

RESEARCH ARTICLE

Pollinators exert selection on floral traits in a pollen-limited, narrowly endemic spring ephemeral

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Abstract

Premise: Floral traits are frequently under pollinator-mediated selection, especially in taxa subject to strong pollen-limitation, such as those reliant on pollinators. However, antagonists can be agents of selection on floral traits as well. The causes of selection acting on spring ephemerals are understudied though these species can experience particularly strong pollen-limitation. I examined pollinator- and antagonist-mediated selection in a narrowly endemic spring ephemeral, *Trillium discolor*.

Methods: I measured pollen limitation in *T. discolor* across two years and evaluated its breeding system. I compared selection on floral traits (display height, petal size, petal color, flowering time) between open-pollinated, and pollen-supplemented plants to measure the strength and mode of pollinator-mediated selection. I assessed whether natural levels of antagonism impacted selection on floral traits.

Results: *Trillium discolor* was self-incompatible and experienced pollen limitation in both years of the study. Pollinators exerted negative disruptive selection on display height and petals size. In one year, pollinator-mediated selection favored lighter petals but in the second year pollinators favored darker petals. Antagonist damage did not alter selection on floral traits.

Conclusions: Results demonstrate that pollinators mediate the strength and mode of selection on floral traits in *T. discolor*. Interannual variation in the strength, mode, and direction of pollinator-mediated selection on floral traits could be important for maintaining of floral diversity in this system. Observed levels of antagonism were weak agents of selection on floral traits.

KEYWORDS

florivory, flower color, herbivory, pollinator-mediated selection, selection gradient, *Trillium*

Plant-pollinator interactions have played a primary role in shaping floral evolution and contributing to the process of angiosperm diversification (Kay and Sargent, 2009; van der Neit et al., 2014). Female reproductive output is frequently limited by insufficient pollen receipt, or the receipt of low-quality pollen (Ashman et al., 2004; Aizen and Harder, 2007). These two components of pollen limitation establish a scenario whereby selection should favor floral traits that attract efficient pollinators to increase pollen receipt. Indeed, stronger pollen-limitation is commonly associated with stronger selection on floral traits (Emel et al., 2017), and this relationship is often

driven specifically by selection that is pollinator mediated (Sletvold and Ågren, 2016; Trunschke et al., 2017; however, see Campbell and Bischoff, 2013). Spring ephemeral plants may be particularly susceptible to pollen limitation, especially in the face of phenological mismatches between plants and pollinators under climate change (Kudo and Ida, 2013; Gezon et al., 2016; Kudo and Cooper, 2019). While selection on floral traits has been measured in spring ephemerals (Irwin, 2000), dissecting the causes of selection in spring ephemerals is rare (however, see Frey, 2004), and the strength of pollinator-mediated selection is unknown.

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Deciphering the extent to which pollinators contribute to selection on floral traits in natural populations is achieved by comparing selection between plants exposed to natural levels of pollination, and those that are supplemented with outcross pollen (Caruso et al., 2010; Parachnowitsch and Kessler, 2010; Sletvold and Ågren, 2010; Lavi and Sapir, 2015; Sletvold et al., 2010, 2016). Supplemental-pollination reduces variation in female fitness among plants that is caused by variable pollinator visitation. If the direction, strength, or shape of selection differs in open-pollinated plants relative to pollen-supplemented plants, then selection is mediated by pollinators. In nearly all cases in which pollinator-mediated selection has been detected using experimental pollen-supplementation experiments, pollinators select for larger floral size, higher flower number, and more conspicuous color traits (intensity, brightness) (Sandring and Ågren, 2009; Parachnowitsch and Kessler, 2010; Sletvold and Ågren, 2010; Sletvold et al., 2010, 2016; Lavi and Sapir, 2015; however, see Caruso et al., 2010). Moreover, temporal variation in the strength or direction of selection on floral traits is common (e.g., Schemske and Horvitz, 1989; Caruso et al., 2003) but whether differences in pollinator-mediated selection underlies such temporal variation is less understood (Sletvold and Ågren, 2010; Sletvold, 2019). It is important to assess pollinator-mediated selection over multiple years because temporal variation in selection has the potential to maintain phenotypic diversity in natural populations (Turelli et al., 2001; Sapir et al., 2021).

The strength of pollinator-mediated selection on floral traits is likely to depend on a population's or species' reliance on pollinators for sexual reproduction. For instance, self-incompatible species often experience more severe pollen-limitation than those that are self-compatible (Burd, 1994; Larson and Barrett, 2000; Bennett et al., 2020) because self-compatible taxa can employ autonomous self-fertilization as a means of reproductive assurance in the absence of sufficient pollination services (e.g., Kalisz et al., 2004). A greater reliance on pollinator-mediated pollen transfer for sexual reproduction should strengthen the magnitude of pollinator-mediated selection on floral traits in self-incompatible taxa. Determining the breeding system of a species or population (self-incompatible vs. self-compatible) is thus important for contextualizing the both the magnitude of pollen limitation and pollinator-mediated selection it experiences.

While pollinators are appreciated as agents of selection on floral traits (e.g., Galen, 1989; Sandring and Ågren, 2009; Parachnowitsch and Kessler, 2010; Sletvold et al., 2010), selection can also be mediated by a variety of forces other than pollinators (Strauss and Whittall, 2006). For instance, antagonists (seed predators, florivores, herbivores) can drive selection on floral traits (Irwin et al., 2003; Parachnowitsch and Caruso, 2008; Sletvold et al., 2015; Jogesh et al., 2017) and abiotic selection can act directly on flowers (Koski and Ashman, 2015). A recent meta-analysis showed that both pollinators and abiotic factors are similarly strong agents of

selection on floral traits, while antagonists are relatively less important (Caruso et al., 2019). Antagonists however could contribute to selection on floral traits that affect a plant's conspicuousness (size, color), or traits that are correlated with chemical deterrents such as floral color (Caruso et al., 2010; McCall et al., 2013; Jogesh et al., 2017; Gélvez-Zúñiga et al., 2018). Finally, signatures of selection on floral traits could also simply reflect differences in resources acquisition between plants (Caruso et al., 2005). Disentangling the causes of selection on floral traits is important for understanding the mechanisms that give rise to the massive diversity of flowering plants.

