THE EVOLUTIONARY ECOLOGY OF ULTRAVIOLET FLORAL PIGMENTATION

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Matthew H. Koski

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This dissertation was presented by

Matthew H. Koski

It was defended on

May 4, 2015

and approved by

Dr. Susan Kalisz, Professor, Dept. of Biological Sciences, University of Pittsburgh

Dr. Nathan Morehouse, Assistant Professor, Dept. of Biological Sciences,

University of Pittsburgh

Dr. Mark Rebeiz, Assistant Professor, Dept. of Biological Sciences,

University of Pittsburgh

Dr. Stacey DeWitt Smith, Assistant Professor, Dept. of Ecology and Evolutionary Biology,

University of Pittsburgh

Dissertation Advisor: Dr. Tia-Lynn Ashman, Professor, Dept. of Biological Sciences, University of Pittsburgh

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Matthew H. Koski, PhD

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The color of flowers varies widely in nature, and this variation has served as an important model for understanding evolutionary processes such as genetic drift, natural selection, speciation and macroevolutionary transitions in phenotypic traits. The flowers of many taxa reflect ultraviolet (UV) wavelengths that are visible to most pollinators. Many taxa also display UV reflectance at petal tips and absorbance at petal bases, which manifests as a 'bullseye' color patterns to pollinators. Most previous research on UV floral traits has been largely descriptive in that it has identified species with UV pattern and speculated about its function with respect to pollination. This dissertation addresses the ecological and evolutionary relevance of UV floral pattern at micro- and macroevolutionary scales. First I use a widespread plant (*Argentina anserina*) to describe the degree to which UV floral pattern varies, and determine the genetic contribution to variation. With the same system, I then use experimental manipulation to test whether and how the UV bullseye pattern mediates plant-pollinator interactions in the field. I then evaluate whether spatial variation in biotic (pollinator) and abiotic selective agents contribute to geographic variation in UV floral traits at regional (Colorado Rocky Mountains elevation gradient) and global (four latitudinal gradients) scales. Finally, I create a molecular phylogeny of the species-rich cinquefoil (*Potentilla*) group to address whether variation in UV pattern among taxa is constrained by evolutionary history, and whether biogeography and bioclimatic factors contribute to interspecific variation. Findings from this dissertation that pollinators contribute to variation in UV pattern, broaden the understanding of the traits that contribute to pollinator-mediated reproductive success of flowering plants. UV irradiance can also impose selection on UV pattern and drive latitudinal trends in floral

pigmentation, extending an ecological rule formulated for animals—Gloger's rule—to plants. Finally, I detected low phylogenetic signal for UV pigmentation in *Potentilla*, but strong biogeographic associations, which together suggest that selection could play a role in shaping UV floral variation among taxa. Overall, this dissertation enhances the understanding of how spatially varying selection regimes contribute to geographic variation and macroevolutionary patterns in a cryptic pigmentation trait in flowers.

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PREFACE

This dissertation would not have been possible without efforts of Dr. Tia-Lynn Ashman, my dissertation advisor. I am deeply grateful for her dedication to my scientific and career development. I would also like to thank the outstanding committee members that have contributed substantially to the development of this dissertation. Much of this work could not have been done without the assistance of a number of undergraduate research assistants; R. M. Dalton, H. Brittingham, T. Kim, C. Kutsek, and B. Chuan. I am thankful to staff at the following field stations, Pymatuning Laboratory of Ecology, Rocky Mountain Biological Laboratory, and herbaria (CM, RM, E, K, RSA, UC, J, and H). I thank Ashman Lab members R. Spigler, R. Govindarajulu, G. A. Meindl, G. A. Gomez, K. A. LeCroy, N. J. Forrester, for influencing the development and presentation of ideas and results, as well as continued friendship. E. York, K. Mazzafferro, B. McTeague, and K. Schuller were hugely important for plant maintenance and guidance in plant care. Finally, I thank my family members who provided encouragement and support every day.

