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An altitudinal cline in UV floral pattern corresponds with a behavioral change of a generalist pollinator assemblage

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Abstract. Spatial variation in pollinator communities or behaviors can underlie floral diversification. Floral traits in the UV spectrum are common and mediate plant–pollinator interactions, but the role of pollinators in driving or maintaining their geographic variation has not been fully explored. We identify an altitudinal cline of increasing relative size of the UV bullseye pattern in *Argentina anserina* (Rosaceae) flowers, and assess whether pollination context contributes to clinal variation. At four sites that varied in altitude, we document the pollinator assemblage, and pollinator preference and visitation behavior. We then determine how pollinator visits affected pollen receipt and export. Finally, we describe how the functional relationship between UV floral phenotype and pollen receipt changed with altitude. Floral UV bullseye size increased with altitude, which corresponded with a change from a hymenopteran- to a dipteran-dominated pollinator assemblage. While dipteran and hymenopteran preferences for bullseye size were similar, flowers with large bullseyes received more foraging visits than those with small bullseyes at higher altitude. The reverse was observed at the lower altitudes; pollinators approached large-bullseye flowers often but rarely foraged. These differences are expected to affect fitness because foraging visits increased pollen export and receipt. Indeed, when natural variation in bullseye size was considered, it had a stronger effect on pollen receipt than other traits (flower size, display, or color). Plants with larger bullseyes tended to receive more pollen at the highest-altitude site, while those with smaller ones received more pollen at the lowest-altitude site. Results suggest that altitudinal changes in preference and behavior of the overall pollinator assemblage, but not differential preferences of pollinator taxonomic groups, could contribute to clinal variation in a UV floral trait for a generalist-pollinated plant.

Key words: Argentina anserina; *clinal variation; Colorado Rocky Mountains, USA; flower color; natural selection; pollinator behavior; UV floral pattern.*

Introduction

Pollinator-mediated selection is widely accepted as a mechanism underlying the diversification of floral form. Spatial covariation between floral phenotype and pollination context (e.g., community composition, preference, visitation behavior) is often considered to be reflective of pollinator-mediated selection. For example, a shift from one dominant pollinator to another across space (e.g., bumble bee to hummingbird) can contribute to phenotypic clines in floral traits (e.g., color; Streisfeld and Kohn 2007). However, plants that are effectively pollinated by diverse pollinator assemblages are common (Waser et al. 1996). While generalist-pollinated plants are predicted to experience lower degrees of directional selection on floral traits relative to those with specialist pollination (Herrera 1988, Johnson and Steiner 2000), complex spatial patterns of selection on floral phenotype have been documented in generalist-pollinated species (Gómez et al. 2009*a*). Whether phenotypic clines in floral traits of generalist-pollinated plants can be driven by shifts in the plant–pollinator interaction, however, is still little understood.

Many floral traits like size, color, and scent are under selection by pollinators (reviewed by Harder and Johnson 2009). While floral color patterns are common among angiosperms (Penny 1983), and can influence pollinator visitation preference (e.g., Koski and Ashman 2014, Peterson et al. 2015) and behavior (e.g., Hansen et al. 2012), whether pattern affects pollination success (e.g., pollen receipt) has received less attention. Notable exceptions include Medel et al. (2003), who showed that selection on petal spots depends on the visitation preferences of pollinators (insects vs. hummingbirds), and Hansen et al. (2012), who demonstrated petal spots on *Lapeirousia oreogena* influenced the likelihood of foraging by flies. Both of these studies, however, assessed color patterns that are apparent to humans, but many flowers emit color stimuli in the UV spectrum, which the majority of pollinating taxa can perceive (Briscoe and Chittka 2001). In particular, UV reflectance (e.g., UV chroma, the contribution of UV reflectance to total reflectance) and a bullseye pattern in the UV spectrum (petal tips reflect while bases

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absorb) are known to increase pollinator attraction (Johnson and Andersson 2002, Koski and Ashman 2014). Thus, to fully evaluate pollinator-mediated floral variation, studies need a multitrait approach that incorporates UV reflectance and pattern.

Changes in altitude can create conditions for clinal variation in plant–pollinator interactions in a number of ways (Galen 1989, Campbell et al. 1997, Totland 2001, Malo and Baonza 2002, Fabbro and Körner 2004, Brunet 2009). First, pollinator assemblages can change with altitude. In montane systems, dipteran pollinators commonly increase in dominance with increasing elevation while hymenopterans increase with decreasing elevation (reviewed in Hodkinson 2005). Thus, for generalist-pollinated plants with broad altitudinal ranges, the types of available pollinators can change markedly over a short distance (Arroyo et al. 1982). Second, pollinator preference can change along an altitudinal gradient, irrespective of community composition. For instance, Totland (2001) found that muscoid flies preferred larger *Ranunculus acris* flowers at lower altitudes, but not higher altitudes. Third, insect behavior once at a flower, not simply floral preferences, can depend on location. For instance, the influence of *Ipomopsis* flower color on the likelihood of hawkmoth probing once at the flower depends on population location (Campbell et al. 1997, Bischoff et al. 2015). Finally, pollinator perception of floral visual signals may change with altitude as a result of abiotic factors. In particular, solar irradiance increases with altitude (Körner 2007), and the light environment can influence the perception of color visual signals (Endler 1992, 1993). Arnold and Chittka (2012) showed that bees displayed greater color discrimination in well-lit environments relative to shaded ones. Thus, understanding which of these aspects of plant–pollinator interactions change with altitude can inform whether they contribute to an altitudinal gradient in floral traits.

