THE CONSEQUENCES OF POLLINATION COMPETITION ON THE REPRODUCTION OF *ASTRAGALUS BIBULLATUS*

by

Ashley Gereben

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Thesis Committee:

Dr. Chris Herlihy, Advisor

Dr. Jeffrey Walck

Dr. Jessica Arbour

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ABSTRACT

Morphological and phenological similarities of flowers may result in species competing for pollinators, and this competition may be particularly intense for rare species. *Astragalus bibullatus* is a federally endangered legume endemic to the cedar glades of middle Tennessee. It co-occurs with a nearby flower homolog, *Pediomelum subacaule*, which is abundant and significantly overlaps in flowering time. In this study, the competitive effects of *P. subacaule* on the reproduction of *A. bibullatus* were assessed by comparing pollinator visitation rates, quantifying the constancy of pollinator taxa, estimating the frequency of interspecific pollination via field stigma collection, and determining the effect of foreign pollen on seed set. The most common pollinator in 2022 for both plants was the eastern bumble bee, *Bombus impatiens,* which visited *P. subacaule* significantly more frequently, indicating that *P. subacaule* may be favored by *Bombus impatiens* and thus reducing pollination of *A. bibullatus*. Despite its low abundance in 2023, blue solitary bees, Megachilidae, had a significantly higher visitation rate to *A. bibullatus,* indicating that blue solitary bees may be essential to *A. bibullatus* pollination. I used a Constancy Index with a scale of 0 (completely inconstant) to 1 (completely constant) and determined the average constancy of all pollinators was 0.899 in 2022 and 0.982 in 2023. To determine the frequency of foreign pollen deposition, 100 stigmas of *A. bibullatus* and *P. subacaule* were collected, and the number and species of pollen grains were recorded. The frequency of foreign pollen grains present in samples was significantly higher in *A. bibullatus* (22% of stigmas) than in *P. subacaule* (4%), which is likely affecting the reproduction of *A. bibullatus*. Racemes of *A. bibullatus* were subjected to treatments of conspecific pollen, mixed pollen, and foreign pollen. Although the sample size was drastically reduced in 2022, the experiment was repeated in 2023 and racemes treated with conspecific pollen had a significantly higher frequency of fruit production (60.71%) than mixed (28.13%) or foreign pollen treatments (3.5%). The results of my study indicate these two plant species are competing for pollination services, consequently reducing pollinator visits to *Astragalus bibullatus* and increasing the frequency of foreign pollen deposition. Future management plans should consider the possible negative effect of *P. subacaule* on pollination and aim to monitor the essential pollinators of *A. bibullatus*, such as eastern bumble bees and blue solitary bees.

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INTRODUCTION

Origin and Significance of the Plant-Pollinator Mutualism

The origin of the plant-pollinator mutualism dates to the early Cretaceous 130-136 million years ago when angiosperms arose in the fossil record (Friis et al., 2006). By the mid-Cretaceous, angiosperms rapidly diversified and became ecologically dominant, which Charles Darwin referred to as “an abominable mystery”, in a quote originating from a famous letter addressed to J.D Hooker (Soltis et al., 2008). Although there were many hypotheses to explain the success of angiosperms, the origin of animal-mediated pollination became a well-supported explanation of this phenomenon. Pollination can be facilitated by three main mechanisms: animals, wind, and water, and the information on the reproduction of early flowering plants has expanded dramatically with the discovery of fossilized floral structures from the early Cretaceous (Friis et al., 2006, 2010; Hu et al., 2007). In a study analyzing the structural characteristics of fossilized pollen, pollen grain structure from the earliest angiosperms were discovered to be most optimized to be pollinated primarily by insects. This evidence aided in constructing a phylogeny that aligns with the hypothesis that animal-mediated pollination, also known as zoophily, is the ancestral mode of plant reproduction (Hu et al., 2007). Furthermore, it supports the idea that early mutualisms encouraged flowering plant and insect coevolution, leading to the diversification of angiosperms (Eriksson & Bremer, 1992; Bao et al., 2019). Today, this mutualism is one of the most well-known and commonly studied symbiotic relationships in ecology.

Most extant flowering plants form mutualisms with pollinators that impact their ecology and evolution. In a study to determine the frequency of zoophilous plant species (Ollerton et al., 2011), 42 surveys of pollination data were collected from published studies and then analyzed. Based on these surveys, the proportion of zoophily as the mode of pollination was found to be 78% in temperate regions and 94% in tropical regions, with an average of 88%. These estimates are vital for ecologists and lawmakers as it directly impacts agriculture and natural plant communities, especially given the recent decline in pollinator populations. Pollinators are sensitive to ecological disturbances such as loss of habitat, climate change, introduced non-native species, and agrochemicals (Potts et al., 2010). In North America alone, over half of the pollinator species are declining and roughly 25% of them are at increased risk for extinction (Kopec & Burd, 2017). These trends are reflected globally with a comprehensive report estimating over 40% of pollinators, primarily bees, are facing extinction. Since most angiosperms depend directly on the service of pollinators, this increases the risk of pollen limitation, which could complicate the conservation of endangered plant species.

Many angiosperms are at risk of extinction due to rapid global changes in climate, habitat, and pollinator abundance. It has been commonly cited that 13% of global flora are threatened with extinction, but recent data collected on tropical endemic plants suggests that 22-47% of angiosperms are threatened with extinction (Walter & Eds, 1998; Pitman & Jorgenson, 2002). Species that are classified as “rare” and “endemic” are a high priority for conservation efforts due to their susceptibility to extinction (Schmeller et al., 2008; Burlakova et al., 2011; Kani, 2011). For a species to be considered “endemic”, it must grow in a single geographic area and may have variable population sizes (Kani, 2011). Moreover, a species is “rare” when it inhabits a small geographical area and has small population sizes (Kani, 2011). Being a rare and endemic species creates challenges that increase vulnerability, such as narrow geographic distribution, small and infrequent populations, and specialized niche demands (Kani, 2011). To protect these vulnerable plants, research on their population and community dynamics is required to provide insight into their specialized ecology. Unfortunately, the specific pollination requirements of endangered and endemic species are often poorly understood, which creates a need for more pollination studies to create successful management plans (Schemske et al., 1994).

Study System

Cedar Glades

The Central Basin of Tennessee contains a habitat called the “cedar glades” or “limestone glades.” The term “glade” describes openings within forests that have rocky and shallow soil, but the cedar glades of middle Tennessee have distinctive characteristics that differentiate them from other types of glades (Baskin et al., 1994). For example, dominant plant types are different among cedar glades and a similar habitat called xeric limestone prairies. Cedar glades are dominated by summer and winter annuals, such as *Sporobolus vaginiflorus*; however, xeric limestone prairies are dominated by perennials, such as *Schizachyrium scoparium* (Baskin et al., 1994). Furthermore, cedar glades are naturally occurring and stable, but other types of glades require some level of management to maintain the habitat.

One of the most important differences that distinguish cedar glades is the presence of endemic flora. Out of the 28 endemic plant taxa found in the cedar glades, 59% of them occur in only three counties of Middle Tennessee: Rutherford, Davidson, and Wilson (Quarterman, 1989). Furthermore, surrounding counties hold approximately 38-46% of cedar glade endemics, which are Williamson, Maury, Bedford, and Marshall County (Quarterman, 1989). These seven counties have been rapidly developing, leading to the destruction of the cedar glades accompanied by the endangerment of rare and endemic plants. Among the growing list of endangered cedar glade endemics is *Astragalus bibullatus*, or Pyne's ground plum. In 1991, *Astragalus bibullatus* was declared to be federally endangered, which has highlighted the need for a conservation management plan for this species (USFWS, 2011).

Astragalus bibullatus- "Limestone Glade Milkvetch" "Pyne's Ground-Plum"

In 1980, Milo Pyne discovered a plant that could not be identified and didn't have representation in local herbaria (Barneby & Bridges, 1987). The collected specimens were brought to Jerry Baskin and then described by Rupert Barneby and Edwin Bridges. *Astragalus bibullatus* (Barneby & Bridges) is a perennial legume in the family Fabaceae and is characterized by racemes of 10-16 light purple flowers and glabrous leaflets (Barneby & Bridges, 1987; Figure 1). The fruits of *A. bibullatus* are red and plum shaped, which attributes to the common name "Pyne's ground-plum." *Astragalus bibullatus* has been historically known and reintroduced to Davidson County, but the current distribution of the plant is restricted to the cedar glades of Rutherford County (Barneby & Bridges, 1987). Within the cedar glades, it occurs on the margins of rocky outcrops that provide partial shade and slightly deeper soil than the center of the glade (Barneby & Bridges, 1987). The microhabitat requirements have not been well described, but a study on the soil mutualisms of *A. bibullatus* indicates that specific microbes and mycorrhizal fungi are necessary for the growth and development of the plants (Baskin & Baskin, 2005; Becknell et al., 2021). *Astragalus bibullatus* flowers in the spring from April-May and fruits in May-June. There is little known about the pollination ecology of this plant, which is the focus of this research.

As a rare and endemic species, *A. bibullatus* is particularly vulnerable to endangerment and faces many challenges. Only about eight naturally occurring populations currently exist, which has sparked an interest in reintroducing this plant into suitable habitats. The cedar glades at Stones River National Battlefield (SRNB) were chosen as suitable sites for seedling reintroduction, which was seemingly an ideal site since it is protected and has a similar habitat as natural populations (Albrecht & McCue, 2010; Albrecht & Long, 2019). In one study, seeds from three natural population sources were grown in a greenhouse and then out-planted into different sites at SRNB in the fall and spring. Several sites had low transplant success despite there being low herbivory rates, suggesting that differences in microsite quality was a major factor that influenced transplant success. Furthermore, many of the plants experienced rapid die-offs indicating the inability to establish necessary soil symbionts such as mycorrhizal fungi or *Rhizobium*. This concept was reflected in a study analyzing the effect of soil microbiome on the growth and reproduction of *A. bibullatus,* which provided strong evidence to suggest that composition of soil microbes is likely a strong barrier to success of reintroduced plants (Becknell et al., 2021).

In another attempt at reintroduction, five populations of *A. bibullatus* were planted at different sites among the cedar glades of the SRNB (Albrecht & Long, 2019). Plants were placed in a xeric-barren or mesic-ecotone microhabitat and half of the plants were caged to exclude herbivory. The plants in the xeric-barren habitats had a much higher survival rate than those planted in mesic ecotones suggesting that habitat type was the primary driver of success (Albrecht & Long, 2019). Furthermore, caged plants had higher survival and flowering rates, due to herbivores consuming the nutritious fruits of the Fabaceae species (Albrecht & Long, 2019; Didinger & Thompson, 2021). Although this reintroduction effort provided information on the barriers to the success of *A. bibullatus*, the reintroduction attempt was unsuccessful as the five populations failed to persist. Despite these previous failures, there have been recent reintroduction attempts at the Savanna, Couchville, and Hall Farm sites; these introduced plants were established with cages to prevent herbivory, which appears successful. (M. Albrecht, pers. comm.).

In addition to site quality and herbivory, drought and genetic drift threaten the success of *A. bibullatus*. Climate change is projected to alter precipitation patterns and drought frequency, which may impact soil moisture and plant success (Sheffield & Wood, 2008; Seneviratne at al., 2010). In a study conducted simulating the effects of droughts and disturbances on *A. bibullatus*, plants exposed to frequent droughts experienced increased mortality, decreased fecundity, and prevention of seedling establishment (Bernardo et al., 2006). Furthermore, drought frequency also determined the probability and time of extinction, and the populations with frequent management and infrequent droughts were least likely to go extinct. Rare and endemic species are at an increased risk for genetic drift and inbreeding depression due to small population sizes

(Reed & Frankham, 2003). This was the case in a study analyzing the isozymes of *A. bibullatus* in which the genetic variability within and between populations was low (Baskauf & Snapp, 1998). Contrastingly, recent studies have found higher levels of genetic variability and no evidence of inbreeding depression (Baskauf & Burke, 2009; Morris et al., 2021).

Pediomelum subacaule- "Whiterim Scurfpea" "Nashville Breadroot"

The cedar glades host another endemic species of Fabaceae, *Pediomelum subacaule* (Torr. & A. Gray) Rydb, that has similar flower morphology and phenology to *A. bibullatus* (Figure 2). *Pediomelum subacaule* is an acaulescent, perennial plant that was first collected by Dr. Roane in Nashville, Tennessee. The collected specimen was then sent to John Torrey and Asa Gray, who published the species description in 1838 (Torrey & Gray, 1940). Thomas Meehan published "Nashville breadroot" as a common name for this species, and it is frequently referred to as "lupine" or "Whiterim Scurfpea." The geographic range of *P. subacaule* is like *A. bibullatus*, with most of the populations residing in the cedar glades of Middle Tennessee. Unlike *A. bibullatus*, *P. subacaule* has a broader distribution with some populations occurring in the glades of Alabama and Georgia (USDA PLANTS, 2023). Furthermore, the population densities vary between these two species, in which *P. subacaule* is more abundant and has higher population densities than *A. bibullatus*. The largest population of *A. bibullatus* has about 1,000 plants, but most sites have fewer than 200 plants (USFWS, 2011). Although there is no published population data on *P. subacaule*, the higher population density is easily observed.

Flowering in *P. subacaule* starts during the first week of April and ends at the beginning of May, and there is an average of a week gap between the opening of the first and last flower on a raceme (Baskin & Quarterman, 1970). The most commonly documented pollinators for this plant are bumble bees (*Bombus*), honeybees (*Apis*), and some butterfly species (Baskin & Quarterman, 1970). Carpenter bees (*Xylocopa*) c visit flowers of breadroot but have not been observed to be effective pollinators. Seeds mature by the end of May, but this is dependent on water availability within the cedar glades (Baskin & Quarterman, 1970).

The occurrence of co-flowering species can have a dramatic effect on plant fertility when there is competition for pollinators (Levin & Anderson 1970; Schemske et al., 1978; Waser, 1978). Interspecific competition for pollination often causes reduced pollinator visits and deposition of foreign pollen, both leading to a reduction in seed set (Waser, 1978; Pleasants, 1980; Thomson et al., 1982; Campbell, 1985; Waser & Fugate, 1986; Brown & Mitchell, 2001; Bell et al., 2005). *Astragalus bibullatus* and *Pediomelum subacaule* are both endemic to the cedar glades and have similar life histories, which make them a good model system for studying the fitness effects of competition for pollinators (Baskin & Quarterman, 1970; Barneby & Bridges, 1987). These “flower homologues” are likely to be competing for pollinator services limiting the success of these plants. Research on this potential hurdle is essential for *A. bibullatus,* as it is federally endangered and has no published studies on its pollination ecology. Consequently, there is a need to quantify pollinator behavior and the effect of pollination competition on plant reproduction, which is the focus of this thesis.

