**Running head:** Pollen color variation

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# On the ecological significance of pollen color: a case study in American trout lily (*Erythronium americanum*)

Authors

Emily J. Austen*[1], Department of Biology, University of Ottawa, Ottawa ON Canada, and Biology Department, Mount Allison University, Sackville NB Canada

Shang-Yao Lin[2], Department of Biology, University of Ottawa, Ottawa ON Canada

Jessica R. K. Forrest[3], Department of Biology, University of Ottawa, Ottawa ON Canada

* Author for correspondence

[1] austen.emily@gmail.com

[2] slin041@uottawa.ca

[3] jforrest@uottawa.ca

## Abstract

Evolutionary ecologists seek to explain the processes that maintain variation within populations. In plants, petal color variation can affect pollinator visitation, environmental tolerance, and herbivore deterrence. Variation in sexual organs may similarly affect plant performance. Within-population variation in pollen color, as occurs in the eastern North American spring ephemeral *Erythronium americanum,* provides an excellent opportunity to investigate the maintenance of variation in this trait. Although the red/yellow pollen-color polymorphism of *E. americanum* is widely recognized, it has been poorly documented. Our goals were thus (1) to determine the geographic distribution of the color morphs, and (2) to test the effects of pollen color on components of pollen performance. Data provided by citizen scientists indicated that populations range from monomorphic red, to polymorphic, to monomorphic yellow, but there was no detectable geographic pattern in morph distribution, suggesting morph occurrence cannot be explained by a broad-scale ecological cline. In field experiments, we found no effect of pollen color on the probability of predation by the pollen-feeding beetle *Asclera ruficollis*, on the ability of pollen to tolerate UV-B radiation, or on siring success (as measured by the fruit set of hand-pollinated flowers). Pollinators, however, exhibited site-specific pollen-color preferences, suggesting they may act as agents of selection on this trait, and, depending on the constancy of their preferences, could contribute to the maintenance of variation. Collectively, our results eliminate some hypothesized ecological effects of pollen color in *E. americanum*, and identify effects of pollen color on pollinator attraction as a promising direction for future investigation.

**Keywords:**

polymorphism, plant-pollinator interaction, pollen-feeding beetle, spring ephemeral, anthocyanin, citizen science, *Asclera ruficollis*, pollen tube, fruit set, UV-B radiation

## Introduction

A central goal of evolutionary ecology is to understand the relative contributions of adaptive and neutral processes to maintaining phenotypic variation. Three general scenarios are expected (Mitchell-Olds et al. 2007). First, trait variation can be maintained through the purely adaptive process of balancing selection if, for example, selection is frequency-dependent (classic examples include sex ratio (Fisher 1930) and self-incompatibility alleles (Wright 1939)), or selection fluctuates over space or time (e.g., Kalisz 1986). Second, under mutation–selection (or migration–selection) balance, the removal of an unfavorable morph by selection is balanced by its reintroduction through mutation/migration. For discrete polymorphisms, this process typically results in a low frequency of the disfavored morph at a given site (e.g., Hoekstra et al. 2004). Third, selectively neutral variation can be maintained, at least temporarily, if factors such as a large population size or overlapping generations buffer against drift. Because two of the processes involve selection, and the third invokes its absence, field observations aimed at determining whether a trait is a target of selection are an important step towards understanding how variation in that trait is maintained.

Easily scored discrete color polymorphisms (e.g., fur color in desert mice: Hoekstra et al. 2004; throat color in side-blotched lizards: Sinervo and Lively 1996; abdomen color in candy-striped spider: Oxford 2005) have provided rich subjects for the study of phenotypic variation. In plants, investigation of color polymorphism has largely focused on the attractive organs of flowers: petals, sepals, and floral bracts. Classic work on a petal color polymorphism in *Linanthus parryae*, for example, concluded that genetic drift maintained polymorphism among finely divided subpopulations (Epling and Dobzhansky 1942, Wright 1943), while later work in the same system uncovered a larger role for selection (Epling et al. 1960, Schemske and

Bierzychudek 2001, 2007). In other systems, flower color has direct or pleiotropic effects on pollinator attraction (Waser and Price 1981, Stanton et al. 1989), environmental tolerance (Warren and Mackenzie 2001, Armbruster 2002, Lacey and Herr 2005), and herbivore deterrence (Irwin et al. 2003). Whether a particular morph outperforms the other in all tested functions and conditions (invoking mutation-selection balance in the maintenance of variation), or the favored morph varies (possibly leading to balancing selection), the color of attractive floral organs in polymorphic species is rarely selectively neutral.

The causes and consequences of color variation in the sexual organs of flowers (stamens and pistils) are less known, but these too can be polymorphic. Consider pollen: yellow is the most common, and probably ancestral, pollen color in the flowering plants (Lunau 2000), but alternatives including white (e.g., *Cichorium intybus*), blue (e.g., *Epilobium angustifolium*), pink (e.g., *Claytonia lanceolata*), and purple-black (e.g., *Phacelia tanacetifolia*) demonstrate that pollen color can evolve. Moreover, pollen color is sometimes polymorphic within flowers (Darwin 1877) and among individual plants (Lau and Galloway 2004, Jorgensen and Andersson 2005). *Erythronium americanum*, a charismatic spring ephemeral of the eastern North American temperate forest, is a striking example of variation among individuals (Fig. 1). Anther and pollen color variation is well known in this species and several of its congeners (Parks and Hardin 1963), with classic accounts variably describing the anthers of *E. americanum* as being typically yellow (e.g., Ker Gawler 1808) or typically red (e.g., Meads 1893). Although *Erythronium*'s unusual pollen color variation has been used to track pollen dispersal within a population (i.e., by counting grains from a known red-pollen donor on the stigmas of yellow flowers: Thomson and Thomson 1989, Thomson and Eisenhart 2003), the ecology and evolution of this variation has not yet been investigated. Such work could, however, advance understanding of the maintenance of color polymorphism in plant sexual organs.

Knowledge of the geographic distribution of morphs can help to refine hypotheses about the mechanisms underlying the maintenance of variation and possible agents of selection. A cline in morph occurrence, for example, could hint that temperature or photoperiod affects morph occurrence (e.g., Jones et al. 1977, Arista et al. 2013, Koski and Galloway 2017), while a patchy distribution could implicate edaphic factors (e.g., Horovitz 1976) or geographic variation in pollinator communities (e.g., Anderson and Johnson 2007). The distribution of *E. americanum* pollen color morphs has not been documented.

Ultimately, pollen color polymorphism must be examined in the context of the many — and sometimes conflicting — tasks that pollen grains perform. First, pollen contributes to the attractive signal of the flower (Lunau 1995, 2000). Second, at the same time, pollen can also contribute to defense against pollen thieves through, for example, camouflage coloration (Hargreaves et al. 2009). Third, the pollenkitt and pollen walls protect the nuclei within from environmental stressors (e.g., desiccation, temperature, and UV-B radiation) encountered during presentation and transport to a receptive flower (Murphy 2006). Fourth, proteins on the pollen grain surface mediate interactions with the stigma of the receiving flower (Murphy 2006). The secondary metabolites responsible for pollen color could affect performance in any of these roles.

Here, we report on a series of studies aimed at uncovering the potential ecological significance of pollen color variation in *E. americanum*. Our first goal was (1) to describe the geographic distribution of the pollen color morphs. Our second goal was (2) to test effects of pollen color on (a) pollinator attraction, (b) pollen theft, (c) UV-B tolerance, and (d) fertilization efficacy. Determining the ecological significance of pollen color variation in *E. americanum* is a first logical step towards identifying putative agents of selection on pollen color, and ultimately, discerning the processes that govern variation in this trait.

## Methods

### Study system

### *Natural history of Erythronium americanum*

*Erythronium americanum* Ker Gawl (Liliaceae) is a long-lived, iteroparous spring ephemeral occupying the temperate forest floor of Eastern North America (Fig. 2). Individuals complete annual growth and reproduction during the ~6-week period between snowmelt and canopy closure; a population's flowering period lasts ~8d (EJA pers. obs.). The perennating corm reproduces clonally through stolon-like "droppers" terminating in a new corm (Robertson 1906). Sexual plants produce two leaves and a single, trimerous hermaphroditic flower. The adaxial surface of the six tepals is yellow (often flecked with red) to the human eye. The syncarpous pistil bears a three-lobed stigma and ~35 ovules (range 24 – 64, Wolfe 1983). Flowers are nodding at night and on cool or overcast days, but for ~3h on warm, sunny days, the petals reflex and the flower pulls into a horizontal position to expose the six pollen-laden anthers (Fig. 1; Meehan 1878). Flowers are protandrous: anther dehiscence is staggered over two days, and pistil lobes reflex to expose the papillose stigmatic surface on or after the second day of anthesis. Individual flowers persist up to ~5d. Flowers are visited by a range of potential pollinators, including generalist (e.g., *Bombus* queens) and specialist (e.g., *Andrena erythronii*) bees. The pollen-feeding beetle *Asclera ruficollis* (Oedmeridae) frequently feeds and mates on *E. americanum* (Arnett 1951). *Erythronium americanum* is weakly self-compatible (Harder et al. 1985), and the strength of self-compatibility varies among populations (Stokes 2012).

*Anther and pollen color variation*

To the human eye, *E. americanum* anther walls and pollen grains range in color from lemon yellow to brick red (Appendix S1: Fig. S1). Yellow anthers always contain yellow pollen; red anthers contain red, medium-orange, or (occasionally, before anther dehiscence) yellow pollen (EJA pers. obs). The red pigment is an anthocyanin (Mark Rausher, pers. comm.). As is common for plant secondary metabolites (Moore et al. 2014), pollen-color variation in *E. americanum* occurs at two levels: (1) the presence/absence of anthocyanin in anther tissue, demarking discrete red-orange (RO) *versus* yellow (YE) morphs, and (2) quantitative variation in pigment concentration within the RO morph (Appendix S1: Fig. S1). We investigated the distribution and ecological effects of the higher-order, discrete variation. The relative magnitudes of genetic *versus* environmental components of discrete pollen-color variation in *E. americanum* have not been established. Based, however, on the regular occurrence of discrete, neighbouring patches exhibiting opposing morphs (suggesting color consistency within genets), and on the stability of patch morphs over years (EJA *personal observation*), we assume a large genetic component.

1. Determining the geographic distribution of anther color morphs

We developed a website (troutlilysurvey.wordpress.com) recruiting citizen scientists to report *E. americanum* anther color at local sites. This study focused on anther color rather than pollen color because anther color is more readily observed from a distance, and, unlike pollen color, it can be observed before anthers dehisce and after all pollen has been removed. During Winter/Spring 2016 and 2017, we advertised the project on the ecolog-L listserv

(listserv.umd.edu/archives/ecolog-l.html) and on the social media pages of native plant societies throughout the species range.

The website linked to a CitSci.org project page where participants submitted data, including the latitude and longitude of the observation site; the approximate number of flowers observed (<50, 50–100, 100–250, 250–1000, >1000); and presence/absence of pure yellow, deep red, and intermediate orange anthers (during 2017, red and orange were grouped into a single category). To aid in identifying study sites for future research, we also asked participants to estimate the frequency of the YE morph. Because we did not enforce a single method for estimating frequency, the frequency data are inappropriate for analysis, but they are available on request to EJA. Participants were invited to submit photographs; 72% of records included at least one photograph. Elevation data were extracted from Google Maps using R package googleway (Cooley 2016).

Before analysis, we removed four records submitted from > 2° latitude or longitude outside the known range of *E. americanum* (Allen & Robertson 2003). We also removed seven records for which the associated photographs were not *E. americanum* (removal of all records lacking a photograph yielded nearly identical results; analysis not shown), and four records for which the observer indicated that neither RO nor YE were present. The remaining data ($N$ = 251 records) were retained for analysis.