In this study, we explore floral pattern variation along an altitudinal gradient using *Argentina anserina* (Rosaceae), a self-incompatible, generalist-pollinated herb. Flowers appear uniformly yellow in the human-visible spectrum, but display a distinct UV bullseye pattern. We sought to determine whether geographic variation in floral UV bullseye size could be functionally linked to changes in plant–pollinator interactions, and subsequently, phenotypic selection. Specifically, we ask: (1) Does the size of the bullseye display an altitudinal cline? (2) Does the dominant pollinator type visiting *A. anserina* change with altitude? (3) Does floral preference or visitation behavior of pollinators vary with size of the bullseye, and/or altitude? (4) Does increased flower visitation lead to increased pollen export and receipt? (5) Does the direction and/or strength of the relationship between bullseye size and pollen receipt change with altitude?

Methods

Study system

Argentina anserina (Rosaceae) is a perennial herb, widely distributed in temperate regions (Rousi 1965). In the Colorado Rocky Mountains (USA) it grows from ~2100 to ~3650 m above sea level (hereafter, MASL), and flowers from June to early August. Populations at low altitude flower ~1–2 weeks earlier than those at high altitude, but there is substantial flowering overlap (M. H. Koski, *personal observation*). Flowers are radially symmetrical, are borne singly on pedicels, and have numerous (30–40) uni-ovulate pistils. They live 1–3 d (M. H. Koski, *personal observation*), and are self-incompatible (Rousi 1965). Flowers in the Colorado Rocky Mountains appear largely pollen-rewarding; they produce little to no nectar (M. H. Koski, *personal observation*). Flowers are uniformly yellow, but have a bullseye pattern in the UV spectrum (Koski and Ashman 2013). The UV-reflective apex of petals is classified as appearing "UV green" to insects while the base appears green (Gumbert et al. 1999). The proportion of the petal that absorbs UV (UV proportion; UVP) is variable and heritable ($H^2 = 0.85$; Koski and Ashman 2013). Yet, petal brightness (total reflectance) and UV chroma (the proportion of reflectance in the UV spectrum) are not genetically correlated with UVP in *A. anserina* (Koski and Ashman 2013). Flowers are visited by generalist insects: small solitary bees, syrphid flies (Koski and Ashman 2014), tabanid, muscid, and bombyliid flies (M. H. Koski, *personal observation*), and bumble bees (Miyinashi et al. 1991). In the Colorado Rocky Mountains, flower-visiting Diptera increase in proportional abundance with altitude due to reduced abundances of other insect orders (Kearns 1992).

Floral traits and altitude

In summer 2012, we sampled floral traits in 12 populations of *A. anserina* in the Gunnison Basin, the West Elk Range, and the San Juan Range of Colorado, USA (Appendices A and B), ranging >1000 m in elevation (2334–3419 MASL). These populations were part of a larger, global study exploring abiotic factors that contribute to latitudinal trends in floral phenotype (Koski and Ashman 2015). Here, we explicitly examine the effects of altitude and biotic interactions in a subset of populations from western Colorado in the West Elk and San Juan Mountains. Populations grew on the sunny edges of ponds, lakes, or streams where soil was moist (mean [range] percentage full sun, 91% [86–100%]; water by weight, 26% [7–55%]). Neither abiotic factor varied with altitude (linear regressions; $b = -0.00007$, $R^2 = 0.12$, $P = 0.3$; $b = -0.00004$, $R^2 = 0.02$, $P = 0.7$, respectively; Appendix A).

In each population, we collected a single flower along linear transects in at least 2-m intervals. Sample size ranged from two to 15, depending on population size

(Appendix A). We photographed flowers in the UV spectrum and scored petal reflectance. Following past work (Koski and Ashman 2013), we digitally analyzed UV photographs in ImageJ (Rasband 1997–2014) to determine UVP and petal area (a proxy for flower size), and used a Jaz spectrometer (Ocean Optics, Dunedin, Florida, USA) to measure reflectance at the petal apex. We binned raw reflectance spectra into 1-nm intervals and calculated total brightness as reflectance between 300 and 700 nm, and UV chroma as $R_{300-400}/R_{300-700}$ using the program CLR (Montgomerie 2008). To determine whether floral traits varied with altitude, we computed population averages and regressed these on altitude. Statistical analyses were performed in R (R Core Development Team; v. 3.0.1) unless otherwise noted. For traits with significant altitudinal effects, we verified that the relationship was not due solely to spatial autocorrelation. We tested for spatial autocorrelation using Mantel's test in the ade4 package (Dray and Dufour 2007). When we detected significant ($P < 0.05$) spatial autocorrelation, we determined the best-fit correlation function (exponential, Gaussian, or spherical; Dormann et al. 2007) by comparing Akaike's information criterion (AIC) values among linear mixed-effects models of each type. We then modeled the mean trait value as a function of altitude using a linear mixed-effects model accounting for spatial autocorrelation (Cressie 1991) using the nlme package (Pinheiro et al. 2015).

Pollinator assemblage and altitude

To determine whether the composition of *A. anserina*'s pollinator assemblage varied with altitude, we collected pollinators at four focal populations: two lower-altitude (2336, 2617 MASL; hereafter L1 [38.51464° N, 106.99503° W] and L2 [38.36533° N, 107.19703° W]), and two higher-altitude populations (3004, 3295 MASL; hereafter H1 [38.11335° N, 106.93162° W] and H2 [38.02602° N, 107.16904° W]) in 2013 (Appendix B). At peak flowering in each population, we netted at least 100 insects foraging on *A. anserina* flowers ($N = 100$–172/population; total $N = 534$ insects). Collections took place between 10:00 and 17:00 hours on 2–5 d from 9 June to 7 July at L1 and L2, and 29 June to 7 July at H1 and H2. We identified insects as dipterans, hymenopterans, lepidopterans, or other (following Fenster et al. 2004). Hymenopterans were small solitary bees, occasionally thread-waisted wasps (Sphecidae), and infrequently, bumble bees (*Bombus*). Dipterans were in the families Muscidae, Anthomyiidae, Tabanidae, Bombyliidae, and Syrphidae. Tachinidae, common flower visitors at higher altitudes in Colorado (Kearns 1992), were not verified, but since these are difficult to distinguish from other groups in the field they may also have been present. Since >95% of the insects collected were hymenopterans or dipterans, we calculated the proportion of flower visitors that were dipterans in each population and used this as an index of the pollinator assemblage. Both groups are known to touch reproductive parts when landing on *A. anserina* flowers (M. H. Koski, *personal observation*), and hereafter are referred to as pollinators. We performed a Spearman's rank correlation between the index and altitude.