Pollinator Behavior and Constancy

The composition and density of a plant community may drastically affect a pollinator’s behavior, and thus, will influence competition between plant species. One of these behaviors is flower constancy, which is the tendency for a pollinator to exclusively visit one species of flower during a foraging trip (Waser, 1986). Flower constancy has been observed in a variety of taxa and typically benefits both the pollinator and the plant. Pollinators have limited cognitive abilities and will benefit from constancy as it reduces the time it takes to find suitable flowers (Marquez, 2009). Furthermore, the cognitive effort used to exploit flowers is reduced, which in turn increases the reward per foraging trip. The occurrence of pollinators foraging on only one plant species is hypothesized to provide an evolutionary advantage, and there are two models proposed to explain constancy. The first model, limited memory hypothesis, proposes that insects have a low capacity of knowledge on morphological traits of flowers, so when a new flower is visited, previous information they knew about other floral types is lost (Lewis, 1986). This hypothesis is supported by studies finding that the handling time of pollinators on new flower morphs is increased.

The second model to explain floral constancy is the “interference hypothesis,” which suggests that bees and other insect pollinators are incapable of retrieving complex information about the morphology of flowers (Heinrich, 1976; Laverty, 1980). If pollinators can't discriminate among flower morphs, they would instead use general traits or “search images” to guide their foraging decisions (Goulson, 1999). The search image model would promote generalism and inconstancy when flowers of similar morphology grow in sympatry. Although generalist pollinators can benefit the plants via facilitation, there is evidence suggesting that generalists are more likely to increase heterospecific movements causing foreign pollen transfer, which reduces plant fitness (Waser, 1978; Thomson et al., 1982).

Flowers often differ in morphological characteristics that influence the behavior and constancy of pollinators, such as corolla color, patterns, and scents. For example, bees have been observed to be most attracted to flowers that are blue to purple and will choose to forage on that color flower even if flowers of other colors provided a greater energetic reward (Raine et al., 2006). Electrophysiological and behavioral studies have explained this phenomenon as a result of a trichromatic vision system that increases perception of UV light and shorter wavelengths of visible light (Autrum & von Zwehl, 1964; Menzel & Blakers, 1976; Peitsch et al. 1992; Giurfa et al., 1997l; Ibara et al., 2001). The perception and preference of blue and purple flowers aligns with the "search image" hypothesis, which states that pollinators will use general properties of a flower to guide their foraging decisions, such as color and symmetry. The indiscriminate movements of pollinators due to a "search image" may decrease pollinator constancy and increase pollination competition among flower homologues.

To predict the frequency of interspecific movements, constancy can be quantified by comparing the ratio of intraspecific movements to all pollinator movements within an area where the study plants coexist. Several equations are used to calculate constancy, but they all utilize the variables 'A', 'B', 'C', and 'D' (Waser, 1986). The 'A' variable represents the intraspecific movements within species 1, and the 'D' variable represents intraspecific movements within species 2. Conversely, the ‘B’ and ‘C’ variables represent interspecific movements from species 1 to species 2 and from species 2 to species 1, respectively. The present study utilized the Constancy Index that produces a value ranging from 0-1, in which a value of 0 would describe a pollinator that makes only interspecific movements and 1 describes a completely constant pollinator (Dafni et al., 2005). The equation for Constancy Index is (A+D)/(A+B+C+D) and is advantageous over other equations as the denominator will never equal 0 and this equation is insensitive to pollinator preferences that may skew the results (Dafni et al., 2005).

In my research system, *A. bibullatus* and *P. subacaule* are both purple and bilaterally symmetrical flowers that are pollinated primarily by bees. Bees tend to prefer flowers of this color and morphology and may be using a general “search image” to prevent discrimination of the flower homologous. In order to predict the frequency of interspecific movements, constancy must be quantified. If *A. bibullatus* is visited by inconstant pollinators, then these interspecific movements are possibly transferring foreign pollen that reduce plant fertility via pollen wastage and stigma clogging.

Competition for Pollination

Pollinators are facing a global decline that may impact plant diversity and coexistence (Kopec & Burd, 2017). With increasing competition for pollinators, there are two potential outcomes on plant coexistence: facilitation or exclusion (Johnson et al., 2022). Competition for pollinators has been predicted to stabilize plant communities since it promotes niche differentiation. On the other hand, pollinators may have imbalanced preferences that lead to the exclusion of unfavorable plant species. A recent experiment was performed to determine which outcome was more likely by subjecting experimental plots of plants with varying densities and identities of plant competitors (Johnson et al., 2022). Half of the groups were supplementally hand pollinated to determine the effects of pollen limitation. Out of the 10 species-pairs that were studied, 9 of them had weakened niche differentiation in the presence of competitors. Competition also had strong effects on plant fitness and seed set due to imbalanced pollinator preference, in which one competitor was more favored than the other. These results support the hypothesis that competition for pollinators is likely to destabilize plant coexistence by favoring common plants species and excluding their rarer competitors.

The consequences of pollination competition can result in a reduced seed set and typically occurs in two forms: reduced pollinator visits and deposition of foreign pollen (Waser, 1978; Pleasants, 1980; Thomson et al., 1982; Campbell, 1985; Waser & Fugate, 1986; Brown & Mitchell, 2001; Bell et al., 2005). The strength of this competition depends on the pollinator's constancy, which is heavily controlled by floral characteristics, such as flower color and size (Giurfa, 1995; Campbell et al., 2010). Flowers of similar morphology are referred to as "flower homologues" and can be considered even more at risk for floral competition due to limitations on a pollinator's ability to distinguish between similar morphs (Barneby & Bridges, 1987). One study that demonstrates the consequences competition has on effective pollinator visits looked at plant fertility of *Mimulus ringens* in mixed vs pure arrays (Bell et al., 2005). When *M. ringens* was planted in mixed arrays with the competitor *Lobelia siphilitica,* there was a significant reduction in seed set compared to plants in pure arrays due to less effective pollination. Not only was *M. ringens* visited by pollinators less frequently in the mixed arrays, but the pollen loads were less effective due to the presence of foreign pollen. A similar result was found in a study comparing seed set of *Stellaria puberia* when in the presence or absence of the competitor *Claytonia virginica* (Campbell & Motten, 1985*)*. They found that pollinator visitation rate was unaffected by presence of a competitor, but there were frequent interspecific movements which reduced the amount of conspecific pollen brought to *C. virginica* stigmas. These results are relevant to this study because *A. bibullatus* and *P. subacaule* have overlapping flowering times and similar morphologies, which may be reducing effective pollinator visits.

Although floral morphology plays a significant role in pollinator foraging, the abundance of available plant species can also influence the intensity of competition. For example, a change in the preference of pollinators may occur if one floral abundance is significantly different than a competing species (Levin & Anderson, 1970). The pollinator will then tend to specialize on the most abundant species to reduce time spent searching for nectar, subsequently increasing the net energy gain during a foraging trip. This behavior is likely to put rare species at a competitive disadvantage when in the presence of an abundant competitor. The study system discussed in this thesis involves a rare and endangered plant species, *A. bibullatus*, and a more common plant species, *P. subacaule*. If pollinators do prefer more abundant plant species and there are no barriers to prevent this effect, then this poses a risk of competitive exclusion of *A. bibullatus.*

Foreign pollen deposition often causes reduced fitness in a plant and the likelihood of this occurring is increased when plant competitors have shared inconstant pollinators. When pollinators are foraging indiscriminately, one potential explanation for a plant's reduced fitness is the incompatible pollen mechanically clogging the stigma or causing pre-mature stigma closure (Waser, 1978). In a study investigating the effect of foreign pollen from invasive species *Lythrum salicaria* on a native species *Lythrum alatum*, the researchers found that the native plant experienced a 28.8% reduction in seed set when treated with a foreign and conspecific pollen mixture (Brown & Mitchell, 2001). The amount of reproductive interference caused by foreign pollen grains supported the conclusion that this seed set reduction was likely the result of changes within the stigma. Similar results were obtained in a study comparing herb competitors *Ipomopsis aggregata* and *Delphinium nelsonii,* in which foreign pollen precedence resulted in stigma lobes closing together (Waser & Fugate, 1986).

In addition to stigmatic interference, pollen allelopathy may occur in which foreign pollen actively produces chemicals that result in a reduced seed set. Pollen allelopathy was confirmed in an experiment involving hand pollinations of varying foreign pollen concentrations onto *Diervilla lonicera* stigmas (Thomson et al., 1982). The results of this experiment indicated that a mixture of 50% foreign pollen onto *D. lonicera* stigmas will lead to complete reproductive failure. Therefore, foreign pollen presence did not act as a natural dilutant, but instead actively inhibited the success of conspecific pollen to a severe degree. Another mechanism that could reduce plant fitness is the creation of inviable and sterile hybrids. *Hibiscus moscheutos* and *Hibiscus laevis* are closely related species that co-flower in sympatry. In a study in which both plants were subjected to hand pollinations of foreign pollen that created fruits, the number of seeds were reduced (Klips, 1999). Moreover, the resulting hybrids from this cross are likely to be unfit for the environment, which reduces the parents’ fitness.

Increasing evidence suggests that competition for pollinators will cause the destabilization of plant communities, which is an effect that magnifies as pollinator abundance declines. Competing plants may experience fitness declines due to pollinators preference affecting visitation frequency. Moreover, inconstant pollinators may be transferring foreign pollen causing reproductive failure. *Astragalus bibullatus* and *P. subacaule* are likely to be competing for pollinators and will experience these consequences. *Pediomelum subacaule* is more abundant than *A. bibullatus,* which may increase the chance of foreign pollen being transferred from inconstant pollinators. Since pollinators often utilize flower color and symmetry to guide foraging decisions, *A. bibullatus* may be receiving pollen from *P. subacaule* that is inhibiting its success. Addressing these concerns is imperative to formulate a proper conservation management plan for *A. bibullatus*.

Experimental Objectives

The goal of this thesis is to increase our current understanding of community interactions that may affect the success of federally endangered *A. bibullatus*. Reproduction of *Astragalus bibullatus* is probably dependent on pollinators to effectively cross-pollinate and produce fertile offspring, but there are currently no published studies on its pollination biology. The presence of a nearby flower homolog, *P. subacaule*, which is more abundant, would increase a pollinators energetic reward if it preferred to forage on it. If this were occurring, it would decrease the pollinator visits to *A. bibullatus* and decrease the percentage of fertilized ovules. Furthermore, if pollinators exhibit inconstant foraging behavior, foreign pollen may reduce plant fitness by stigma clogging, pollen allelopathy, or by the creation of inviable hybrids. To quantify the effect of pollination competition on the reproduction of *A. bibullatus*, the present study had three main objectives. First, I investigated which pollinators visit both plant species and the impact that the presence of a plant competitor had on the frequency of visitation to *A. bibullatus*. This first objective consisted of field studies monitoring the pollination of mixed populations of *A. bibullatus* and *P. subacaule* to calculate and then compare visitation rates. My second objective aimed to calculate pollinator constancy to determine the frequency of interspecific movements. This objective also involved field experiments and quantifying constancy using a Constancy Index, which compared the relative frequencies of intraspecific to interspecific movements. If pollinators are exhibiting a low constancy, then there is a potential of foreign pollen deposition. For my third objective, I quantified the reproductive costs of inconstant pollinator movements which would result in interspecific pollen flow. To accomplish this objective, I counted seed sets after applying hand-pollination treatments that consisted of either conspecific, mixed, or foreign-only pollen loads. Furthermore, I collected *A. bibullatus* and *P. subacaule* stigmas and observed them under the microscope to determine the frequency of foreign pollen deposition in natural populations. Overall, the three objectives served to guide the conservation plan for *A. bibullatus* by supplying data on its pollinators and how it’s reproduction may be impacted by a nearby flower homolog.

METHODS

Study Area

The study was conducted at Flat Rock Cedar Glades and Barrens State Natural Area in Rutherford County, Tennessee. As a lab group, we used the following sites of *A. bibullatus*: Davenport West, Airport, Alexander, Savanna, and Hall Farm (Table 1). Previous field observations have shown that *P. subacaule* co-flowers with *A. bibullatus* at these sites, making them practical for my investigations, even though the abundance of flowers varies annually among the sites. To determine which sites were best suited for each experiment, we visited the sites in late March of each year of the study to assess the density of *A. bibullatus* plants and their proximity to *P. subacaule*.

Airport was the most densely populated site for both *A. bibullatus* and *P. subacaule* plants, which appeals to pollinators to result in high pollinator activity. Airport is along Factory Road in a state protected area. Furthermore, the site is near the road, relatively open, and is easy to navigate. Due to these characteristics, this site was chosen for collecting visitation and constancy data in both field seasons. Since pollinator activity was high, many stigmas of both species were collected and preserved to determine the frequency of interspecific pollen transfer. In the 2023 field season, this site was also utilized to repeat my hand pollination experiment to have an additional treatment and larger sample size.

Savanna is in a rocky, open, grassy area, while Alexander has deeper soil and is surrounded by more woody plants. These sites were used in the 2022 field season for the hand pollination experiment, but the sample size was limited as these sites have the lowest density of *A. bibullatus*. Furthermore, stigmas were collected and preserved at these sites to determine the presence and frequency of foreign pollen.

Competition for Pollinators- Visitation Rates

The field experiment for quantifying visitation rates took place during peak flowering to ensure that most plants would have open flowers available for pollination. In the 2022 field season, observations began on April 7th and ended on April 24th. During this time frame, two observers were able to collect 20 h of visitation data. In the 2023 season, *A. bibullatus* had remarkably less racemes compared to the previous year; this decrease in flower abundance resulted in only 12 h of visitation for *A. bibullatus*. On the other hand, *P. subacaule* racemes were very abundant and 22 h of visitation data were obtained with the help of three different observers.

To determine the pollinator visitation rate of both plant species, I used a small section of the Airport site that included representative members from both species. After the boundaries of the plot were defined, the relative density of each plant species was determined by counting the number of racemes with open flowers. During 1 h time increments, data were collected on both plant species simultaneously by recording pollinator visits on visitation rate sheets that included information on the type of pollinator, duration at each flower, and movements between flowers in the plots. Pollinator movements were recorded using the following system: initial visits to a flower in the plot (V), movement from one raceme to another on the same plant (MTF- “Move to new flower”), movement from one raceme to another on a different plant of the same species (MTP- “Move to new plant”), movement from one raceme to another on a different species (MTS- “Move to new species”), and movement out of the plot range (MTR- “Move to new range”). To ensure accurate identification of pollinators, I created a field guide including insects that have been previously encountered in the glades that may pollinate *A. bibullatus* or *P. subacaule*.