Given that the probability of observing a locally rare pollen morph probably increases with sample size, we first examined effects of the number of flowers observed on the probability of polymorphism (i.e., presence of both RO and YE) in a logistic generalized linear model. For ease of interpretation, we simplified the number of flowers observed to categories ≤100 ($N$ = 190 populations) or >100 ($N$ = 61 populations). Next, to examine geographic patterns in morph distributions, we analyzed occurrence of the YE morph at a site in a logistic generalized linear model with predictors latitude (range: 35.218–47.102 °N), longitude (range: 63.233–89.444 °W), and elevation (range: 0–1607 m; log-transformed for analysis). We again included number of flowers observed (simplified to categories ≤100 or >100) as a predictor to account for sample size. Because occurrence of one morph does not preclude occurrence of the other, we also analyzed occurrence of the RO morph (i.e., presence of red and/or orange at site) using the same model structure. Analyses of polymorphism and of morph occurrence were performed in R (R Core Team 2016), and morph distribution was plotted using R packages maps (Becker et al. 2016) and mapplots (Gerristen 2014).

## 2. Testing the ecological significance of pollen color morphs

We performed field experiments at eight sites polymorphic for pollen color (Table 1, Fig. 2, max five sites per experiment) to test four ecological hypotheses concerning possible advantages of one pollen color morph over the other: (a) one morph may be more attractive to pollinators, (b) one morph may better deter pollen-feeding beetles, (c) one morph may be more tolerant of UV-B exposure, or (d) one morph may be better able to effect fertilization and fruit maturation. In all experiments, we made comparisons between the YE and RO pollen morphs, choosing the most pigmented individuals available to represent RO, and always using flowers collected at the site. Experiments testing hypotheses (a) and (b) were conducted during 2016, (c) during 2015, and (d) during 2015 and 2017. Data were analyzed in R (R Core Team 2016; see details below).

### *(a) Pollinator attraction*

We hypothesized that pollinators might preferentially visit one pollen morph. For example, YE may be more apparent to pollinators because at the peak spectral sensitivities of at least one pollinator (*Bombus impatiens*), anthers of the YE morph are more easily distinguished from a background of green *E. americanum* leaves and dry leaf litter than are those of the RO morph (Appendix S1: Fig. S2). Innate color preferences in pollinators may also result in increased visitation to the YE morph (Schiestl and Johnson 2013). Alternatively, the RO morph may enhance the attractive signal of red-flecked tepals (Lunau 1995). Even with no long-term morph preference, bees can learn pollen colors (Muth et al. 2016), and short-term color constancy by pollinators could facilitate assortative mating. By increasing the phenotypic frequency of a recessive-homozygote morph, assortative mating can increase opportunity for another mechanism to maintain polymorphism (Pérez i de Lanuza et al. 2012). Alternatively, assortative mating can destabilize polymorphism by causing mate-limitation in rare morphs (Pryke 2010). To test for effects of pollen color on pollinator attraction, we observed visitation to mixed pollen-color arrays at five study sites (Table 1).

Arrays comprised four flower patches, spaced 30 cm from one another on a square grid. Each patch consisted of 2 – 4 cut flowers (number depended on flower availability at the site), each with at least three freshly-dehisced anthers, held individually in 15 mL floral picks (hereafter, vials) spaced 4 cm from one another in a square grid of insulation foam (Appendix S1: Fig. S3). Each vial also contained a single *E. americanum* leaf. The foam bases were covered in leaf litter. All flowers within a patch were of the same pollen morph, and each array consisted of two YE and two RO patches. Each patch was equidistant from one patch of the same morph and one of the alternate morph (Appendix S1: Fig. S3). This design mimicked the naturally patchy distribution of pollen morphs.

Arrays were positioned in areas where both pollen morphs occurred naturally, and were observed for an average of 90 min between midday and early afternoon (min = 30, max = 210, total = 24 h 15 min; Table 1) per observation day. All observations were performed within the first four dry, sunny days of the flowering season at a given site (range: 1–4 days per site). Using a digital voice recorder, we recorded the identity of each visitor, the number of flowers it visited within each patch, the sequence in which it visited patches within a bout, and whether it was foraging for pollen or for nectar. We did not follow visitors after they left the array; thus, any returns by the same individual were recorded as separate bouts. We excluded *Asclera ruficollis* (Oedemerideae) from these observations (see below), and male solitary bees seeking mates rather than floral rewards (these either navigated through the array without landing on flowers, or landed directly on female bees). Prior to analysis, we categorized visitors into five classes based on identity and the reward for which they were most often seen foraging on *E. americanum*. Pollen foragers included *Apis mellifera*, *Bombus* queens, and females of large solitary bees (e.g., *Andrena*, *Osmia*). Nectar foragers included two classes: bombyliid flies, and nectar-foraging solitary bees (i.e., small solitary bees such as *Ceratina*, parasitic bees (*Nomada*), and nectar-foraging males of large solitary bees).

We analyzed visits to patches within bouts, treating each pollinator bout at the array as a unique observation. The proportion of visits within a bout to RO patches was analyzed in three binomial generalized linear models (Gelman and Hill 2007: 116). The first two models tested for effects of site on pollen-morph preference, and of pollinator class on pollen-morph preference, respectively.  We calculated deviance to compare the fit of each of these two models to that of a simple model including intercept only (Gelman and Hill 2007: 100). Because not all pollinator classes were observed at all sites (see results, Appendix S1: Table S1), interactive effects were analyzed in a separate model for the best-represented sites and pollinator classes only (i.e., excluding site BAW, and excluding classes *Apis* and *Bombus*). Models were fit using function glm( ) in R, and least-squares means were calculated using package lsmeans (Lenth 2016).

We tested the potential for pollinator-mediated assortative mating by analyzing pollinator movements between nearest-neighbor patches within a bout (i.e., excluding diagonal movements across the array, Appendix S1: Fig. S3). Each movement between nearest-neighbor patches was classified as “same” if the visitor travelled between patches of the same morph, and “different” if it travelled between pollen morphs. The frequency of “same” visits within a bout was analyzed in three binomial generalized linear models (Gelman and Hill 2007: 116): the first two tested for site effects on the tendency towards constancy, and for visitor class effects, respectively. We calculated deviance to compare the fit of each of these two models to that of a simple model estimating an intercept only (Gelman and Hill 2007: 100). We fit models using function glm( ) in R.

### *(b) Deterrence of pollen-consuming beetles*

*Asclera ruficollis* (Oedemeridae) are frequent visitors to *E. americanum* flowers (Arnett 1951). Although they facilitate limited cross-pollination in some strongly pollen-limited spring ephemerals (e.g. *Hepatica acutiloba* [= *Anemone acutiloba*], Murphy and Vasseur 1995), these beetles move infrequently between *E. americanum* flowers (EJA *personal observation*), and so likely contribute more to vector-mediated selfing than to cross-pollination in our study system. Moreover, like all oedemerids, adult *A. ruficollis* feed on pollen grains (Marshall 2006). For these reasons, visitation by *A. ruficollis* is likely a net detriment to male fitness in *E. americanum*. We hypothesized that *A. ruficollis* might prefer the YE morph, because the YE morph may be more readily recognized as pollen, or because RO anthers may be less easily discriminated from the leaf litter background (Appendix S1: Fig. S2). We tested the pollen morph preference of *A. ruficollis* in a choice experiment.

In the choice experiment, beetles were captured by hand and chilled in a cooler with ice packs for up to 10 minutes. After chilling, beetles were placed individually in 30 cm × 30 cm × 30 cm white mesh cages along with two vials in modeling-clay bases. Each vial contained one *E. americanum* leaf and flower (one of each pollen morph, collected from the local population). Vials were positioned 10 cm from one another in the center of the cage floor. We observed the position of the beetles (on tepals or anthers, or elsewhere in cage) after five and 10 minutes. Eight beetles were observed for an additional 20 min; just one of these moved to the second flower during the total 30 min period (we recorded the first flower visited as its flower choice). Otherwise, following the 10 min observation, we released the beetle at least 20 m from the experimental set-up, and introduced a new chilled beetle to the cage. We tested a total of 68 beetles across three study sites (Table 1), using up to four cages simultaneously per site.

For those beetles making a flower choice during the 10 min observation period, the probability of choosing the RO morph was analyzed in a mixed-effects binomial generalized linear model (Gelman and Hill 2007: 256) using function glmer( ) in R package lme4 (Bates et al. 2015). We initially included cage ID as a random effect. When AIC comparison to a null model indicated that the random effect did not improve model fit (Gelman and Hill 2007: 525), we next fit a model including site ID as a fixed effect using function glm( ), and compared its fit to a model with intercept only (Gelman and Hill 2007: 100).

### *(c) UV-B tolerance*

The anthers of both pollen morphs are UV-B absorbing (Appendix S1: Fig. S4). However, while anthocyanins can protect tissue from mutagenic UV-B radiation (Takahashi et al. 1991), they are less effective in this role than are dihydroflavonols (their metabolic precursors) or flavonols (the alternate products made from dihydroflavanols) (Solovchenko 2010; see Wessinger & Rausher 2012 for metabolic pathway). We therefore hypothesized that the YE morph would exhibit more germination and tube growth following UV-B exposure than would the RO morph (which has diverted some dihydroflavonols to the anthocyanin pathway). To test this hypothesis, we exposed (or protected) pollen grains to UV-B radiation, and then applied them to the stigmas of cut flowers in the lab, as described below. We observed pollen tube growth ~20h later as a measure of pollen function, assuming that UV-B damage to the vegetative nucleus of the pollen grain would impede pollen germination or pollen tube growth (Feng et al. 2000).

Undehisced anthers of both pollen morphs were collected at KSR (Table 1) early in the morning, before flower opening. Three anthers (one from each of three flowers of a single morph) were placed together in a small petri dish, along with a moist piece of paper towel folded to ~1cm$^3$ to maintain humidity. Dishes were covered with either a UV-B transmitting filter (low-density polyethylene), or a UV-B blocking filter (Mylar) (c.f. Torabinejad et al. 1998). Four petri dishes (one transmitting and one blocking for each pollen morph) were grouped together to form a ‘block’, and blocks were placed directly beneath a UV-B bulb (Repti-Glo 13W UVB 100) for 3h (the maximum duration of midday exposure we would expect an anther to receive before being stripped by pollinators). Anthers covered by a UV-B transmitting filter received four times the UV-B photon flux as those with a blocking filter (mean ± SD = 4.1± 0.5 µmol/m$^2$/s *versus* 1.1± 0.1 µmol/m$^2$/s; paired $t_{17} = 29.0$, $P < 10^{-15}$, measured using SpectroSense meter, Skye

Instruments); the UV-B exposed anthers received a flux comparable to that measured midday at KSR (3.9 µmol/m$^2$/s, EJA *personal observation*). Exposures were repeated five days during early May, with three to four UV-B lamps (blocks) per day and 18 blocks in total. Almost all anthers fully dehisced during exposure.

Immediately after exposure, pollen grains were scraped from the anthers within a dish, mixed together using fine forceps, and applied to the stigmas of recipient flowers. Pollen recipients were collected from the field population two days prior to anther exposure, emasculated, and stored in flower picks at room temperature in the lab. We visually confirmed stigma receptivity (papillose texture) before use. We applied pollen to recipient stigmas in a paired design (Cruzan 1990): one pollen treatment was applied to one stigma lobe, a second treatment to a second lobe, and the third lobe was excised as a place-marker. Because pollen tubes generally grow down stylar channels immediately below the lobe to which they were applied in *Erythronium* (Cruzan 1990), we could assign pollen tubes to pollen treatment, and thereby compare function in a common test arena. Four pairs (‘contests’) were constructed within each block: (1) both pollen morphs exposed to UV-B, (2) both morphs protected from UV-B, (3) exposed and protected YE pollen, and (4) exposed and protected RO pollen. We applied ~100 pollen grains per lobe using a needle tool (mean ± SD = 89 ± 17, $N$ = 17 counts of RO pollen). After ~20h (range: 17–24h) at room temperature, the pistils of recipient flowers were transferred to 70% Et-OH. Each contest within each block was repeated on a YE and a RO recipient; when preliminary analysis revealed that contest outcomes did not differ by recipient morph (data not shown), we restricted dissections (see below) to one randomly selected recipient per contest.