Pollinator-mediated selection has the potential to be an important driver of floral evolution in *Trillium* (Melanthiaceae). *Trillium* species of Eastern North America often bloom early in the spring, and multiple species are known to experience strong pollen-limitation of fruit and seed set (Irwin, 2000; Knight, 2003, 2004). Phenotypic selection has been measured in two *Trillium* species to date, and both experienced selection for larger vegetative and floral size traits (Irwin, 2000). The causes of selection were unknown, and may have been driven by pollinator visitation, resource variation, or both (Irwin, 2000). Herbivory on *Trillium* species is common and an important determinant of population demography (e.g., Knight 2004). However, whether floral traits mediate the likelihood of herbivore (or florivore) damage has not yet been examined in any *Trillium* species to date. Breeding systems in *Trillium* vary from self-compatible to self-incompatible across taxa (Ohara et al., 1990; Steven et al., 2003), thus an understanding of the breeding system of the study species is important for addressing the likelihood that pollen limitation may result in pollinator-mediated selection.

Trillium discolor Wray ex. Hook. (Melanthiaceae) is a narrow endemic restricted to the upper Savannah River drainage in the southeastern United States. I evaluated whether *T. discolor* is self-compatible and able to autonomously self-fertilize, and measured pollen limitation across two seasons. I experimentally tested for pollinator-mediated selection on floral traits using a pollen-supplementation experiment. Finally, I evaluated the potential for natural levels of antagonism from herbivores and florivores to shape selection on floral traits. I addressed the following questions: (1) To what degree is seed production pollen-limited? (2) Do pollinators exert selection on floral display traits? (3) Do florivores and herbivores contribute to selection on floral traits?

MATERIALS AND METHODS

Study system and sites

Trillium discolor Wray ex. Hook, commonly known as mottled wakerobin, pale yellow trillium, or small yellow toadshade (Case, 2002) is a sessile *Trillium* species that is narrowly endemic to the upper Savannah River drainage of

the southeastern United States. It occurs in 4 counties in South Carolina, 4 counties in Georgia, and one county in North Carolina. It is globally vulnerable, critically imperiled in North Carolina and Georgia, and apparently secure in South Carolina. Within its narrow range, it is locally common in forested drainages and surrounding upland forests. Threats to *T. discolor* are largely from development, as much of the upper Savannah River drainage has experienced damming. Flowers are borne singly on plants from April to early May, and capsules with ant-dispersed seeds (Miller and Kwit, 2018; Miller et al., 2020) set in June and July. Petals are adaxially white to cream while abaxial petal color varies from white to maroon at the petal base (M. Koski, this study). The strongly scented flowers persist for over a week in most cases, and are dichogamous (M. Koski, personal observation). Anthers dehisce 2 to 4 days prior to the opening of the tri-lobed stigma. However, there is substantial overlap between the period of pollen presence on anthers and stigmatic opening. Thus, the potential for intrafloral pollen transfer exists (M. Koski, personal observation). While other sessile *Trillium* are self-incompatible (e.g., Sawyer, 2010), the breeding system of *T. discolor* has not been examined.

This study took place in the Clemson Experimental Forest along the southern edge of the Seneca Creek Branch of Lake Hartwell in Oconee County, South Carolina, USA. In the study area, *T. discolor* is locally common in high density along drainages into Lake Hartwell, and in lower density in drier areas on the forest floor (Appendix S1). I selected three focal patches with >50 reproductive individuals for this study (Carmen: 34.6885, -82.8851; Kayak: 34.6801, -82.8856; Quarry: 34.6801, -82.8832). Patches were between 160 and 800 m apart, but *T. discolor* plants were common between focal patches. Floral visitors observed during field work included flies (*Drosophila*), click beetles (*Conodermus*), stoneflies (Order Plecoptera) and web-spinners (order Embioptera) (M. Koski, personal observation). Lepidoptera larvae in the Noctuidae family were observed consuming petal tissue and reproductive organs, and deer consumption of aboveground tissue was common.

In other systems beetle and small dipteran pollinators have been shown to affect pollen dispersal at a much greater distance than the largest distance between patches studied in *T. discolor* (Young, 1986; Inouye et al., 2015). In other *Trillium* species, deer affect long-distance seed dispersal (over several hundred meters; Vellend et al., 2003). The focal patches are thus likely to be connected by both pollen and seed movement, and were treated as a single population in this study.

Breeding system determination

To evaluate the breeding system of *T. discolor*, I conducted self-pollinations by hand to test for self-compatibility, and excluded pollinators without manual self-pollen transfer to test for autonomous autogamy. These treatments were

performed in two of the three patches (Quarry and Carmen). Across the flowering season in 2020 and 2021, I used bridal veil cloth and a zip-tie to exclude pollinators from 20 plants prior to floral anthesis. After stigmatic opening, I removed the bridal veil from 10 randomly selected flowers, manually self-pollinated them, then replaced the bridal veil (self-pollination treatment). On 10 separate individuals, I removed and replaced the bridal veil without conducting self-pollen transfer (autonomous autogamy treatment). I collected reproductive material in June when other plants were producing capsules and scored fruit set (0 or 1) and seed number if a fruit was present. Three of the plants used to test for autonomous autogamy and one plant that was manually self-pollinated were lost to flooding or tornado damage.

Pollen limitation and pollinator-mediated selection

I selected reproductive individuals and assigned them to an outcross pollen-supplement treatment or open-pollination treatment in early April. I paired supplemented and open-pollinated individuals spatially by tagging one randomly selected reproductive individual as 'S' (pollen-supplemented), and another within 30 cm as 'C' (open-pollinated control) with lab tape at the base of the stem. Across the three patches in 2020, I tagged 66 control and 64 supplement plants, and in 2021 I tagged 47 open-pollinated and 47 supplement plants. In 2020, three plants were lost to tornado damage which left parts of two patches (Kayak, Carmen) inaccessible in 2021. I did not record whether the same exact plant was included in both years of the study. However, because I analyzed data separately within each year (see Statistical Analyses below) the potential for repeated measures on the same individual across years does not pose statistical concern.

I monitored plants at least every three days and recorded the date of floral anthesis. In 2020, one patch (Carmen), was identified as a focal site after the onset of floral anthesis. Thus, flowering date was not scored at that patch in 2020. I measured a suite of vegetative and floral traits on each plant: stem diameter, leaf width, leaf length, height to the base of the flower (floral display height), sepal width, sepal length, petal length, petal width, and petal color. All dimensions were measured using digital calipers. Leaf, sepal, and petal width were recorded at the widest point. I scored petal color discretely as either white, light purple, purple, or maroon. Because plants produce a single flower, and measuring petal reflectance requires destructive sampling, I did not measure petal reflectance on each individual in the study. Instead, I objectively evaluated reflectance of each visually assessed color category (see Petal Spectral Reflectance section below). In some cases, I recorded two colors for an individual because color was intermediate. I converted each category to a numeric value from 0 (white) to 3 (maroon) in order to assess phenotypic correlations between color and other traits. Individuals

with two color scores received intermediate values (e.g., a white/light purple individual received a color score of 0.5).

