these bee species were common to both focal plant species. A qualitative comparison of size and pollen location from specimens demonstrated that *P. virens* experiences not only a greater species level diversity in floral visitors, but also a greater diversity of size and pollen-grooming behavior among those visitors.

The remaining floral visitors observed and collected were flies and butterflies. Two species of fly (Diptera) were collected from the two focal plant species: a beefly (Bombyliidae) visiting *P. secundiflorus* and *P. virens* and a hoverfly (Syrphidae) visiting only *P. virens*. Rarely, butterflies (Lepidoptera), most likely from family Lycaenidae, were also seen landing on the lip of the corolla of *P. virens* and a swallowtail (family Papilionidae) was recorded visiting *P. secundiflorus* (Dr. J. Mitton, pers. comm.). For both the Lycaenid and the Bombyllid visitors, the absence of observed contact with anthers suggests that the latter visitors exploit both *Penstemon* species by extracting nectar without pollinating either species, a phenomenon termed nectar robbing. Hummingbirds were also present at the study site but were never observed interacting with either focal species.

Table 1. Floral Visitors to *P. secundiflorus*

Floral Visitor	Number Caught	Size Class
Hymenoptera (Apoidea)		
<i>Hylaeus paraproopsis</i> sp. 1*	1	Small
<i>Lasioglossum dialictus</i> sp.1*	3	Small
<i>Lasioglossum dialictus</i> sp. 2*	1	Small
<i>Lasioglossum hemihalictus</i> sp. 2	1	Medium
<i>Lasioglossum lasioglossum</i> sp. 1	1	Medium
<i>Lasioglossum sisymbrii</i> *	6	Medium
<i>Lasioglossum trizonatum</i> *	1	Medium
Diptera		
<i>Bombyliidae</i> spp.	1	Medium

Floral Visitors collected from *P. secundiflorus* June 15-19, 2016 with relative size class. Visitors marked with an asterisk were collected from both