Pollinator response to floral bullseye size

To evaluate whether pollinator preference and behavior depended on bullseye size, altitude, pollinator type, or a combination of these factors, we constructed experimental arrays of flowers manipulated to have small and large bullseyes, and presented these at each of the four focal populations. We manipulated UVP of flowers collected from plants in situ, and manipulations were guided by the range seen in 2012 (34–77% UV absorbing; see *Results*). Specifically, we created flowers with small (~25% UV absorbing) and large bullseyes (~80% UV absorbing). As in past work (Koski and Ashman 2014), we created large bullseyes by painting a mixture of sunscreens (Parsol MXC + Parsol 1789 sunscreens; DSM Nutritional Products, Heerlen, Netherlands) dissolved in duck preen gland fat (Marryat, Basel, Switzerland) over petals (also see Johnson and Andersson 2002, Peter and Johnson 2008, Campbell et al. 2010) and painting the tips of petals with UV-reflective yellow paint (Fish Vision UV Lure Paint; Reel Wing Decoys, Fargo, North Dakota, USA). We created small bullseyes by painting yellow UV-reflective paint on ~75% of the petals and applying the sunscreen mixture to the base of petals. To control for potential olfactory effects of different amounts of paint/sunscreen used in each treatment, we applied yellow paint to petal undersides of the large-bullseye flowers, and the sunscreen solution to petal undersides in the small-bullseye flowers. Koski and Ashman (2014) verified that the reflectance of manipulated petals was similar to natural *A. anserina* flowers.

We placed three flowers of each bullseye size in water-filled microcentrifuge tubes arranged alternately and ~15 cm apart in circular arrays (see Koski and Ashman 2014) and observed insect visitation. We scored preference from the phenotype of the first flower chosen by a pollinator when arriving at an array (Fenster et al. 2006), and then we characterized its behavior as "approach" if it hovered over but did not make contact, or "forage" if it landed and made contact with reproductive parts of the flower (Hansen et al. 2012). Though approaches did not result in contact with a flower, we refer to both approaches and forages collectively as visits when discussing results. We observed nine to 12 arrays ranging 45–90 min each in each focal population. Every 15 min, we changed the order of the flowers within an array such that small and large bullseyes occupied different places, to eliminate spatial bias. Visits were enumerated within each array by behavior type (approach, forage), pollinator type (hymenopteran, dipteran), and bullseye size (small, large).

We analyzed visit count data using a generalized linear mixed model (procedure glimmix in SAS; SAS Institute, Cary, North Carolina, USA) with a Poisson distribution. The number of visits to each flower type per array was the response variable. Altitude (low or high), bullseye (small or large), pollinator type (hymenopteran or dipteran), behavior (approach or forage), and all two, three, and four-way interactions were fixed effects. Population (L1, L2, H1, H2) nested within altitude (low or high), and array replicate nested within population were random effects. We were particularly interested in whether altitude affected the pollinators visiting the artificial arrays, and for this, evaluated the pollinator type × altitude effect. We assessed the bullseye size × pollinator type effect to determine whether pollinator types displayed differential floral preference for the bullseye phenotype. We assessed whether overall pollinator preferences changed with altitude via the bullseye size × altitude effect. Finally, to address whether bullseye size affected pollinator behavior, and whether this depended on altitude, we evaluated the bullseye size × behavior, and the bullseye size × behavior × altitude terms, respectively. In addition, we included a preplanned contrast to specifically determine whether, within a given altitude, the number of "functional" visits, i.e., only foraging visits, differed between small- and large-bullseye flowers.

Relationship between visit number and pollen receipt and export

We characterized whether increased foraging visits by hymenopterans and dipterans increased female (outcross pollen receipt) and male (pollen export) metrics of pollination success. To assess outcross pollen receipt, we emasculated virgin flowers in water-filled microcentrifuge tubes and assembled them in arrays of 15–30 cut flowers. These were placed at three populations (L1, L2, H2) where flowers were allowed to receive zero to four foraging visits from either hymenopterans, dipterans, or a mix of both. After visitation, we recorded the number of pollen grains on five stigmas per flower. Pooling flowers across arrays ($N = 93$ flowers), we characterized pollen receipt via multiple linear regression with average outcross pollen grains/stigma modeled as a function of the number of hymenopteran and dipteran foraging visits. We also performed regression with the addition of visits squared to evaluate nonlinearity.

To assess male function, we collected flowers pre-anthesis in L1 and L2. From each flower, we removed one undehisced anther, stored it in ethanol, and counted the remaining anthers. After anthers began to dehisce, we arranged 11–16 flowers in each array ($N = 53$ flowers) and allowed zero to five foraging visits by pollinators. After visitation, we stored the remaining anthers in ethanol. We used acetolysis to liberate pollen from anther sacs (Kearns and Inouye 1993) and enumerated the grains in the undehisced anther and the post-visitation anthers with the aid of a hemocytometer and light microscopy. We estimated total pollen produced per flower from the product of the pollen in the undehisced anther and the number of anthers. We calculated the proportion of pollen exported as (total pollen − remaining pollen)/total pollen and regressed the proportion pollen removed on the number of foraging visits. Only two visits were made by dipterans, so we could not assess pollen export by each group separately. We tested for a nonlinear relationship by including the squared term of visit number in the regression; it was not significant, so we report only the linear regression results.