1.0 INTRODUCTION

Flower color has served as an important model for dissecting the ecological and evolutionary processes that drive phenotypic diversification (Rausher 2010). For decades, it has been known that pollinators, one of the most important agents of natural selection on floral traits, perceive colors very differently than humans. Ultraviolet (UV) reflectance from flowers, and UV 'nectar guide' patterns that are apparent to pollinators (Briscoe and Chittka 2001) have been described in many groups of flowering plants (e.g., Guldberg and Atsatt 1975; Harborne and Nash 1984; Reiseberg and Schilling 1985) but their ecological relevance has received little attention. In the following thesis, I (1) describe the degree of floral UV pigmentation variation and estimate its heritability (Chapter 1), (2) experimentally test how floral pigmentation variation affects plant-pollinator interactions (Chapter 2), (3) evaluate the extent to which the biotic (pollination) and abiotic (bioclimatic variables) contribute to phenotypic variation across space within a species (Chapters 3 and 4), and (4) discern factors that contribute to variation in UV pigmentation diversity at a macroevolutionary scale using phylogenetic comparative methods (Chapter 5).

In the flowering plant, *Argentina anserina*, flowers apparently uniform in color to humans display marked UV pattern variation, and this variation is heritable in the broad sense. I found high support from observational and experimental studies that both pollinators and the abiotic environment, namely UV irradiance, can impose selection on UV floral pigmentation. First, variation in UV floral pattern affects pollinator attraction from a distance, with the presence of a UV 'bullseye' pattern increasing pollinator attraction to flowers. Second, a larger area of UV absorption on petals reduces negative effects of UV irradiance on pollen viability. Spatially variable selection from both agents can shape broad scale geographic patterns of phenotypic variation within a species. In particular, pollinators contribute to altitudinal variation in UV pattern

in the Colorado Rocky Mountains, and UV irradiance contributes to global latitudinal trends of increasing UV absorptive floral areas towards more equatorial regions. Finally, in the diverse *Potentilla* genus, UV pigmentation pattern diversity is shaped by a variety of factors including evolutionary history, associations with human-visible flower color, and abiotic parameters of species' ranges. An integrative approach form the micro- to macroevolutionary scale has provided evidence for the ecological relevance of floral UV pigmentation, and the factors that shape phenotypic diversity of a previously understudied floral trait. Additionally, this work highlights the importance of abiotic factors in shaping floral pigmentation traits.

2.0 QUANTITATIVE VARIATION, HERITABILITY, AND TRAIT CORRELATIONS FOR ULTRAVIOLET FLORAL TRAITS IN *ARGENTINA ANSERINA* (*ROSACEAE*): IMPLICATIONS FOR FLORAL EVOLUTION

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2.1 INTRODUCTION

From the perspective of most insects, flower color is a combination of light reflected in both the human-visible (400-700nm) and ultraviolet (UV, 300-400nm) spectrum (Briscoe and Chittka 2001). Spatial color variation on petals is common among angiosperms (Penny 1983) and variation in the degree of spectral UV reflectance within flowers (UV pattern) has been of particular interest since it is cryptic to humans (Horovitz and Cohen 1972; Guldberg and Atsatt 1975; Skogin and Zakar 1976; Ingerson 1983; Rieseberg and Schilling 1985; Dyer 1996; Jones et al. 1999; Naruhashi and Ikeda 1999). Since most insects perceive UV, floral pattern in the UV spectrum has ignited interest in both the proximate physiological mechanisms underlying pattern, as well as the evolutionary processes that lead to pattern variation between species. A common pattern is for the bases of petals to absorb UV and the apices of petals to reflect UV, forming a 'bulls-eye' (Fig. 1). Such a pattern may function as a nectar guide for pollinators—directing them to floral rewards, and increasing pollinator efficiency (Daumer 1956; Thompson et al. 1972). Or, simply the presence of UV reflection may increase pollinator visitation and plant fitness (Johnson and Andersson 2002; Peter and Johnson 2008; Rae and Vamosi 2012; but see Jones and Buchmann 1974; Campbell et al. 2010). Moreover, variation in the relative area of floral UV absorption between congeners has been hypothesized to be important for reinforcing reproductive isolation between species with similar flower color in the human-visible spectrum, and similar floral morphology (Skogin and Zakar 1976).