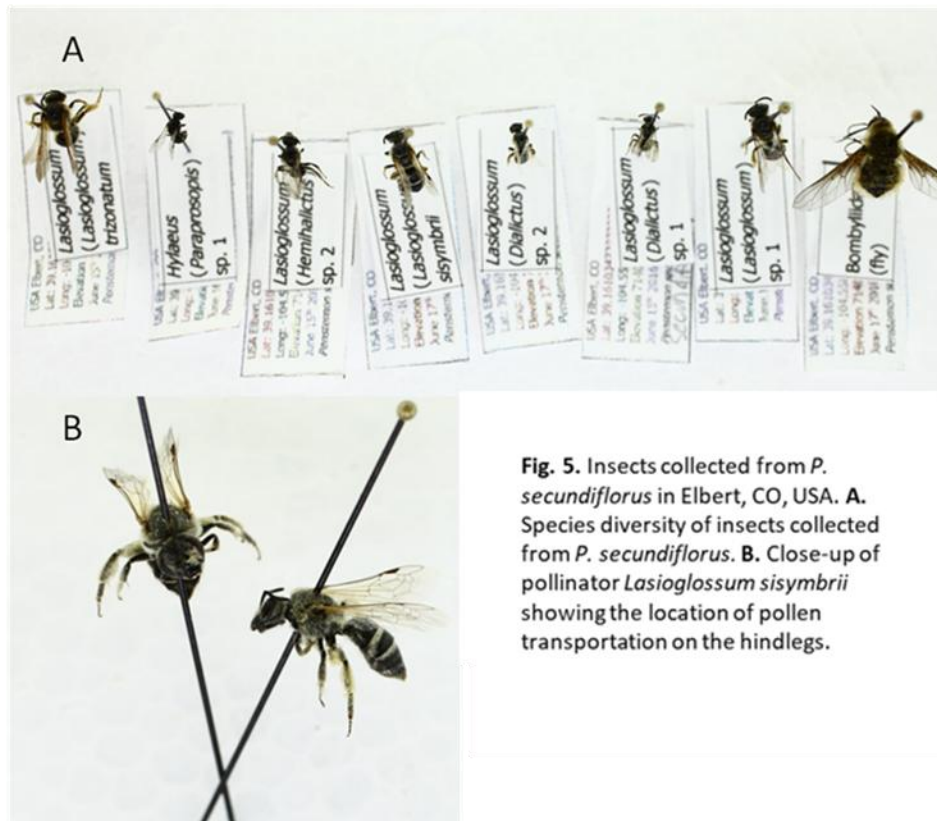


Table 2. Floral Visitors to *P. virens*

Floral Visitor	Number Caught	Size Class
Hymenoptera (Apoidea)		
<i>Andrena andrena</i> sp. 1	1	Large
<i>Anthophora</i> sp. 1	2	Large
<i>Apis mellifera</i>	4	Large
<i>Augochlorella aurata</i>	4	Medium
<i>Bombus huntii</i>	2	Large
<i>Bombus rufocinctus</i>	1	Large
<i>Bombus centralis</i>	3	Large
<i>Halictus tripartitus</i>	3	Small
<i>Hoplitis truncata</i>	1	Medium
<i>Hylaeus paraprosoptis</i> sp. 1*	1	Small
<i>Lasioglossum sisymbrii</i> *	8	Medium
<i>Lasioglossum dialictus</i> sp. 1*	8	Small
<i>Lasioglossum dialictus</i> sp. 2*	1	Small
<i>Lasioglossum hemihalictus</i> sp. 1	2	Small
<i>Lasioglossum trizonatum</i> *	1	Medium
<i>Osmia osmia</i> sp. 1	1	Medium
<i>Osmia osmia</i> sp. 2	5	Medium
<i>Osmia osmia</i> sp. 3	1	Medium
<i>Osmia osmia</i> sp. 4	1	Medium
<i>Osmia osmia</i> sp. 5	1	Medium
Diptera		
<i>Syrphidae</i> spp.	2	Medium

Floral Visitors collected from *P. virens* June 15-19, 2016 with relative size class. Visitors marked with an asterisk were collected from both focal species.



Fig. 6. Species diversity insects collected from *P. virens* at Elbert, CO, USA

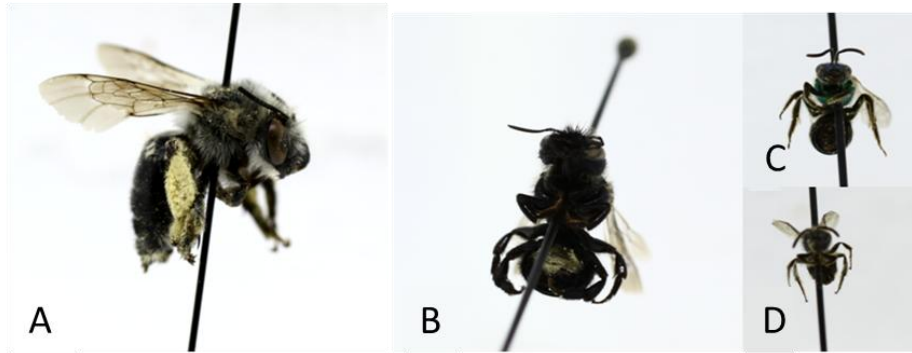


Fig. 7. A-D. Macroscopic view of locations for pollen transport on bees collected from *P. virens*. **A.** *Andrena* sp 3 with pollen groomed into scopa on the hindlegs. **B.** *Osmia* sp. 2 with pollen groomed onto abdomen. **C.** *Augochlorella aurata* pollen on abdomen and hindlegs. **D.** *Dialictus* sp. 1 with minimal pollen on abdomen and hindlegs.

Bagged Experiments. Peroxidase tests revealed that both stigmas from *P. secundiflorus* and *P. virens* were receptive 48-72 hours after buds opened. Eight days after conducting a series of manual crosses, none of the experimental plants yielded any fruit, including those outcrossed within the same species as a control. Unfortunately, on the ninth day, a hail storm damaged the experimental plants, and, subsequently, no fruits matured from either the control (intraspecific) or experimental (interspecific) crosses.