Relationships between natural variation in floral traits and pollen receipt

In the four focal populations, we examined the relationship between pollen receipt and natural variation in five floral traits: UVP, flower size, brightness, UV chroma, and number of open flowers. We randomly selected 66–75 plants per population at least 2 m apart. From a single flower per plant we measured petal brightness, UV chroma, flower size, and UVP in all populations except L2, where we only measured the latter two traits. Additionally, we scored the number of open flowers per plant at all populations. We collected one spent flower per plant. From this flower we collected five styles, softened them with KOH, and stained them with decolorized aniline blue (Allied Chemical and Dye Company, New York, New York, USA; Kearns and Inouye 1993). We enumerated conspecific pollen grains per stigma under a Zeiss Fluorescent microscope (Zeiss, Oberkochen, Germany). The average number of pollen grains received per pistil was calculated for each flower. Within each population, we calculated relative pollen receipt as mean pollen/pistil per plant divided by the population average pollen/pistil, and calculated the z score of each floral trait as (trait_x − mean trait value)/standard deviation trait_x. In each population, we estimated partial regression coefficients (β_i') from a multiple linear regression of relative pollen receipt on all standardized floral traits (Lande and Arnold 1983). Residuals from regressions were normally distributed; thus P values obtained adequately assess significance ($P < 0.05$; Mitchell-Olds and Shaw 1987). Trait correlations with UVP were low and nonsignificant after Bonferroni correction in all populations (Appendix C). However, there were significant positive correlations between petal size, chroma, and brightness parameters in L1 and H1 (Appendix C). Multicollinearity was negligible in the regression models, so correlations among traits did not violate model assumptions; variance inflation factors (VIF) for predictor variables were 1.01–1.47, falling far below thresholds indicating multicollinearity (e.g., 10–30; O'Brien 2007), and mean VIFs for models ranged 1.0–1.3.

To test whether the relationship between floral traits and pollen receipt varied among populations, we used analysis of covariance (ANCOVA; Galen and Newport

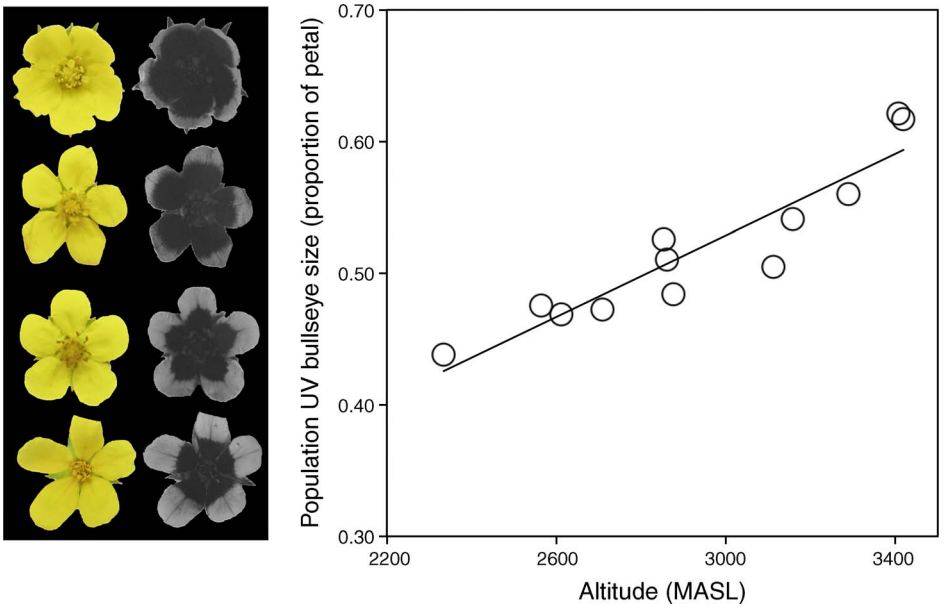

FIG. 1. Mean size of the UV bullseye (the proportion of the petal that absorbs UV) in *Argentina anserina* increases with altitude (m above sea level; MASL) across 12 populations in Colorado, USA (bullseye size $= 1.5 \times 10^{-4}$(MASL) $+ 6.6 \times 10^{-3}$, $P < 0.0001$, $R^2 = 0.83$). Representative flowers in the human-visible (yellow) and UV (grayscale) spectrum are provided to show the variation in bullseye proportion size ranging from 0.35 to 0.62.

1987, Dudley 1996, Caruso 2000, Parachnowitsch and Kessler 2010) on the combined data set, where relative pollen receipt was modeled as a function of population, standardized trait values, and the population-by-trait interaction using SAS (GLM procedure). Because petal UV chroma and brightness were not measured in L2, we performed two separate ANCOVAs: (1) all traits from L1, H1, and H2, and (2) all populations, but no data on UV chroma and brightness.

RESULTS

Floral traits, pollinator assemblage, and altitude

UV bullseye size varied from 34% to 77% of the flower (UVP range 0.34–0.77), and population mean UVP increased with altitude; UVP $= 1.5 \times 10^{-4}$(MASL) $+$ 0.0066 ($R^2 = 0.83$, $P < 0.001$, $N = 12$; Fig. 1). In contrast, variation in other floral traits (petal size, brightness, and UV chroma) was not associated with altitude (b range: -1.46×10^{-5} to 5.8×10^{-4}; all $R^2 < 0.19$; $P > 0.16$). There was significant spatial autocorrelation for UVP (Mantel's test $r = 0.39$, simulated $P = 0.015$), and the best fit-correlation function (exponential, Gaussian, or spherical) was exponential ($AIC_{exp} = -36.7$; $AIC_G = -34.8$; $AIC_s = -35.9$). Inclusion of spatial autocorrelation in the regression model, however, did not reduce the influence of altitude on bullseye size; UVP $= 1.5 \times 10^{-4}$(MASL) $+ 0.09$; $P < 0.001$. The pollinator assemblage at the four focal populations varied with altitude: the proportion of pollinators visiting *A. anserina* flowers that were dipterans increased from 11% to 66% from the lowest to highest altitude site ($r_S = 0.95$, $P = 0.05$).