The preserved pistils were dissected to observe pollen tubes. The styles were slit longitudinally from the excised lobe to the top of the ovary, and then butterflied using insect pins and stained with one drop each of acetocarmine and 0.1% aniline blue (Cruzan 1989; Appendix S1: Fig. S5). Under a dissecting scope, we qualitatively scored contest outcomes, assigning ‘lose’ to any pollen treatment yielding at least 25% fewer pollen tubes than the other, and ‘win’ to the opposing treatment. If treatments differed by less than 25%, we scored the outcome as a ‘tie.’ We developed a simulation in R to verify that the 25% threshold was sufficient to detect a true difference in tube growth, allowing sampling error in the number of grains applied (Appendix S2). The distribution of win-lose-tie outcomes was analyzed independently for each contest type using a Bradley-Terry test (Agresti 2002: 436), implemented using R package BradleyTerry2 (Turner & Firth 2012), to determine whether one pollen treatment won more often than the other.

### *(d) Fertilization and fruit maturation*

We hand-pollinated flowers at four sites (Table 1) to test the capacity of each morph to effect fruit maturation. At each site, we located 11 – 40 pairs of unopened *E. americanum* flower buds (Appendix S1: Table S2). Pairs comprised two flowers of the same pollen morph within 1 m of each other and roughly matched for size and developmental stage. Inter-pair distance was ≥ 15 m. We gently pried apart the tepals to remove anthers, and enclosed the entire plant (two leaves plus flower) in a fine mesh bag to prevent pollinator access. Following at least two sunny, warm days, we returned to pollinate the bagged flowers (Appendix S1: Table S2). We examined stigma texture prior to pollination, and pollinated only if papillose. Freshly dehiscing YE and RO anthers were collected from the surrounding population (≥10 m from recipient flowers, and from one another), and recipients were pollinated by brushing anthers against stigmas. Each flower received pollen from a single unique donor; one member of each pair received YE pollen, and the other RO. In total, 49 YE recipients received YE pollen; 50 YE received RO pollen; 50 RO received YE pollen; and 53 RO received RO pollen. We resealed the mesh bags following pollination, and returned ≥ 4 weeks later to collect fruit (Appendix S1: Table S2). Fruit were dissected to count filled seeds and ovules (all sites except KSR).

The effects of donor and recipient pollen color morphs on fruit set probability and on seed set (seeds / (seeds + ovules)) were tested in binomial generalized linear models (Gelman and Hill 2007: 116) including site as a fixed effect using function glm( ) in R. We compared nested models by AIC (Gelman and Hill 2007: 525).

## Results

### 1. Geographic distribution of anther color morphs

More than 180 citizen scientists submitted color morph occurrence data for 266 populations, 251 of which were retained for analysis (see Methods). Forty-two populations (17%) were monomorphic YE, 100 (40%) were monomorphic RO, and the color morphs co-occurred in the remaining 109 (43%). When >100 flowers were observed, the probability of polymorphism increased to 62%, compared to 37% when ≤100 flowers were observed (logistic generalized linear model: $b_{>100\ \mathrm{fls}} = 1.02 \pm 0.30$, $z = 3.35$, $P < 0.01$). Considering monomorphic and polymorphic populations together, neither the occurrence of RO nor occurrence of YE varied with latitude, longitude, or elevation, but the probability of reporting the YE morph increased when > 100 flowers were observed (logistic generalized linear model: $b_{>100\ \mathrm{fls}} = 0.71 \pm 0.32$, $z = 2.20$, $P = 0.03$) (Fig. 2, Table 2).

## 2. Ecological significance of pollen color morphs

### *(a) Pollinator attraction*

We observed 378 visitors to the *E. americanum* arrays (Appendix S1: Table S1). Nectar foragers (216 visitors; 57%) were slightly more common than pollen foragers (162; 43%). Most visitors (273; 72%) foraged on just one patch within the array. An intercept-only model detected no universal preference for one pollen morph over the other (frequency of visit to RO (95% CI) = 0.516 (0.476 – 0.557)). However, across all sites, bombyliid flies under-visited RO (Fig. 3a, Appendix S1: Table S3), and across all pollinator classes, pollinators at two of five sites over-visited RO (Appendix S1: Table S4). Study site explained more variance in proportion of visits to RO (ANOVA, deviance = 12.64, df = 4, $P = 0.01$) than did pollinator class (ANOVA, deviance = 7.70, df = 4, $P = 0.10$).

Within the best-represented sites and pollinator classes, the RO morph was under-visited by some pollinator classes at some sites, and over-visited by other classes at other sites (Fig. 4, Appendix S1: Table S5). The model including additive effects of both site and pollinator class provided a better fit than did models excluding site (ANOVA, deviance = 19.18, df = 3, $P < 0.01$) or pollinator class (ANOVA, deviance = 8.15, df = 2, $P = 0.02$), and a better fit than a model including an interaction between pollinator class and site (ANOVA, deviance = 8.68, df = 6, $P = 0.19$).

Eighty-eight visitors made at least one movement between nearest-neighbor patches (i.e., excluding $N = 44$ diagonal movements across the array); 153 such movements were observed in total ($N = 85$ moves between "same" morph patches, and $N = 68$ moves between patches of different morphs). There was no universal tendency towards assortative movements by pollen morph in a model fit with intercept only (probability of assortative movement (95% CI) = 0.556

(0.476 – 0.633)), and neither site (ANOVA, deviance = 1.38, df = 3, $P = 0.71$) nor pollinator class (ANOVA, deviance = 3.86, df = 4, $P = 0.43$) affected the probability of assortative movements.

### *(b) Deterrence of pollen-consuming beetles*

Forty-four of 68 *A. ruficollis* beetles (65%) visited a flower during the choice trials. Of these 44, 19 visited the RO flower (43%). The probability of choosing RO did not vary by cage ($\Delta$AIC = 2, df = 1) or by site (ANOVA, deviance = 2.5, df = 2, $P = 0.28$), and the distribution of beetles between pollen morphs was indistinguishable from random choice (probability of RO (95%CI) = 0.43 (0.28 – 0.59)). A *post hoc* power analysis indicated that at least 311 beetles would have to be tested to conclude that the observed distribution of color choices differed significantly from 50% (Appendix S3). The 22 beetles observed directly on anthers were evenly split between the pollen morphs (Fig. 3b).

### *(c) UV-B tolerance*

A ‘tie’ was the most frequent outcome across all pollen tube contests. When pollen morphs were exposed to UV-B radiation and competed against each other within pistils, the ratio of RO ‘wins’ to YE ‘wins’ did not differ from 1 (Bradley-Terry coefficient = 0.22, $N = 18$, $P = 0.51$; Fig. 3c). Other contest types also yielded ratios indistinguishable from 1 (Appendix S1: Fig. S6). Thus, no one pollen treatment performed better than another.

### *(d) Fertilization and fruit maturation*

Ninety-three of 202 hand-pollinated flowers set fruit. Fruit set probability did not vary by site, or by recipient morph, donor morph, or their interaction (Fig. 3d, Appendix S1: Table S6). In contrast, donor pollen-color morph, recipient morph, and site interacted to affect seed set (Appendix S1: Table S7), but variation among cross-types within sites was small, and there was no indication that RO pollen performed better (or worse) than YE pollen (Appendix S1: Fig. S7). Thus, the two pollen color morphs are seemingly equally able to effect fruit maturation.

## Discussion

Our multi-faceted investigation has ruled out some hypothesized ecological effects of pollen color in *E. americanum*. First, the citizen science data revealed an absence of broad-scale geographic patterns in morph distribution, suggesting that morph occurrence is not readily attributable to broad-scale ecological clines in, for example, temperature or photoperiod (Fig. 2, Table 2). Second, pollen-feeding beetles exhibited no morph preference (Fig. 3b), and pollen germination and tube growth of both morphs was unaffected by UV-B exposure (Fig. 3c), ruling out two hypothesized agents of selection on pollen color. Third, neither fruit set nor seed set varied with the color of donor pollen (Fig. 3d), indicating that pollen color does not affect sexual function, despite prior reports that YE pollen is deformed (“déformé”, Lamoureux 2002: 152). Fourth, we found no evidence of pollinator-mediated assortative mating by pollen color. Eliminating these possibilities helps to direct future work.

In contrast, data here do lend support to the hypothesis that pollen color affects pollinator attraction to a flower. Specifically, we detected among-site variation in pollinator visitation to RO patches (Appendix S1: Table S3), and site-dependent pollen morph preferences by some pollinator classes (Fig. 4, Appendix S1: Table S5). The cause of site-specific preferences is unclear. There was no obvious association between local morph frequency and pollinator morph preference, but our power to detect such an association was restricted by small sample size (*N* = 5 sites) and a limited range of morph frequencies (Table 1). Site-specific preferences by some classes could also be explained by among-site variation in the surrounding plant community (Geber and Moeller 2006), but this explanation awaits further testing in *E. americanum*.

Regardless of underlying cause, and depending on the relative effectiveness of the pollinator classes (Ivey et al. 2003, Sahli and Conner 2007, Parker et al. 2016), the observed site- and class-specific pollinator preferences could lead to selection on pollen color. If pollinator preferences do lead to selection, among-site variation in pollinator preferences could maintain local pollen color variation via migration–selection balance. Although the ant-dispersed seeds of *E. americanum* are unlikely to travel more than a few meters (c.f. Pudlo et al. 1980, Cain et al. 1998, Kalisz et al. 1999), pollen grains may disperse further (Thomson and Thomson 1989), and occasional long-distance seed dispersal (e.g., via adherence with mud to vertebrate feet) is inferred from the post-glaciation migration rates of woodland herbs (Cain et al. 1998). The migration–selection balance scenario requires fixed among-site variation in pollinator-mediated selection, but, alternatively, pollinator preferences could vary over seasons within a site, or among microsites within a season. If preferences do vary over time or among microsites, then pollinators could mediate the maintenance of variation in pollen color via fluctuating selection. Indeed, overlapping generations, as occur in *E. americanum*, relax the conditions under which weak fluctuations in selection can maintain polymorphism (Ellner & Hairston, Jr. 1994; Turelli et al. 2001). Either scenario suggests that pollinators may be key to understanding pollen color variation in this species.

Effects of pollen color on pollinator behavior have been reported in other systems in which among-individual variation in pollen color has been investigated. In *Campanulastrum* *americanum*, halictid — but not *Bombus* — visitors discriminated against the female-phase flowers of one of two pollen-color morphs (Lau and Galloway 2004), and in *Nigella degenii*, pollinator preference for pollen-color morphs varied among observation dates (Jorgensen et al. 2006). The occurrence of variable pollinator preferences in all three systems in which among-plant pollen color polymorphism has been investigated suggests that these may be particularly important agents in pollen color evolution. In *N. degenii*, pollen color also affected pollination success, with the purple-pollen morph siring more seeds per single-donor cross than the yellow morph (Jorgensen et al. 2006). Our experiments with *E. americanum* (Fig. 3d, Appendix S1: Fig. S7) demonstrate that effects of pollen color on fruit- and seed-set are not universal.

