The Role of Pollinator Preference in the Maintenance of Pollen Color Variation

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The Role of Pollinator Preference in the Maintenance of Pollen Color Variation

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Running title: Pollinator preference maintains pollen color variation

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Abstract:

Background and Aims

Pollinators often drive the evolution of floral traits, but their capacity to influence the evolution of pollen color remains unclear. Pollen color in *Campanula americana* is variable and displays a longitudinal cline from prevalence of deep purple in western populations to white and light-purple pollen in eastern populations. While selection for thermal tolerance likely underlies darker pollen in the west, factors contributing to the predominance of light pollen in eastern populations and the maintenance of color variation within populations throughout the range are unknown. Here we examine whether pollinators contribute to the maintenance of pollen color variation in *C. americana*.

Methods

In a flight cage experiment, we assessed whether *Bombus impatiens* foragers can use pollen color as a reward cue. We then established floral arrays that varied in the frequency of white- and purple-pollen plants in two naturally occurring eastern populations. We observed foraging patterns of wild bees, totaling over 1,100 individual visits.

Key Results

We successfully trained *B. impatiens* to prefer one pollen color morph. In natural populations, the specialist pollinator, *Megachile campanulae,* displayed a strong and consistent preference for purple-pollen plants regardless of morph frequency. *Megachile* also exhibited a bias toward pollen-bearing male-phase flowers and this bias was more pronounced for purple-pollen. The other main pollinators, *Bombus* spp. and small bees, did not display pollen color preference.

Conclusions

Previous research found that *Megachile* removes twice as much pollen per visit as other bees and can deplete pollen from natural populations. Taken together these results suggest that *Megachile* could reduce the reproductive success of plants with purple pollen, resulting in the prevalence of

light-colored pollen in eastern populations of *C. americana.* Our research demonstrates that pollinator preferences may play a role in the maintenance of pollen color variation in natural populations.

Key words

Bombus, *Campanula americana*, *Campanulastrum americanum*, floral traits, geographic cline, *Megachile*, plant-pollinator interaction, pollen color, pollen depletion

Introduction

Natural selection and genetic drift can decrease phenotypic variation in populations, especially for traits related to fitness or if a population is small (e.g. Wright 1943; Schemske and Bradshaw 1999). However, in plants, petal color variation has been reported in a number of populations, even when one color has an apparent selective advantage (i.e. higher pollinator visitation; Stanton 1987; Campbell *et al.* 2010). While the maintenance of variation in petal color has been well studied (e.g. Rebelo and Siegfried 1985; Jones and Reithel 2001; Gigord *et al.* 2001; Eckhart *et al.* 2006; Thairu and Brunet 2015; Twyford *et al.* 2018), until recently variation in pollen color has received less attention (Jorgensen *et al.* 2006; Koski and Galloway 2018; Austen *et al.* 2018; Wang *et al.* 2018). In addition, we still lack knowledge of the role of pollinator-mediated selection on the maintenance of pollen color variation.

In many species, pollen color is determined by flavonoid and/or carotenoid compounds that accumulate in the pollen grains (Wiermann and Vieth 1983; Mo *et al.* 1992; Okinaka *et al.* 2003; Tanaka *et al.* 2008). The presence and amount of flavonoid compounds has been correlated with variation in pollen germination and tube growth rates (Mo *et al.* 1992; Ylstra *et al.* 1992). In species polymorphic for pollen color, variation in pollen viability between color morphs has important evolutionary implications. For example, in polymorphic *Epimedium pubescens*, green pollen has higher germination rates than yellow pollen, but mixed pollen loads have lower siring success than either type alone (Wang *et al.* 2018). These results suggest that there is likely selection against polymorphic populations in *E. pubescens*. Flavanoids are also suggested to confer protection against environmental stressors (Winkel-Shirley 2002) and a growing body of work has found that patterns of pollen color variation are correlated with the abiotic conditions of a population (Jorgensen and Andersson 2005; Jorgensen *et al.* 2006; Koski and Galloway 2018).

Pollen color is likely also under pollinator-mediated selection. Insect pollinators are selective when foraging, using floral cues such as flower size, corolla length, nectar reward,

polarization patterns, and petal or pollen color (Lunau 1991; Foster *et al.* 2014; Nicholls *et al.* 2017). Therefore, pollinators can exert selective pressure on specific floral characteristics (Brown and Clegg 1984; Schemske and Horvitz 1984; Castellanos *et al.* 2003), including pollen color. For example, solitary bee pollinators showed a site-specific pollen color preference in a dramatic red/yellow pollen color polymorphism in *Erythonium americanum* (Austen *et al.* 2018). In addition, pollinator preferences for floral traits fluctuate depending on trait frequencies. For example, bee pollinators displayed a frequency-dependent preference for petal spot morphs in *Clarkia xantiana*. *Hesperapis regularis* (Melittidae) preferentially visited arrays that mimicked the natural morph frequency, while other pollinators preferentially visited arrays that contained a greater frequency of morphs that were the minority in the natural population (Eckhart *et al.* 2006).

Visual systems and learning processes play key roles in the behaviors of foraging insects and can aid in the development of pollinator preferences (Gumbert 2000). While pollen preferences exist in honeybees, the preference has been linked to odor (Pernal and Currie 2002), and it is unclear how much of a role vision plays in discriminating pollen-based rewards. Most bee species have trichromatic color vision, with photoreceptors sensitive to green, blue, and ultraviolet wavelengths (Briscoe and Chittka 2001). Floral color cues can help bees distinguish potential resources from the background (Jones and Buchmann 1974). Even if pollinators can discern the color differences, they may not have the visual acuity to distinguish smaller structures, like pollen, as a floral cue. Researchers have used artificial flowers and colored disks to demonstrate that pollinators can associate color with pollen reward quality (Nicholls and Hempel de Ibarra 2014). However, more research is needed to determine if insect pollinators can or do develop a pollen color preference in plant species with variable pollen color.

The American Bellflower (*Campanula americana*) is an herbaceous plant commonly found throughout eastern North America (Barnard−Kubow *et al.* 2015). It is insect pollinated by members of several bee families: Apidae, Megachilidae, and Halictidae (Lau and Galloway 2004;

Koski *et al.* 2018*a*). In *C. americana*, pollen color is variable (ranging from white to deep purple) and heritable (Koski and Galloway 2018). Pollen color variation displays a longitudinal cline where westerly populations have a prevalence of purple pollen, likely due to abiotic selection for heat stress resistance, and plants in eastern populations have mostly light-purple or white pollen (Koski and Galloway 2018). Factors contributing to the predominance of white and light-purple pollen in the eastern populations and the overall maintenance of color variation in populations throughout the range remain unclear. We examined pollinator-mediated mechanisms for the pollen color variation by asking the following questions: 1) Are bees able to use pollen color as a visual cue in *C. americana*? 2) Do natural bee pollinators exhibit a preference for pollen color? 3) If so, does the preference vary based on pollen color frequencies?