Pollinator response to floral bullseye size

We observed 293 insect visits at artificial arrays (148 at low-altitude sites; 145 at high-altitude sites). The number of visits by hymenopterans and dipterans depended on altitude, and reflected assemblage changes noted on natural *A. anserina* flowers (pollinator type $\times$ altitude, $P < 0.0001$; Tables 1a and 2a, Fig. 2a). Specifically, at low altitude, visits by Hymenoptera were more than eight times those of Diptera, but at high altitude, dipterans visited flowers 37% more frequently than hymenopterans (Fig. 2a). The high frequency of Hymenoptera at low altitude accounts for the overall higher number of visits by Hymenoptera at our arrays (pollinator type, $P < 0.0001$). Visitation preference did not depend on pollinator type (bullseye size $\times$ pollinator type, $P > 0.35$; Table 1a), and evidence for overall preference based on bullseye size was weak (bullseye size, $P = 0.079$; Table 1a) and marginally dependent on altitude (bullseye size $\times$ altitude, $P = 0.056$; Table 1a). The number of approaches and foraging visits to large UV bullseyes was two times that of small ones at high altitude, but only 30% higher at low altitude (Fig. 2b). Bullseye size affected pollinator behavior (bullseye size $\times$ behavior, $P = 0.015$; Table 1a), but the strength of the effect depended on altitude (bullseye size $\times$ behavior $\times$ altitude, $P = 0.016$). Approaches increased with UV bullseye size at both altitudes; however, the effect of bullseye size on foraging visits depended on altitude (Fig. 2b). When considering foraging visits alone, large-

TABLE 1. (a) Fixed effects from generalized mixed-model testing, (b) preplanned contrast between bullseye sizes for foraging visits only within altitudes, and (c) random effects included in the model.

Traits and interactions	$F_{1, 320}$	P
a) Fixed effects		
Altitude†	1.75	0.265
Bullseye	3.1	0.079
Pollinator type	23.48	<0.0001
Behavior	3.38	0.067
Bullseye × altitude	3.7	0.056
Pollinator type × altitude	50	<0.0001
Behavior × altitude	2.49	0.116
Bullseye × pollinator type	0.85	0.356
Bullseye × behavior	6.01	0.015
Behavior × pollinator type	3.18	0.075
Bullseye × pollinator type × altitude	0.13	0.723
Bullseye × behavior × altitude	5.91	0.016
Bullseye × pollinator type × behavior	3.61	0.058
Pollinator type × behavior × altitude	1.32	0.251
Bullseye × pollinator type × behavior × altitude	2.45	0.118
b) Contrast: bullseye size for foraging visits only		
Low altitude	3.13	0.077
High altitude	6.78	0.01
c) Random effects		
Population (altitude)‡	$Z = 0.078$	0.28
Array (population)‡	$Z = 2.46$	0.007

Note: The mixed model in (a) tests the effects of altitude (low vs. high), bullseye size (small [~25% absorbing] vs. large [~80% absorbing]), pollinator type (hymenopteran vs. dipteran), behavior (approach vs. forage), and their interactions on the number of visits to *Argentina anserina* flowers in floral arrays.

† $F_{1,3.53.}$

‡ For the random effects, the test statistic is Z rather than F.

bullseye flowers received twice as many as small-bullseye flowers at high altitude (behavior × bullseye contrast, high altitude, $P = 0.01$, Table 1b, Fig. 2b), but this pattern was reversed at the low altitudes where large-bullseye flowers received 63% fewer foraging visits than did small ones, though the difference was only marginally significant (behavior × bullseye contrast, low altitude, $P = 0.077$; Table 1b, Fig. 2b). The behavioral change in response to bullseye size from low to high altitude was similar among Hymenoptera and Diptera (bullseye × pollinator type × behavior × altitude, $P = 0.118$; Table 1a). For example, at low altitude, 25% of the Hymenoptera encounters with large-bullseye flowers were foraging visits, and 10% of Diptera encounters were foraging visits. At high altitudes, though, Hymenoptera foraged on 36% of their encounters with large-bullseye flowers, while Diptera foraged on 60% of their encounters with large-UV flowers. Thus, both pollinator types increased the likelihood of foraging at large bullseyes from low to high altitude.

Relationship between visit number and pollen receipt and export

Pollen receipt per pistil displayed a nonlinear increase with the total number of foraging visits (pollen/pistil = $1.12(\text{visits}) - 0.23(\text{visits})^2 + 0.40$; $R^2 = 0.12$, $P < 0.001$; Fig. 3a). Multiple linear regression showed that foraging

TABLE 2. (a) Altitude (meters above sea level, MASL) and the proportion of dipteran pollinators visiting *A. anserina* flowers in the four focal populations in the Colorado Rocky Mountains, USA, and (b) relationship between floral traits and pollen receipt.