Discussion

Penstemon secundiflorus exhibits significantly larger flowers and longer reproductive organs than *P. virens*, lending support to hypothesis (1) that differences between flower morphologies of *P. virens* and *P. secundiflorus* prevents pollen transfer across species in a manner that results in mechanical isolation. A mechanical barrier could occur either through the pollen-carriers themselves (e.g. a mismatch between pollen placement and pollen deposition on pollinator bodies, with respect to flowers of different plant species) or through a pollen-tube/pistil incompatibility (e.g. pollen tubes of one species cannot grow to sufficient length to pass through the pistil and fertilize ovules in the ovary of the other species; see Tong and Huang,

2016). Further research would be needed to determine whether mechanical isolation occurs primarily through the pollinators or through pollen-tube/pistil incompatibility. If the pollinators are depositing pollen across species, the pollen-tube/pistil incompatibility may be unidirectional. Consequently, I cannot rule out the possibility of asymmetric hybridization, i.e., from paternal *P. secundiflorus* to maternal *P. virens* without further research.

The combined floral trait and floral visitor data (Figures 3-7, Tables 1 and 2) partially support hypothesis (2) that sustained selection for distinctive floral traits has resulted in ethological isolation. The corollas of *P. secundiflorus* were consistently larger than those of *P. virens* and approach the size of *Penstemon* species known to be pollinated by hummingbirds (e.g. those having corolla lengths of 20-26 mm; Lange & Scott, 1999). Nectar production, which can increase visitation frequency and is associated with a wide range of pollinators (Thomson et al., 1989), varied between both species. Part of this variability can be attributed to differences in abiotic factors since nectar production is affected by environmental conditions such as water availability, temperature, soil quality, or the variability may represent physiological differences (Boose, 1997). Considering the close proximity of these species and the number of individuals sampled, it is likely that physiological characteristics are responsible for the variation in nectar production. It can thus be concluded that *P. secundiflorus* has the potential to produce nectar more consistently when conditions are favorable. However, even though *P. secundiflorus* produced nectar more frequently, the volumes produced do not approach volumes associated with bird-syndrome species (Lara and Ornelas, 2008; K. Beckrich, pers. obs.).

Despite the significant differences in floral traits, there is some overlap in pollinator taxa, which creates the potential for pollen transfer across species (see Tables 1 and 2). The populations of *P. secundiflorus* and *P. virens* observed in this study shared several species of

Lasioglossum and one species of *Hylaeus*. Bees from the genus of *Lasioglossum* were the most frequent floral visitors to both *P. virens* and *P. secundiflorus*, which is noteworthy considering that previous studies situated in western North America have found bees from the genera *Osmia* and *Anthophora* to be most commonly associated with species of *Penstemon* (Tepedino et al., 2011; Wilson et al., 2006). While both genera were collected visiting *P. virens*, they were much less abundant than *Lasioglossum*, and were entirely absent from *P. secundiflorus*. Floral visitors collected from *P. virens* exhibited much greater species diversity. Whether this is symptomatic of the greater abundance of *P. virens* in the study plots facilitating a more representative sample of floral visitors or indicative of truly greater species diversity of floral visitors cannot be determined without controlling the relative species abundance in each plot, which was beyond the scope of this study.

The few shared pollinators between the focal species likely do not contribute significantly to pollen transfer between *P. secundiflorus* and *P. virens*. However, further research should explore the efficiency and behavior of these shared pollinators, i.e., how much pollen do they carry and how much of that pollen is deposited on the female stigma? How many types of pollen do they carry? Do they forage primarily for nectar or pollen? Without knowing the respective pollen-loads needed for individuals of *P. virens* or *P. secundiflorus* to set seed or the efficiency and the behavior of their shared pollinators, it cannot be determined how significantly the partial ethological barriers documented here contribute to RI.

The bagged experiments failed to resolve hypothesis (3), that eliminating mechanical and ethological barriers will result in hybridization. The absence of seed set following manual cross-pollination indicates that these flowers had either been damaged prior to fertilization, did not receive sufficient pollen loads, or had very low seed-set rates. Future work should repeat this

experiment with larger sample sizes and in a controlled environment to eliminate the risk specimen loss.