The dependent variable in this experiment was the visitation rate and was calculated using the following equation: Rate = (number of pollinator visits/number of flowering racemes/hour). The independent variables were the plant species, *A. bibullatus* or *P. subacaule*, and pollinator type, identified at the family, genus, or species level. The data sets comparing the two plant species were first evaluated via a Shapiro-Wilk test to determine the appropriate two sample tests. Most of the data sets were not normally distributed, and thus, a non-parametric Wilcoxon rank sum test was performed. All necessary analyses for this thesis were performed using the R Statistical Software (v4.1.2; R Core Team 2021; Gereben, 2022). Since multiple Wilcoxon rank sum tests were performed, the Benjamini-Hochberg procedure was applied to each test to correct for the increased family-wise error rate. The adjusted alpha levels range from 0.0029 to 0.05 and p-values that are less than their adjusted significance level are denoted with an asterisk (*).

Pollinator Constancy

Pollinator foraging movements can be categorized as intraspecific or interspecific movements and the ratio of these movements determines pollinator constancy. An inconstant pollinator has a high probability of facilitating the transfer of interspecific pollen, which is data on the types of pollinator movements. This data was collected simultaneously with the visitation data as the codes MTF and MTP indicate intraspecific movements, and MTS indicates interspecific movements. These movements were then assigned to categories that included each possible movement within the study area: A, B, C, or D. I utilized the Constancy Index formula, which is calculated as Constancy = (A+D)/(A+B+C+D). The Constancy Index is the most appropriate equation to use compared to other indexes because the denominator will never be zero and flower preferences cannot potentially skew the results.

The present study took place during peak flowering and there was 20 h of data collection in the 2022 field season. Samples (hours) that had no pollinator visits were excluded from the analysis, which reduced the overall sample size for each pollinator taxa. The number of samples were 14, 16, and 8 for eastern bumble bees, carpenter bees, and both solitary bee families, respectively. In the 2023 field season, 12 h of data was obtained for blue solitary bees and 11 h for carpenter bees. Since this experiment had a categorical independent variable (pollinator type) and a continuous dependent variable (constancy), a one-way ANOVA was used to determine if there is a significant difference of constancy among the pollinator types from both seasons individually and combined. The analysis only included taxa with adequate sample sizes, including eastern bumble bees, carpenter bees, green solitary bees, and blue solitary bees.

Hand Pollination Experiment

Pollinators that bring mixed pollen loads to stigmas could inhibit plant reproduction by pollen allelopathy, stigma clogging, or stigma closure. The aim of this part of my project was to quantify the effects of mixed pollen on reproductive output.

This experiment started by selecting plants that would be hand pollinated. In the 2022 season, the Alexander and Savanna sites were used due to another experiment taking place in the Airport site. Prior to peak flowering, these sites were visited to determine the most obtainable sample size with the available plants. In the 2023 season, the Airport site was used due to more flower availability.

Three treatments occurred in the 2022 season and 20 racemes were used per treatment. Prior to flowering, mesh bags were placed over budding racemes to prevent natural pollination. Once the flowers opened in April, the plants were hand-pollinated with the appropriate treatment. The three treatments used in the 2022 field season were as follows: conspecific pollen only, foreign pollen only, and a mix of foreign and conspecific pollen. The treatment with mixed pollen involved two sub-treatments to determine if the order of pollen transfer affected reproductive output: conspecific pollen before foreign pollen and foreign pollen before conspecific pollen. To ensure proper pollen transfer, anthers from *A. bibullatus* and *P. subacaule* were removed from nearby flowers and then rubbed onto the stigma surface of the treatment flowers. The flowers required being “tripped,” which required pulling the keel down to expose the stigma and anthers (Figure 1). Once the stigma was exposed, anthers were rubbed onto the stigma of one flower on the raceme and then discarded. This process was repeated two more times to have a total of three pollinated flowers per bagged raceme. The mesh bags covering the racemes were temporarily removed for hand pollination and immediately replaced after hand pollination. In the 2023 season, four treatments with 30 racemes per treatment were used. The treatments were conspecific pollen only, foreign pollen only, a mix of foreign and conspecific pollen, and no pollen. The procedures were the same as the previous field season, but five flowers were pollinated on each raceme.

In late May, *A. bibullatus* had begun fruiting and fruits were mature by the first week of June. The fruits from bagged racemes were collected and brought to the laboratory to be counted. After counting the number of fruits and seeds, the 2022 field data was analyzed with a two-way ANOVA to determine the effect of treatment and site on seed set. Since the data from 2023 were all obtained from one site, a one-way ANOVA was performed to determine the effect of treatment on seed set. Both sets of data were followed by a post-hoc Tukey's HSD test to determine differences among the treatments. Additionally, linear discriminant analysis was performed to provide data visualization and classification among the treatments.

Stigma Collection and Pollen Grain Analysis

The natural frequency of interspecific pollen transfer was estimated by preserving 100 stigmas each of *A. bibullatus* and *P. subacaule*. Prior to field collection, glycerin jelly was mixed as a medium for preserving the stigmas. To prepare this jelly, 10 g of unflavored gelatin was placed in a 250 mL beaker. Then, 35 mL of water and 30 mL of glycerol was added to the beaker and stirred. The beaker was placed on a hot plate to warm and dissolve the mixture and it was continuously stirred during the heating process. After about 10 minutes, the gelatin was dissolved, and the mixture was removed from the heat. The liquid glycerin was then poured into empty petri dishes filling them each halfway with the jelly. Once the jelly had solidified and cooled, the dishes were covered and dated.

During peak flowering, I collected flowers at the Airport, Alexander, and Savanna sites. The slides were prepared by using a laboratory spatula to add a small square of glycerin jelly to the right side of the slide. Racemes were chosen randomly and only one flower from each raceme was pulled for collection. Furthermore, flowers were collected from all areas of the sites to prevent bias in data collection. The flowers were tripped to expose the reproductive structures, which were removed by forceps. Since the stigma and anthers are difficult to distinguish and separate in the field, all the reproductive structures were placed on top of the glycerin jelly and the stigma was later identified underneath the microscope. Cover slips were placed on top of the jelly and reproductive structures to secure and protect the specimen. Once secure, a lighter was placed underneath the slide to slowly melt the glycerin jelly into a liquid. After the jelly had cooled and resolidified, the slide was labelled with the plant species, date, and site location. The slides were cleaned with Kimtech glass cleaning wipes and then placed in a slide box for later use in the laboratory.

To count the pollen grains on the stigmas, the prepared slides were placed under a light microscope and focused with the 10x objective lens. Once the stigma was identified, the number of conspecific grains were counted and recorded that were touching the stigmas. To ensure an accurate count, pictures were taken of the slides and each pollen grain was marked on the picture after it had been counted. If foreign pollen grains were present, the number of foreign pollen grains on the stigma and entire slide were recorded. After the pollen grains on all stigmas were counted, the mean number of conspecific pollen grains on stigmas was determined to be not normally distributed, so a non-parametric Wilcoxon sum rank test was performed. The frequency of foreign pollen was compared by assigning either 0 for foreign pollen absent or 1 for foreign pollen present. The data were then statistically tested via Wilcoxon sum rank test. Furthermore, when foreign pollen was present, the number of foreign pollen grains was compared via a Wilcoxon sum rank test.

The pollen of *A. bibullatus* and *P. subacaule* are distinguishable underneath the microscope due to the size and shape of the grains. To quantify the size and shape differences, 20 pictures of the pollen grains for each species were taken and then uploaded into the Fiji-Image J software (Schindelin et al., 2012). The pictures were taken at 400x total magnification with a field of view of 0.45 mm. This measurement was used as the scale for the image by dragging a line across the diameter of the light, selecting “set scale” underneath the analyze tab, and entering 0.45 mm for the known distance. The length, width, and area of 60 pollen grains were measured. The data were statistically analyzed with a MANOVA test to determine if there were differences between the two species across measurements. Furthermore, a linear discriminant analysis test was performed to determine how well these measurements can discriminate the pollen species.

RESULTS

Visitation Rate

April 2022

In the 2022 field season, 20 h of data were collected on visitation rates for both *A. bibullatus* and *P. subacaule.* Of the 1,143 visits recorded, 350 were to *A. bibullatus* racemes and 793 were to *P. subacaule* racemes. The most common insect visitor to both plant species were carpenter bees (*Xylocopa*), which comprised 68% of all visits to *A. bibullatus* and 59% to *P. subacaule* (Figure 3). Eastern bumble bees (*Bombus impatiens*) were the next most common group making up 22% of visits to *A. bibullatus* and 31% of *P. subacaule.* Solitary bees (Halictidae and Megachilidae) made up 6% and 4% of visits to *A. bibullatus*, respectively. Similarly, Halictidae and Megachilidae made up 5% and 5% of visits to *P. subacaule*, respectively.

Carpenter bees had the highest visitation rate of both plant species, with an average of 0.45 for *A. bibullatus* and 0.60 for *P. subacaule*, which is indicated by the red points in Figure 4. As shown in Figure 4 and similar plots, the median is represented by a bold line, the outliers are open circles, the ends of the box are the first and third quartiles of the data set, and the dashed lines indicate the range via a minimum and maximum. A Shapiro-Wilk test was used to determine normality of each data set, and the distribution of carpenter bee data was abnormal and right skewed (P<0.001; Figure 4). Although the mean visitation was greater in *P. subacaule* than in *A. bibullatus,* no significant difference occurred in visitation rate among the two plant species (W=160, P=0.272). Although carpenter bees were the most frequent visitor to both plant species, they were not observed to actively pollinate. Fabaceae plants require to be “tripped” in which the pollinator lands on the keel to expose the anthers and style (Larkin & Graumann, 1954). Instead, carpenter bees would insert their proboscis into the back side of the flower without exposing the reproductive structures, a commonly documented behavior known as “nectar robbing” (Somanathan et al., 2019).

The visitation rate of carpenter bees varied throughout the field season with a low visitation frequency at the beginning of the field season, 7 April 2022, that gradually increased for both plant species (Figure 5). The highest daily visitation rate of carpenter bees to *A. bibullatus* was 1.67 and occurred on 20 April 2022, which was likely due to the high abundance of flowers attracting the bees. As *A. bibullatus* flowers became less abundant, carpenter bees switched to visiting the more abundant *P. subacaule*, resulting in a dramatic decrease in visits to *A. bibullatus.* The highest recorded visitation rate to *P. subacaule* was 2.69 and occurred on 15 April 2022.

Eastern bumble bees had an average visitation rate of 0.10 for *A. bibullatus* and 0.39 for *P. subacaule* (Figure 6). The visitation rates between the two plants were significantly different, suggesting a pollinator preference for *P. subacaule* (W=105, **P*=0.008). Unlike carpenter bees, eastern bumble bees were observed to land on the keel of flowers to expose reproductive structures when foraging for nectar. Furthermore, the bumble bees often had visible pollen baskets providing further evidence of their role as a pollinator.

Bumble bees started visiting flowers early in the season and visitation peaked for *A. bibullatus* on 14 April 2022 with a rate of 0.21 (Figure 7). For *P. subacaule*, the highest recorded visitation rate was 0.84 which also occurred on 14 April 2022. After peak visitation, a dramatic decrease occurred in visitation to both plants, likely due to cloudy and cold weather. By 20 April 2022, bumble bees started foraging again but only visited *P. subacaule*.

Two families of solitary bees visited both plant species, Megachilidae and Halictidae. Blue solitary bees (Megachilidae) had an average visitation rate of 0.02 to *A. bibullatus* and 0.06 to *P. subacaule* (Figure 8). The difference in Megachilidae visitation rate among the two plant species was not statistically significant (*P*=0.255). Green solitary bees (Halictidae) visited *A. bibullatus* and *P. subacaule* at average rates of 0.065 and 0.059, respectively, which did not differ between the two plant species (*P*=0.456; Figure 9).

The visitation rate of solitary bees during the field season was highly variable. At the beginning of data collection (7 April 2022), visitation remained low for blue solitary bees (Figure 10). On 15 April, they began to forage frequently on *P. subacaule* but not on *A. bibullatus*. On 20 April 2022, they were foraging on both plant species, but towards the end of the season, they were only visiting *P. subacaule.* Green solitary bees followed a different trend and did not consistently forage until about April 15th (Figure 11). Towards the end of April, there were unusually high visitation rates of green solitary bees to *A. bibullatus*. On 23 and 24 April 2022, green solitary bees had an average visitation rate of 0.18 and 0.60 to *P. subacaule* and *A. bibullatus*, respectively. At this point, flowering plants of *A. bibullatus* were less abundant than its competitor, *P. subacaule,* which may suggest that green solitary bees prefer *A. bibullatus*.

April 2023

In the 2023 field season, I collected 22 h of visitation data on *P. subacaule* and 12 h on *A. bibullatus*. Of the 1,189 visits recorded, 132 were to *A. bibullatus* and 1,057 were to *P. subacaule*. Blue solitary bees were the most common visitors to *A. bibullatus* and the second most common to *P. subacaule*, comprising 84% and 22% of visits, respectively (Figure 12). Carpenter bees were the most common visitors to *P. subacaule* and the second most common to *A. bibullatus*, comprising 68% and 12%, respectively. For both plant species, green solitary bees were the least common visitors making up 2% of visits to *A. bibullatus* and 5% to *P. subacaule*. Eastern bumble bees rarely visited compared to the 2022 field season, so there was not enough data to analyze.

Carpenter bees had an average visitation rate of 0.32 for *A. bibullatus*, which was significantly lower than 2.18 for *P. subacaule (*W=41, **P*<0.001; Figure 13). Compared to the 2022 field season, the visitation rate for *A. bibullatus* in 2023 was not significantly different (W=132, *P*=0.627; Figure 14). On the other hand, carpenter bees had an average visitation rate of 0.6 for *P. subacaule* in 2022, which is significantly less than 2.18 in 2023 (W=102, **P*=0.002; Figure 15).

Data collection on visitation occurred between 11 April 2023 and 20 April 2023, and the visitation rate of carpenter bees varied throughout. The highest visitation of carpenter bees to *A. bibullatus* occurred around peak flowering on 11 April 2023 (Figure 16). Within two days, the visitation rate to *A. bibullatus* dropped to zero. Consequently, the visitation rate to *P. subacaule* was 2.9 on 11 April 2023 and began to rise after visitation to *A. bibullatus* decreased to zero by 13 April 2023. The highest recorded daily average was 5.8 which occurred on 15 April 2023, which then steadily decreased until the end of the data collection period.