Trait type	Population			
	L1	L2	H1	H2
a) Population feature				
Altitude (m)	2336	2617	3004	3295
Proportion Diptera	0.11	0.34	0.66	0.66
b) Floral trait				
Bullseye size	−0.75**	−0.06	0.07	0.13†
Flower size	0.32*	0.08	0.02	0.12†
Floral display	0	0	0.02	0.04
Brightness	−0.18	NA	−0.09	0.02
UV chroma	−0.38*	NA	0.1	−0.02

Notes: Populations include two lower-altitude (2336, 2617 MASL; L1 [38.51464° N, 106.99503° W] and L2 [38.36533° N, 107.19703° W]), and two higher-altitude populations (3004, 3295 MASL; H1 [38.11335° N, 106.93162° W] and H2 [38.02602° N, 107.16904° W]). In (b), the relationships between floral traits and pollen receipt are measured as standardized regression coefficients (β_i'). In L2, brightness and UV chroma were not measured (NA). The number of plants used for regression analyses are as follows: 60–76 plants (L1), 67 plants (L2), 68 plants (H1), and 66 plants (H2).

* $P < 0.05$; ** $P < 0.0001$; † $P = 0.06$–0.08.

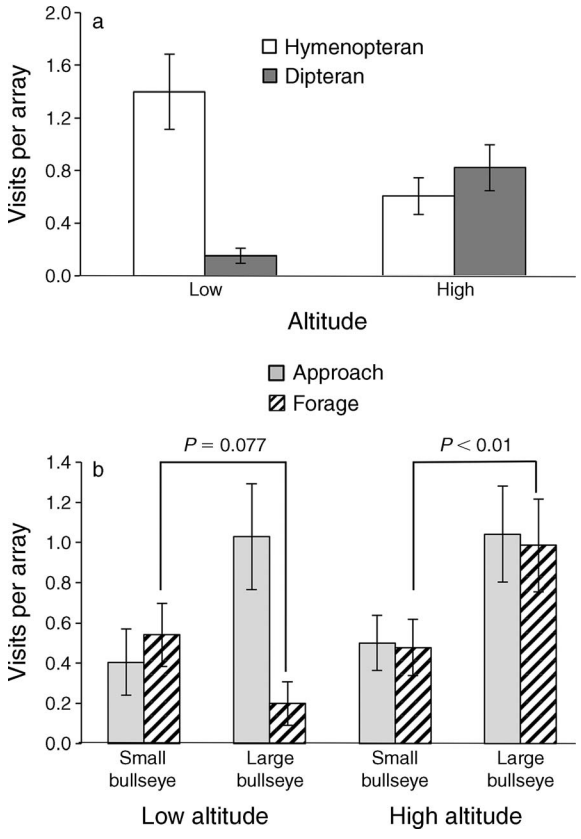

FIG. 2. (a) The number of visits to *A. anserina* flowers per floral array by hymenopterans (white bars) and dipterans (gray bars) at low- and high-altitude populations, and (b) the number of approach (gray) and forage visits (hatched) to flowers with small bullseyes (~25% absorbing) and large bullseyes (~80% absorbing) at low- and high-altitude sites. Counts presented are back-transformed least squares means ± 1 SE from a generalized linear mixed model with a Poisson distribution. *P* values compare the number of foraging visits between flower types within an altitude class.

visits by both hymenopterans and dipterans contributed to outcross pollen receipt (pollen/pistil = 0.34(hymenopteran visits) + 1.65(dipteran visits) + 0.66; $R^2 = 0.11$, $P < 0.01$). Over the range of visits observed, the proportion of pollen exported increased linearly with the number of foraging visits received (proportion pollen exported = 0.10(visits) + 0.25; $R^2 = 0.37$, $P < 0.0001$; Fig. 3b).

Relationship between natural variation in floral traits and pollen receipt

The relationship between pollen receipt and UV bullseye size varied significantly among the three populations in which all traits were measured (bullseye size × population, $F_{2, 175} = 28.31$, $P < 0.0001$; Fig. 4). This result can be extended to the four populations even in the absence of data on UV chroma or brightness ($F_{3, 260} = 30.78$, $P < 0.0001$), as bullseye size was not significantly correlated with either (Appendix C). Specifically, in L1, flowers with smaller bullseyes received larger pollen loads ($\beta'_{bull} = -0.75$; $P < 0.0001$; Table 2, Fig. 4a), but in H2, larger bullseyes tended to receive more pollen ($\beta'_{bull} = 0.134$, $P = 0.08$; Table 2, Fig. 4d). However, no relationship was seen in L2 or H1 (Table 2, Fig. 4b, c). Of the other traits studied, flower size and UV chroma influenced pollen receipt, but only in some populations (Table 2), and only for the latter trait was the population variation significant (L1, H1, H2: $F_{2, 175} = 6.04$, $P < 0.01$). Specifically, individuals with lower UV chroma received more pollen in L1 ($\beta'_{UVc} = -0.38$, $P < 0.05$) but not in H1 or H2 (Table 2).

DISCUSSION

Our work demonstrates altitudinal variation in the size of the UV floral bullseye, and a coincident altitudinal change in the composition and behavior of pollinator assemblage. This suggests that generalist pollinator fauna may affect evolutionary change in