Methods

Study system

The American bellflower (*Campanula americana* L., Campanulaceae) is an herbaceous annual or biennial plant found at forest edges throughout the eastern United States (Fig. 1). *Campanula americana* is protandrous and capable of self-fertilization. Flowers open in the male phase, where pollen is presented on pollen-collecting hairs along the style. Flowers transition to the female phase after pollen is removed and the stigmatic lobes open (Koski *et al.* 2018*b*). The reflectance of all pollen colors peaks at 460 nm, and white/light-purple pollen has higher reflectance than purple pollen (Koski and Galloway 2018). Petal color is also variable but does not co-vary with pollen color (see Results). Petals have peak reflectance in the violet range at 439 nm with an average of 30% reflectance (+/- 5.13 sd).

Campanula americana is insect pollinated and is visited by a variety of pollinators including *Bombus* spp. (Apidae), *Megachile campanulae* (Megachilidae) and small solitary bees (including *Augochlorella* spp. and *Lasioglossum* spp. in the Halictidae, and *Ceratina* spp. in the Apidae; Lau and Galloway 2004; Koski *et al.* 2018*a*). All insect pollinators forage for nectar but

M. campanulae and the small bees also forage for pollen. Per visit, *Bombus* are significantly more effective pollinators than *M. campanulae* and small bees. *Megachile campanulae* removes more pollen per visit than *Bombus* spp. and small bees, and small bees deposit less pollen per visit than the other pollinator taxa (Koski *et al.* 2018*a*).

Pollen color as a visual cue

To determine whether pollinators have the ability to distinguish differences in pollen color in a natural system, we trained *Bombus impatiens,* a natural pollinator of *C. americana*, to use pollen color as a reward cue. We used two *B. impatiens* colonies (BioBest® and Natupol®) consisting of female workers and a queen. One colony was kept in an agricultural landscape at the College of Wooster's field station, Fern Valley. The other colony was kept in a residential area in Wooster, OH. Outside of experimental trials, the bees were free to forage in the surrounding area and we provided them with sugar water and pollen. However, we withheld food and prevented natural foraging for 24h prior to training and testing days to ensure foraging. To identify bees, we caught individuals in the flight cage and labeled them by applying small dots of acrylic paint on the thorax between their wings.

We used *C. americana* plants from six populations in Alabama, Indiana, Kansas, Minnesota, Pennsylvania, and Wisconsin (Koski *et al.* 2017). Plants were grown from seed at the University of Virginia and transported to Wooster OH where they were kept in a greenhouse at Ohio State University Agricultural Technical Institute. We recorded the pollen color and petal color for all plants used in this study. We scored pollen and petal color from 1-7 (hereafter color score) using Sherwin Williams' Interior Color Answers paint sample #119, ranging from white to deep purple.

We set up two displays of *C. americana* in a flight cage (Coleman™ Instant Screenhouse; 3x3m mesh tent)—one display had four plants with deep purple pollen (color scores 5-7; Fig. 1) and the other, presented at the same time, had four plants with white pollen (color scores 1 or 2; Fig. 1). We tested if petal and pollen color co-varied by comparing the petal color score of white-

pollen plants and purple-pollen plants with an independent sample t-test. All female-phase flowers were removed and the location of each display within the tent was randomized daily to ensure that the bees were not learning to forage by location. Light conditions varied slightly due to cloud coverage (sunny to slightly overcast), although the trials were not conducted on rainy or cold days.

We trained the foragers use pollen as a reward cue by arbitrarily making purple-pollen flowers rewarding and white-pollen flowers non-rewarding. To do this, we removed the nectar from each flower and filled the nectaries with 20 µl water (white-pollen flowers) or 20 µl of a 1:3 sucrose water mixture (purple-pollen flowers). The sucrose solution was within the range of *C. americana*'s nectar sugar concentration in the greenhouse, but less concentrated than the greenhouse mean (57.7%; Koski and Galloway, unpublished data). During the training session, we allowed the bees to forage on the *C. americana* displays inside the flight cage. We tracked individual bees as they foraged and recorded pollen color and bee ID. A full training session for a bee consisted of at least six visits. Each bee was conditioned for a minimum of four training sessions before the testing session.

In the testing session, the floral displays were the same as in the training session, however, all flowers were non-rewarding and filled with 20 µl of water. We then recorded the foraging visits of previously trained bees. Bees with incomplete training were not permitted to forage. To assess whether *B. impatiens* learned to use pollen color as a reward cue we conducted G-tests for Goodness of Fit (DescTools package, R v.1.0.143). We compared the number of observed first visits to each pollen color morph to the expected number of visits (50%) for training session one and the testing session. A lack of preference for pollen color in the first training session, but a preference for purple pollen in the testing session indicates that *B. impatiens* can learn to associate purple pollen with a nectar reward.

We also modeled pollinator perception of petal and pollen color to assess the degree to which pollen contrasts from petals of *C. americana*. To estimate the average petal color of plants

used in flight cage and field array experiments, we measured spectral reflectance from 71 flowers across the six source populations from which arrays were constructed (n=7-14/pop) using an Ocean Optics Spectrophotometer with a UV-VIS Deuterium light source (Ocean Optics, Dunedin, FL). The average petal reflectance was calculated using the 'aggspec' function in R (pavo package). We measured spectral reflectance of pollen for 2-5 plants with five color categories (described in Koski and Galloway 2018). We modeled the perceived distance between petal and pollen color using two separate insect visual systems—*B. impatiens* and *Osmia rufa*. *Bombus impatiens*' color photoreceptors have peak sensitivity at 347, 424 and 539nm (Skorupski and Chittka 2010). While the photoreceptor sensitivity for *Megachile campanulae* is unknown, the Megachilidae species, *O. rufa*, also has trichromatic vision with peak sensitivities at 344, 432, and 560nm (Peitsch *et al.* 1992).

We measured contrast between each pollen color category and the average petal for *B. impatiens* and *O. rufa*. For each pollinator type we measured photons of light captured by each of the three photoreceptors (quantum catch) using spectral inputs (average petal and pollen of each color morph) with Standard Illuminant D65, and a green background with the 'vismodel' function using the pavo package in R (Maia *et al.* 2013). We visualized the relative locations of petals and pollen in hexagonal insect color perceptual space using the 'colspace' function (Chittka and Menzel 1992). Finally, we measured Euclidean distances between mean petal color and each pollen color class in hexagonal space (chromatic contrast), as well as long-wavelength photoreceptor distance (achromatic contrast) with the 'coldist' function.

Pollen color preferences in natural populations

To determine if wild pollinators have a pollen color preference, we selected two naturally occurring populations of *C. americana* in northeast Ohio. The first site, along the Chuckery Trail in the Cascade Valley Metro Park (Akron, OH; 41°06'50.5"N 81°31'12.6"W), had a large and widespread *C. americana* population. The second site, located along a natural trail (40°42'32.3"N 81°58'54.2"W) within the Killbuck Marsh Wildlife Area in Shreve, OH, had occasional clumps

of *C. americana*. We scored pollen color in the populations using the same method as the experimental plants (see *Pollen color as a visual cue*). Pollen color in both populations ranged from white to purple with a mean pollen color score of 2.63 (Chuckery Trail, n = 286) and 2.83 (Killbuck, n = 36; Supplemental Material Fig. S1).