Interspecific variation in floral UV pattern and/or the intensity of UV reflectance has been documented within plant communities (Guldberg and Atsatt 1975; Ingerson 1983; Dyer 1996; Jones et al. 1999), as well as among related taxa (Brassicaceae; Horovitz and Cohen 1972; *Bidens;* Skogin and Zakar 1976; *Viguera;* Rieseberg and Schilling 1985; *Potentilla*; Naruhashi and Ikeda 1999). However, there has been less of a focus on intraspecific variation, despite qualitative variation for UV pattern being noted in natural populations (Cruden 1972; Naruhashi and Ikeda 1999). The best description of intraspecific variation is for human-selected cultivars of *Brassica rapa* (Brassicaceae), in which the area of UV absorption (UV-absorbing area, Fig. 1a) relative to flower size (UV proportion Fig. 1a) was variable and heritable (Yoshioka et al. 2005; Syafaruddin et al. 2006). However, we lack a detailed understanding of the patterns of variation in UV proportion and its heritability in wild populations, and therefore have little foundation for understanding the evolutionary potential of UV patterns. For instance, differences in standing variation among populations (or taxa) could reflect past selection or drift and subsequently modifies evolutionary potentials. Moreover, if UV proportion is variable within populations, and associated with fitness, then there is the potential for natural selection to occur.

Color contrast on petals in both the UV and visible spectrum is ubiquitous (Penny 1983), and a number of studies have pinpointed the genetic basis for variation in petal color pattern (Almeida et al. 1989; Lister et al. 1993; Jorgenson 1995; Suzuki et al. 2000). Interestingly, while genetic correlations among floral organ size or reward traits are common (Connor and Via 1993; Connor and Sterling 1995; Caruso 2004; Ashman and Majetic 2006), we know little about covariance of spectral properties of distinct parts of petals (e.g., UV absorbing petal base vs. UV reflective petal apex; Fig. 1a-b; however see Hodges et al. 2002). We may expect positive correlations within petals due to shared developmental programs, and thus spectral properties at the base and apex of petals may evolve in concert. For example, Allen et al. (2008) suggest that

reduced developmental compartmentalization can lead to higher constraint to adaptive evolution of buttefly eye-spot patterns. Covariance between traits is key to predicting the pattern of evolution by natural selection (Blows and Hoffman 2005). Therefore, an assessment of the within-flower (i.e., spatial) correlation in color will adress the likelihood that specific petal regions can evolve independently and thus provide insight into the constraints on evolution of color in patterned flowers.

While pollinators have been purported to be selective drivers of floral UV traits, other agents of selection may also be important, as has been demonstrated for flower color in the human-visible spectrum (reviewed by Strauss and Whittall 2006). Although UV-absorptive portions of petals can manifest from unique epidermal cell shape (Gorton and Vogelmann 1996) they commonly result from UV-absorbing pigments (Thompson et al. 1972; Harborne and Nash 1984; Reisberg and Schilling 1985). Specifically, flavonoids absorb UV light, and their presence or absence can determine UV pattern in flowers (Thompson et al. 1972; Harborne and Nash 1984; Reisberg and Schilling 1985, Gronquist et al. 2001). Flavonoids are products of the anthocyanin pathway which gives rise not only to floral pigments (Grotewold 2006), but also phenolic compounds that protect against antagonists (reviewed in Treutter 2005), or protect tissues/DNA from abiotic stress (UV radiation, Jansen et al. 1998; Cold, Rivero et al. 2001). Interestingly, in at least 23 genera, petal and vegetative pigmentation are correlated (Onslow 1925) and these underlying biochemical associations can have important ecological and evolutionary outcomes—namely, indirect selection on flower color (Strauss and Whittall 2006). Most examples for which there is indirect selection on petal color are from species with anthocyanin polymorphisms. In these cases, pigmented morphs (anthocyanin +) often tolerate abiotic stress better than non-pigmented morphs (anthocyanin -) (Warren and Mackenzie 2001; Coberly and Rausher 2003). To our knowledge, no studies to date have assessed whether variation in floral patterning is correlated