The average visitation rate of blue solitary bees for *A. bibullatus* and *P. subacaule* was 3.00 and 0.54, respectively (Figure 17). The data sets were normally distributed for *A. bibullatus* and *P. subacaule* (*P*=0.063, *P*=0.123), and the t-test results supported that blue solitary bees had a significantly higher visitation rate to *A. bibullatus* than *P. subacaule* (t=3.06, **P*=0.005). Compared to the 2022 field season, blue solitary bees visited *A. bibullatus* and *P. subacaule* significantly more in 2023 (W=0.5, **P*<0.001; W=71, **P*<0.001; Figure 14 and Figure 15). Notably, the visitation to *A. bibullatus* drastically increased from its average of 0.02 in 2022.

On the first day of data collection, 11 April 2023, blue solitary bees visited *A. bibullatus* and *P. subacaule* at an average rate of 3.12 and 0.65, respectively (Figure 18). The visitation rate to both plants dropped close to zero on 13 April 2023 but increased the following days. The highest recorded daily average of *A. bibullatus* occurred on 18 April 2023 with a rate of 8.00. Furthermore, the highest daily average rate of *P. subacaule* was 0.75 and occurred on 20 April 2023.

Green solitary bees were the least common visitors to *A. bibullatus* and *P. subacaule* with an average visitation rate of 0.11 and 0.10, respectively, which was not significantly different (W=97.5, *P*=0.161; Figure 19). Compared to the 2022 field season, visitation rates did not significantly differ from the 2023 season for *A. bibullatus* and *P. subacaule* (W=141, *P*=0.332; W=215.5, *P*=0.915; Figure 14 and Figure 15). Green solitary bees started foraging on 15 April 2023 exclusively to *P. subacaule* (Figure 20). The highest daily average rate for *P. subacaule* occurred on 20 April 2023 at 0.24. Green solitary bees only visited *A. bibullatus* on 18 April 2023, which they visited at an average rate of 0.50.

In addition to the common pollinator taxa previously described, there were frequent observations of other pollinators visiting both plants. For example, there were visits of honeybees, butterflies, and moths. Although these pollinators didn't visit frequently enough to be analyzed separately, they did contribute to the visitation rate of all pollinators. In 2022, the average visitation rate of all pollinators was 0.63 for *A. bibullatus*, which was not significantly less than 1.01 for *P. subacaule* after the adjusted significance level (W=127, *P*=0.026; Figure 21). In 2023, the average visitation rate of all pollinators was 3.49 for *A. bibullatus* and 2.82 for *P. subacaule*, which was not significantly different (W=137, *P*=0.681). Visitation rates for *A. bibullatus* and *P. subacaule* were significantly higher in 2023 than 2022 (W=212, **P*<0.001; W=354, **P*<0.001).

Constancy

April 2022

In 2022, carpenter bees had an average constancy of 0.844, which is the lowest average among the insect taxa examined (n=14) (Figure 22 and Table 2). Eastern bumble bees had an average constancy of 0.899 (n=15), blue solitary bees had an average constancy of 0.894 (n=9), and green solitary bees were completely constant with no interspecific movements documented (1.0) (n=8). The constancies among insect visitor groups were significantly different (*P*=0.029). A post hoc Dunn test determined the significance among group-group comparisons and supported that green solitary bees had a significantly higher constancy than blue solitary bees, carpenter bees, and eastern bumble bees (Table 3). There was no difference in the following groupings: eastern bumble bees-carpenter bees, eastern bumble bees-blue solitary bees, or carpenter bees-blue solitary bees.

April 2023

Due to a small number of *A. bibullatus* racemes, constancy data was limited this field season. Carpenter bees and blue solitary bees had an average constancy of 0.996 (n=11) and 0.969 (n=12), respectively (Figure 23). The average constancy between carpenter bees and blue solitary bees was not statistically different (W=84, *P*=0.135). There was insufficient data for constancy analysis on green solitary bees.

Hand-Pollination Experiment

April 2022

Racemes treated with only conspecific pollen produced an average of 12.78 seeds per fruit, which was the highest average among treatments (Figure 24). Of the 7 surviving racemes, 6 of them produced 9 fruits. Hand pollinated plants with a mix of conspecific pollen and foreign pollen produced an average of 11.44 seeds per fruit. Racemes in Treatment 2a (initially pollinated with conspecific pollen and then foreign pollen) had only 2 out of 10 racemes survive, and they produced 2 fruits with 14 seeds on average. On the other hand, treatment 2b (initially pollinated with foreign pollen and then conspecific pollen) had 8 out of the 10 plants survive and produce fruit. A total of 16 fruits were collected, and they had an average of 11.13 seeds per fruit. Foreign pollinated racemes had 20 initial racemes and 17 of them were at the Savanna field site and survived the storm. The 3 racemes at the Alexander site were excluded from analysis as it was difficult to distinguish if the cause of no fruit production was due to the pollen treatment or weather conditions. Of the surviving plants at Savanna, 11 racemes produced 19 fruits with an average of 7.21 seeds per fruit.

A two-way ANOVA determined the effect of treatment and site location on seed production. The site location was not a statistically significant factor affecting seed production (P=0.061). The pollen treatment affected seed production (P=0.019), and a post hoc Tukey's HSD revealed that fruits from treatment 3 had significantly lower seed production than treatment 1 (P=0.037). Due to the significant reduction in sample size and inability to accurately determine the cause of no fruit production, the statistical analyses for this data are based on two assumptions. The first assumption is that most racemes died at the Alexander site, and the only ones to survive were 4 racemes of treatment 1. Many other racemes with treatment 1 at the Alexander site that produced no fruit were excluded since the effect was likely due to the large amount of precipitation and not the pollination treatment. The second assumption is that all the racemes at the Savannah sites survived. The Savannah site is open and exposed to sunlight, which would have evaporated excess moisture from the flower bags. The flowers were still in good condition following the storm, and their seed count was included in the analysis. Because of these assumptions, the effect of the treatments may not be accurately quantified. A primary goal of the 2023 field season was to repeat this experiment in a different site with a larger sample size to conclusively determine the effect of foreign pollen on reproduction.

April 2023

The frequency of fruit production varied significantly based on hand pollination treatment in 2023. Racemes treated with conspecific pollen had the highest frequency of fruit production at 60.71%, which was significantly higher than the mixed, foreign, and no pollen treatments (*P*=0.003; *P*<0.001; *P*<0.001; Figure 25). The mixed pollen treatment was split into two sub-treatments with type A racemes being initially pollinated with conspecific pollen and type B being initially pollinated with foreign pollen; there was no significant difference in the fruit production of the sub-treatments (*P*=0.741). Of the racemes treated with mixed pollen, 28.13% produced fruit, which was significantly lower than the conspecific pollen treatment (*P*=0.003). Furthermore, 3.5% of foreign pollen racemes produced fruit and 0% of non-pollinated racemes produced fruit, which was significantly less frequent than the mixed pollinated racemes (*P*=0.038; *P*<0.001).

Racemes treated with only conspecific pollen produced 37 fruits with an average of 11.73 seeds per fruit (Figure 26). Hand-pollinated racemes receiving mixed pollen produced an average of 10.59 seeds per fruit. Racemes in sub-treatment type A produced 16 fruits with 10 seeds on average. On the other hand, type B pollinated racemes produced 6 fruits with an average of 12.17 seeds per fruit. The average seed set among sub-treatments A and B was not statistically different (*P*=0.715). Foreign-pollinated racemes only produced 2 fruits with an average of 8 seeds per fruit. The number of seeds produced did not differ significantly among treatments (*P*=0.674).

Stigma Slides- Absence or Presence of Foreign Pollen

Of the 200 stigma slides prepared, 80 *A. bibullatus* and 91 *P. subacaule* stigmas were unobstructed and used for analysis. The average number of conspecific pollen grains on or touching the stigmas of *A. bibullatus* was 63.11 and was significantly higher than the average 30.97 conspecific pollen grains found on *P. subacaule* samples ($P<0.001$; Figure 27). There were 22% of *A. bibullatus* slides with foreign pollen present and only 4% of *P. subacaule* slides, which was significantly different among the two species ($P<0.001$; Figure 28). When foreign pollen was present on the slide, the average number of foreign pollen grains was 8.45 on *A. bibullatus* slides and 17.75 on *P. subacaule* slides, which was not significantly different ($P=0.473$; Figure 29).

Measurement of Pollen Grains

The primary characteristics used to distinguish the pollen were size and shape. For example, the pollen of *A. bibullatus* is smaller than *P. subacaule* and has more of an oval shape. To standardize the size and shape of the pollen, 60 grains of each species had their length, width, and area measured. All measurements were analyzed via a MANOVA and were statistically different among species ($P<0.001$). Furthermore, a post hoc ANOVA determined each measurement was significantly different among species (length $P=0.013$, width $P<0.001$, and area $P<0.001$; Table 4). A Linear Discriminant Analysis test was used to confirm if these traits can discriminate between the pollen of *A. bibullatus* and *P. subacaule*. The LDA model was able to predict the correct species 100% of the time when given measurements. Furthermore, there was no overlap between the discriminant function values between the two species (Figure 30).

DISCUSSION

Astragalus bibullatus and *P. subacaule* were primarily visited by carpenter bees, eastern bumble bees, and solitary bees, indicating that they compete for pollinators. Carpenter bees had a high visitation rate and were the most common visitors to both plant species in 2022. Throughout both field seasons, carpenter bees were never observed to enter the front of the flower but instead drilled holes into the back of the flower to steal nectar. This nectar-robbing behavior depletes the available nectar supply for that flower and likely deters future pollinator visits. In 2022, the most common insect visitors observed to pollinate were eastern bumble bees. Eastern bumble bees go through the front of the flower during foraging, which formed visible pollen baskets. Eastern bumble bees also had a higher visitation rate to *P. subacaule*, indicating they may prefer to forage on this species. This preference is likely caused by a higher relative flower density of *P. subacaule* because visitation rates of carpenter and bumble bees increased with flower availability. For example, *A. bibullatus* racemes started senescing around April 19th, corresponding with a decrease in visitation but a drastic increase in visitation to its competitor, *P. subacaule*. The only exception to this pattern was the visitation rate of green solitary bees, which increased as the flower abundance of *A. bibullatus* decreased.

In 2023, the relative abundance of *A. bibullatus* was very low compared to the previous field season. According to unpublished long-term data, populations of *A. bibullatus* tend to have two good flowering years followed by a low flowering year (M. Albrecht, pers. comm.). Likely due to this decrease and unseasonably warm weather, the visitation rates of pollinators drastically differed in 2023. For example, eastern bumble bees rarely visited either species, so I could not compare the visitation rates between plants. On the other hand, carpenter bees were very abundant and significantly preferred *P. subacaule* over *A. bibullatus*. Compared to the previous year, carpenter bees visited *P. subacaule* more frequently than in 2022, which may be due to the much higher *P. subacaule* relative abundance. Blue solitary bees had a significantly higher visitation rate to both plant species in 2023 and preferred *A. bibullatus*. Since the relative abundance of *A. bibullatus* was much lower in 2023, these results support that blue solitary bees likely prefer *A. bibullatus*.

Pollinators may have fixed affinities for one flower due to physiological dependencies or preferences, referred to as oligolecty (Waser, 1986; Praz, 2008). In contrast, pollinators may follow the optimal diet theory and choose to forage on the more abundant and rewarding species (MacArthur & Pianka, 1966). For my study, carpenter bees and bumble bees support the latter hypothesis of foraging behavior as they had a higher visitation rate to the more abundant species. Johnson et al. (2022) found similar results and concluded that different abundances of two species often result in imbalanced pollinator preferences and the exclusion of the rare species. The exception I found was blue solitary bees, which preferred *A. bibullatus* despite its low abundance; these results suggest that blue solitary bees may have a fixed preference for *A. bibullatus*. Previous studies and observations have found that oligolecty is common for bees in the family Megachilidae (Praz, 2008). This pattern of specialization is either caused by physiological constraints, such as differences in the nutritional quality of floral rewards, or by neurological limitations preventing the proper handling of certain flowers. Since blue solitary bees preferred and consistently visited *A. bibullatus* in both seasons, future studies should focus on the role of blue solitary bees in the reproduction of *A. bibullatus*. Furthermore, monitoring blue solitary bee populations should be incorporated into the management plan of *A. bibullatus*.

Floral constancy impacts the frequency of foreign pollen deposition, which can result in a reduced seed set (Waser, 1978; Thomson et al., 1982). In 2022, carpenter bees, eastern bumble bees, and blue solitary bees moved between species, but most movements were intraspecific. Unlike the more common visitor taxa, green solitary bees were completely constant, but they rarely visited and probably had minimal impact on reproduction. The inconstant transitions between plants are likely due to morphological similarities and similar relative abundances of both plant species, which increases the likelihood of indiscriminate foraging. In a related study, Bell et al. (2005) designed experimental arrays of pure and mixed morphologically similar flowers and found similar results. *Mimulus ringens* and *Lobelia siphilitica* planted in mixed arrays experienced several interspecific movements of pollinators, resulting in occasional foreign pollen transfer and reduced seed set. The inconstant transitions between *A. bibullatus* and *P. subacaule* may have a similar effect, but future studies are required to quantify those consequences.

In 2023, the data collected for constancy was limited due to few *A. bibullatus* racemes to monitor. Based on the available data, carpenter bees and blue solitary bees had a higher constancy than the previous year. The difference in constancy between field seasons is likely due to the decrease in the relative abundance of *A. bibullatus* racemes, which encourages foraging solely on the more common *P. subacaule*.

Although, blue solitary bees foraged on *A. bibullatus* more frequently and had a constancy of 0.969.

The effect of the hand pollination treatment was difficult to determine in the 2022 field season due to a dramatic decline in sample size. In a previous study, Brown & Campbell (2005) found that *Lythrum alatum* treated with mixed pollen loads resulted in a 28.8% lower seed set than those treated with conspecific pollen. The results of my study were different since *A. bibullatus* racemes treated with conspecific pollen did not differ in seed set from those treated with mixed pollen loads. On the other hand, racemes treated solely with foreign pollen did produce significantly fewer seeds than the other two treatments, which did align with the results of Brown & Campbell (2005). Racemes treated with foreign pollen were predicted to produce no fruits or seeds, but several of my samples produced seemingly viable seeds. Some fruits also contained abnormal fuzzy seeds that were presumed aborted. The production of seeds, when pollinated with foreign pollen, was unexpected because these plants are in different genera and are unlikely to hybridize. Since the anthers of *A. bibullatus* were not removed before the treatment of foreign pollen to the stigmas, it is feasible that seed production was the result of self-pollination.