In each population, we established arrays of twelve potted *C. americana* plants to evaluate pollen color preference of insect visitors. Each array was 60 cm x 90 cm and individual plants were placed 30 cm from each other. To assess the influence of pollen color morph frequency on color preference, we set up 6P:6W arrays with an equal number of purple (n_p =6) and white (n_w = 6) pollen morphs. We also set up purple-skewed arrays (8P:4W; n_p = 8; n_w = 4) and white-skewed arrays (4P:8W; n_p = 4; n_w = 8). For the white-pollen morphs, we used plants with a color score of 1 or 2 (rarely 3), and the purple-pollen morphs had a color score of 5-7 (rarely 4; Fig. 1; Table S1). We positioned each array adjacent to the natural populations to ensure that local pollinators were accustomed to foraging on the plant. Arrays were initiated by mid- to late-morning so that data collection occurred before or during peak pollinator activity (Evanhoe and Galloway 2002). Each array type was repeated on five days—three days at Chuckery Trail and two days at Killbuck (n = 15 arrays; Table S1). We used a randomized block design to determine the order of arrays. We used the same stock of plants for these arrays as we used in the flight cage study.

For each plant in the arrays, we reduced floral display size to two male-phase flowers and two female-phase flowers. Males were identified by the presence of pollen on the style and females by the reflexed three-lobed stigma and no pollen remaining on the style. When a plant had more than two flowers in the male or female phase, we excluded the extra flowers by covering them with a split drinking straw. We observed pollen levels throughout the day. If a male flower was depleted of pollen, we would uncover a new male flower if available. However, if 30% of male-phase flowers were stripped of pollen, we ended data collection for that array. We observed pollinators, defined as floral visitors making contact with the style. For each insect, we

recorded the plant position and flower sex phase for all visits in an array. We also collected foraging data as bees transitioned between plants and flowers within the array, however pollinators were shooed away after ten consecutive transitions between flowers. We replicated arrays until each array type received at least 30 visits from each of three pollinator groups: *Bombus* spp. (hereafter *Bombus)*, *Megachile campanulae* (hereafter *Megachile*), and small bees (including *Augochlorella* spp., *Lasioglossum* spp. and *Ceratina* spp.). Data were collected between July and August 2017, the natural flowering time of plants in northeastern Ohio.

To answer whether naturally-occurring pollinators displayed a preference for different pollen color morphs and whether the preference depended on morph frequency, we used a generalized mixed linear model with a Poisson distribution (SAS v. 9.4, PROC GLIMMIX). In each array replicate, we totaled the number of first visits made by a pollinator to each color morph and floral sex-phase. First visits represent the initial choice made by a pollinator upon entering an array. We modeled the number of first visits as a function of array type (i.e., 'morph frequency'; white biased, mixed, purple biased), pollinator type (*Bombus*, *Megachile*, small bee), pollen color morph (purple, white), and floral sex phase (male, female). All two-way and three-way interactions were included. Four-way interactions were not significant and were removed from the model. Array replicate nested within array type was modeled as a random effect. We did not have the replication to test for site-specific effects. There was a significant pollinator type by pollen color morph interaction, so we assessed which pollinator type(s) displayed a color preference using a SLICE statement in SAS. We generated least-squares means from models and back-transformed them to visualize the data. We also conducted this analysis using the first male-phase flower (pollen-bearing) each pollinator visited. The results were very similar between the two models.

We used a similar model to test whether color-morphs experience differential pollinator visitation taking into account entire pollinator foraging bouts. In this model the response was the number of total visits to each floral color morph and flower-sex phase by each pollinator type in

each array. We removed all visits from the dataset that resulted from movement of a pollinator between flowers on the same plant. Visits resulting from movement between flowers on the same plant were removed because these were unlikely to reflect a choice made by a pollinator based on floral traits. Again, we assessed differences between groups within significant interactions terms using a SLICE statement in SAS and visualized the data as noted above.

Results

Pollen color as a visual cue

Pollen and petal color for the plants used in this study did not co-vary (mean petal color for white-pollen plants = 5.15, sd = 0.24; mean petal color for purple-pollen plants = 5.13, sd = 0.36; $t = 0.18$, $df = 28$, $p = 0.86$). In the flight cage study, we completed four training sessions and one testing session for 20 different *B. impatiens* foragers. The bees displayed no initial preference for pollen color during training session one (T1 Fig. 2; $G = 0.20$, $df = 1$, $p = 0.65$). However, by the final training session, T4, 17 of 20 foragers visited the rewarding purple-pollen morph first. During the testing session, when all plants were unrewarding, 16 of the 20 foragers (80%) visited a purple-pollen plant first (Fig. 2; $G = 7.71$, $df = 1$, $p = 0.005$).

Results from the color vision model demonstrated that the perception of pollen and petal color was largely the same for *Bombus* and *Osmia* (Figs. 3, S2). Petals of *C. americana* fall within the 'blue' area of hexagonal color space for both pollinator types, indicating quantum catch of the mid-wavelength photoreceptor is higher than the shorter- and long-wavelength receptors. All pollen color morphs are in the 'blue-green' range of hexagonal color space so pollen excites the long-wavelength green receptor more than the petal does. White pollen displays the highest chromatic and achromatic contrast from the petal, and both chromatic and achromatic contrasts from the petal decline with increasing darkness of pollen (Figs. 3, S2).

Pollen color preferences in natural populations

We recorded 1,108 pollinator visits by *Bombus* (98), *Megachile* (428), and small bees (582) to the floral arrays (Fig. S3). All array types had similar number of visits (array effect in all models $p > 0.6$; Table 1, S2). However, there were more *Megachile* and small bee visits compared to *Bombus*, with only two *Bombus* visits recorded at the Killbuck population (pollinator type effect in all models $p < 0.001$; Tables 1, S2, S3)

For all traits, pollen color preferences varied by pollinator group with *Megachile* displaying a strong and consistent preference for plants with purple pollen (pollinator type*pollen color; all models $p < 0.01$; Figs. 4, S4, Tables 1, S2, S3). *Megachile* demonstrated a preference for purple-pollen plants on their first visit to the array (Fig. 4A, Table S2), their first visit to a male-phase flower (Fig. S4, Table S3), and across their foraging bout (Fig. 4B, Table 1). In contrast, *Bombus* and small bees showed no pollen color preference in their first visit, their first male-phase visit, or within a foraging bout (Figs. 4, S4, Tables 1, S2, S3). In all models, there was no difference in frequency-dependent pollen morph preference among pollinators (pollinator type*pollen color*array type; all models $p > 0.6$; Tables 1, S2, S3). Both *Megachile* and small bees had a significant preference for male-phase flowers both within a foraging bout and for their first visit (Fig. 5A, Tables S2, S4). In contrast, *Bombus* showed no sex-phase preference (Fig. 5A). Interestingly, the bias for male phase flowers was stronger for purple pollen compared to white pollen (Fig. 5B; sex phase*pollen color $p < 0.05$; Tables 1, S4).