Upon stigmatic opening, I outcross-pollinated each individual in the supplement treatment using pollen collected from 2 to 4 separate individual donors by dragging dehisced anthers across the stigmatic surface. Pollen donors were collected >5 m from each focal plant to reduce the likelihood of crossing within a genet.

Upon fruit set and ripening, I collected capsules. For any plant that failed to set fruit, I scored fruit set as '0' while those producing fruit were scored as '1'. I dissected capsules and counted the number of fully formed seeds for each individual setting fruit. Seeds were bulked by site and randomly dispersed back into each site after counting.

Petal spectral reflectance

To confirm that petal color scored categorically by eye was associated with differences in spectral reflectance, I measured reflectance from the abaxial base of 4 to 14 petals of each of the four defined color classes. Each petal was collected from a separate non-experimental plant in 2020. I used an Ocean Optics spectrometer (Ocean Optics, Dunedin, Florida, USA) equipped with a deuterium-tungsten UV-NIR light and positioned the probe at a 45-degree angle to capture spectral reflectance from the petal base. Using the *pavo* package in R, I smoothed reflectance curves (*prospec*) with a parameter of 0.5 between 300 and 700 nm and extracted two objective metrics to quantify petal reflectance: violet chroma ($R_{\lambda 300-415}/R_{\lambda 300-700}$) and red chroma ($R_{\lambda 605-700}/R_{\lambda 300-700}$) to capture variation observed in the visible spectrum. I correlated petal-color class scored quantitatively 0 to 3 (white to maroon) with violet chroma and red chroma using Pearson product-moment correlation. I did not model color using an insect visual system as the primary pollinators are unknown.

Antagonist damage

I scored the presence of herbivore and florivore damage throughout each season, observing each plant at least twice a week. Insect herbivores consumed portions of leaves, while deer frequently consumed the majority of above-ground tissue. Any plant with >10% of leaf tissue damaged by herbivores was scored as experiencing herbivory. Deer herbivory was detectable because the plant tag persisted at the base of the stem after deer consumption. Florivores were primarily lepidopteran larvae which were observed consuming sepal, petal, pistil, and anther tissue. I scored any plant with holes in sepal or petal tissue, pistils removed, anthers removed, or active lepidopteran feeding as experiencing florivory. A percentage of 24.8% and 24.7% of plants experienced either florivory, herbivory, or both, in 2020 and 2021, respectively. Antagonist damage was incurred by a similar number of open-pollinated and pollen-supplemented plants in each year

(2020: 17 open-pollinated, 15 supplemented; 2021: 12 open-pollinated, 9 supplemented).

Statistics

Pollen limitation

All statistical analyses were performed using R (version 3.6.3; R Core Team, 2020). I tested for pollen limitation of seed number using a separate model for each year. In each year many plants did not produce any seed (73% in 2020; 39% in 2021). Because seed number was zero-inflated, I modeled it as a function of treatment (open-pollinated vs. pollen-supplemented) using a zero-inflated Poisson regression (*glmmTMB*). This approach models seed number using a Poisson distribution and excess zeros using a binomial distribution with a logit link. I generated back-transformed estimated marginal means and standard errors for seed number from the zero-inflated Poisson model using the *emmeans* package (Searle et al., 1980).

Trait correlations

I measured phenotypic correlations among all vegetative and floral traits measured using Pearson product moment correlations with the *rcorr* function in the *Hmisc* package (Harrell, 2022). I evaluated trait correlations separately for each year of the study.

Pollinator-mediated selection

I measured pollinator-mediated selection on floral display traits separately for each year using seed number as the fitness metric. I estimated selection using separate models for two continuous floral traits in 2020 (display height, petal size) and three continuous traits in 2021 (display height, petal size, flowering date). I characterized petal size by multiplying petal length by petal width. Separately within each treatment (open-pollinated, pollen-supplemented) and year, I generated Z-scores of each phenotypic value and calculated relative seed number for each plant by dividing individual seed number by mean seed number. None of the traits differed between the pollination treatments in either year of the study ($F_{1,80.01-127} = 0.04-3.09$, $P = 0.083-0.89$; data not shown).

If pollinators impose selection on floral traits, then the relationship between a given trait and fitness will differ between open-pollinated and pollen-supplemented plants. I therefore tested for interactions between treatment and each standardized floral trait on relative seed number to test for pollinator-mediated selection. I included the squared term of each continuous floral trait and its interaction with treatment to test for pollinator-mediated nonlinear selection (Lande and Arnold, 1983). Standardized leaf width (Z-score)

was included in each model to control for the influence of vegetative size on seed number. Relative seed number was zero-inflated in each year, so I used zero-inflated Poisson models. Poisson distributions for the conditional (non-zero) portion of each model provided better fits than Gaussian distributions based on lower AIC values. A significant treatment \times trait interaction in the conditional portion of the model indicates that pollinators exerted selection via seed number. A significant treatment \times trait interaction for the zero-inflated portion of the model indicates that pollinators exerted selection by impacting the likelihood of producing seed.

I then ran the models separately within pollination treatments in each year to generate linear and quadratic selection gradients in the open-pollinated (β_{OP} , γ_{OP}) and hand-pollinated (β_{HP} , γ_{HP}) groups. I again used zero-inflated Poisson models. I report doubled nonlinear selection gradients and associated standard errors following Stinchcombe et al. (2008).

I calculated pollinator-mediated linear ($\Delta\beta_{poll}$) and quadratic ($\Delta\gamma_{poll}$) selection on each floral trait by subtracting linear and quadratic selection gradients measured from the conditional portion of the zero-inflated model in the pollen supplemented group from those in the open-pollinated group. I calculated standard errors of $\Delta\beta_{poll}$ and $\Delta\gamma_{poll}$ as $\sqrt{SE_{B_{OP}}^2 + SE_{B_{HP}}^2}$ and $\sqrt{SE_{\gamma_{OP}}^2 + SE_{\gamma_{HP}}^2}$ respectively (Chapurlat et al., 2019). It is important to note that while zero-inflated models have been used to estimate selection on floral traits in other studies (e.g., Campbell and Bischoff, 2013) the vast majority of studies on pollinator-mediated selection estimate selection using linear models assuming a Gaussian distribution. Thus, estimates of selection in this study should not be directly compared to others.