Other fitness effects of pollen color in *E. americanum* may have escaped detection by the experiments presented here. The pollinator and pollen-feeding beetle preference experiments, for example, were performed at sites where morphs occur at near-equal frequencies, and therefore precluded detection of frequency-dependent effects. It is unlikely that pollinators would prefer a rare morph of a rewarding species like *E. americanum* (Smithson and MacNair 1997). Pollinator over-visitation to a common morph, however, could increase the selfing rate of the rare morph. Provided self-incompatibility, inbreeding depression and pollen discounting are weak, the rare morph may then benefit from the automatic transmission advantage of selfing (Epperson and Clegg 1987, Rausher et al. 1993). It is also conceivable that pollen-feeding beetles would over-visit a common morph, providing an advantage to the rare morph. Such frequency-dependent selection may be detectable only when morph frequencies are perturbed outside of a neutral range (Oxford 2005).

In addition, our experiments were not designed to detect indirect selection on pollen color, as may arise through correlation between pollen characters and other floral traits (Sarkissian and

Harder 2001), or through correlation with anthocyanin expression in other tissues (Armbruster 2002). Berardi et al. (2016) investigated flower and leaf anthocyanin profiles of Solanaceae varying in flower color and found no cross-tissue correlation, but the red mottled leaves and flecked tepals of *E. americanum* suggest the possibility nonetheless merits investigation in this species.

Our understanding of the processes that maintain variation in floral organs rests largely on the study of short-lived, semelparous species (e.g., *Linanthus parryae, Ipomoea purpurea, Antirrhinum majus, Raphanus raphanistrum*). Extending the range of life histories examined may provide new insight. In long-lived, extensively clonal plants like *E. americanum*, acquired somatic mutations could contribute to polymorphism. First, somatic mutations affecting flowers can spread through clonal reproduction before they are even expressed, potentially enabling a higher frequency of flowers (ramets) expressing a detrimental morph than would otherwise be expected. Second, trade-offs between investment in fruit production *versus* clonal growth (e.g., Van Drunen and Dorken 2012) could further accelerate the clonal spread of a morph with reduced sexual success (i.e., reduced investment in fruit), potentially leading to discordance between ramet morph frequencies and genet morph frequencies (Yakimowski and Barrett 2014). Third, peculiarities of somatic mutations could help maintain the wildtype. Depending on the shoot meristem layer in which they arise, somatic mutations can be excluded from gametes such that sexual reproduction restores the wildtype (Klekowsi 1988, 2003). In *Ranunculus repens*, for example, somatic mutations affecting petal number are transmitted during vegetative reproduction, but not through seeds (Warren 2009), and fewer than 50% of induced somatic mutations affecting anther color in maize were transmitted through pollen grains (Dawe and Freeling 1990). Thus, the frequency of alternate morphs in a population of clonal plants (e.g., pollen morph frequencies in an *E. americanum* population) could depend on the frequency of somatic mutation affecting the trait of interest, the strength of trade-offs between clonal growth and sexual reproduction, and local relative frequencies of clonal and sexual recruitment. In *E. americanum*, identifying the locus (or loci) regulating the RO/YE polymorphism would aid in testing possible roles of somatic mutation.

Genus *Erythronium* is a compelling system for the study of pollen color evolution. At least two other *Erythronium* species are polymorphic for pollen color (*E. grandiflorum* and *E. umbilicatum*), and others are monomorphic with yellow (e.g., *E. albidum*), white (e.g., *E. citrinum*), or pink-purple (e.g., *E. dens-canis*) pollen. The genus is divided into three clades (Clennett et al. 2012), and pollen color variation occurs within each. Establishing lifetime fitness consequences is difficult in long-lived, iteroparous perennials like *Erythronium*, but as demonstrated here, effects of pollen color on particular components of performance can nonetheless be tested. Moreover, the anthocyanin pathway is well described and highly conserved (Wessinger and Rausher 2012), making a genomic approach to studying the gain and loss of pollen pigmentation tractable. Mayr (1966: 162) boldly stated, “Cases of neutral polymorphism do not exist.” In the years since, evolutionary biologists have come to assume the neutrality of certain genetic polymorphisms (RFLPs, AFLPs, microsatellites, etc.), but published cases of neutral phenotypic polymorphism remain scarce (see Twyford et al. 2018 for a recent example), and Mayr’s statement has largely held true for color polymorphism in the attractive organs of short-lived, semelparous plants. With pollen polymorphism occurring at nearly 50% of sites surveyed by citizen scientists, and both morphs frequently well represented (Table 1), *E. americanum* and its congeners promise rich opportunity to extend understanding of the processes maintaining color polymorphism to a greater range of floral organs and plant habits.

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**Table 1:**

Study site locations and sample sizes for field experiments testing the ecological significance of pollen color variation in *Erythronium americanum*.

| Site[1] | Lat. (°) | Long. (°) | freq. YE[2] | *N* 2a[3] | *N* 2b[4] | *N* 2c[5] | *N* 2d[6] |
|---|---|---|---|---|---|---|---|
| BAW | 40.1913 | –76.5639 | 0.20 | 120 | 0 | 0 | 0 |
| SWS | 42.4768 | –76.4506 | 0.30 | 170 | 8 | 0 | 0 |
| HYP | 42.5162 | –74.1500 | 0.20 | 340 | 20 | 0 | 0 |
| KSR | 44.0340 | –79.5356 | 0.40 | 0 | 0 | 18 | 26 |
| MCW | 45.3637 | –75.6739 | 0.35 | 360 | 0 | 0 | 0 |
| GAT | 45.5072 | –75.8133 | 0.40 | 525 | 40 | 0 | 80 |
| DOR | 45.8428 | –64.5231 | 0.5 | 0 | 0 | 0 | 24 |
| ODL | 45.9558 | –66.6675 | 0.5 | 0 | 0 | 0 | 72 |

Notes

1. KSR = Koffler Scientific Reserve (University of Toronto, King City, ON Canada: west-side trails); BAW = Bellaire Woods (Lancaster Co., PA USA); SWS = Sapsucker Woods Sanctuary (Cornell University, Ithaca, NY USA); HYP = Huyck Preserve & Biological Research Station (Rensselaerville, NY USA: Lake Trail West); MCW = McCarthy Woods (National Capital Region, Ottawa, ON Canada); GAT = Gatineau Park (National Capital Region, Gatineau, PQ Canada: Sugarbush Trail & Mulvihill Lake Trail); DOR = Dorchester Cape (private property, NB Canada); ODL = Odell Park (Fredericton, NB Canada).

2. Estimated frequency of flowers with YE (yellow) pollen morph; freq. RO = 1 – freq. YE. Based on impressions after >1 day working at a site; the clonal nature of *E. americanum* and

time constraints precluded quantitative sampling. All estimates by EJA.

3. Minutes of pollinator observation (experiment 2a)

4. Number of *Asclera ruficollis* choice trials (experiment 2b)

5. Blocks of UV-B exposure trials (experiment 2c)

6. Number of hand pollinations (experiment 2d)

**Table 2:**

Effects of site latitude, longitude, elevation, and number of flowers observed (≤ 100 or > 100) on the presence of each of two anther color morphs in populations of *Erythronium americanum.* Site locations centered such that intercept represents latitude and longitude of the southern-most and western-most populations, respectively. Coefficients (SE) estimated by binomial generalized linear models; bold font denotes coefficient is statistically different from zero (*P* < 0.05); * indicates *P* < 0.10. *N* = 251 populations. Data reported by citizen scientists.

| Model term | YE anthers[1] | RO anthers[1] |
|---|---|---|
| Intercept | 0.487 (0.900) | **2.122 (1.055)** |
| Latitude (°N) | –0.012 (0.053) | –0.061 (0.074) |
| Longitude (°E) | 0.021 (0.029) | –0.039 (0.037) |
| Elevation (m, log-transformed) | –0.075 (0.139) | 0.035 (0.157) |
| Number of flowers (> 100) | **0.711 (0.323)** | 0.793 (0.473)* |

Notes

[1] YE anthers lack anthocyanins in anther walls and are pure yellow in color; walls of RO anthers are pigmented by anthocyanins and are red-to-orange in color. Populations can be monomorphic or polymorphic for anther color.

## Figure Captions

**Figure 1:** Yellow-anthered and red-anthered *Erythronium americanum.* Pollen color (visible on dehiscing anther surfaces) corresponds to anther wall color. Photo credit (L): Emily Austen; (R): Shang-Yao Peter Lin.

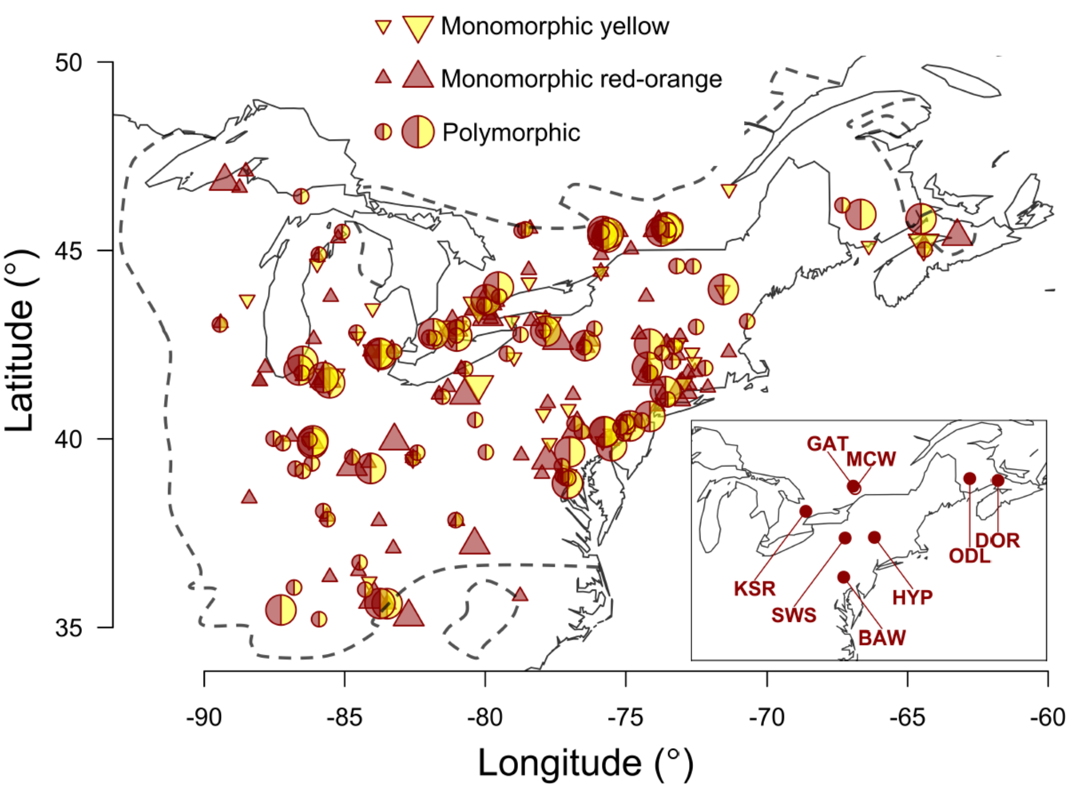
**Figure 2:** Occurrence of anther-color morphs (presence/absence) within *Erythronium americanum* populations, as reported by citizen scientists. Observation sites are plotted against the species distribution (dashed line; after Allen & Robertson 2003). Small symbols: ≤ 100 flowers observed; large symbols: > 100 flowers observed. Inset: sites at which experiments were conducted (Table 1).