Discussion

Our study examined the role of pollinator preference in the prevalence of light pollen in eastern populations of *C. americana*. Using a flight cage experiment, we found that *Bombus* have the ability to perceive differences in pollen color and use pollen color as a visual cue while foraging (Fig 2). Data from our field study demonstrated that the specialist *Megachile* bee had a strong and consistent preference for purple pollen (Fig. 4). *Megachile*'s purple preference was not dependent on the frequency of pollen colors in the arrays and was observed in both sites (Figs. 4,

S4, Tables 1, S2-S4). In contrast, *Bombus* and small bees did not show a pollen color preference in any of the arrays regardless of pollen morph frequencies. Similar to previous studies, both *Megachile* and small bees showed a bias toward male-phase flowers, but *Megachile*'s bias for male-phase flowers was stronger in purple-pollen plants than white-pollen plants (Fig. 5; Tables 1, S4). A concurrent study in the same populations found that *Megachile* removed nearly twice as many pollen grains per visit to male-phase flowers than small bees or *Bombus* (~10,500 grains compared to around ~5,700 grains for small bees and ~5,000 for *Bombus*), but deposited significantly fewer pollen grains than *Bombus* (Koski *et al.* 2018*a*). Because of *Megachile*'s strong biases for male-phase flowers and purple pollen, it could reduce the reproductive success of plants with purple pollen, resulting in light pollen across the range of *C. americana*, and the potential to shape geographic variation in pollen color.

Visual abilities of bees to distinguish pollen color variation

Visual acuity in insect pollinators is generally considered to be low (e.g. *Bombus* visual acuity is estimated at 0.36 cycles per degree, Jander and Jander 2002) and prior to our study, it was not known whether insect pollinators are able distinguish and respond to subtle pollen color variation in a natural system. Our flight cage results demonstrated that *Bombus* were able to use pollen color as a reward cue in *C. americana*. Individual bees initially displayed no preference in pollen color (Fig. 2), but by the fourth training session most foragers exhibited a notable preference for purple pollen. This preference continued into the testing session when both pollen color morphs were unrewarding (Fig. 2). Ideally, we would have trained *Bombus* workers to prefer the white-pollen phenotype too. Logistically however, we could not train some *Bombus* workers on purple-pollen as the rewarding phenotype and others on white-pollen, because each worker experienced four complete training sessions and we could not control which worker foraged at any given time. Yet, we believe *Bombus* workers could have been trained to prefer white-pollen phenotype for two reasons 1) *Bombus* workers showed no initial preference for pollen color in both the flight cage study and in the natural populations (Figs. 2 & 4) and 2) based

on the vision modeling the white-pollen phenotype is more distinct from the petal color background than the purple-pollen phenotype (Fig. 3). Our results demonstrating that the color of pollen can be learned by *Bombus* workers is an important first step for understanding whether pollinators can exert selection on this trait in a natural system.

Color vision models demonstrated that in relation to average petal color, white pollen is more distinct than purple pollen (Figs. 3, S2) when viewed by both *Bombus impatiens* and *Osmia rufa* (Megachilidae). Since *B. impatiens* learned to associate purple pollen with a reward, it is likely to be able to utilize the even more obvious white pollen in foraging decisions as well. These results, in combination with our field study results, show that pollinating bees can perceive pollen color variation in *C. americana* and associate it with a reward. To the best of our knowledge, ours is one of the first studies to demonstrate that bees can distinguish and learn to prefer a given pollen color morph using naturally-occurring pollen color variants.

Implications of pollen color foraging preferences in natural populations

Pollinator-mediated selection on floral traits is often due to pollinators that are both efficient and abundant (Fenster *et al.* 2004). Yet, in many populations the most abundant pollinator is not always the most efficient pollinator. In fact, when in low abundance, efficient pollinators likely do not exert significant selective pressures and therefore don't influence floral trait evolution. For example, in *Heterotheca subaxillaris* some of the most efficient pollinators are generally rare and as a result of low importance to seed production, whereas the most important pollinators are less effective but more abundant (Olsen 1996). Similarly, the influence of both pollinator foraging behavior and abundance could drive the prevalence of white and light-colored pollen in eastern populations of *C. americana*.

Specifically, we hypothesize that the abundant specialist pollinator, *Megachile*, is exerting selection against purple pollen. While *Bombus* is the most efficient pollinator per visit (Koski *et al.* 2018*a*), we observed fewer *Bombus* visits than either *Megachile* or small bees, with *Bombus* visits nearly non-existent at the Killbuck population (Fig. S3). In contrast, *Megachile* and

small bees were common at both sites in all array types. *Megachile* always preferred purple-pollen plants and had a stronger male-phase flower bias when a plant had purple pollen compared to white pollen (Figs. 4, 4B). Since *Megachile* removes nearly twice as much pollen per visit as either *Bombus* or a small bee (Koski *et al.* 2018*a*), we hypothesize that *Megachile*'s preference for purple pollen is reducing the male fitness of purple-pollen plants. In *Claytonia virginica*, specialist *Andrena erigeniae* also removes significantly more pollen from flowers than any other pollinator and populations with high *A. erigeniae* visitation produce fewer seeds (Parker *et al.* 2016). *Megachile*'s strong preference towards purple-pollen plants and its male-phase bias may similarly deplete purple pollen from *C. americana* populations.

The role of pollinators in the maintenance of intraspecific pollen color variation

Our research, along with previous research in this system, can start to elucidate why populations with variable pollen color are found throughout the range of *C. americana* as well as the prevalence a light-colored pollen in the east of the range. In western populations, more abundant deep-purple pollen is favored by selection due to its resistance to heat stress, whereas the germination of white pollen is reduced under high temperatures (Koski and Galloway 2018). Greater thermal tolerance of purple pollen may be conferred by elevated flavonol content since some flavonols are crucial for pollen germination (Mo et al. 1992), this has yet to be tested in *C. americana*. Therefore, abiotic selection is predicted to drive *C. americana* populations to purple pollen. However, we demonstrate that pollinators have the ability to discern intraspecific pollen color variation and that an abundant wild pollinator prefers one pollen color over another. Previous research has found that opposing selective pressures maintain petal color variation in *Claytonia virginica* populations (Frey and Williams 2004). Similarly, our results suggest that opposing selection between abiotic factors and pollinator preferences help to maintain pollen color variation in *C. americana.*

While we found no frequency-dependent preference (i.e. *Megachile* always prefers purple-pollen plants,) the evolutionary implications of pollen color preference could still be