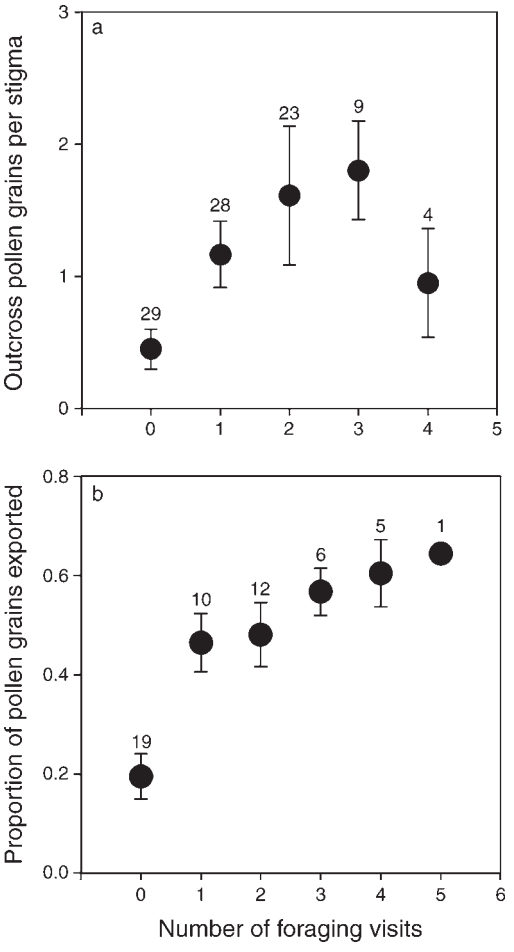

FIG. 3. The association between the number of foraging visits by pollinators and (a) outcross pollen receipt per stigma, and (b) the proportion of pollen grains removed from *A. anserina* flowers. Values are means ± 1 SE and sample sizes are given above each point.

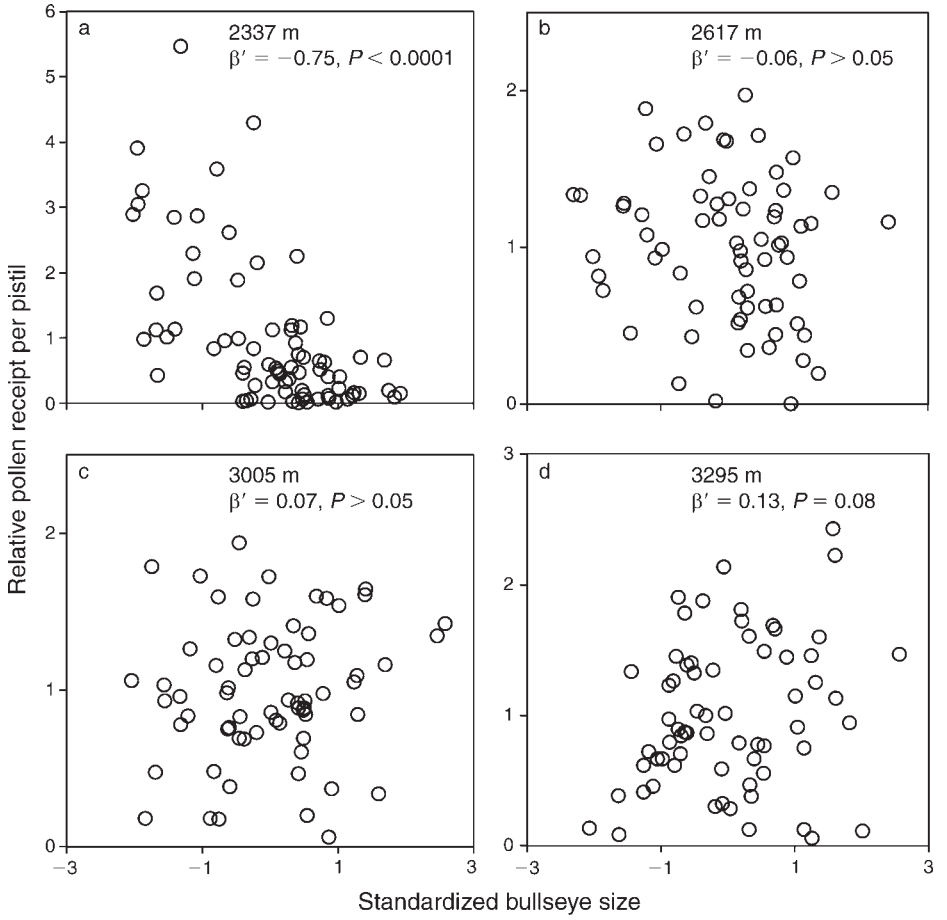

FIG. 4. Standardized pollen receipt per pistil (mean pollen receipt per pistil relative to the population average pollen receipt per pistil) plotted against UV bullseye size (standardized UVP; the proportion of the petal that absorbs UV) in four populations of *A. anserina* with varying altitude. Panels (a–d) correspond with two lower-altitude (2336, 2617 MASL; L1 [38.51464° N, 106.99503° W] and L2 [38.36533° N, 107.19703° W]), and two higher-altitude sites (3004, 3295 MASL; H1 [38.11335° N, 106.93162° W] and H2 [38.02602° N, 107.16904° W]), respectively. The partial regression coefficient β′ for bullseye size (UVP) and *P* value for each are reported in each panel. For full regression model results, see Table 2.

flowers through subtle and complex shifts in plant–pollinator interactions. Moreover, this work joins other recent studies stressing that a holistic view of floral phenotype, including traits cryptic to humans (e.g., Junker and Parachnowitsch 2015, Peterson et al. 2015), is required to understand pollinator-mediated floral evolution.

In generalist-pollinated plants, multiple taxa with various morphologies and floral preferences are effective pollinators (Gómez and Zamora 1999), leading some to conclude that directional pollinator-mediated selection on floral traits will be weak and that spatial shifts in pollinator community composition are unlikely to contribute to geographic variation in floral phenotypes (Herrera 1988, Johnson and Steiner 2000). Our results suggest, however, that in generalist-pollinated *A. anserina*, clinal variation in the floral UV bullseye results, at least in part, from changes in behavior of the overall pollinator assemblage. While an increase in UV bullseye size corresponded with an increase in dipterans and decrease in hymenopterans in *A. anserina*'s pollinator assemblage, the two pollinator groups did not display differential preference for bullseye size. Thus, the shift in the pollinator community composition alone may not be sufficient to contribute to clinal variation. Instead, both pollinator classes preferred large bullseyes at high altitude; they approached and foraged twice as much on large-bullseye flowers (Fig. 2b). In contrast, at low altitude, pollinators were attracted to large-bullseye flowers but rarely foraged; only 16% of the encounters with large-bullseye flowers resulted in foraging, whereas 57% of the encounters with small bullseyes resulted in foraging (Fig. 2b). This resulted in small-bullseye flowers receiving over twice as many foraging visits as large-bullseye flowers, despite a similar "attraction" to large-bullseye flowers (i.e., in approach behavior). These results suggest an advantage for the small-bullseye phenotype at low altitude, and for the large bullseye at high altitude, which is in accordance with clinal variation observed.