In 2023, the hand pollination experiment was more successful, and most racemes survived. The effect of the treatments on fruit production was significant, and those treated with conspecific pollen produced fruit more frequently than the other treatments. Since there was a decline in fruit production when foreign pollen was present, foreign pollen likely caused reproductive interference. When fruit production did occur, there was no significant difference in the seed set; these results conflict with the data from the previous field season, which makes it difficult to determine the effect of foreign pollen on the seed set. Racemes that received no pollen did not produce fruits or seeds, indicating that the mesh bags used in the experiment were effective at excluding pollinators.

Pollen grains of *A. bibullatus* and *P. subacaule* differed in size and shape, which allowed for easy identification of the pollen under the microscope. Foreign pollen was found significantly more frequently on *A. bibullatus* stigmas than on *P. subacaule*, despite relatively equal observed interspecific movements between the species. The frequent foreign pollen may cause stigma clogging, stigmatic closure, or pollen allelopathy, reducing female fitness of *A. bibullatus* (Waser, 1978; Thomson et al., 1982; Waser & Fugate, 1986). Furthermore, *A. bibullatus* had significantly more conspecific pollen produced that remained on the stigma, which would result in reduced male fitness of *A. bibullatus* as pollen is not transferred to new flowers.

This study supports that *Astragalus bibullatus* competes with *Pediomelum subacaule* for pollinators, which may be affecting the reproductive success of *A. bibullatus*. The data collected on constancy was limited in 2023 due to insufficient available racemes for observation. Furthermore, the visitation and constancy data were collected only from the Airport population since it was the largest and had the most active pollinators, but data from multiple sites could have provided more insight into pollinator activity. The mesh bags to deter pollinators in my hand pollination experiment were a weakness of my experimental design since excess water buildup in the bags prevented the flowers from fruiting. In future studies, it would be advisable to come up with another way to prevent natural pollination or to perform an experiment in a greenhouse. Despite these flaws, pollination competition does pose a risk to the reproduction of *A. bibullatus,* and the data from this study should inform future management practices.

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APPENDIX A: TABLES

Table 1: Latitude and longitude coordinates for the populations of *Astragalus bibullatus* used by the Herlihy-Walck lab group.

Population Name	Latitude	Longitude
Airport	35.85559	-86.29003
Alexander	35.85639	-86.29013
Davenport West	35.85784	-86.29589
Hall Farm	35.86812	-86.28599
Savanna	35.85911	-86.295

Table 2: Average constancy values of the primary insect visitor taxa in April 2022. Green solitary bees were determined to be completely constant (1), while other groups had documented interspecific movements and constancy ranged from 0.844-0.899.

Pollinator Taxa	Calculated Constancy	
Eastern bumble	0.899	n = 15
Carpenter	0.844	n = 14
Blue solitary	0.894	n = 9
Green solitary	1.000	n = 8
Average of all samples:	0.899	n = 46

Table 3: Post hoc Dunn test results following a significant Kruskal-Wallis test on insect visitor constancies. Green solitary bees had a significantly higher constancy than three other insect groups, but there was no significant difference among carpenter bees, eastern bumble bees, and blue solitary bees.

	Blue solitary	Carpenter	Eastern bumble
Carpenter	0.576845 $P = 0.2820$		
Eastern bumble	-0.313006 $P = 0.3771$	-1.056728 $P = 0.1453$	
Green solitary	-2.106632 $P = 0.0176$	-2.953446 $P = 0.0016$	-2.092937 $P = 0.0182$

Table 4: Post hoc ANOVA test results following a significant MANOVA test on pollen grain measurements. Length, width, and area were all statistically different between *A. bibullatus* and *P. subacaule*.

	Length	Width	Area
Astragalus bibullatus	$\bar{X} = 0.023$ mm	$\bar{X} = 0.019$ mm	$\bar{X} = 2.998\ e^{-4}\ mm^2$
Pediomelum subacaule	$\bar{X} = 0.036$ mm	$\bar{X} = 0.030$ mm	$\bar{X} = 6.271\ e^{-4}\ mm^2$
P-value (post hoc ANOVA)	$P = 0.013$	$P < 0.001$	$P < 0.001$

APPENDIX B: FIGURES

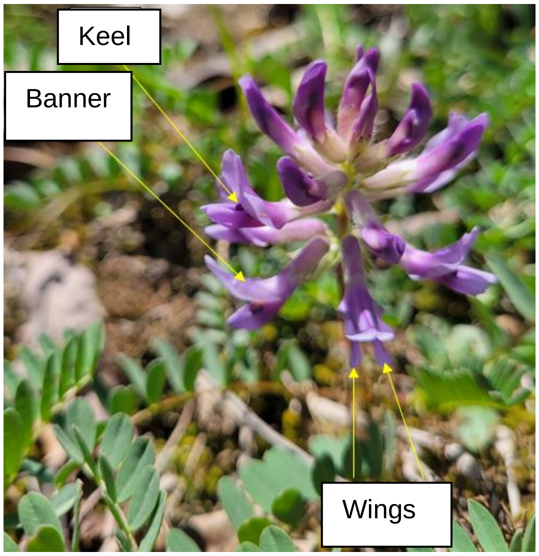

Figure 1: Photograph of early flowering *Astragalus bibullatus* raceme taken on March 30th, 2023. The arrows point to the morphological features in the flower: the banner, wings, and keel.

Figure 2: Photograph of flowering *Pediomelum subacaule* raceme taken on April 10th, 2023.

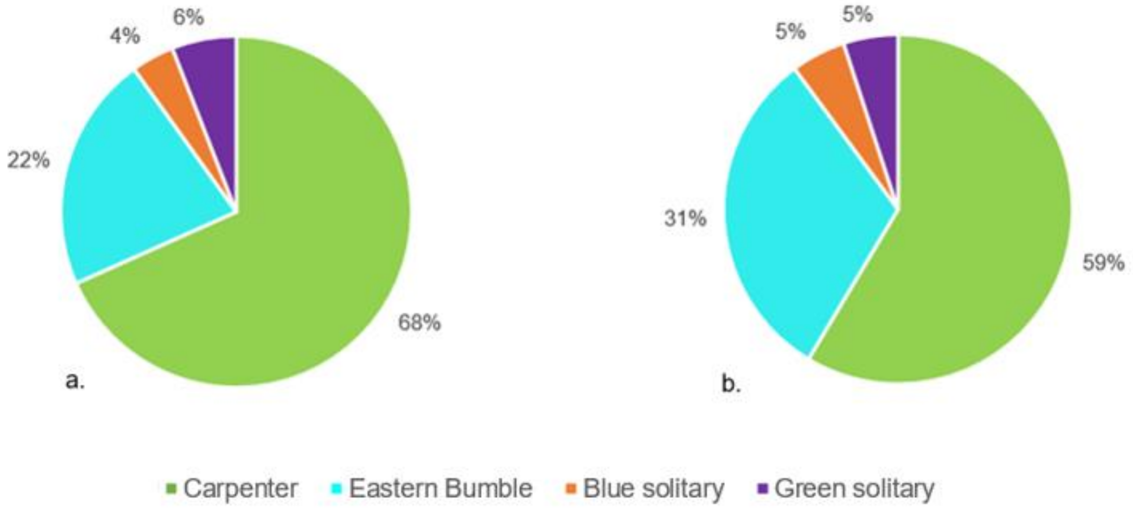

Figure 3: The proportion of insect visitors to *Astragalus bibullatus* (a) and *Pediomelum subacaule* (b) racemes during the 2022 field season. Carpenter bees were the most common visitors making up 68% and 59% of visits to *A. bibullatus* and *P. subacaule*, respectively.

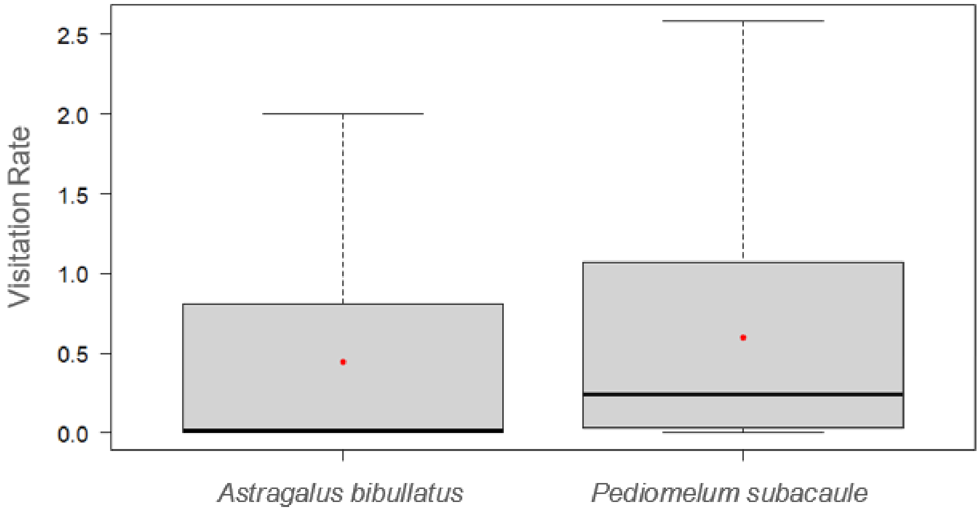

Figure 4: Visitation rate (#visits/#racemes/hour) of carpenter bees in April 2022. The visitation rates between *A. bibullatus* and *P. subacaule* were not statistically different (*P*=0.2723).

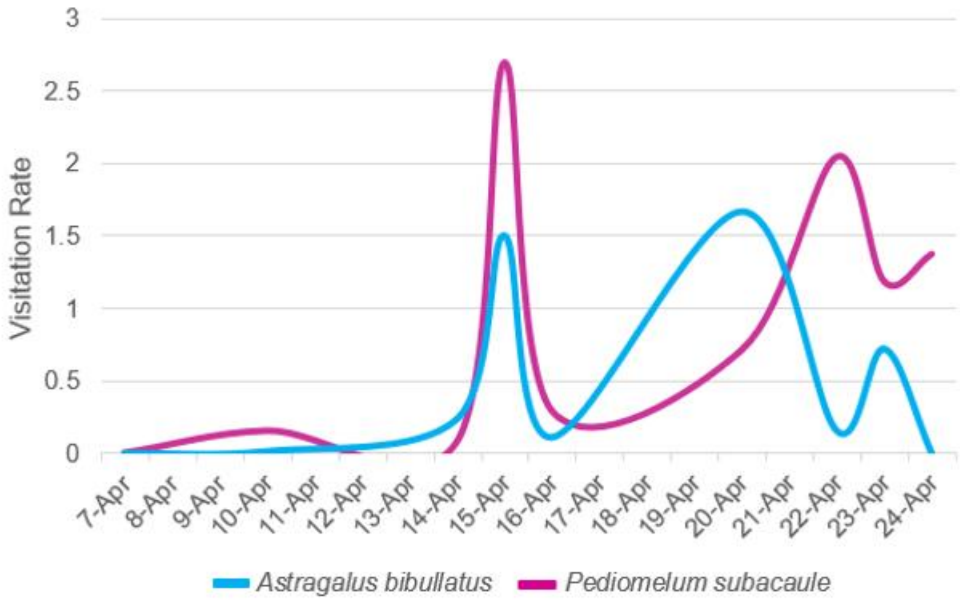

Figure 5: The visitation rate of carpenter bees throughout the 2022 field season. Peak activity for *A. bibullatus* and *P. subacaule* was around April 15th, and then visitation fluctuated until the end of the season.

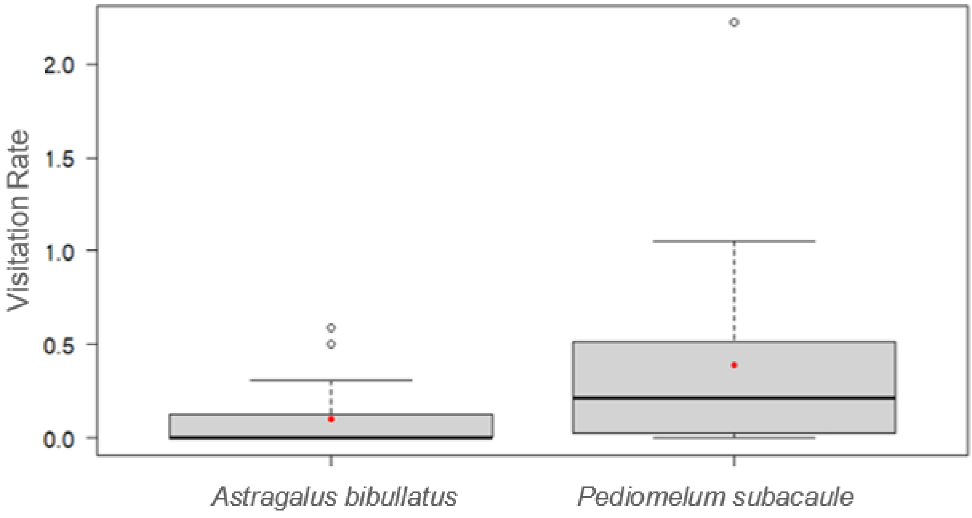

Figure 6: Visitation rate (#visits/#racemes/hour) of eastern bumble bees in April 2022. The visitation rates between *A. bibullatus* and *P. subacaule* were statistically different (*P*=0.0082).

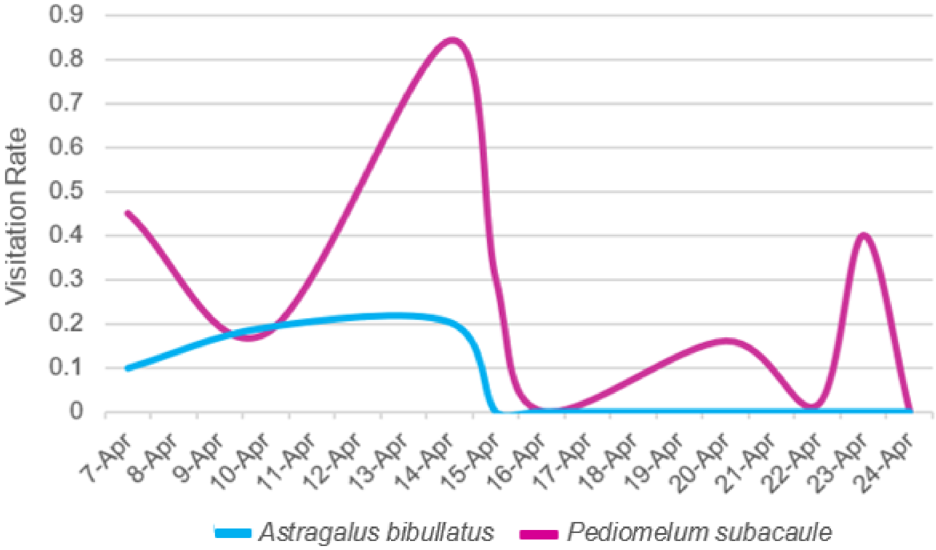

Figure 7: The visitation rate of eastern bumble bees throughout the 2022 field season. Peak activity for *A. bibullatus* was on April 14th, and then dramatically decreased. Peak visitation to *P. subacaule* was on April 14th, but it drastically reduced for several days.