**Figure 3:** Pollen color (YE: yellow, RO: red-orange) and performance in *Erythronium americanum*. **(a)** With the exception of nectar-foraging bombyliid flies, which tended to visit YE more often than RO (error bars exclude the horizontal line at Proportion of visits to RO = 0.5), visitors to mixed-morph arrays of *E. americanum* did not exhibit a universal preference for one morph over the other across study sites. Proportions (±95% CI) are least-squares means estimated by a binomial GLM; *N* = 378 floral visitors over >24h of observation at five study sites; values below bars are observations per pollinator class. “Solitary” on the abscissa = solitary bee; for pollen foragers, this includes females of large bees (e.g., *Andrena carlinii*, *A. erythronii*, *Osmia* sp.); for nectar foragers, this category includes males of large bees normally observed foraging for nectar (e.g., *Andrena carlinii*, *A. erythronii*, *Osmia* sp.), male and female small bees (e.g., *Ceratina*), and male and female cleptoparasites (e.g., *Nomada* sp.). “Fly” = bombyliid. **(b)** *Asclera ruficollis* beetles did not express a pollen morph preference during choice trials; this

result holds whether beetles observed on anthers are analyzed together with or separately from beetles on tepals. *N* = 44 beetles observed on a flower. **(c)** Following 3h UV-B exposure, pollen-color morphs did not differ in their capacity to grow pollen tubes in paired contests within *E. americanum* pistils. A pollen type was the contest "loser" if it yielded at least 25% fewer pollen tubes than the alternate pollen type within the same pistil; the alternate pollen type was the "winner." If treatments differed by less than 25%, the outcome was judged a tie. *N* = 18 contests. **(d)** Ninety-three of 202 hand-pollinated flowers set fruit. Neither donor nor recipient morph affected the probability of fruit set.

**Figure 4:** Proportion of visits to patches of *Erythronium americanum* flowers with red-orange (RO) pollen in mixed arrays of flowers with RO and yellow pollen by visitor class and by study site. Proportions are least-squares means (± 95% CI) estimated by binomial generalized linear model; bars that exclude the dashed horizontal line at proportion = 0.5 are statistically different from 50% visitation to RO. Solitary bee (pollen) = pollen-foraging solitary bees, i.e., females of large bees (e.g., *Andrena carlinii, A. erythronii, Osmia* sp.). Solitary bee (nectar) = nectar-foraging solitary bees, i.e., males of large bees normally observed foraging for nectar (e.g., *Andrena carlinii, A. erythronii, Osmia* sp.), male and female small bees (e.g., *Ceratina*), and male and female cleptoparasites (e.g., *Nomada* sp.). H = site HYP, S = site SWS, M = MCW, G = site GAT; see Table 1. * = 95% CI excludes 0.5.

Fig. 1



▽ Monomorphic yellow
△ Monomorphic red-orange
◐ Polymorphic
50
45
40
35
Latitude (°)
-90
-85
-80
-75
-70
-65
-60
Longitude (°)
GAT
MCW
KSR
SWS
HYP
ODL
DOR
BAW

Fig. 2

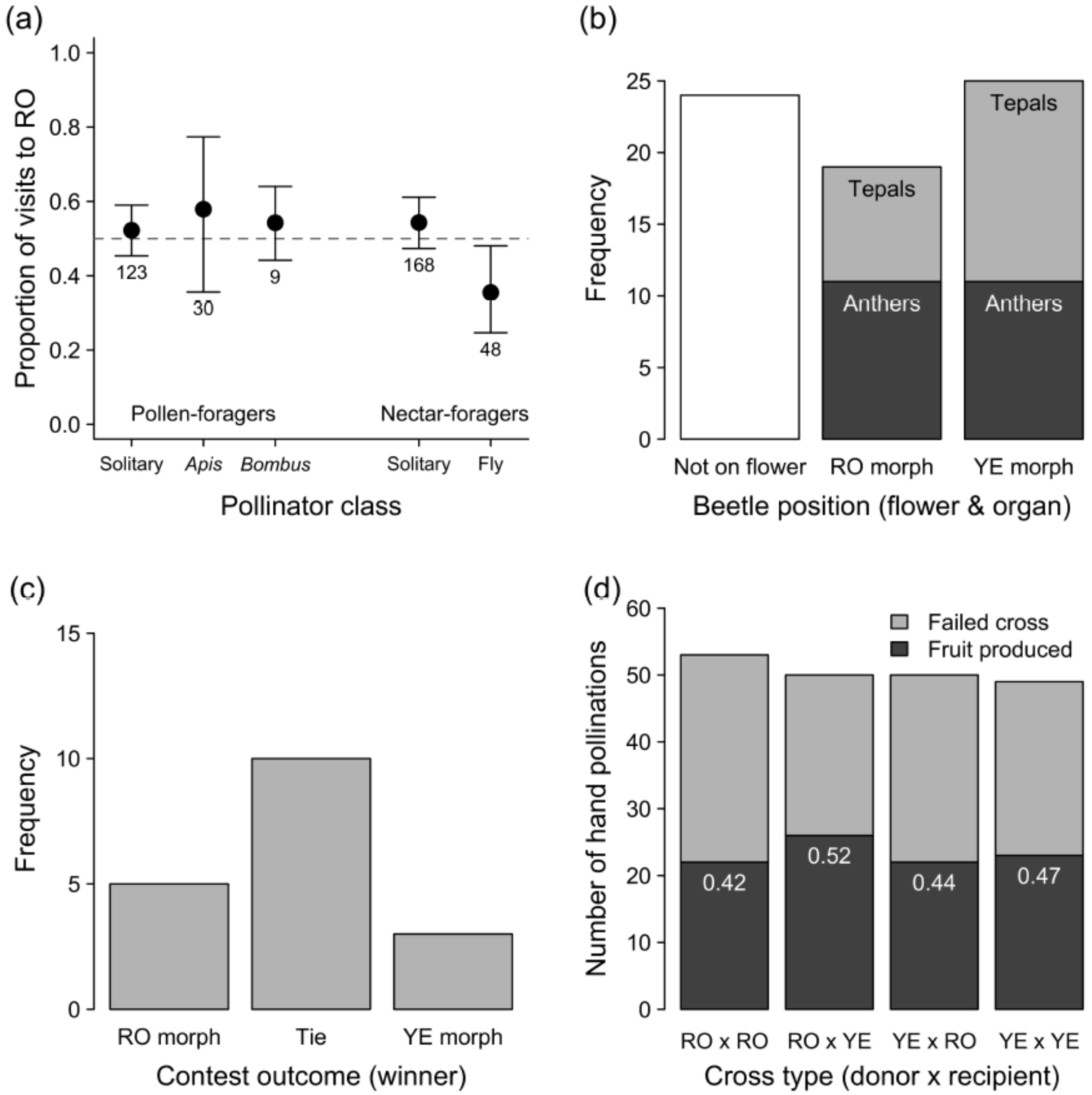

(a)
1.0
0.8
0.6
0.4
0.2
0.0
Proportion of visits to RO
123
30
9
168
48
Pollen-foragers
Nectar-foragers
Solitary
Apis
Bombus
Solitary
Fly
Pollinator class
(b)
25
20
15
10
5
0
Frequency
Tepals
Anthers
Tepals
Anthers
Not on flower
RO morph
YE morph
Beetle position (flower & organ)
(c)
15
10
5
0
Frequency
RO morph
Tie
YE morph
Contest outcome (winner)
(d)
60
50
40
30
20
10
0
Number of hand pollinations
Failed cross
Fruit produced
0.42
0.52
0.44
0.47
RO x RO
RO x YE
YE x RO
YE x YE
Cross type (donor x recipient)

Fig. 3

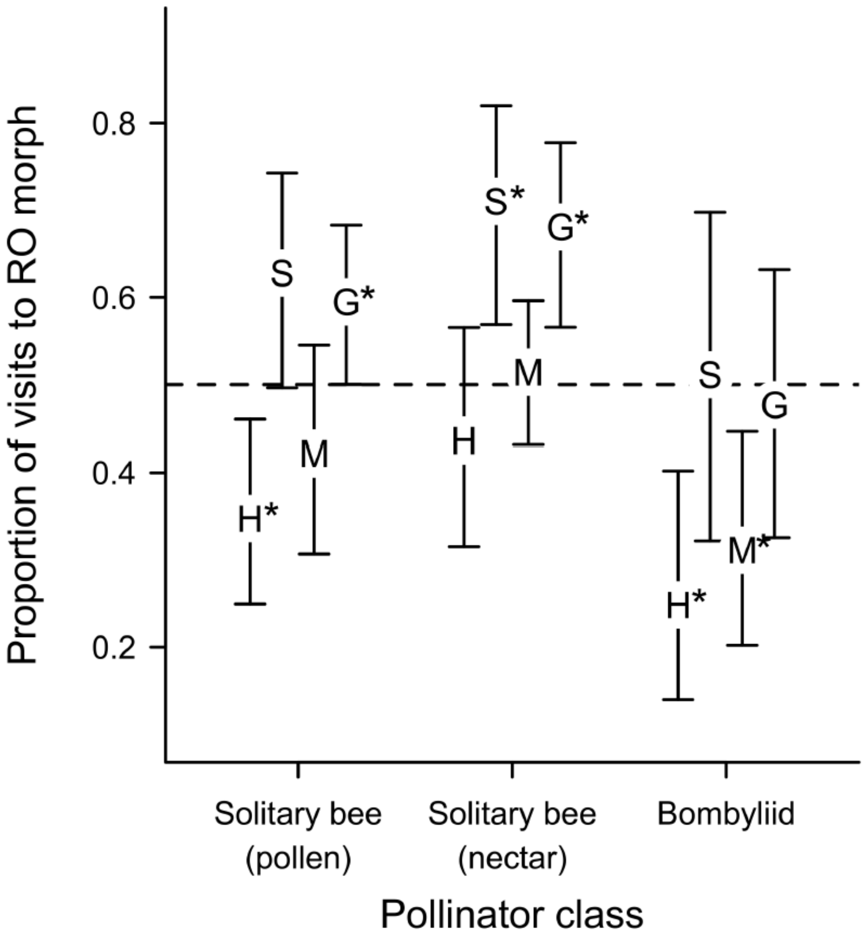

Proportion of visits to RO morph
0.8
0.6
0.4
0.2
S
G*
H*
M
S*
G*
H
M
S
G
H*
M*
Solitary bee (pollen)
Solitary bee (nectar)
Bombyliid
Pollinator class

Fig. 4

# Appendix S1: Supplementary Figures & Tables

Austen, Lin & Forrest, Pollen color variation in *Erythronium americanum*

## Appendix S1: Figure S1

**Fig. S1:** Anther color variation in *Erythronium americanum* can be described by a two level model. At the first level, a discrete break occurs between red-orange (RO) anthers expressing any amount of red pigment (an anthocyanin, M. Rausher *pers. comm.*), and yellow (YE) anthers expressing none. The second level describes quantitative variation within the RO morph, yielding a colour range from deep brick red to pale orange (L to R).  Pollen color typically corresponds to the anther wall color, though RO anthers sometimes contain YE pollen, particularly before anther dehiscence. Pictured are 34 anthers collected one per flower from a natural population of *E. americanum* in southern Ontario, Canada.