The present study contradicts some of the findings from previous research which established pollination syndromes in the genus of *Penstemon*. The small, blue flowers of *P. virens* and their infrequent nectar production match well with prior research on bee-syndrome species. However, small flower size has been shown to predict smaller bee taxa (Wilson et al., 2004) which is not consistent with the heterogeneous collection of bees from *P. virens* in this study. This suggests that pollination syndromes may be useful when applied generally to pollinator behavior and less appropriate for predicting specific pollinator taxa or pollinator functional groups.

Furthermore, pollination syndromes cannot adequately explain the array of floral traits shown in *P. secundiflorus* (Figures 3 and 4), particularly in the context of the observed floral visitors. If pollination syndromes alone had been used to predict floral visitors to *P. secundiflorus*, two syndromes would have been predicted; first, that bees should contribute minimally to the species diversity of floral visitors to *P. secundiflorus* due to the pink floral color, which has been shown to deter bee visitation (Bergamo et al., 2016; Castellanos et al., 2004), or, alternately, that *P. secundiflorus* should be visited by a greater frequency of large-bodied insects because of the larger flower size (Wilson et al., 2004). The collection of floral visitors in tandem with analysis of floral traits demonstrates that both predictions are inaccurate and that flower size in both focal species failed to predict floral visitor size. This reveals gaps in our current understanding of pollination syndromes in the genus *Penstemon*, particularly the role of pollinator functional groups in driving selection for flower size.

The combination of floral traits and floral visitors to *P. secundiflorus* ultimately raises more questions than it answers. It is possible that *P. secundiflorus* has adapted certain traits to attract a more generalized group of pollinators, but this is not supported by homogenous associated floral visitors. It is also possible that *P. secundiflorus* exhibits traits corresponding to a pollination syndrome that has not yet been extensively documented in the genus or that it is potentially shifting from one pollinator taxon or functional group to another. Multiple shifts from predominantly bee-pollinated to predominantly hummingbird-pollinated have been documented in the genus (see Wolfe et al., 2006). Furthermore, the frequency and persistence of bee-to-bird pollination syndromes in *Penstemon* can be explained through underlying genetic causes and selective pressures. Red and pink flower color has been linked to loss-of-function mutations in regulatory and gene-coding sequences and decreased bee visitation, thus making a trait reversal highly unlikely from a genetics perspective and reducing the selection pressures for bee-syndrome flowers (Wessinger and Rausher, 2012; Castellanos et al., 2003). However, no hummingbirds were observed visiting either species, so future research should investigate how blue-to-red color shifts also affect visitation rates from other pollinator taxa known to visit species of *Penstemon*, such as butterflies or flies.

These findings emphasize the need for additional fieldwork documenting pollinator behavior in response to floral traits in order to determine the accuracy of pollination syndromes.

Conclusions

Results from the present study support hypothesis (1) that significant morphological differences contribute to RI in sympatric populations of *P. virens* and *P. secundiflorus*. Furthermore, floral visitor data partially support hypothesis (2) that ethological barriers facilitate

RI, both species exhibited distinct floral traits and correspondingly little overlap in pollinators. Hypothesis (3) must be re-evaluated with more robust sample sizes and, ideally, in a controlled, experimental setting. Future research is needed to quantify the efficiency of these overlapping pollen vectors to determine if they are reliable pollinators which contribute to gene flow between the focal species. The available data suggest that mechanical barriers contribute most significantly to RI, while pollinator specialization reduces the potential for, but does not exclude the possibility of, gene flow between species. There may also be additional pre- or post-zygotic barriers to hybridization not addressed in this study which could be explored through further work that incorporates more manual crossing experimentation. My future research will focus on expanding the study site to different localities, thus determining if ethological and mechanical barriers vary geographically or along an elevation gradient, evaluating pollen-tube/pistil incompatibilities between the focal species, and gauging the potential for hybridization by repeating the manual crosses.

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