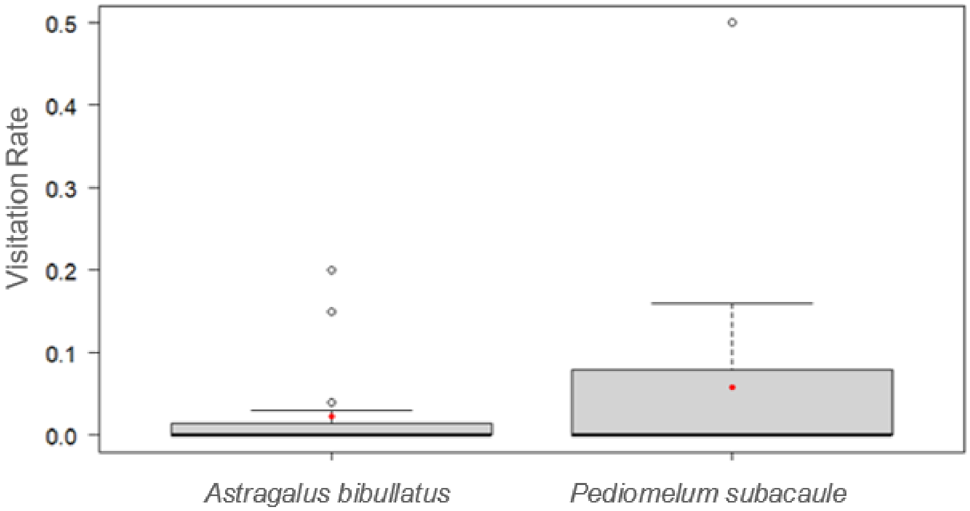

Figure 8: Visitation rate (#visits/#racemes/hour) of blue solitary bees in April 2022. The visitation rate of blue solitary bees to *P. subacaule* was not significantly greater than visitation to *A. bibullatus* (*P*=0.2551).

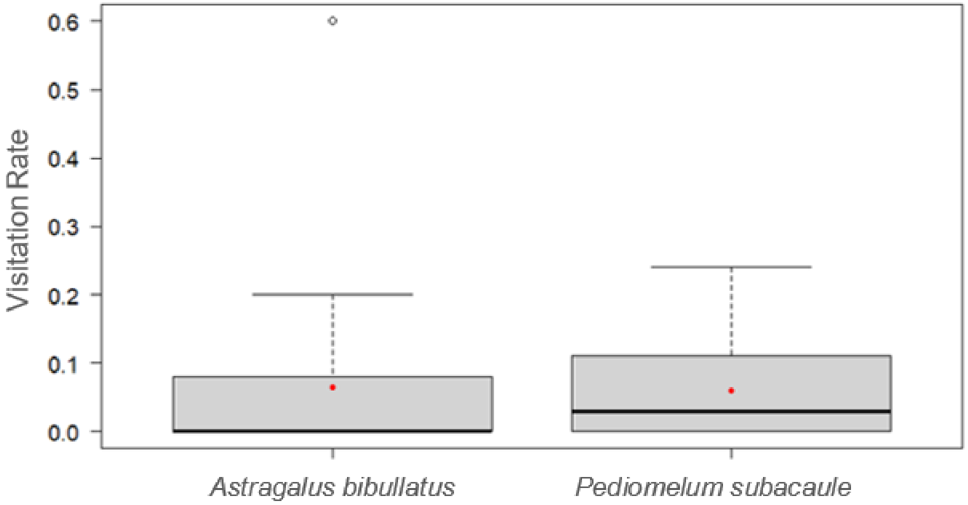

Figure 9: Visitation rate (#visits/#racemes/hour) of green solitary bees in April 2022. The visitation rate of green solitary bees to *P. subacaule* was not significantly different than visitation to *A. bibullatus* (*P*=0.4556).

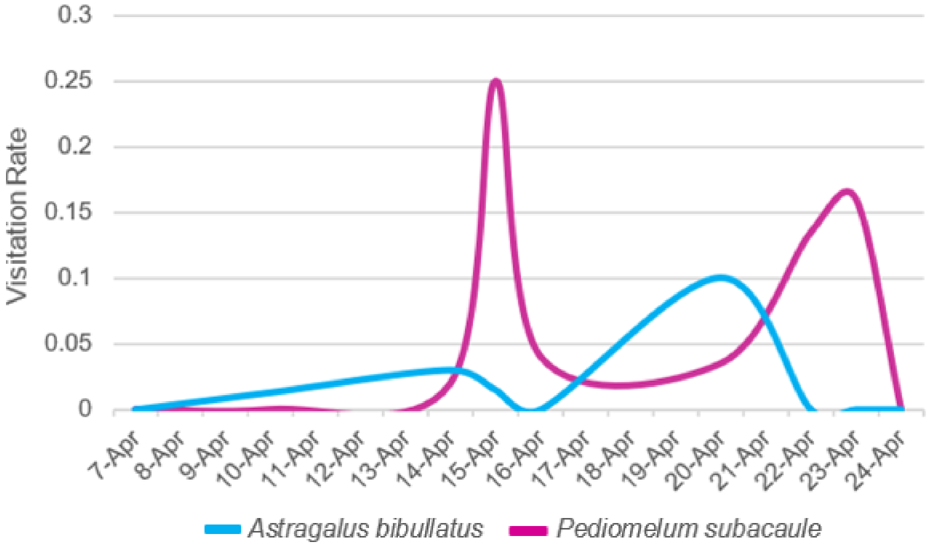

Figure 10: The average daily visitation rate of blue solitary bees throughout the 2022 field season. Peak activity for *A. bibullatus* was on April 20th and then decreased the remaining season. Peak visitation to *P. subacaule* was on April 15th, but it drastically reduced for several days until increasing after April 20th.

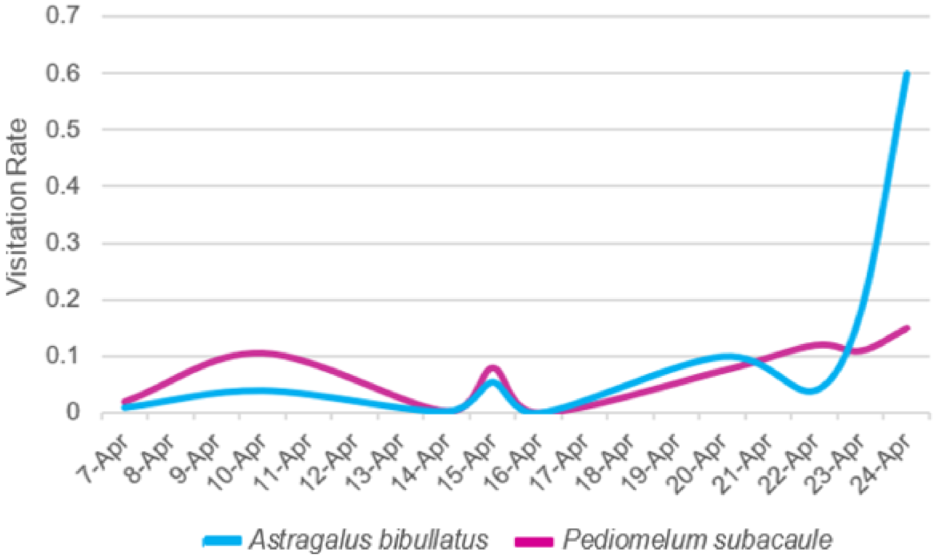

Figure 11: The average daily visitation rate of green solitary bees throughout the 2022 field season. Visitation didn't start until April 15th, and then after April 20th the visitation to both plants increased.

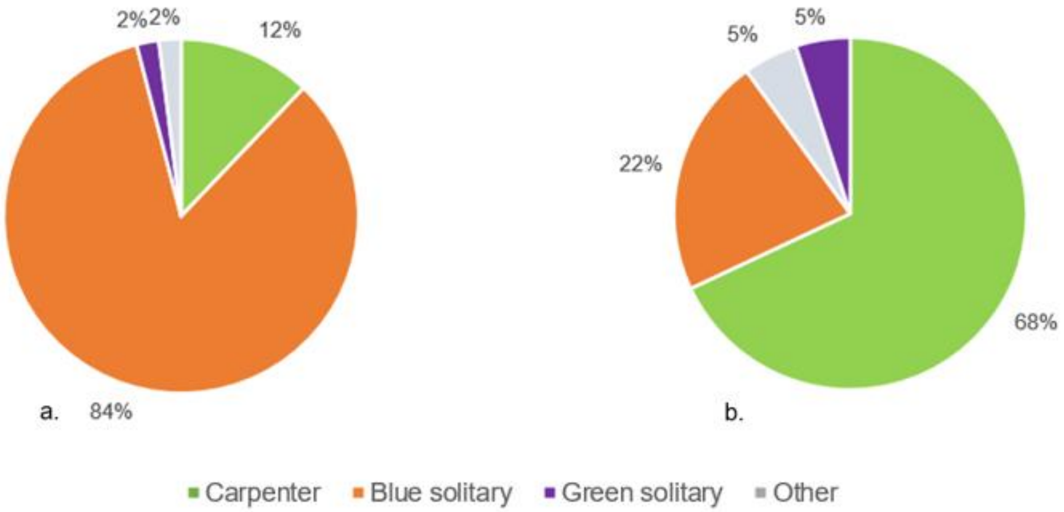

Figure 12: The proportion of insect visitors to *Astragalus bibullatus* (a) and *Pediomelum subacaule* (b) racemes during the 2023 field season. Blue solitary bees were the most common visitors to *A. bibullatus* comprising 84% of the visits. Carpenter bees were the most common visitors to *P. subacaule* comprising 68% of the visits.

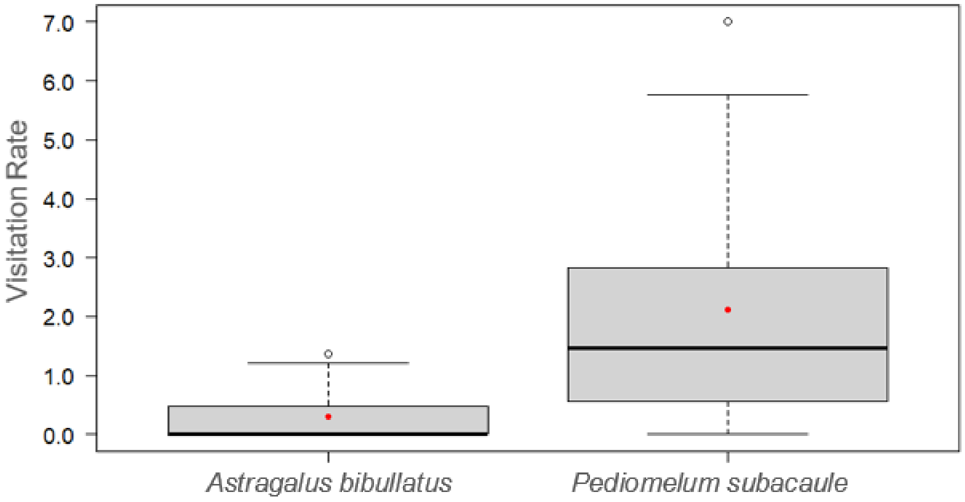

Figure 13: Visitation rate (#visits/#racemes/hour) of carpenter bees in April 2023. The visitation rate from carpenter bees to *P. subacaule* was significantly greater than visitation to *A. bibullatus* (*P*<0.001).

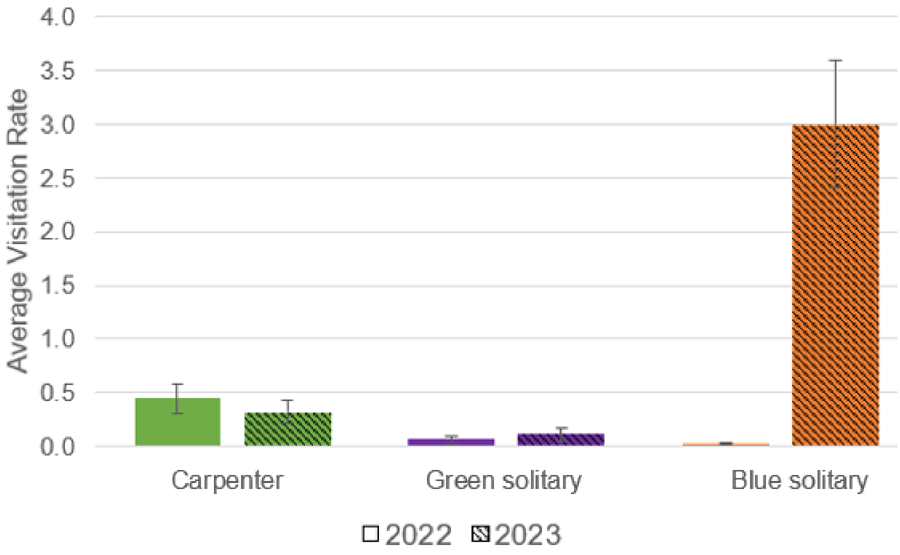

Figure 14: Average visitation rates of common pollinator taxa to *A. bibullatus* in 2022 vs 2023. Blue solitary bees visited significantly more frequently in 2023 than 2022 (*P*<0.001).

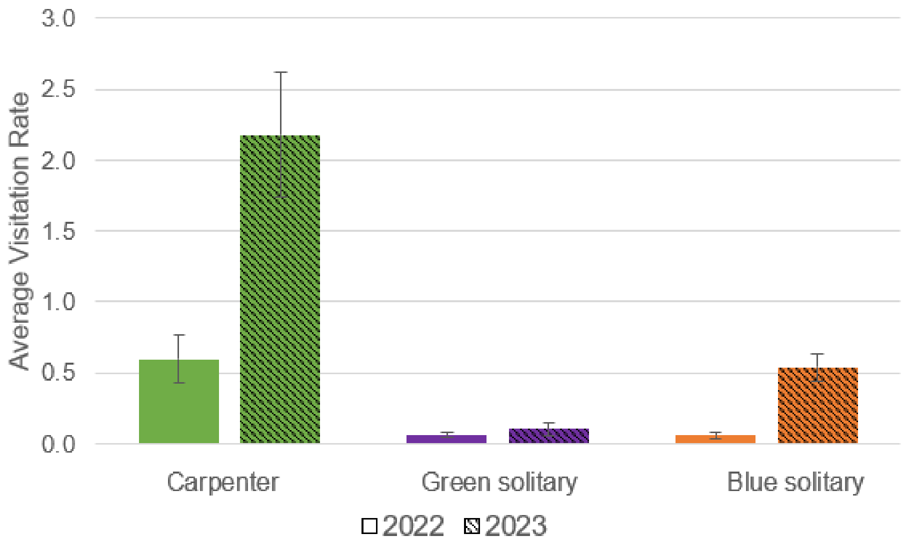

Figure 15: Average visitation rates of common pollinator taxa to *P. subacaule* in 2022 vs 2023. Carpenter and blue solitary bees visited significantly more frequently in 2023 than 2022 (*P*<0.001, *P*=0.002).