# Appendix S1: Figure S2

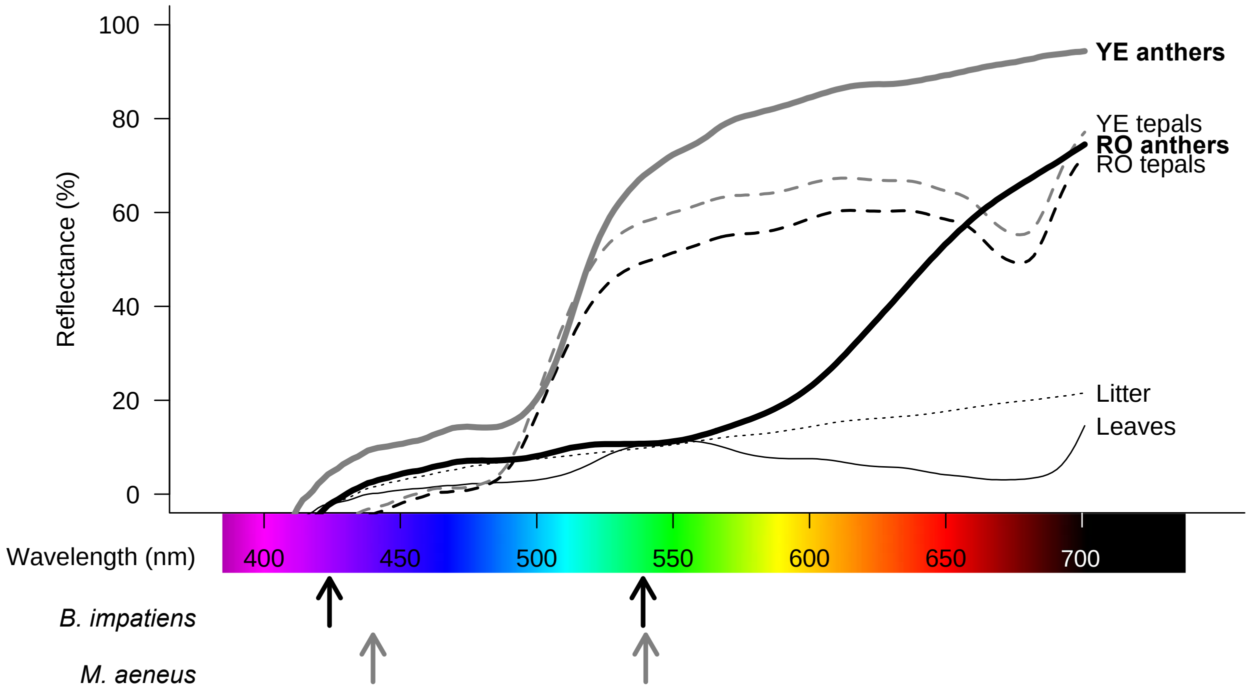


**Figure S2:** Reflectance spectra of yellow (YE) and red-orange (RO) *Erythronium americanum* anthers, as compared to the spectra of *E. americanum* tepals from flowers with YE and RO anthers, *E. americanum* leaves, and dry leaf litter collected from forest floor (primarily *Acer*, *Fagus*). All spectral measurements made using OceanOptics *Jaz* spectrometer, and processed using SpectraSuite software (OceanOptics 2011). Each spectrum depicts the average of five independent samples. Undehisced anthers were measured on each of five flowers per morph; tepals were measured at four positions per flower for these same ten flowers (tip and base of adaxial surface of one randomly selected inner and outer tepal per flower). Spectra were recorded at three randomly selected positions on each of five *E. americanum* leaves, and at three random positions within each of five leaf litter samples. All material was collected at site GAT (see Table 1) and measured in the lab. Colors along abscissa portray the human-visual spectrum (image modified from http://www.giangrandi.ch/optics/spectrum/visible-a.png; Accessed May 19 2017). Black and grey arrows along abscissa depict peak spectral sensitivities of the bumblebee *Bombus* *impatiens* (Skorupski & Chittka 2010) and the pollen-feeding beetle *Meligethes aeneus* (Nitidulidae) (Döring et al. 2012), respectively (*M. aeneus* arrow at 440nm is an approximate peak). (Comparable data unavailable for *Asclera ruficollis*). *Bombus impatiens* and *M. aeneus* both exhibit a peak at ~540nm, and both species also exhibit a third peak in the UV-range (not shown).

**Literature cited:**

Döring, T.F., M. Skellern, N. Watts, and S.M. Cook. 2012. Colour choice behaviour in the pollen beetle *Meligethes aeneus* (Coleoptera: Nitidulidae). Physiological Entomology 37:360–368.

Skorupski, P., and L. Chittka. 2010. Photoreceptor spectral sensitivity in the bumblebee, *Bombus impatiens* (Hymenoptera: Apidae). PLoS ONE 5:e12049

## Appendix S1: Figure S3

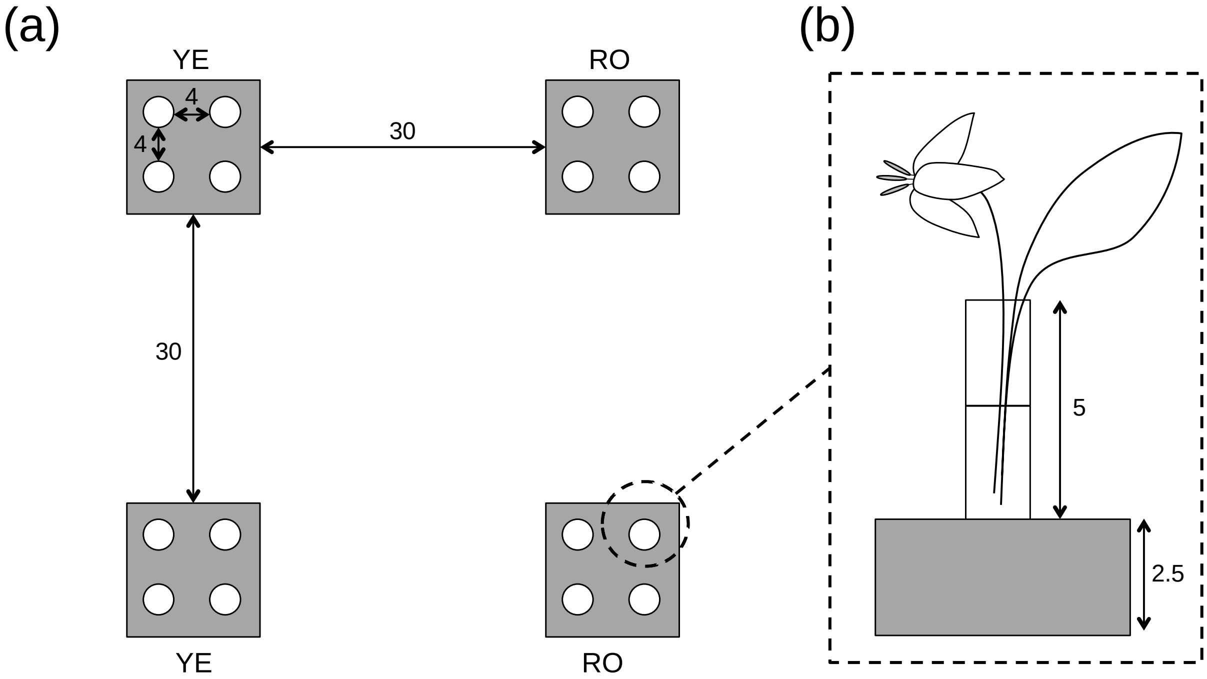


**Fig. S3:** Schematic of mixed array used to observe visitation to yellow (YE) and red-orange (RO) *Erythronium americanum* pollen morphs. All distances are cm. **(a)** Arrays consisted of four foam grids ('patches'), each holding four 15 mL vials. Between 2–4 vials per patch contained a single cut flower + leaf. All flowers within a patch were of the same pollen morph, and each patch was equidistant from another of the same morph, and one of the alternate morph. **(b)** Single cut *Erythronium americanum* flower and leaf within a vial.

## Appendix S1: Figure S4

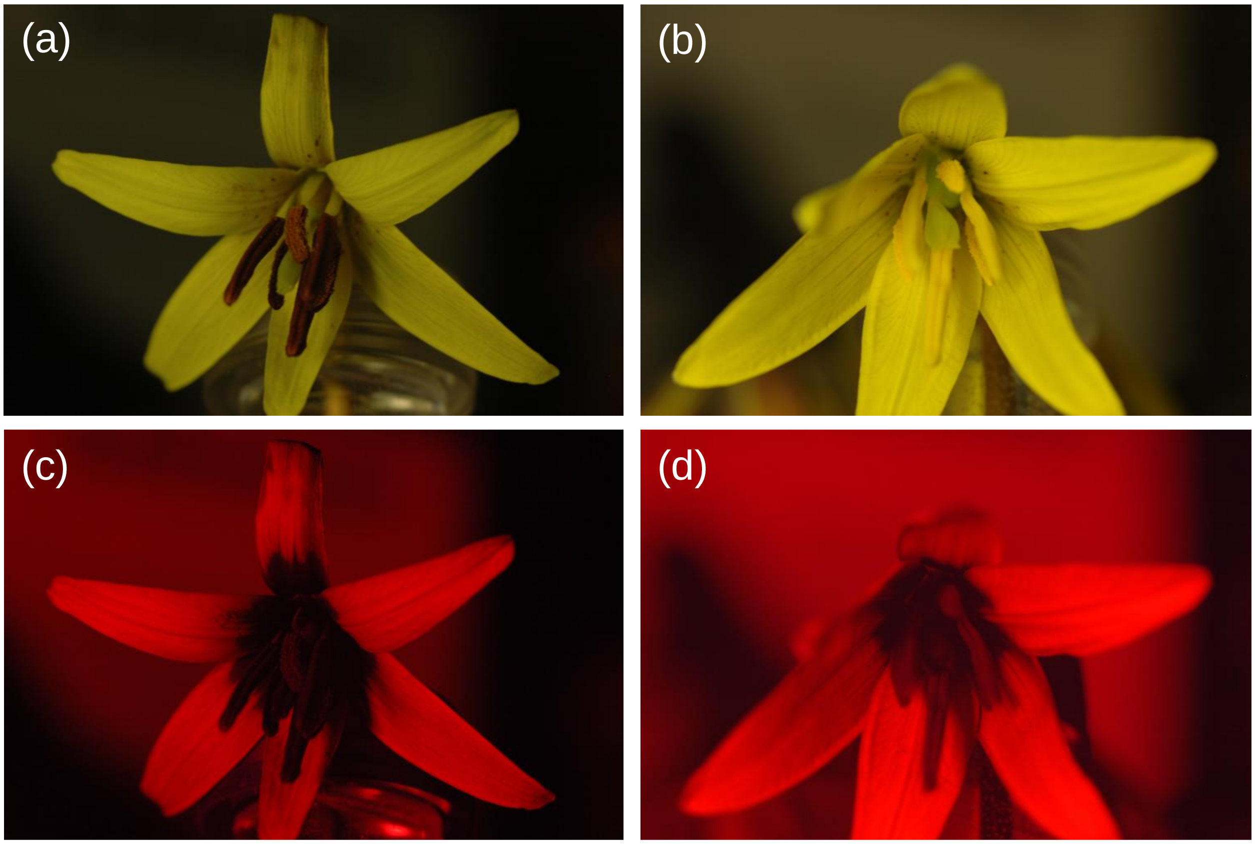


**Fig. S4:** Red-anthered (a, c) and yellow-anthered (b, d) *Erythronium americanum* flowers in the human-visible spectrum (a, b) and in the UV-B spectrum (c, d). Flowers were illuminated by a Repti-Glo 13W UVB 100 bulb. A Nikon D70s camera and a Nikon Nikkor 75mm f4 enlarger lens were used to capture all photographs. For images (c, d), the camera was equipped with a Baader U-filter (Baader Planetarium, Denmark), and set to a ~30s exposure. Both red and yellow anthers are UV-B absorbing; a UV-B absorbing "bulls eye" is also visible at the center of both flowers.