I scored petal color categorically, so did not treat it as a continuous trait for estimates of phenotypic selection. I binned petal color into one of three categories (white, light purple, and purple/maroon combined). Because maroon was exceedingly rare in each year (see results), I combined it with purple. I modeled relative seed number as a function of treatment, petal color, and their interaction. A significant color \times treatment interaction indicates pollinator-mediated selection on petal color. I again used a zero-inflated Poisson model to estimate selection on color. I assessed the overall treatment \times color interaction using ANOVA (car package) with III Sums of Squares, and generated back-transformed estimated marginal means of relative seed number for each color class and treatment combination using the *emmeans* package. Post-hoc comparisons between pollination treatments within color class were also assessed in the *emmeans* package.

Test of antagonist-mediated floral selection

I evaluated whether antagonists impacted selection on floral traits by testing whether the direction or strength of

selection differed between plants damaged by herbivores and florivores compared to those that were undamaged (Caruso et al., 2010). Because only four of the damaged plants in each year produced seed, the datasets were not amenable to zero-inflated models using relative seed number as a metric of fitness. Thus, I modeled fruit set as a binomial response variable using a generalized linear model. Within each year, I modeled fruit set as a function of pollination treatment, and the interaction of each relativized floral trait value with damage score using a separate model for each trait. As in the models testing for pollinator-mediated selection, standardized leaf width was included to account for variation in fitness associated with plant size. I also included pollination treatment in the model because antagonist damage occurred on plants in both treatments, and all plants were included in the analysis. A significant trait \times damage interaction would indicate that antagonists impose selection (Caruso et al., 2010). The interaction between damage and the nonlinear terms of each trait were evaluated but none were significant and were removed from the final model presented.

RESULTS

Breeding system

None of the manually self-pollinated plants set fruit, and none of the flowers bagged to eliminate pollinators set fruit. Thus, *T. discolor* was self-incompatible, and autonomous autogamy did not occur.

Pollen limitation

Plants were pollen-limited in both years of the study, though pollen limitation was more severe in 2020. In 2020, the likelihood of producing at least one seed was higher in pollen-supplemented plants ($Z_{zero-inflated} = -3.01$, $P = 0.002$; Figure 1A) and pollen-supplemented plants made significantly more seeds than open-pollinated plants ($Z_{poisson} = 4.90$, $P < 0.0001$; Figure 1B). Pollen-supplemented plants made 12.17 (± 0.65 SE) seeds while open-pollinated plants made only 1.59 (± 0.71 SE) seeds, on average. In 2021, the likelihood of producing seed was higher in pollen-supplemented plants ($Z_{zero-inflated} = -2.44$, $P = 0.015$, Figure 1A) and while average seed number tended to be higher in pollen-supplemented plants (14.1 ± 0.78 SE) than open-pollinated plants (12.6 ± 1.0 SE), the difference was not significant ($Z_{poisson} = 1.15$, $P = 0.25$; Figure 1B).

Variation in floral color

Petals scored as darker by eye had lower violet chroma (Figure 2B), and higher red chroma (Figure 2C) than those scored as lighter, indicating that visually scored phenotypes

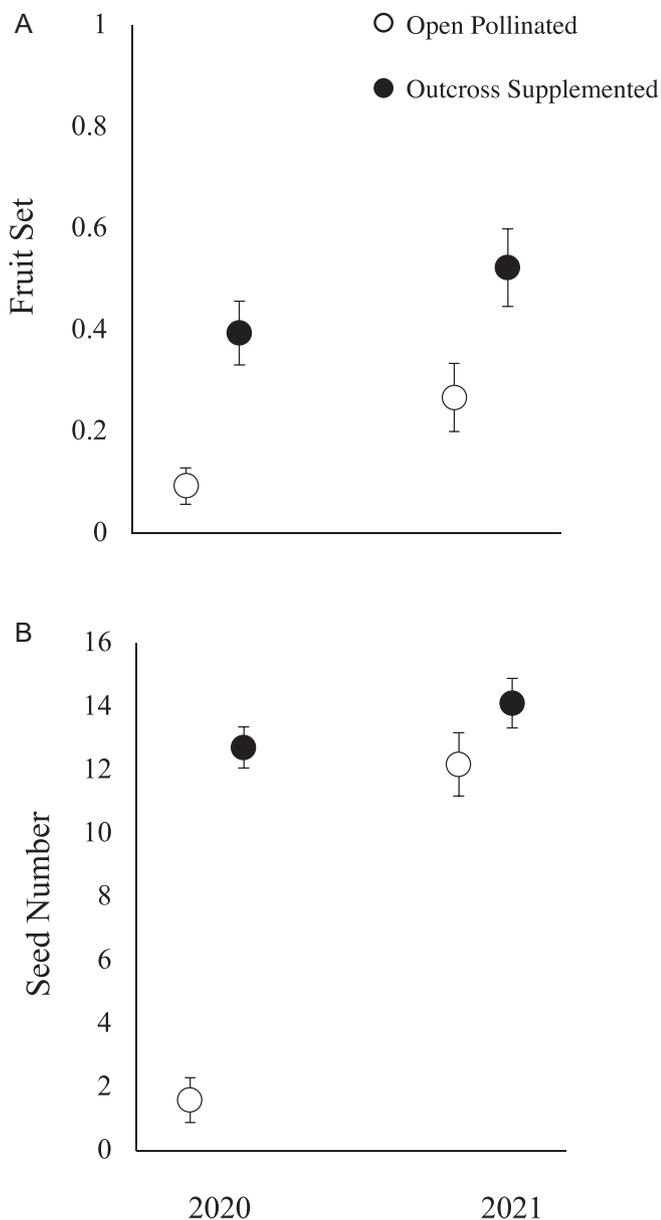


FIGURE 1 The effect of outcross pollen supplementation on (A) fruit set and (B) seed number per fruit in *Trillium discolor* in 2020 and 2021. Points in panel B depict back-transformed estimated marginal means from zero-inflated Poisson models with one standard error.

were consistent differences in spectral reflectance. Light purple petals were the most frequent color morph (38.1%), followed by white (34.3%), purple (18.1%), white/light purple intermediate (6.6%), and finally, maroon (2.8%).

Phenotypic correlations

Vegetative traits (stem diameter, leaf width, leaf length) were significantly positively correlated with one another in both years (2020: $r = 0.59-0.82$; 2021: $r = 0.63-0.78$, all $P < 0.001$; Appendices S2, S3). Floral size traits (petal width, petal length, sepal width, sepal length, anther height, pistil

height, flower display height) were also positively correlated (2020: $r = 0.26-0.69$; 2021: $r = 0.37-0.76$, all $P < 0.001$). Flower color score, however, was largely uncorrelated with vegetative and floral traits. It was only weakly correlated with petal length ($r = 0.18$, $P = 0.037$) in 2020 (Appendix S2). Julian date of first flower was modestly correlated with flower color score ($r = 0.32$, $P = 0.003$; Appendix S2) in 2021.