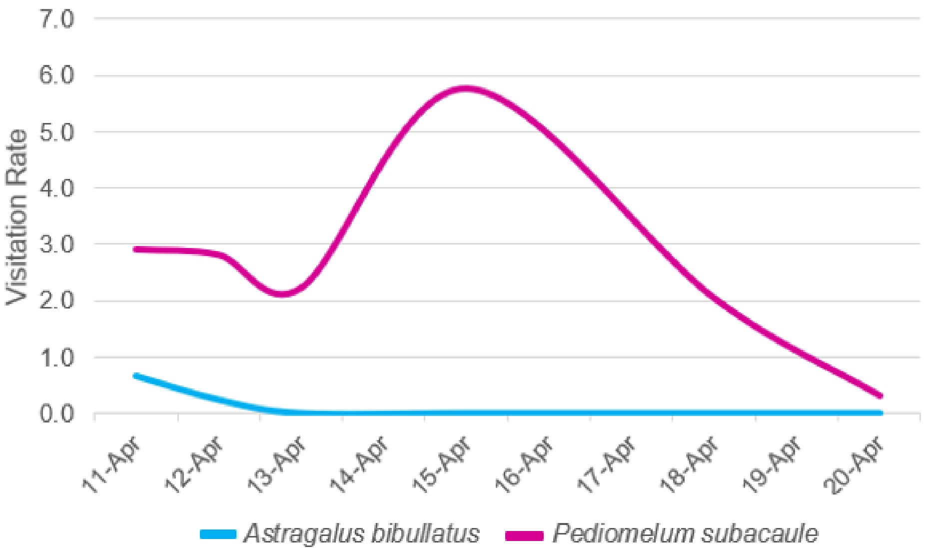

Figure 16: The average daily visitation rate of carpenter bees throughout the 2023 field season. Peak activity for *A. bibullatus* was around April 11th, and then dramatically decreased to zero by April 13th. Visitation to *P. subacaule* peaked on April 15th and then gradually decreased until the end of the season.

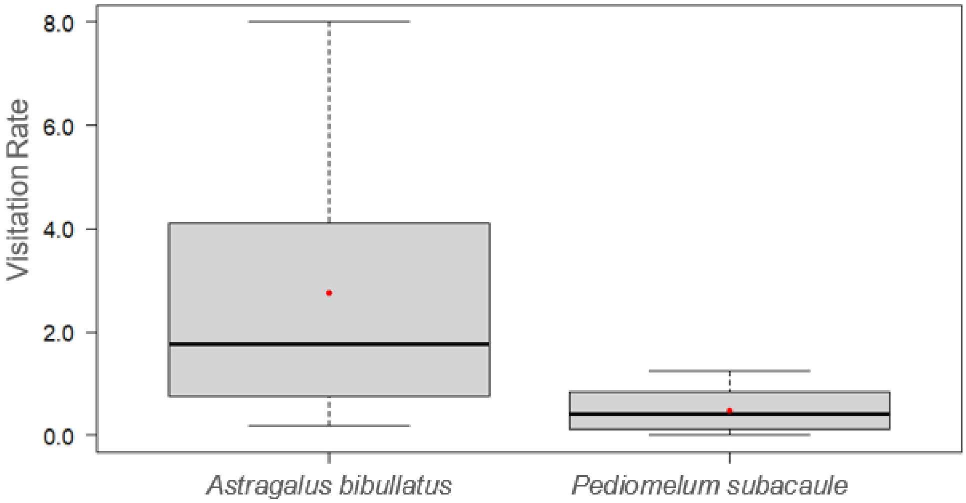

Figure 17: Visitation rate (#visits/#racemes/hour) of blue solitary bees in April 2023. The visitation rate of blue solitary bees to *A. bibullatus* was significantly greater than visitation to *P. subacaule* (*P*=0.005).

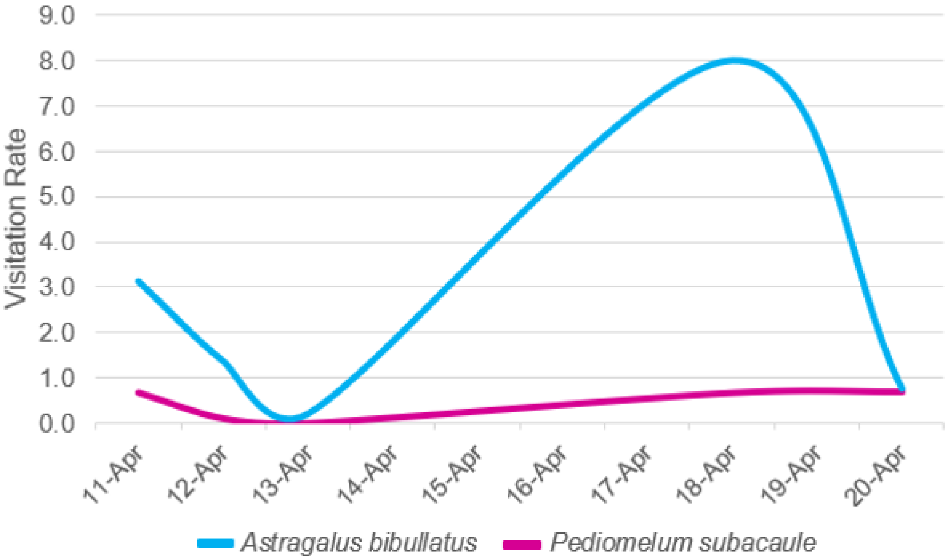

Figure 18: The average daily visitation rate of blue solitary bees throughout the 2023 field season. Visitation to *A. bibullatus* steadily increased until peak activity on April 18th. Visitation to *P. subacaule* remained low throughout the season and peaked on April 20th.

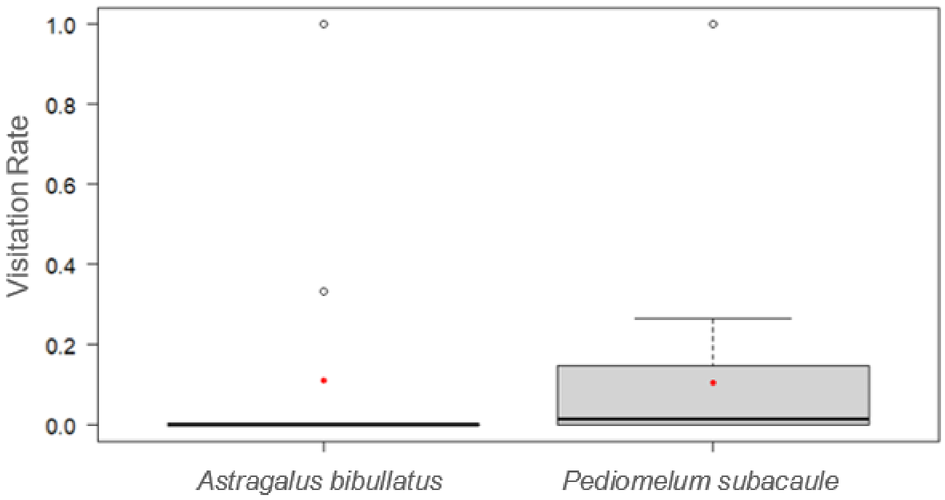

Figure 19: Visitation rate (#visits/#racemes/hour) of green solitary bees in April 2023. The visitation rates of green solitary bees between *A. bibullatus* and *P. subacaule* were not significantly different (*P*=0.161).

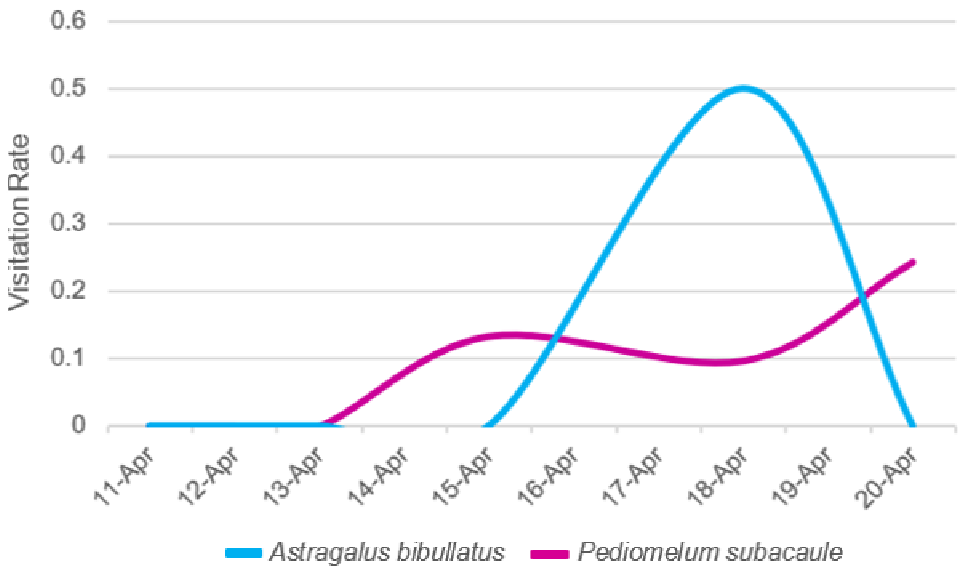

Figure 20: The average daily visitation rate of green solitary bees throughout the 2023 field season. Visitation to *A. bibullatus* remained at zero until peak activity on April 18th. Visitation to *P. subacaule* remained low throughout the season and peaked on April 20th.

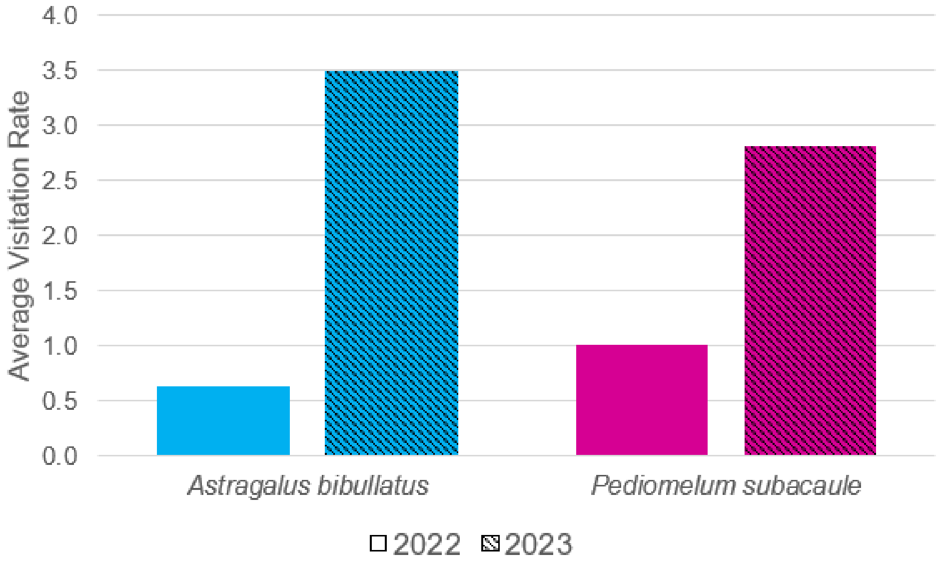

Figure 21: The average visitation rate of all pollinators for *A. bibullatus* and *P. subacaule* in 2022 and 2023. The average visitation rate for 2023 was significantly higher than 2022 for both plants (*P*<0.001; *P*<0.001). Furthermore, the average visitation of *P. subacaule* in 2022 was significantly higher than *A. bibullatus* in 2022 (*P*=0.03).

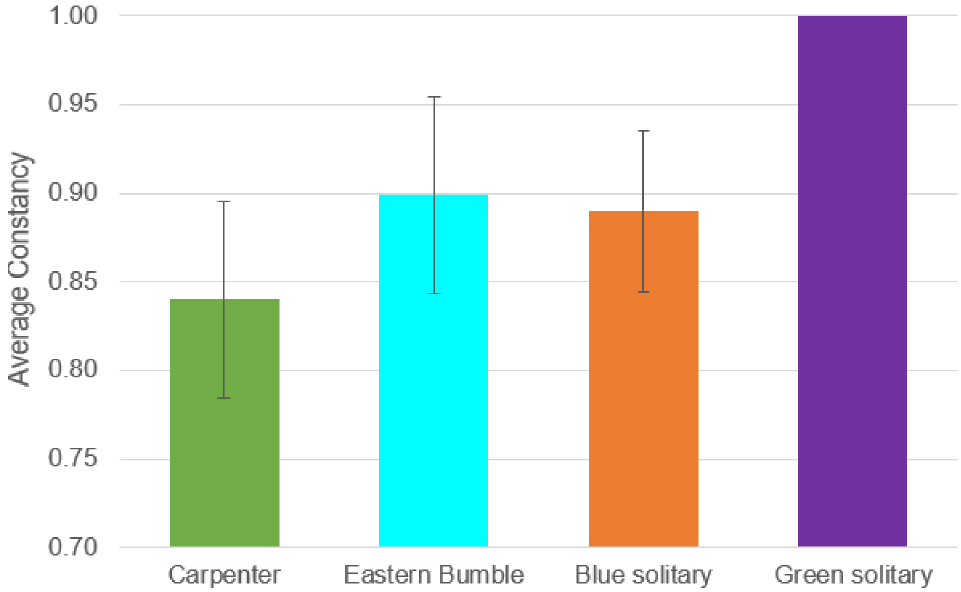

Figure 22: The average constancy of pollinator taxa in 2022. Green solitary bees had an average constancy of 1, which is significantly higher than all other pollinator taxa (*P*=0.029). The lowest constancy was 0.84 for carpenter bees. Eastern bumble bees and blue solitary bees had an average of 0.899 and 0.894, respectively.