The change in pollinator response to bullseye size could be explained by a number of potential mechanisms. First, the efficiency of the bullseye pattern as a guide may be context dependent. The light environment can affect insects' perception of colors and color contrast (Endler 1992, 1993, Arnold and Chittka 2012), and clear-sky irradiance tends to increase with altitude, as does the proportion of solar irradiance comprising UV wavelengths (Körner 2007). Perhaps at low altitudes, pollinators were less able to detect UV color contrast on large-bullseye flowers, and were thus deterred from foraging. Second, bullseye size may be associated with an unmeasured attractive trait like floral scent that influenced pollinator behavior. This explanation, however, would require that a correlation be present in low- but not high-altitude populations. Third, species composition within the broad classes of flower visitors scored in this study (Hymenoptera and Diptera) may have shifted. For example, bee species richness can decrease with altitude (Hoiss et al. 2012). Thus, the Hymenoptera that showed behavioral preference for small bullseye size at low altitude may have dropped from the pollinator assemblage at higher altitudes. Finally, recent work by Peterson et al. (2015) shows that pollinators discriminate against the locally unfamiliar UV phenotype in *Mimulus*. Thus, pollinator discrimination against rarer large-bullseye phenotypes at low altitude could be due to previous pollinator experience.

Naturally occurring plants with smaller UV bullseyes had larger stigmatic pollen loads at the lowest altitude, while those with larger-bullseye flowers tended to have larger loads at the highest altitudes (Fig. 4). This change in the relationship between bullseye size and pollen receipt corresponds to the change in pollinator behavior observed in the artificial floral arrays, especially since increased foraging visits leads to increased outcross pollen receipt (Fig. 3). We caution, however, that while higher pollen loads often beget greater seed output (Shore and Barrett 1984, Waites and Ågren 2004), this may not always be the case (e.g., Campbell et al. 1991), as incompatible grains may make up part of the pollen load (Silander and Primack 1978). However, our functional assays indicate that not only outcrossed pollen receipt but also pollen export increased with increasing foraging visits, so it is likely that selection will act on bullseye size via male as well as female function. While our study is one of only a few that has examined intraspecific spatial variation for floral traits of generalist-pollinated plants (Herrera et al. 2006, Gómez et al. 2009*a*, *b*), it clearly supports the idea that specialization is not a necessary precursor to pollinator-mediated selection. In these other studies, the preference and/or morphology of the locally dominant pollinator corresponded with floral variation (Herrera et al. 2006, Gómez et al. 2009*a*, *b*). In the present study, floral diversification in a generalist-pollinated plant may result from varying behavioral responses to floral phenotype. We suggest that future work aimed at disentangling mechanisms of pollinator-mediated selection in generalist-pollinated plants should incorporate not only composition of pollinator assemblages, their preferences, and visitation rates, but also their behaviors once attracted to flowers (e.g., Bischoff et al. 2015).

This study also makes clear that if we are to understand how floral color mediates plant–pollinator interactions and contributes to floral diversification, we need to incorporate floral patterns, including those in the UV spectrum. In this study, bullseye pattern was the only trait that changed predictably with altitude, and it tended to be more linked with pollen receipt than other floral traits measured, like flower size and reflectance. Thus, while our phenotypic evaluation was only focused on flower size, color, and pattern (not scent or shape; Junker and Parachnowitsch 2015), by including all of these we were able to isolate bullseye size as a functionally relevant trait, and putative target of selection. Thus, this is the first study to demonstrate the functional role of natural variation in the UV-specific bullseye, despite the commonness of UV floral patterns (e.g., Penny 1983, Dyer 1996). Since UV patterns or petal "nectar guides" are likely to be as widespread as those that are visible to humans, there is a clear need for more studies of this kind (see Waser and Price 1985, Medel et al. 2003, Hansen et al. 2012, de Jager and Ellis 2014) to determine the generality of our findings.

Finally, a full understanding of the factors that determine clinal variation in floral pattern phenotypes requires a perspective that incorporates multiple agents of selection. For example, Koski and Ashman (2015) showed that an abiotic factor, UV irradiance, contributes to latitudinal variation in UV bullseye size in *A. anserina*. Altitude was included as a covariate in that analysis, and its effect on bullseye size was found to vary by region (Koski and Ashman 2015), suggesting that ecological factors other than just an altitudinal increase in UV exposure must be involved in shaping variation. However, altitudinal variation in bullseye size in the Colorado Rocky Mountains is consistent with both pollinator and UV-mediated selection. The dissection of flower–pollinator interaction described in this study provides a first glimpse at the types of biotic interactions that can contribute to altitudinal gradients in UV bullseye size. While florivory was not a common occurrence in the populations of *A. anserina* studied here (M. H. Koski, *personal observation*), it too may play a role in some locations, as UV-absorbing compounds in floral tissue can deter florivores (Gronquist et al. 2001). In fact, the relative importance of antagonistic, mutualistic, and abiotic agents of selection on floral UV pattern should be examined to evaluate their relative contributions to floral diversification in *A. anserina* and other systems.

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Supplemental Material

Ecological Archives

Appendices A–C are available online: http://dx.doi.org/10.1890/15-0242.1.sm