387 context dependent. For instance, in populations with a high frequency of purple pollen,
388 *Megachile*'s purple preference may have little impact since they may not deplete all the purple
389 pollen from the population. It is also important to note that we only measured preference in two
390 eastern populations even though all three pollinator groups are common throughout *C.*
391 *americana*'s range (Koski *et al.* 2017). While pollen color preference did not vary in Ohio
392 populations based on morph frequencies, preference may vary across the range. In a Virginia
393 population of *C. americana*, light pollen is preferred by small bees when only male-phase flowers
394 are available (Lau and Galloway 2004) and site-specific pollen color preferences have been
395 observed in other systems (Austen *et al.* 2018).
396 Our results do not rule out the role of neutral genetic processes and dispersal limitation in
397 the observed pollen color cline and population-level variation. However, given the strong and
398 consistent preference of the specialist *Megachile* pollinator for purple pollen, it seems likely that
399 *Megachile* visits are imposing a selective pressure on pollen color that is in opposition to abiotic
400 selection. Previous research in other *C. americana* populations supports our interpretation that
401 pollen color variation is not solely driven by neutral genetic structure. For example, nearly all
402 populations have pollen color variation, even small populations (Koski and Galloway 2018). In
403 addition, *C. americana*'s northward pattern of post-glacial migration would be expected to cause
404 a latitudinal not longitudinal cline due to population genetic structure associated with migration
405 (Barnard-Kubow *et al.* 2015).
406 In conclusion, our study suggests that opposing selection may maintain floral trait
407 variation and contribute to observed geographic patterns in floral traits. *Megachile* are relatively
408 inefficient pollinators of *C. americana*, preferentially visiting male-phase flowers and removing
409 twice has much pollen as the other pollinators while depositing less than *Bombus*. Since
410 *Megachile* have a strong and consistent preference for purple pollen they are likely depleting
411 purple pollen from natural populations. This preference may result in selection against plants with

purple pollen. However, selection against purple pollen is opposed by abiotic selection favoring purple pollen since it is more heat resistant. These opposing selective forces may help to maintain pollen color variation throughout *C. americana*'s range, with a prevalence of white and light-color pollen in the eastern part of the range where abiotic selection is likely relaxed.

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Supplementary data

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: Sample sizes and phenotype information for the floral arrays. Table S2: Fixed effects and least square means of the generalized mixed linear model for first flower a pollinator visited upon entering the array. Table S3: Fixed effects and least square means of the generalized mixed linear model for first male-phase flower a pollinator visited upon entering the array. Table S4: Least square means from the generalized mixed linear model for visitation across a foraging bout. Fig. S1: Pollen color frequencies in the two natural populations, Chuckery Trail

and Killbuck in Ohio, USA. Fig. S2: The average *Campanula americana* petal and pollen color of five color morphs placed in hexagonal color space for *Osmia rufa* (Megachilidae). Fig. S3: The number of each pollinator group that visited arrays at Chuckery Trail and Killbuck populations. Fig S4: The number of first visits to a male-phase flower upon entering an array for the primary pollinator groups.

Table 1. Analysis of number of flowers visited in a foraging bout by three types of pollinators to arrays of *C. americana* that differed in the frequency of flowers with purple- and white- pollen. Fixed effects of the generalized mixed linear model where the response is the number of visits in a foraging bout (see Fig. 4B, 5; Table S4). Array replicate nested within array type was modeled as a random effect. Floral pollen morph frequency (6P:6W, 8P:4W, and 4P:8W) = Array type, pollinator group (*Bombus* spp., *Megachile campanulae,* and small bees= Pollinator type, pollen color = Pollen color, flower sex phase = Sex phase, model degrees of freedom = Num. df, and denominator degrees of freedom = Den. df.

Effect	Num. df	Den. Df	F-value	P-value
Array type	2	12	0.41	0.671
Pollinator	2	136	122.08	<0.001
Pollen color	1	136	10.55	0.015
Sex phase	1	136	14.81	0.002
Array type*Pollinator	4	136	2.66	0.035
Array type*Pollen color	2	136	56.71	<0.001
Array type*Sex phase	2	136	8.51	0.003
Pollinator*Pollen color	2	136	8.76	0.003
Pollinator*Sex phase	2	136	2.85	0.024
Sex phase*Pollen color	1	136	4.99	0.027
Array type*Pollinator*Pollen color	4	136	0.54	0.707
Array type*Pollinator*Sex phase	4	136	1.51	0.202
Array type*Sex phase*Pollen color	2	136	5.77	0.004
Pollinator*Sex phase*Pollen color	2	136	0.70	0.500

Figure captions

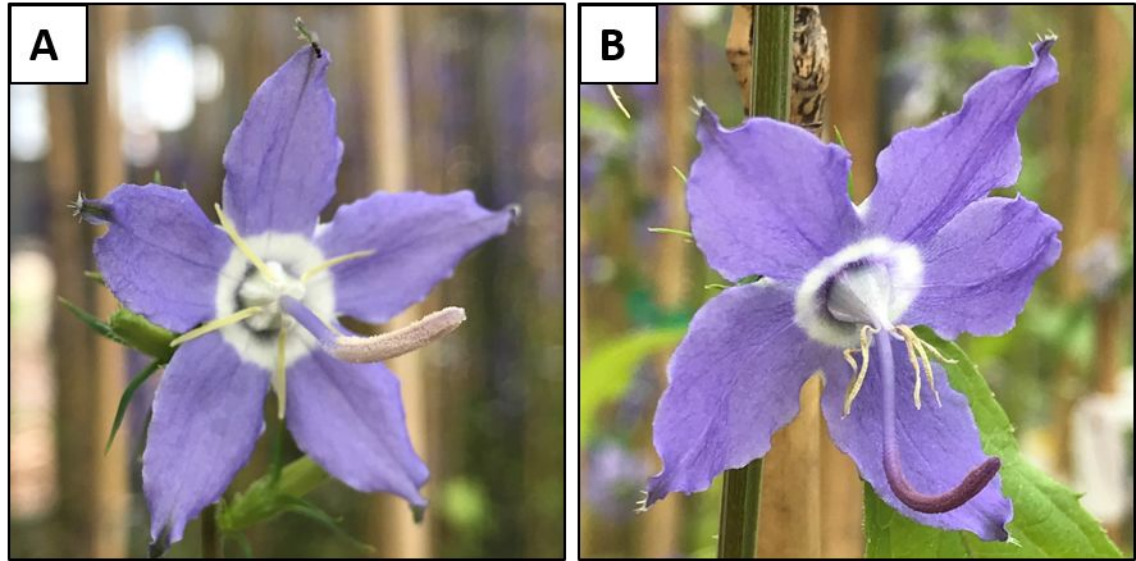
Figure 1: Male-phase *Campanula americana* flowers with pollen present on unreceptive style. A) Flower with white pollen (color score =1; see methods). B) Flower with deep-purple pollen (color score = 6; see methods). Photo credit: M.H.K.