Pollinator-mediated selection

In 2020, pollinators exerted selection favoring smaller petals (Table 1, Figure 3A) and tended to exert selection favoring shorter display height (Table 1, Figure 3A). Pollinators also exerted disruptive selection on display height (Table 1, Figure 3B). Zero-inflated portions of each model indicated that pollinators did not impose selection by impacting the likelihood of producing seed (Table 1). More lightly pigmented flowers had higher relative fitness than darkly pigmented flowers in the open-pollinated group, but there was little association between petal color and relative fitness in the pollen-supplemented group (Figure 4A) which resulted in a marginally significant interaction between pollination treatment and flower color (Table 1). This result indicates that pollinators selected for lighter flowers.

In 2021, pollinators again favored shorter floral displays and tended to favor smaller petals (Table 2, Figure 3A). Pollinators also impacted the mode of selection on display height and petal size (Table 2, Figure 3B). Specifically, pollinators imposed disruptive selection on display height and petal size. As in 2020, there was no evidence that pollinators exerted selection by impacting the likelihood of producing seed (Table 2). More darkly pigmented flowers had higher fitness in the open-pollinated group than the pollen-supplemented group (Treatment \times Color, $P = 0.05$; Figure 4B), indicating pollinators exerted selection that favored darker flowers in 2021.

Antagonist-mediated selection

There was no evidence that antagonists imposed selection on floral traits with fruit set as the fitness metric. Neither the strength nor direction of selection differed between plants that were damaged by antagonists and those that were not damaged (Appendix S4).

DISCUSSION

Trillium discolor was incapable of self-fertilization and was pollen-limited in two separate years, suggesting that there is strong potential for pollinator-mediated selection to act on floral display traits. Indeed, both the direction and mode of selection on several floral traits in open-pollinated plants differed from pollen-supplemented plants, supporting that

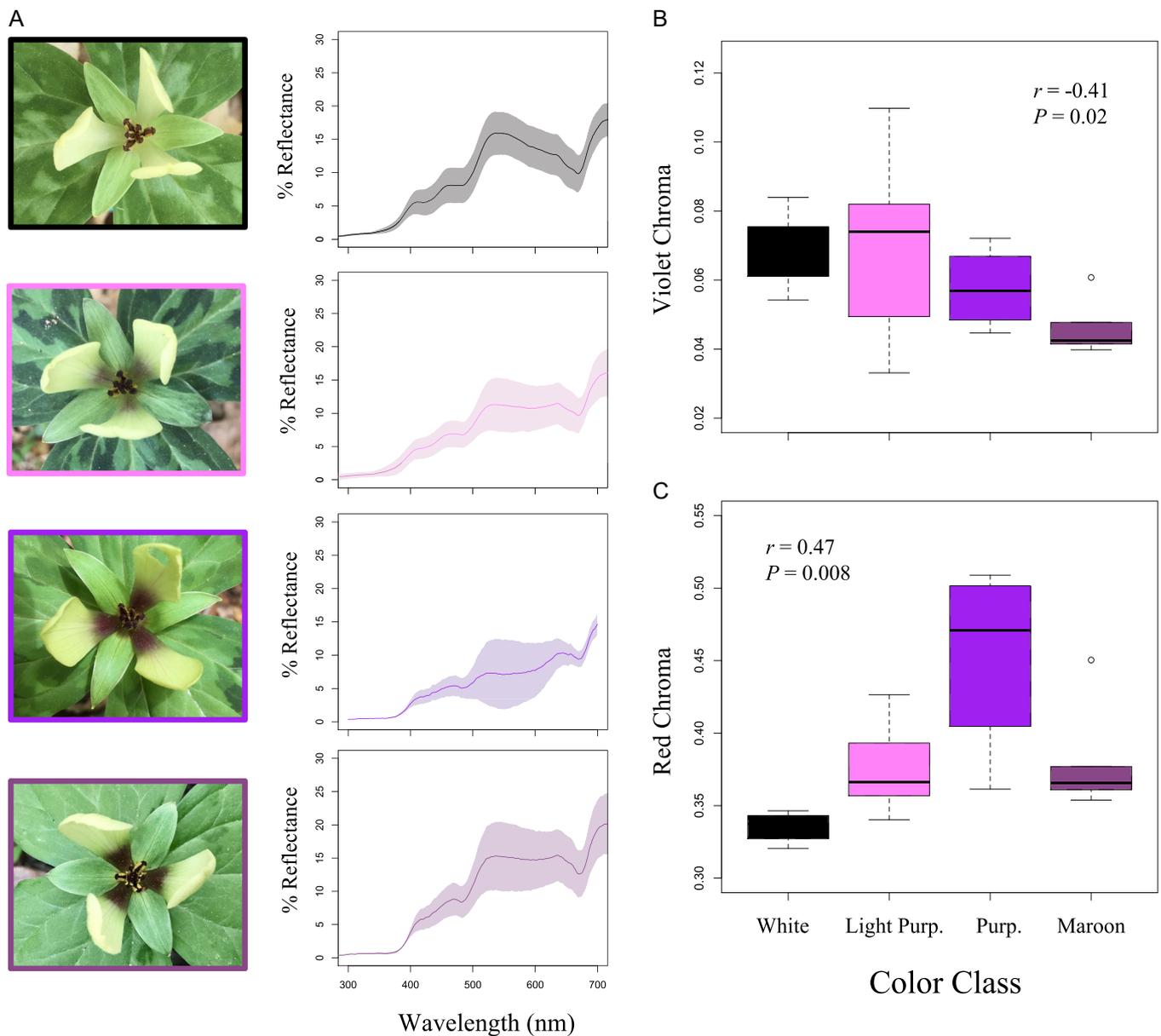


FIGURE 2 (A) Images and spectral reflectance of flowers of *Trillium discolor* from four different color classes (white, light purple, purple, and maroon). Lines in spectral reflectance plots depict average reflectance across all flowers measured within a given color class ($N = 4-14$) and shaded areas depict one standard error. Box plots of (B) violet chroma ($R_{\lambda_{300-415}}/R_{\lambda_{300-700}}$) and (C) red chroma ($R_{\lambda_{605-700}}/R_{\lambda_{300-700}}$) of flowers in each color class, with correlation coefficients between color class (scored 0–3) and each chroma metric provided.

pollinators were important agents of selection. Moreover, the strength and direction of pollinator-mediated selection differed between years for some traits. Temporal variation in pollinator-mediated selection could thus be important for maintaining diversity in floral traits in this system. Antagonist damage was common, but antagonists did not alter selection on floral traits. Thus, antagonists appear to be less important than pollinators for exerting selection on floral traits in this system. Results provide insight into drivers of selection in early spring ephemerals, and are in line with a recent meta-analysis showing stronger pollinator-mediated selection than antagonist-mediated selection on floral traits (Caruso et al., 2019).