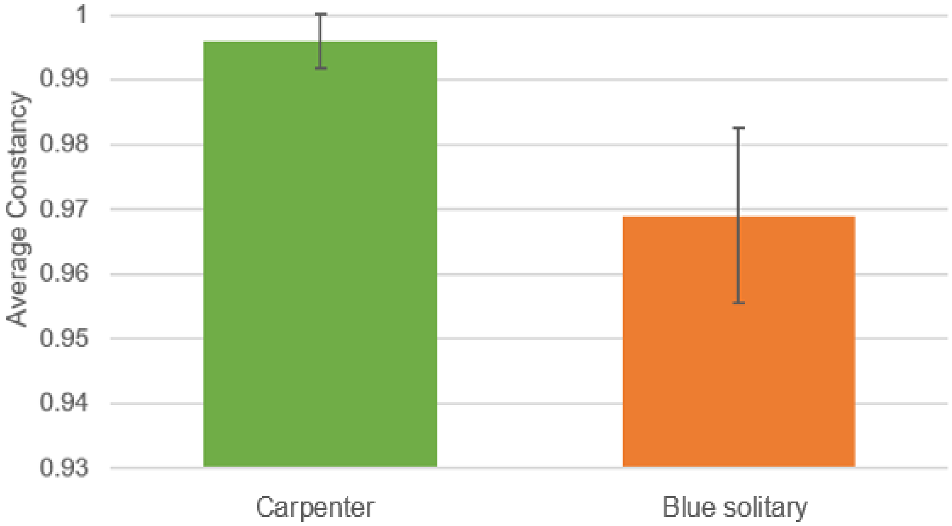

Figure 23: The average constancy of pollinator taxa in 2023. Carpenter bees had an average constancy of 0.996, which was not significantly different than 0.969 for blue solitary bees (*P*=0.14).

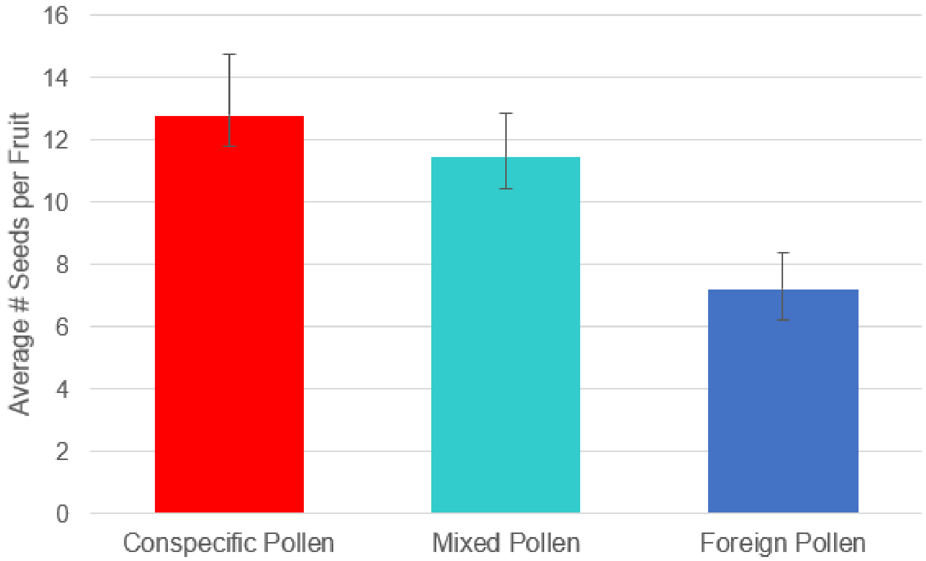

Figure 24: Average seed production among hand pollination treatments. Plants treated with conspecific pollen had an average of 12.78 seeds per fruit, mixed pollen plants had 11.44 seeds per fruit, and foreign pollen plants had 7.21 seeds per fruit.

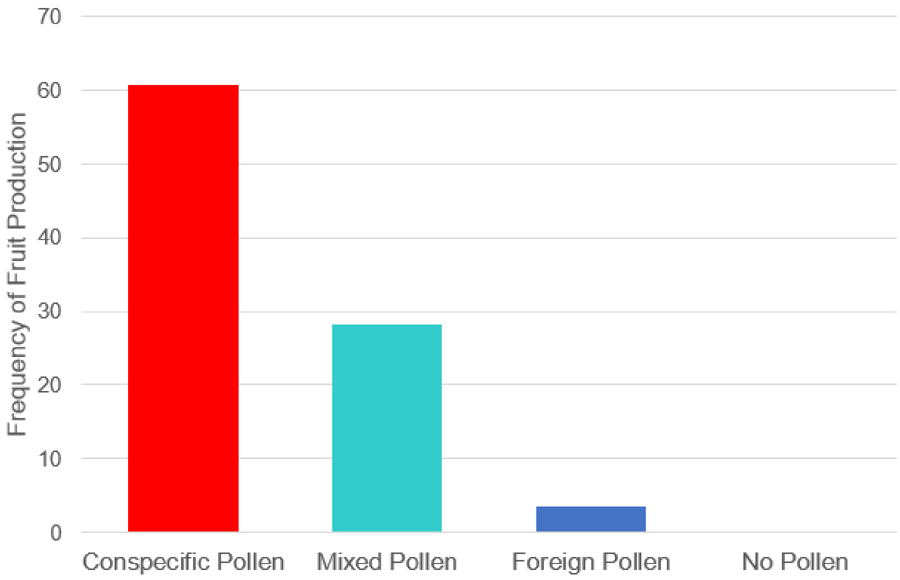

Figure 25: Frequency of fruit production among hand pollination treatments. 60.71% of racemes treated with conspecific pollen produced fruit, which was significantly higher than the mixed, foreign, and no pollen treatments (*P*=0.003; *P*<0.001; *P*<0.001). Furthermore, 28.13% of mixed pollen racemes produced fruit, which was significantly higher than the foreign and no pollen treatments (*P*=0.038; *P*<0.001).

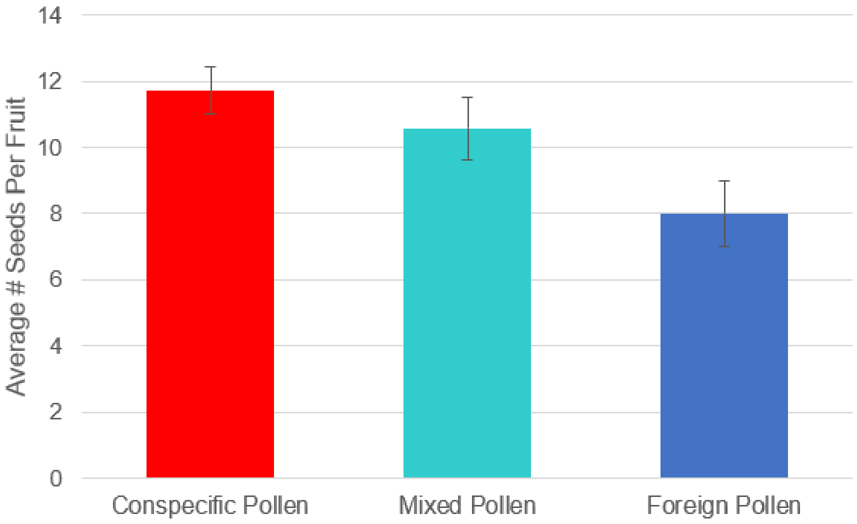

Figure 26: Average seed production among hand pollination treatments. Racemes treated with conspecific, mixed, and foreign pollen had an average seed set of 11.73, 10.59, and 8 seeds per fruit, respectively. There is no significant difference between the seed production among treatments (*P*=0.674).

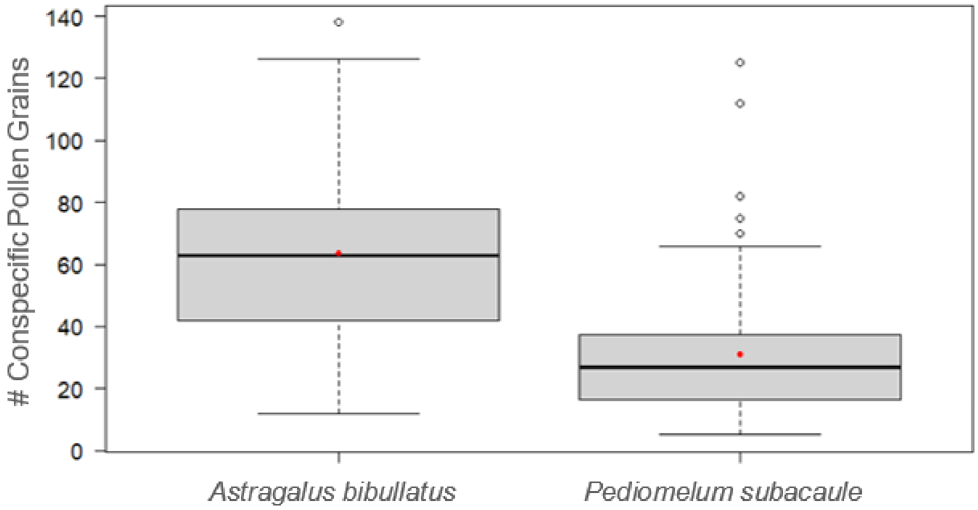

Figure 27: Boxplot distribution of the number of conspecific pollen grains found on *A. bibullatus* and *P. subacaule* stigmas. The average number of conspecific pollen grains on *A. bibullatus* was 63.11 and 30.97 on *P. subacaule*, which was statistically different (*P*<0.001).

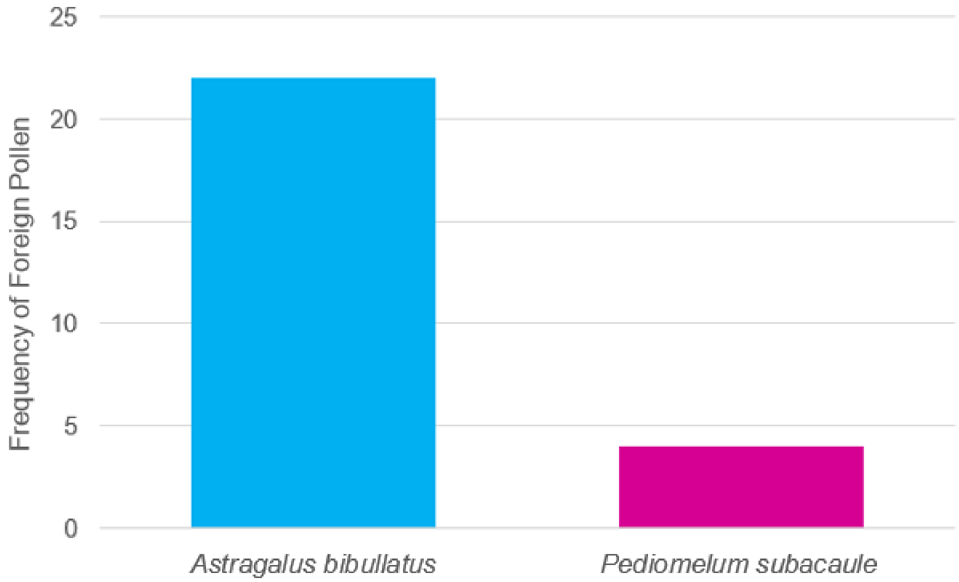

Figure 28: The frequency of foreign pollen present on *A. bibullatus* and *P. subacaule* stigmas. 22% of *A. bibullatus* had foreign pollen present and only 4% of *P. subacaule* had foreign pollen. This difference in frequency was statistically different (*P*<0.001).

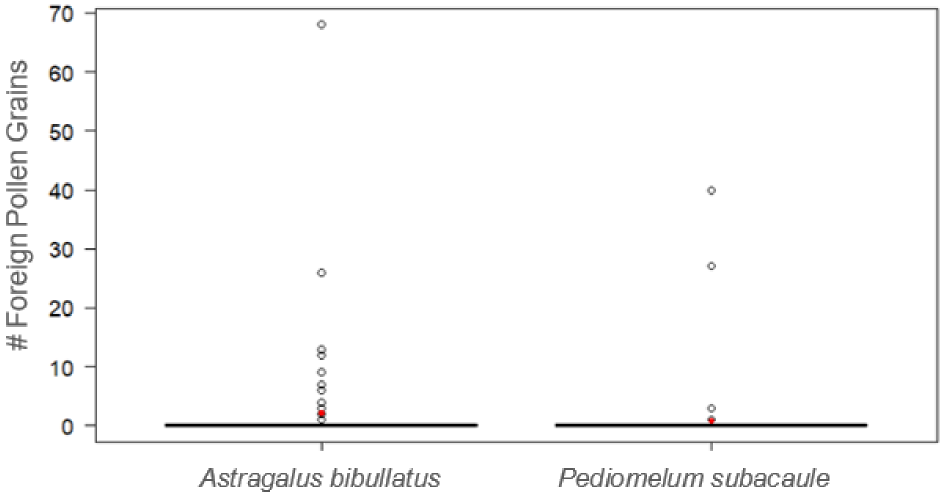

Figure 29: Boxplot distribution of the number of foreign pollen grains found on *A. bibullatus* and *P. subacaule* stigmas when present. The average number of foreign pollen grains on *A. bibullatus* was 8.45 and 17.75 on *P. subacaule*, which was not statistically different (*P*=0.4733).

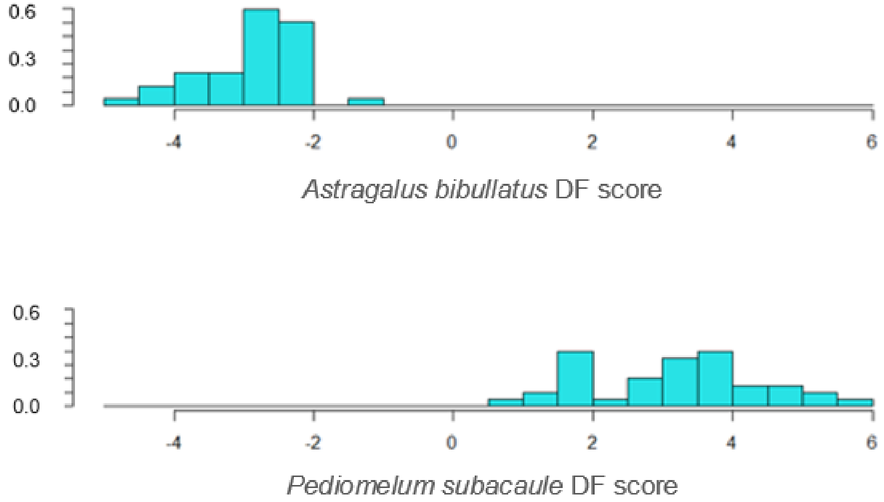

Figure 30: Linear discriminant function values of *A. bibullatus* and *P. subacaule*. There is no overlap between DF values and the model can predict the species of pollen based on length, width, and area with 100% accuracy.