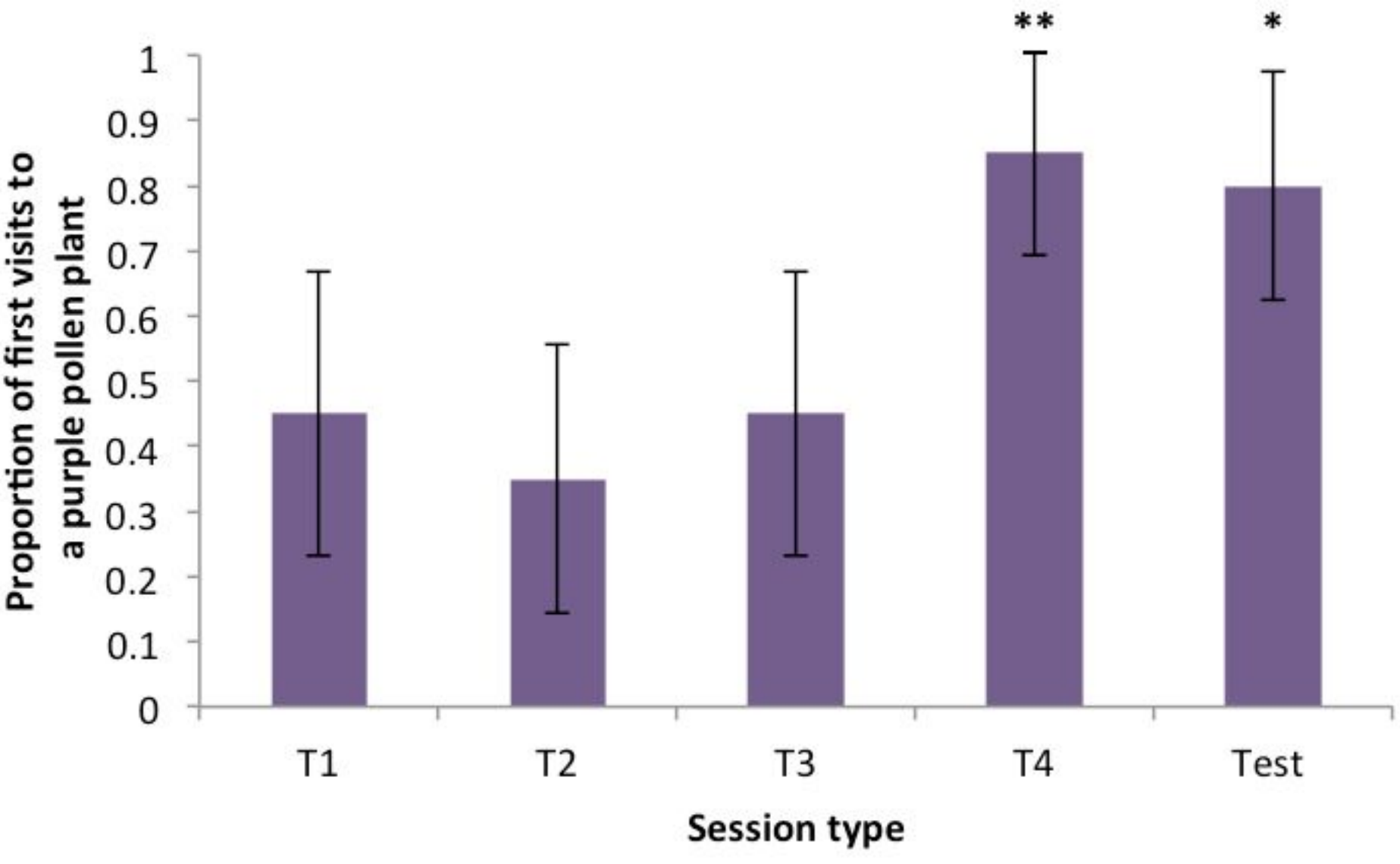
Figure 2: Proportion of purple-pollen plants *Bombus impatiens* foragers visited first during the training sessions (T1-T4) and the testing session (Test). In training sessions, plants with purple-pollen plants were rewarding and those with white-pollen plants were not. In the test session, both color morphs were non-rewarding. Asterisks indicate significant over-visiting of purple-pollen plants. Error bars represent the binomial 95% confidence interval. *P<0.05. **P<0.01.

Figure 3: A, B) The average *Campanula americana* petal and pollen color of five color morphs placed in hexagonal color space for *Bombus impatiens*. C) Chromatic (ds) and achromatic (dl) distance between each pollen color morph and the average petal color.

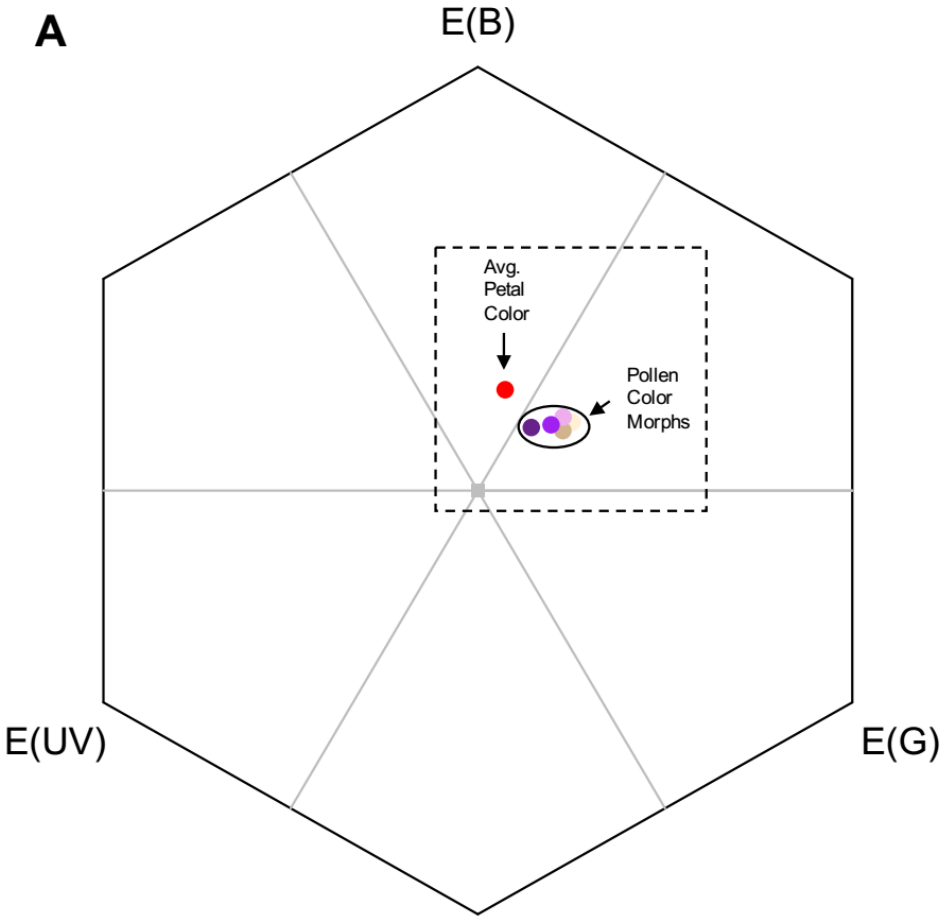
Figure 4: Pollen color visitation of the primary pollinator groups, *Bombus, Megachile,* and small bees. Displayed are the least square means (± 1 se) for A) the first pollen color morph a pollinator visited upon entering the array and B) across a pollinator's foraging bout. Asterisks represent a preference for purple pollen for *Megachile.* ***P<0.001.