Breeding system and pollen limitation

Self-incompatible taxa experience more severe pollen-limitation than self-compatible taxa which possess the capacity for reproductive assurance (Bennett et al., 2020). Pollen limitation of fruit set was, on average, more severe for self-incompatible *T. discolor* compared to self-compatible populations of other *Trillium* species (*T. erectum* L. and *T. grandiflorum* (Michx.) Salisb.; Irwin, 2000). However, pollen limitation in *T. discolor* was also more severe than found for self-incompatible populations of *T. grandiflorum* (Knight, 2003). Strong pollen-limitation could be driven by low pollinator visitation rates to *T. discolor* relative to other

TABLE 1 Linear (β) and nonlinear (γ) estimates of selection on continuous floral traits (Display height, Petal size) in open-pollinated (OP) and hand outcross-supplemented (HP) plants of *Trillium discolor* using seed number as a metric of female fitness in 2020 using zero-inflated Poisson models. Leaf width was used in the model for each trait to control for variation in fitness explained by vegetative size. Pollinator-mediated selection ($\Delta \beta_{\text{poll}}$ and $\Delta \gamma_{\text{poll}}$) was measured as the difference in selection between OP and HP and its significance was evaluated by the interaction of trait and pollination treatment. Standard errors are in parentheses. Petal color was scored quantitatively so selection gradients were not calculated. However, a significant interaction between color class and pollination treatment indicates pollinator-mediated selection. Selection estimates in bold are significant.

		β_{OP}	β_{HP}	$D \beta_{\text{poll}}$	P	γ_{OP}	γ_{HP}	$D \gamma_{\text{poll}}$	P
Poisson	Display height	2.96 (1.07)	-0.13 (0.18)	-2.83 (1.08)	0.06	2.30 (2.20)	0.18 (0.12)	2.12 (2.20)	0.12
	Petal size	-3.94 (1.2)	0.47 (0.29)	-4.41 (1.23)	<0.0001	16.44 (3.78)	-0.24 (0.16)	16.68 (3.78)	<0.0001
	Petal color	—	—	—	0.066	—	—	—	—
Zero-inflated	Display height	-2.36 (1.72)	-0.18 (0.42)		0.58	1.64 (2.82)	-0.22 (0.48)		0.5
	Petal size	20.2 (16.77)	0.08 (0.66)		0.08	61.5 (53.58)	0.10 (0.46)		0.14
	Petal color	—	—	—	0.79	—	—	—	—

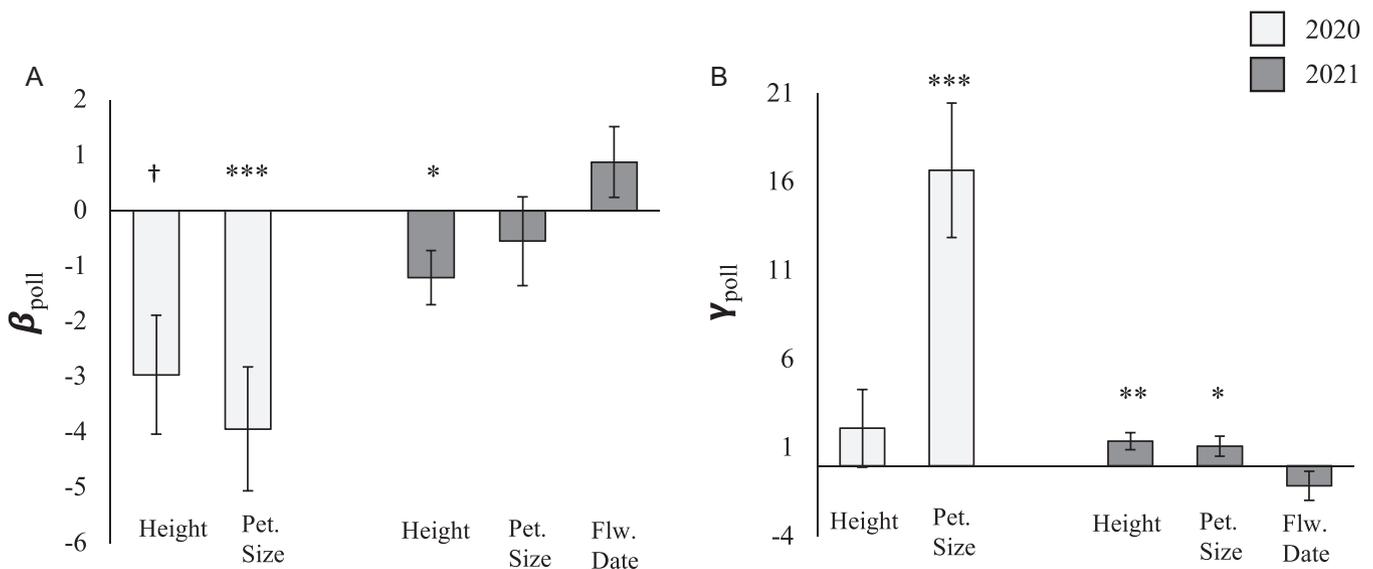


FIGURE 3 Pollinator-mediated linear (A) and quadratic (B) selection on floral traits across two years in *Trillium discolor*. Bars show the difference between selection gradients in open-pollinated plants and pollen-supplemented plants with one standard error. Selection gradients were estimated from the conditional portion of zero-inflated Poisson models (see Materials and Methods). Height = floral display height, Pet. Size = Petal size as length \times width, and Flw. date = Julian date of floral anthesis. $\dagger = 0.06$, $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$.

Trillium species, or temporal mismatches between *T. discolor* phenology and its pollinators. Observations of flower-visiting insects on *T. discolor* (mostly small flies and beetles) were exceedingly rare while conducting fieldwork. A deeper investigation into the primary pollinators of *T. discolor* and linking flowering time with and pollinator emergence will be important for explaining the strong pollen-limitation in this system. Regardless, pollen limitation sets the stage for pollinator-mediated selection to be potentially strong (Sletvold and Ågren, 2016; Trunschke et al., 2017).