## Appendix S1: Figure S5

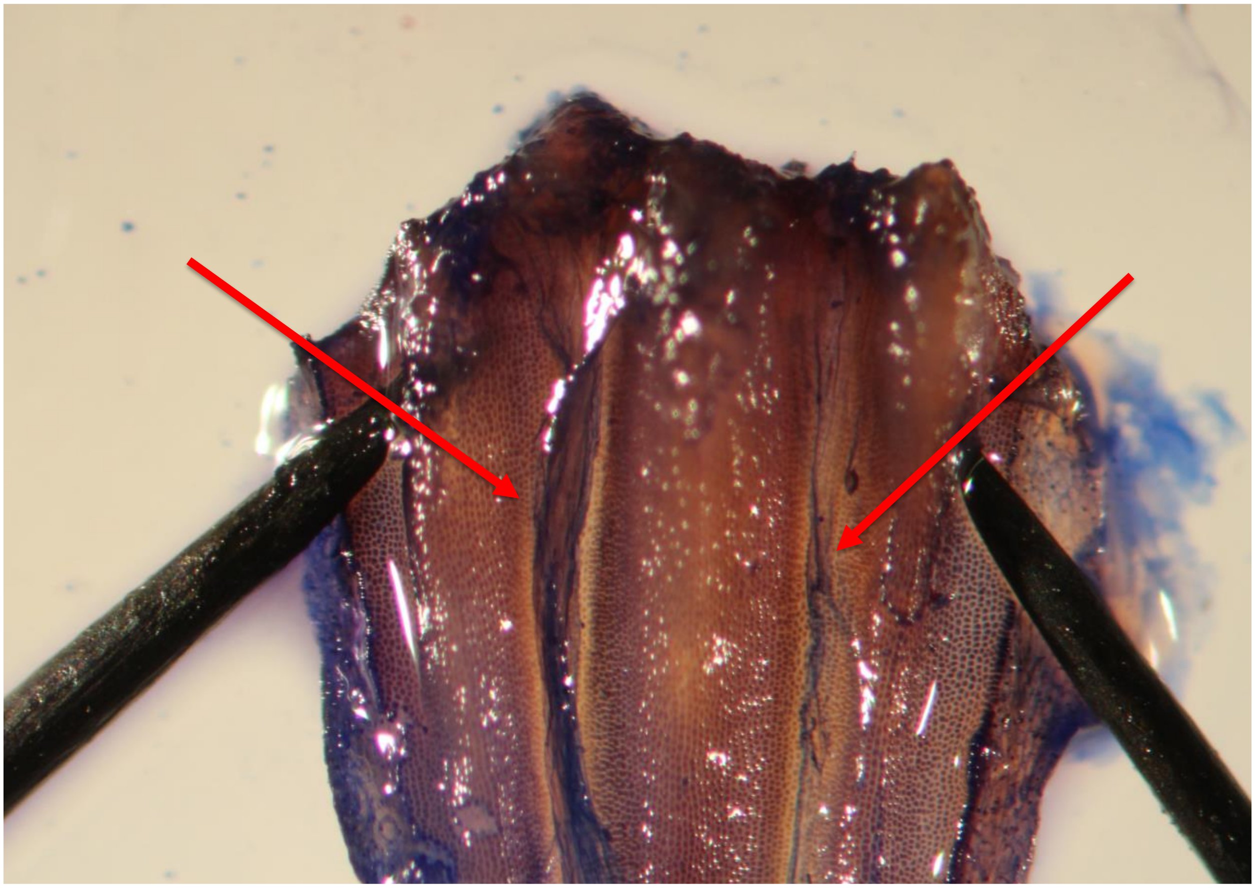


**Fig. S5:** Dissected *Erythronium americanum* pistil (stigma (top) + upper portion of style (bottom)) viewed under a dissecting scope. Pollen tubes growing down from the stigma lobes are stained with acetocarmine and 0.1% aniline blue, and are indicated by red arrows. In this pistil, the pollen type applied to the left lobe was judged the contest ‘winner’, with the pollen applied to the right lobe producing at least 25% fewer pollen tubes.

# Appendix S1: Figure S6

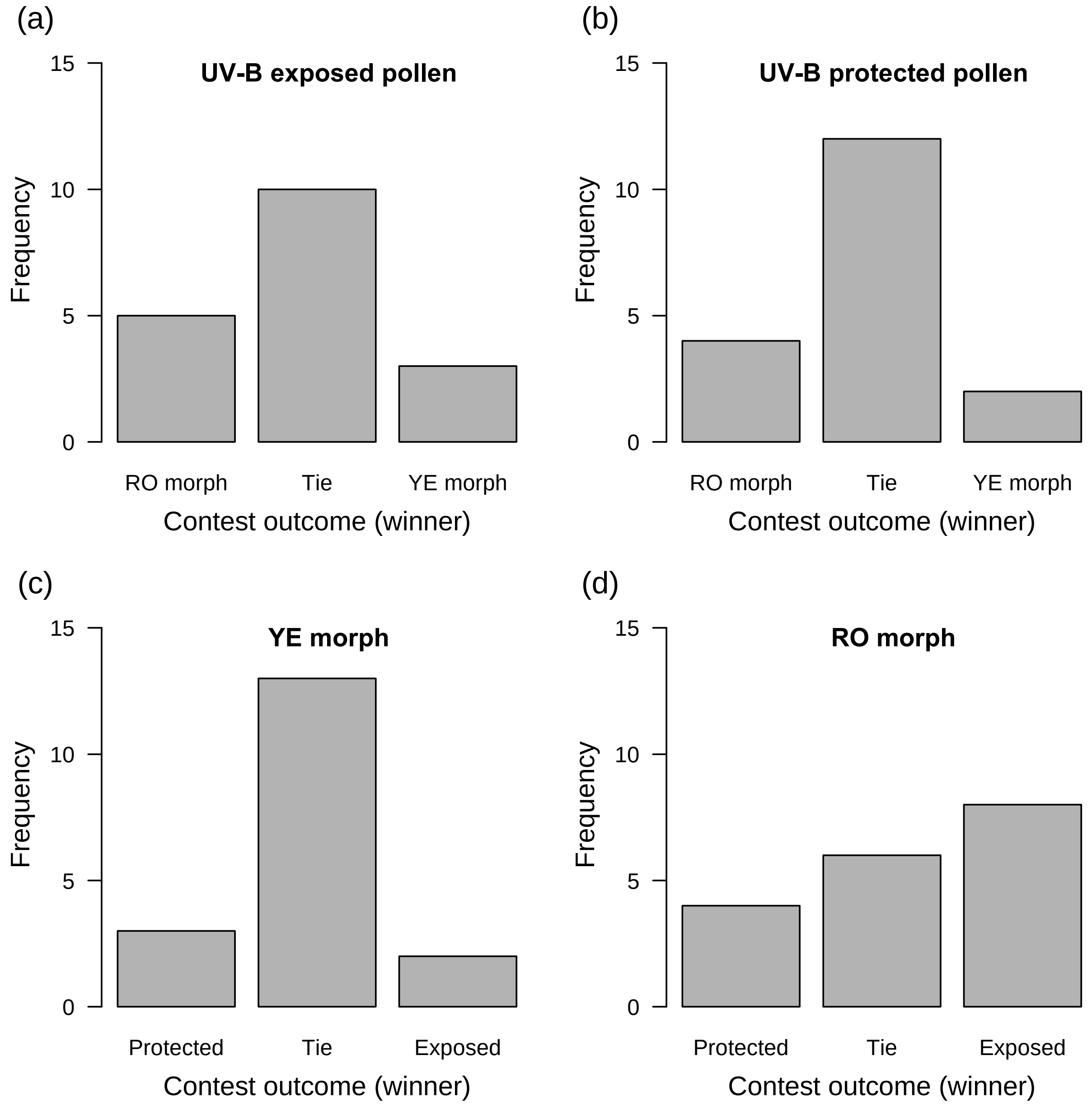


**Fig. S6:** Outcomes of paired pollen tube growth contests within *Erythronium americanum* pistils. Pollen of two types was applied to distinct stigma lobes of cut flowers, and pollen tube growth was compared between lobes ~20h later. A pollen type was the contest “loser” if it yielded at least 25% fewer pollen tubes than the alternate pollen type within the same pistil; the alternate pollen type was the “winner”. If treatments differed by less than 25%, the outcome was judged a tie. The ratio of wins never differed from 1 (Bradley-Terry (B-T) test), indicating that no pollen type performed better than the other in any contest type. Note that panel (a) is reprinted from Fig. 3c in the main manuscript to facilitate comparisons across contest types. **(a)** Red-orange pollen competed against yellow pollen, both morphs exposed to UV-B radiation. Bradley-Terry coefficient = 0.22, $N = 18$, $P = 0.51$. **(b)** Red-orange pollen competed against yellow pollen, both morphs were protected from UV-B radiation during lab exposure. B-T coefficient = 0.22, $N = 18$, $P = 0.51$. **(c)** Yellow pollen protected from UV-B radiation competed against yellow pollen exposed to UV-B radiation. B-T coefficient = 0.11, $N = 18$, $P = 0.74$. **(d)** Red-orange pollen protected from UV-B radiation competed against red-orange exposed to UV-B radiation. B-T coefficient = –0.45, $N = 18$, $P = 0.19$.

## Appendix S1: Figure S7

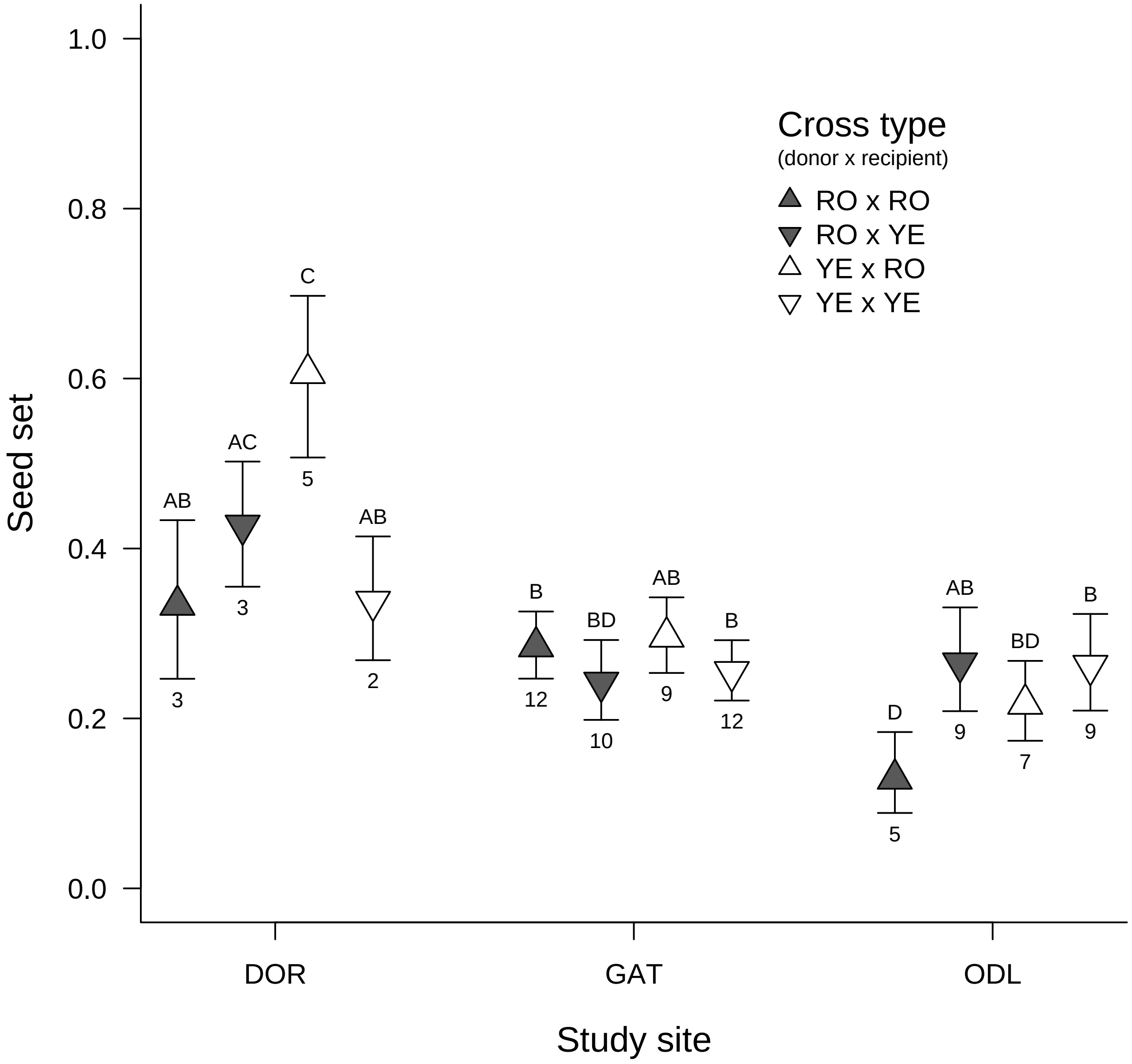


**Fig. S7:** Proportion of ovules setting seed (least squares mean ± 95%CI, estimated by binomial generalized linear model, see Table S7) following single-donor hand pollinations of *Erythronium americanum* at three study sites. Pollen donors and pollen recipients are classified by pollen-color morph (RO = red-orange; YE = yellow). Points sharing a letter are not statistically different from one another in pairwise contrast. Values below points are sample size (i.e., number of fruit produced from a total of 6 replicate pollinations per cross type at DOR, 20 replicates at GAT, and 18 replicates at ODL).