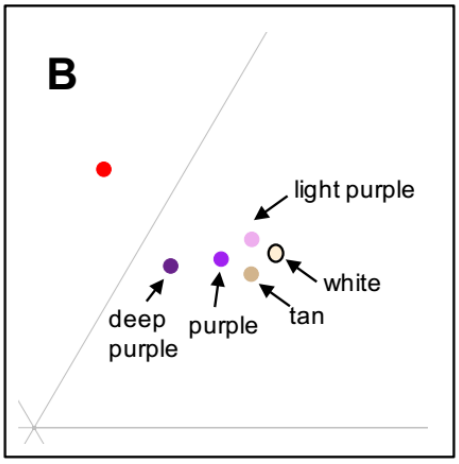
Figure 5: Flower sex phase visitation of the primary pollinator groups, *Bombus, Megachile,* and small bees. Displayed are the least square means (± 1 se) for visitation across a foraging bout A) by each pollinator type and B) by pollen color. Asterisks represent a preference for male-phase flowers. *P<0.05. ***P<0.001.


A
B


**
*
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Proportion of first visits to a purple pollen plant
T1
T2
T3
T4
Test
Session type

Bombus impatiens

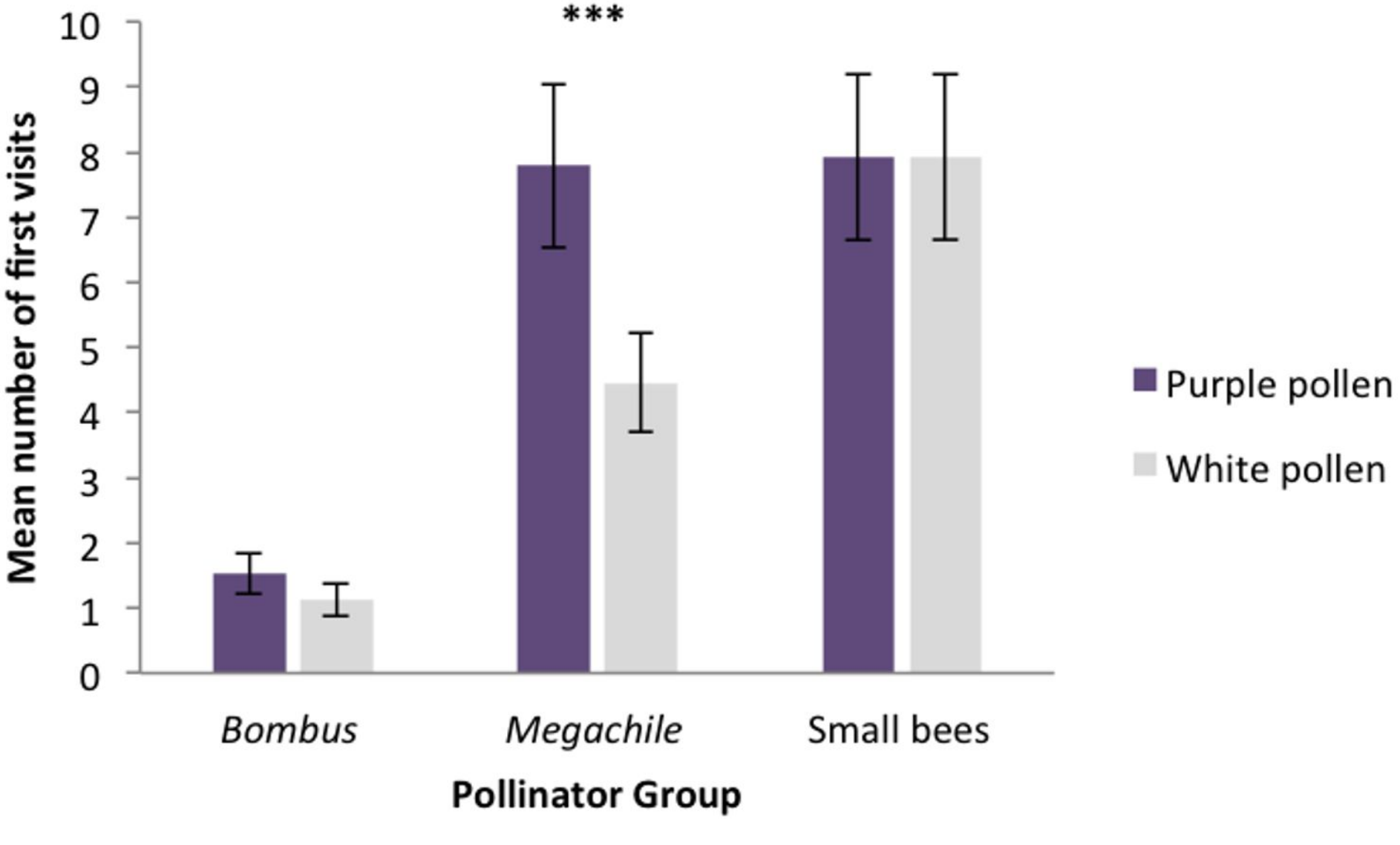




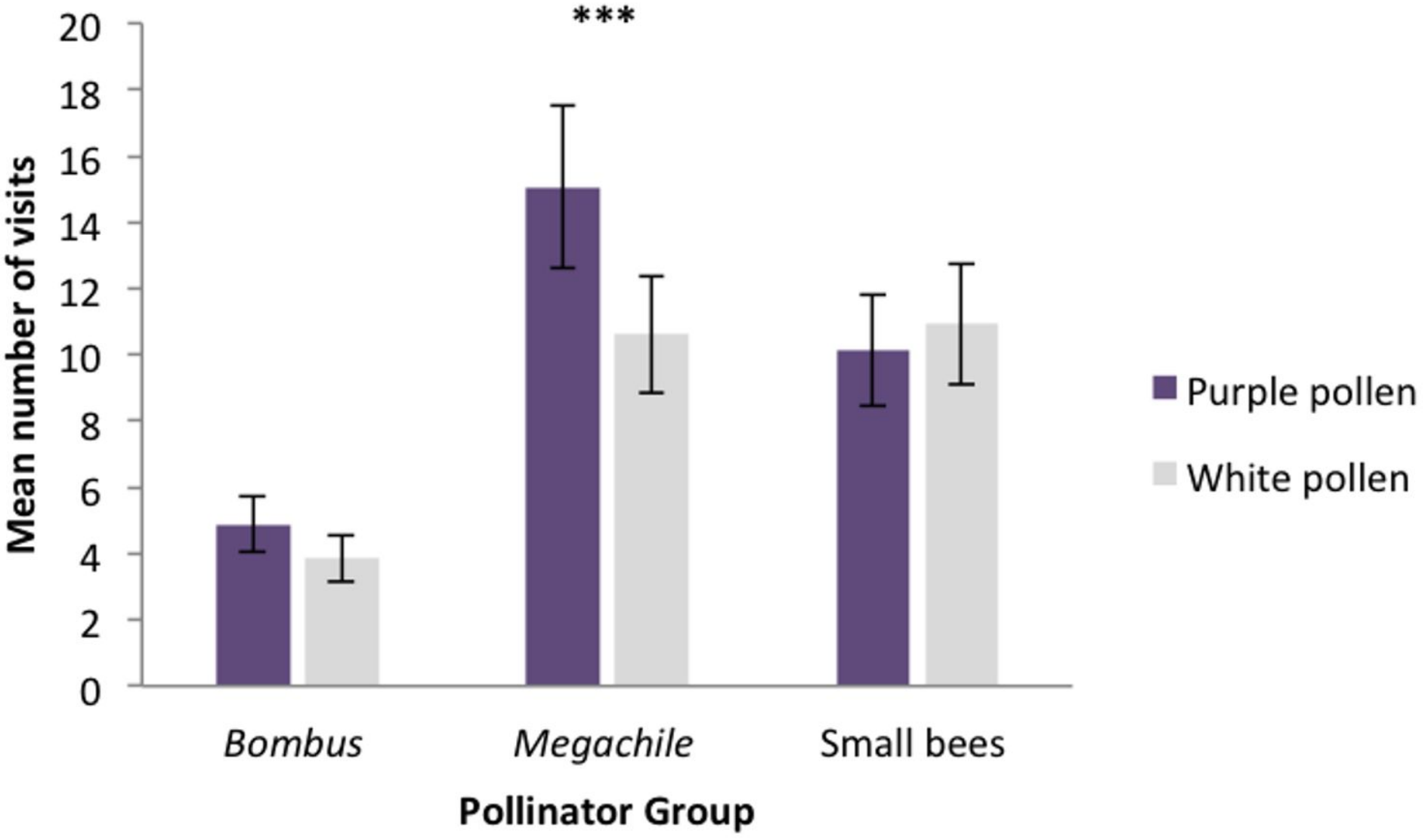
C

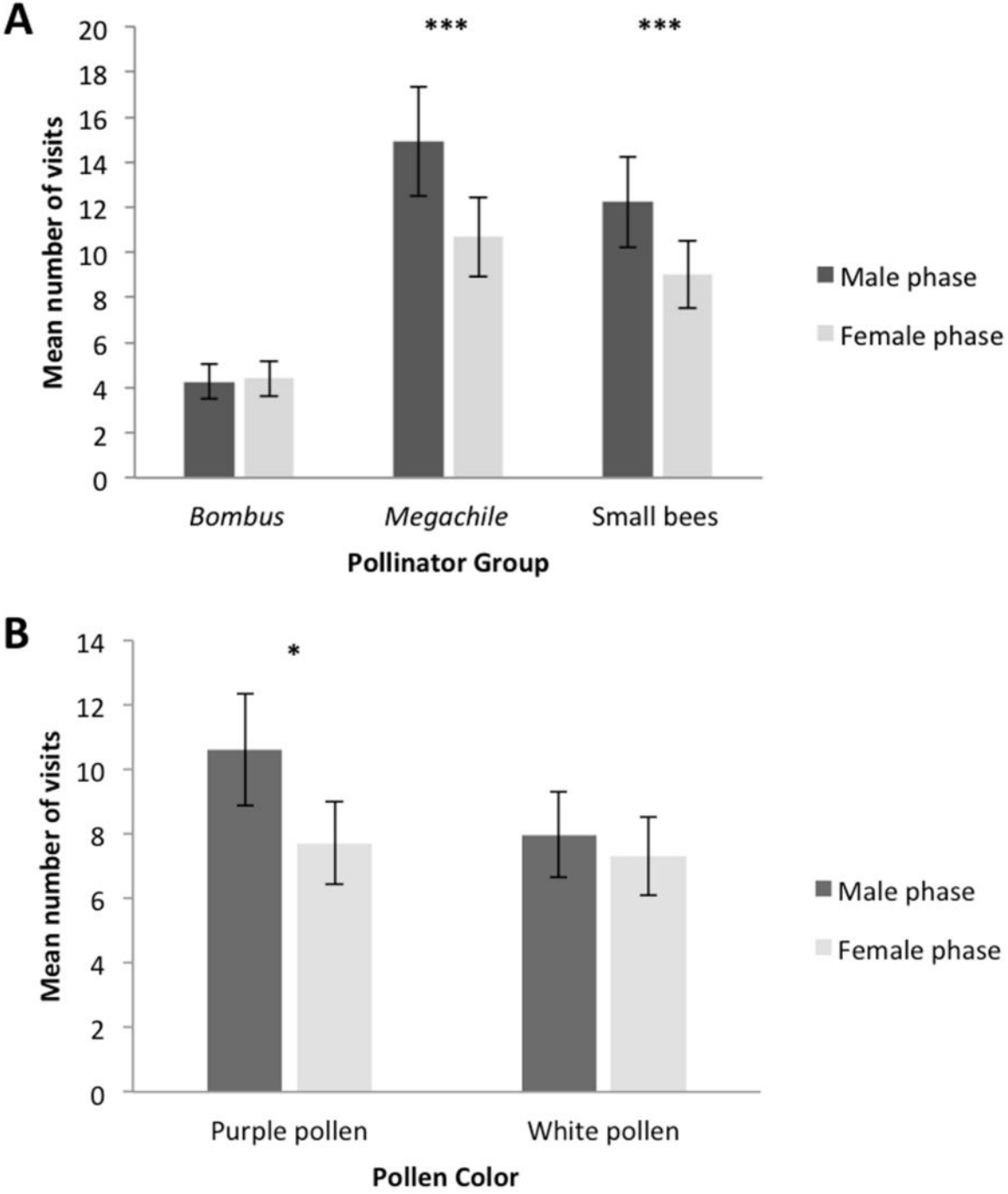
Pollen Color	dS from Petal	dL from Petal
White	0.176	0.102
Tan	0.167	0.094
Light Purple	0.152	0.095
Purple	0.137	0.049
Deep Purple	0.109	0.016

A


***
Mean number of first visits
0
1
2
3
4
5
6
7
8
9
10
Bombus
Megachile
Small bees
Pollinator Group
Purple pollen
White pollen

B


***
Mean number of visits
0
2
4
6
8
10
12
14
16
18
20
Bombus
Megachile
Small bees
Pollinator Group
Purple pollen
White pollen


A
Mean number of visits
20
18
16
14
12
10
8
6
4
2
0
***
***
Bombus
Megachile
Small bees
Pollinator Group
Male phase
Female phase
B
Mean number of visits
14
12
10
8
6
4
2
0
*
Purple pollen
White pollen
Pollen Color
Male phase
Female phase