While *T. discolor* was pollen-limited, fruit set among outcross-supplemented plants was still low (39% in 2020 and ~50% in 2021). This result could be driven by two potential factors. First, resource availability could limit the

likelihood of producing a fruit, even when provided with ample outcross pollen. Second, S-alleles diversity could be low, limiting mate availability (e.g., Young and Pickup, 2010). Effectively small populations of self-incompatible plants have been found to have significantly lower seed production than those that are effectively large due to low S-allele diversity (Busch and Schoen, 2008). *T. discolor*, like all *Trillium* species, spreads vegetatively via rhizomes. Thus, while care was taken to cross-pollinate focal plants with donors that were >5 m away, the potential for crossing between individuals sharing S-alleles exists. Together, both pollinator-limitation and self-incompatibility are likely important contributors to the strong pollen-limitation observed in these systems.

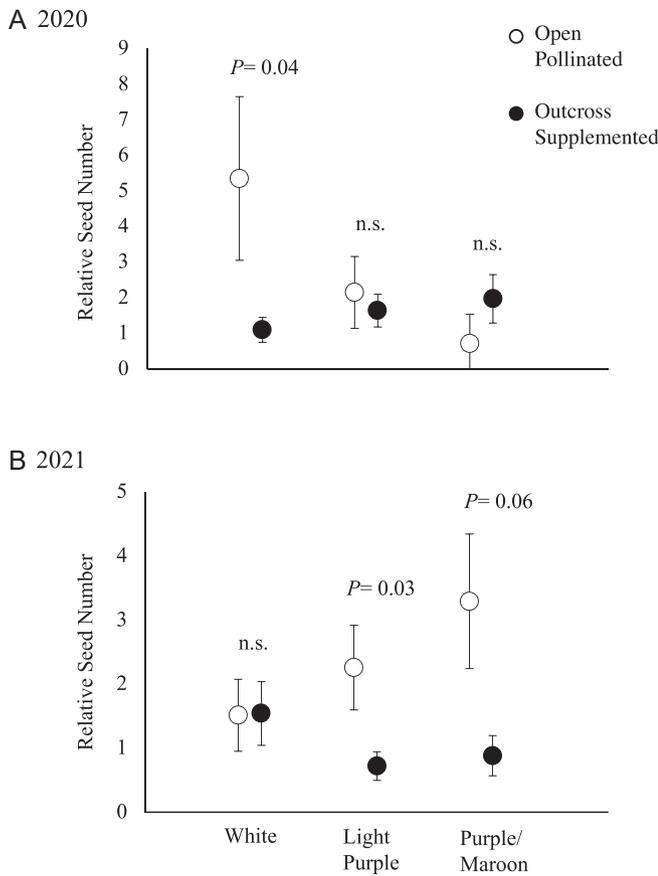


FIGURE 4 Relative seed number for three petal color classes in open-pollinated and pollen-supplemented plants in (A) 2020, and (B) 2021. Points represent back-transformed estimated marginal means from zero-inflated Poisson models with one standard error. *P*-values denote significance of post-hoc tests for differences between pollination treatments within color class. n.s. = not significant.

Pollinator-mediated selection

Pollen limitation should increase variance in female reproductive output and thus the opportunity for phenotypic selection by pollinators (e.g., Emel et al., 2017; Trunschke et al., 2017). The pollen-supplementation experiment conducted in *T. discolor* indicated that selection on floral traits by pollinators occurred in two separate years. Pollinators favored plants with shorter floral display heights and smaller petals in both years. In both years, pollinators exerted disruptive selection on display height and petal size. Disruptive selection is hypothesized to be crucial for the maintenance of genetic diversity in natural populations (Rueffler et al., 2006). Reporting of quadratic selection gradients for floral traits is far less common than reporting of linear selection gradients, and in many cases quadratic selection gradients are underestimated in the literature (Stinchcombe et al., 2008, Caruso et al., 2019). However, both stabilizing pollinator-mediated selection on flower number in *Penstemon digitalis* Nutt. ex Sims [Plantaginaceae] (Parachnowitsch and Kessler, 2010), and disruptive pollinator-mediated selection on flower size in *Iris atropurpurea* Baker [Iridaceae] (Lavi and Sapir, 2014) have been reported. Disruptive selection in particular should be crucial for the co-occurrence of distinct floral morphs (e.g., sex morphs, color morphs) in natural populations (e.g., Rymer et al., 2010; Briscoe Runquist et al., 2017), but more estimates of nonlinear pollinator-mediated selection are required to determine the degree to which pollinators mediate disruptive selection on floral traits across plants.

Pollinator-mediated selection favored more darkly pigmented flowers in the second year of the study but in the previous year pollinator-mediated selection tended to be in

TABLE 2 Linear (β) and nonlinear (γ) estimates of selection on quantitative floral traits (Display height, Petal size, Flowering date) in open-pollinated (OP) and hand outcross-supplemented (HP) plants of *Trillium discolor* using seed number as a metric of female fitness in 2021 using zero-inflated Poisson models. Leaf width was used in the model for each trait to control for variation in fitness explained by vegetative size. Pollinator-mediated selection ($\Delta \beta_{\text{poll}}$ and $\Delta \gamma_{\text{poll}}$) was measured as the difference in selection between OP and HP and its significance was evaluated by the interaction of trait and pollination treatment. Standard errors are in parentheses. Petal color was scored quantitatively so selection gradients were not calculated. However, a significant interaction between color class and pollination treatment indicates pollinator-mediated selection. Selection estimates in bold are significant.

		β_{OP}	β_{HP}	$D \beta_{\text{poll}}$	<i>P</i>	γ_{OP}	γ_{HP}	$D \gamma_{\text{poll}}$	<i>P</i>
Poisson	Display height	-0.79 (0.33)	0.42 (0.36)	-1.21 (0.49)	0.02	0.92 (0.36)	-0.48 (0.34)	1.4 (0.49)	<0.01
	Petal size	1.00 (0.615)	1.55 (0.49)	-0.55 (0.79)	0.12	-0.28 (0.34)	-1.4 (0.44)	1.12 (0.56)	0.01
	Flowering date	0.98 (0.59)	0.10 (0.25)	0.88 (0.64)	0.24	-1.68 (0.76)	-0.56 (0.32)	-1.12 (0.82)	0.85
	Petal color	—	—	—	0.05	—	—	—	—
Zero-inflated	Display height	-0.86 (0.60)	-1.23 (3.17)	0.37 (3.23)	0.78	1.18 (1.24)	-5.16 (7.72)	6.34 (7.82)	0.45
	Petal size	-1.00 (1.07)	-7.78 (9.02)	6.78 (9.08)	1	0.76 (0.70)	-40.38 (44.14)	41.14 (44.15)	1
	Flowering date	-0.23 (0.45)	-2.67 (1.69)	2.44 (1.75)	1	-0.18 (0.58)	-0.90 (1.78)	0.72 (1.87)	1
	Petal color	—	—	—	0.28	—	—	—	—

the opposite direction. Pollinators have been shown to exert selection for less bright petal color in one system (Caruso et al., 2010) but brighter colors and more stark color contrasts between portions of petals in another (Sletvold et al., 2016). Both spatially and temporally fluctuating variation on floral traits has been observed in other systems (Sletvold and Ågren, 2010), and is a primary factor that could contribute to the maintenance of variation in natural populations (Turelli et al., 2001; Sapir et al., 2021).