# Appendix S1: Table S1

**Table S1:** Number of visitors to mixed arrays of *Erythronium americanum* flowers with yellow (YE) and red-orange (RO) pollen by class and study site.

| | **Pollen foragers** | | | **Nectar foragers** | | |
|---|---|---|---|---|---|---|
| **Site** | *Apis* | *Bombus* (Queen) | Solitary bee[1] | Solitary bee[2] | Bombyliidae | **Total** |
| BAW | 0 | 0 | 1 | 1 | 3 | **5** |
| SWS | 0 | 1 | 32 | 14 | 1 | **48** |
| HYP | 30 | 4 | 27 | 28 | 6 | **95** |
| MCW | 0 | 1 | 18 | 98 | 25 | **142** |
| GAT | 0 | 3 | 45 | 27 | 13 | **88** |
| **Total** | **30** | **9** | **123** | **168** | **48** | **378** |

Notes

[1] Females of large bees, e.g., *Andrena carlinii, A. erythronii, Osmia* sp.

[2] Males of large bees, e.g., *Andrena carlinii, A. erythronii, Osmia* sp., male and female small bees, e.g., *Ceratina*, and male and female cleptoparasites, e.g., *Nomada* sp.

# Appendix S1: Table S2

**Table S2:** Number of replicates, and dates of emasculation and bagging, pollination, and fruit collection at each of four sites during single-donor pollination experiment to test fertilization efficacy of yellow and red-orange pollen-color morphs of *Erythronium americanum*.

| **Site**[1] | **Year** | ***N* pairs (flowers)**[2] | **Emasculation**[3] | **Pollination**[3] | **Fruit collection**[3] |
|---|---|---|---|---|---|
| KSR | 2015 | 11 (26) | May 4, 5<br>(124, 125) | May 7, 8<br>(127, 128) | June 19<br>(170)[4] |
| GAT | 2017 | 40 (80) | April 27, 28<br>(117, 118) | April 29, May 3, 4<br>(119, 123, 124) | June 9<br>(160) |
| ODL | 2017 | 36 (72) | May 10<br>(130) | May 13, 14<br>(133, 134) | June 15<br>(166) |
| DOR | 2017 | 12 (24) | May 8<br>(128) | May 14, 18<br>(134, 138) | June 14<br>(165) |

Notes

[1] See Table 1 in main manuscript for site details.

[2] Each pair comprised two recipient flowers (= individuals) of same pollen morph; one received yellow pollen and the other red-orange pollen. At KSR, 12 flowers were grouped into triplets instead of pairs. Within a triplet, one flower received each of yellow, red, and orange pollen. With the exception of KSR (five yellow pairs/triplets + 6 red-orange pairs/triplets), pairs were equally split between yellow and red-orange morphs.

[3] Values in parentheses are day-of-year.

[4] Developing fruit were observed May 28 2015 (day-of-year148). All fruit developing at this time were matured by June 19, and no flowers that had failed to initiate fruit by May 28 went on to subsequently mature fruit by June 19.

# Appendix S1: Table S3

**Table S3:** Effect of visitor class on proportion of visits to red-orange pollen (RO) patches of *Erythronium americanum* in mixed arrays of RO and yellow (YE) pollen morphs. Coefficients estimated by binomial generalized linear model fit without intercept; back-transformed least-squares means (LSM) are estimated from the fitted model. *N*= 378 visitors observed at five study sites. Bold text indicates coefficient is statistically different from zero (and, consequently, 95%CI of back-transformed LSM excludes 0.5).

| Visitor class | Coefficient (SE) | z | *P* | LSM (95% CI) |
|---:|:---:|:---:|:---:|:---:|
| Solitary bee (pollen)[1] | 0.09 (0.14) | 0.63 | 0.53 | 0.522 (0.453 – 0.590) |
| *Bombus* | 0.32 (0.46) | 0.69 | 0.49 | 0.579 (0.357 – 0.780) |
| *Apis* | 0.17 (0.21) | 0.82 | 0.41 | 0.542 (0.442 –0.641) |
| Solitary bee (nectar)[2] | 0.17 (0.14) | 1.21 | 0.23 | 0.543 (0.473 – 0.611) |
| **Bombyliid** | **–0.60 (0.27)** | **–2.25** | **0.02** | **0.354 (0.243 – 0.478)** |

Notes

[1] Solitary bee (pollen) = pollen-foraging solitary bees, i.e., females of large bees (e.g., *Andrena carlinii, A. erythronii, Osmia* sp.).

[2] Solitary bee (nectar) = nectar-foraging solitary bees, i.e., males of large bees (e.g., *Andrena carlinii, A. erythronii*, *Osmia* sp.), male and female small bees normally observed foraging for nectar (e.g., *Ceratina*), and male and female cleptoparasites (e.g., *Nomada* sp.).

# Appendix S1: Table S4

**Table S4:** Effect of study site on proportion of visits to red-orange pollen (RO) patches of *Erythronium americanum* in mixed arrays of RO and yellow (YE) pollen morphs. Coefficients estimated by binomial generalized linear model fit without intercept; back-transformed least-squares means (LSM) are estimated from the fitted model. *N*= 378 visitors observed at five study sites. Bold text indicates coefficient is statistically different from zero (and, consequently, 95%CI of back-transformed LSM excludes 0.5).

| **Site**[1] | **Coefficient (SE)** | **z** | ***P*** | **LSM (95% CI)** |
|---|---|---|---|---|
| HYP | –014 (0.15) | –0.94 | 0.35 | 0.466 (0.396 – 0.537) |
| **SWS** | **0.65 (0.26)** | **2.46** | **0.01** | **0.656 (0.536 – 0.765)** |
| MCW | –0.15 (0.15) | –0.98 | 0.33 | 0.463 (0.390 – 0.537) |
| BAW | –0.41 (0.91) | –0.44 | 0.66 | 0.400 (0.081 – 0.801) |
| **GAT** | **0.38 (0.17)** | **2.19** | **0.03** | **0.593 (0.510 – 0.672)** |

Notes

[1] Site codes correspond to sites in Table 1 of main text.

# Appendix S1: Table S5

**Table S5:** Effects of study site and visitor class on proportion of visits to red-orange pollen (RO) patches of *Erythronium americanum* in mixed arrays of RO and yellow (YE) pollen morphs. Coefficients estimated by additive binomial generalized linear model. *N*= 334 visitors observed at four study sites (classes *Apis* and *Bombus,* and site BAW, excluded). Bold text indicates coefficient is statistically different from zero.

| Term | Coefficient (SE) | z | *P* |
|---:|:---:|:---:|:---:|
| **Intercept (Solitary bee (pollen)[1] at HYP[3])** | **–0.63 (0.24)** | **–2.62** | **0.01** |
| Solitary bee (nectar)[2] | 0.37 (0.24) | 1.56 | 0.12 |
| Bombyliid | –0.48 (0.33) | –1.44 | 0.15 |
| **Site SWS[3]** | **1.15 (0.35)** | **3.33** | **<0.01** |
| Site MCW[3] | 0.31 (0.29) | 1.08 | 0.28 |
| **Site GAT[3]** | **1.01 (0.29)** | **3.54** | **<0.01** |

Notes

[1] Solitary bee (pollen) = pollen-foraging solitary bees, i.e., females of large bees (e.g., *Andrena carlinii, A. erythronii, Osmia* sp.).

[2] Solitary bee (nectar) = nectar-foraging solitary bees, i.e., males of large bees (e.g., *Andrena carlinii, A. erythronii, Osmia* sp.), male and female small bees normally observed foraging for nectar (e.g., *Ceratina*), and male and female cleptoparasites (e.g., *Nomada* sp.).

[3] Three-letter site codes correspond to sites in Table 1 of main text.

# Appendix S1: Table S6

**Table S6:** Comparison of models estimating effects of recipient pollen morph (yellow: YE; red-orange: RO), donor pollen morph, site, and the interaction of recipient and donor morphs on fruit set probability following hand pollination. N = 202 hand pollinations. Bold text = best-fit model.

| Model | ΔAIC | df | Residual Deviance |
|---:|:---:|:---:|:---:|
| **Null (intercept only)** | **0.0** | **1** | **278.76** |
| Recipient | 1.1 | 2 | 277.83 |
| Donor | 2.0 | 2 | 278.74 |
| Recipient + Donor | 3.0 | 3 | 277.80 |
| Recipient + Donor + Site | 2.0 | 6 | 270.81 |
| Recipient + Donor + Site + (Recipient × Donor) | 3.8 | 7 | 270.56 |

## Appendix S1: Table S7

**Table S7:** Effects of study site and donor and recipient pollen-color morphs (RO = red-orange; YE = yellow) on seed set following single-donor hand pollinations of *Erythronium americanum*. Coefficients are estimated by binomial generalized linear model. *N* = 86 fruit from 176 hand pollinations; seed set data unavailable for KSR. Bold text indicates coefficient is statistically different from zero. See Fig. S8 for least square means of each site × donor morph × recipient morph.

| **Term** | **Coefficient** | **z** | ***P*** |
|---|---|---|---|
| **Intercept (RO Donor, RO recipient, Site DOR)** | **–0.69 (0.22)** | **–3.20** | **0.001** |
| Donor (YE) | 0.40 (0.27) | 1.50 | 0.134 |
| **Recipient (YE)** | **1.12 (0.30)** | **3.76** | **< 0.001** |
| Site GAT | –0.23 (0.24) | –0.96 | 0.337 |
| **Site ODL** | **–1.22 (0.30)** | **–4.00** | **< 0.001** |
| **Donor (YE) × Recipient (YE)** | **–1.50 (0.38)** | **–4.00** | **< 0.001** |
| **Donor (YE) × Site GAT** | **–0.62 (0.31)** | **–1.98** | **0.048** |
| Donor (YE) × Site ODL | 0.49 (0.38) | 1.31 | 0.192 |
| **Recipient (YE) × Site GAT** | **–1.07 (0.33)** | **–3.21** | **0.001** |
| Recipient (YE) × Site ODL | –0.50 (0.40) | –1.26 | 0.207 |
| **Donor (YE) × Recipient (YE) × Site GAT** | **1.52 (0.43)** | **3.49** | **<0.001** |
| Donor (YE) × Recipient (YE) × Site ODL | 0.86 (0.51) | 1.70 | 0.088 |