I am unable to address why pollinator-mediated selection on petal color tended to differ between years with the data at hand. The strength and direction of pollinator-mediated selection can be highly dependent on non-pollinator biotic interactions, resource availability, and co-flowering community composition (Sletvold, 2019). Furthermore, differences in pollinator-mediated selection across space can result from local differences in the frequency of dominant pollinators (e.g., Gómez et al., 2008). In *T. discolor*, floral anthesis was one week earlier in 2020 (mean Julian date = 95.3 ± 3.2 SD) than 2021 (mean Julian date = 102.3 ± 3.4 SD) on average. If different pollinator classes (e.g., flies vs. beetles) differ in both phenology and preference for color, this could contribute to inter-annual differences in pollinator-mediated selection.

My study only estimated selection via metrics of female fecundity which is the case for most selection studies in plants (Caruso et al., 2019). However, selection on floral traits can also act through male fitness (e.g., Caruso et al., 2005; Hodgins and Barrett, 2008; Sahli and Conner, 2011). Selection via male fitness is expected to be stronger than selection via female fitness in populations that are not pollen-limited (Hodgins and Barrett, 2008). However, selection via female and male fitness are frequently correlated in studies that estimate both sexual functions (Ashman and Morgan, 2004). Selection analyses that include metrics of male fitness like paternity or pollen export in *T. discolor* have the potential to reveal patterns of pollinator-mediated selection that are not demonstrated in the current study.

Antagonist-mediated selection

There was no evidence that natural levels of florivory and herbivory altered patterns of selection on petal size. I caution however, that the tests for antagonist-mediated selection were limited by the sample size of individuals that experienced antagonism (~25% of plants in the study each year). Additionally, plants that were consumed entirely by deer prior to flowering were missing floral trait data and were thus not included in selection analyses. These plants did not express floral traits prior to death and represent 'the invisible fraction' (Grafen, 1988), which has been shown to contribute quite strongly to patterns of selection (Mojica and Kelly, 2010). Regardless of these caveats, the data at hand do not support that selection on floral traits was mediated by antagonists.

In other systems, plants with larger floral displays experienced elevated levels of florivory (Asikainen and

Mutikainen, 2005; Teixido et al., 2011) potentially because they were more conspicuous, or generated more nectar or pollen resources though the degree to which this resulted in selection for smaller flower was not tested. However, most strong selection on floral traits has been found for seed-consuming antagonists (Parachnowitsch and Caruso, 2008; Caruso et al., 2010; Bartkowska and Johnston, 2012) rather than strict herbivores florivores that only consume petal tissue (Bartkowska and Johnston, 2012). The level of florivory was highly variable among plants in *T. discolor*, ranging from small holes in petals to complete consumption of sepals, petals, anthers, and pistils. In populations where pistil consumption is common, florivores would have a stronger potential to impact selection. Likewise, herbivory ranged from minor leaf damage (likely by insect herbivores) to full consumption of above-ground tissue by deer. Deer grazing is common in understory plants, and has important demographic consequences for other *Trillium* species (Knight, 2004). Selection on floral traits mediated by large grazers like deer would most likely be correlative selection through grazer choice for larger plants (e.g., Gomez, 2003). However, detecting selection by deer would require experimental enclosures and may still face issues associated with 'the invisible fraction' for plants grazed prior to flowering.

CONCLUSIONS

This work provides one of the first experimental pollen-supplementation studies to test for pollinator-mediated selection on floral traits in a spring ephemeral. While pollen limitation (Williams and Winfree, 2013; Gezon et al., 2016) and selection (Irwin, 2000; Frey, 2004) have been reported in spring ephemerals, the degree to which pollinators exert selection on floral traits has remained elusive. I found that *T. discolor* was incapable of self-fertilization which likely contributed to strong pollen-limitation, setting the stage for pollinator-mediated selection. The strength direction of pollinator-mediated selection on floral color differed between two years. Such interannual differences in selection could be important for the maintenance of phenotypic diversity in *T. discolor*. Antagonists have been shown to be important agents of selection on spring ephemerals (Frey, 2004). While there was some evidence that the likelihood of experiencing antagonist damage was higher for plants with smaller petals, antagonists did not impose selection on any floral trait measured. Thus, pollinators are more likely to be consistent agents of selection than antagonists across seasons. In spring ephemerals, the onset of blooming is strongly influenced by climate change (Kudo et al., 2004; Gezon et al., 2016; Petruski et al., 2019), in some cases putting spring ephemerals at risk of phenological mismatch with their pollinators (Kudo and Cooper, 2019). Spring ephemeral systems such as *T. discolor* thus provide compelling systems in which to examine mechanisms that drive interannual variation in pollen limitation and pollinator-mediated selection.

AUTHOR CONTRIBUTIONS

M.H.K. conceived of the study, collected, and analyzed the data, and wrote the manuscript.

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DATA AVAILABILITY STATEMENT

All phenotypic values, fitness metrics, and code are available in the Zenodo data repository: <https://doi.org/10.5281/zenodo.7258367> (Koski, 2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Aerial image of study area and *Trillium discolor* presence. Aerial image of the study area (South shore of the Seneca Creek Branch of Lake Hartwell in the Clemson Experimental Forest), with known location of *Trillium discolor* plants (white polygons), and the three patches with a high density of reproductive individuals used to estimate phenotypic selection.

Appendix S2. Phenotypic trait correlations, 2020. Pearson product-moment correlations between traits measured on *Trillium discolor* in 2020 above the diagonal, and significance of correlation below the diagonal. Measurements across three sites within 500 to 800 m of one another were pooled.

Appendix S3. Phenotypic trait correlations, 2021. Pearson product-moment correlations between traits measured on *Trillium discolor* in 2021 above the diagonal, and

significance of correlation below the diagonal. Measurements across three sites within 500 to 800 m of one another were pooled.

Appendix S4. Statistical tests of the impact of antagonist damage on selection. Results from linear models testing whether antagonist damage impacted selection on floral traits *T. discolor* across two years. Fruit set (0 or 1) was modeled with a binomial distribution. Flowering date was not evaluated in the model of data from 2020. A significant interaction between antagonist damage and a trait indicates that antagonists mediate selection.

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