with vegetative biochemistry, but such a correlation could affect the evolution of UV proportion. For instance, if intraspecific variation in UV proportion is due to gross differences in plant biochemistry, then we may expect a positive correlation between the concentration of UV-absorbing compounds in the leaf tissue and UV proportion. If, on the other hand, variation in UV proportion is due to petal-level regulatory variation (e.g., Almeida et al. 1989), then we would not expect this correlation. Determining which of these is the case is a first step in identifying the potential for direct or indirect response to selection of UV proportion.

To begin to understand the evolutionary potential of floral UV pattern we must first characterize the extent of intraspecific phenotypic and genotypic variation, and the covariation of UV pattern with floral spectral properties in a wild species. To this end, we address questions regarding floral UV pattern and spectral properties in two taxa within the *Argentina anserina* (Rosaceae) aggregate, a widespread perennial with a distinct UV bulls-eye pattern. These two taxa are likely to have distinct evolutionary histories and thus comparing them provides a general assessment of the patterns of variation. We sought to answer the following questions: 1) what is the degree of phenotypic variation for UV proportion and UV–absorbing area (Fig. 1a) within and among wild populations? 2) to what degree are UV-absorbing area, UV proportion, and spectral properties of petals relevant to insect visual systems (UV reflectance, UV chroma, brightness, green chroma; Fig. 1c) heritable? 3) do measures of the bulls-eye pattern (UV-absorbing area and UV proportion; Fig. 1) covary with direct quantitative assessments of reflectance in the UV (absolute UV reflectance, UV chroma), visible (green chroma), or overall spectrum (brightness)? 4) are the petal bases and apices spectrally constrained? and 5) does the concentration of UV-absorbing compounds in vegetative tissue positively correlate with floral UV proportion?

2.2 METHODS

2.2.1 Study system

The *Argentina anserina* (L.) Rydb. species aggregate (Rosaceae, formerly *Potentilla* L.) is a globally-distributed perennial herb that inhabits the edges of lakes, streams, and marshes, as well as roadsides and pastures (Rousi 1965). The species aggregate is comprised of a number of potential taxa (Rousi 1965). Here we focus on two, *Argentina pacifica* Rydb. and *A. anserina* L. s. str. (hereafter *A. anserina*). *Argentina pacifica* occurs along the Pacific coast of North America whereas *A. anserina* occurs inland in North America and in Northern Europe (Rousi 1965). The taxa are interfertile (M. Koski, unpublished) and both reproduce sexually through seed and clonally through plantlets along stolons (Ockendon and Walters 1970; Eriksson 1986). Hermaphroditic, largely self-incompatible flowers are borne singly in the crown or along stolons from May to September in the Northern Hemisphere (Rousi 1965; Ockendon & Walters 1970; Eriksson 1987), and are visited by small bees, syrphid flies (M. Koski, unpublished), and bumblebees (Miyanishi et al. 1991). Flowers appear uniformly yellow to humans but are considered 'UV-green' from the perspective of bees (Gumbert et al. 1999; Arnold et al. 2010). In addition, they have strong within-flower variation in the degree of UV reflection (Fig. 1). In particular, petals reflect UV from the apex, but not the base, forming a bulls-eye pattern (Fig. 1a,b). Harborne and Nash (1984) found that a flavonol glycoside is responsible for UV-absorption in petals of *A. anserina*, and that both yellow coloration and UV-reflection of petals are conferred by carotenoids. In other *Potentilla* taxa surveyed by Harborne and Nash (1984) various glycosylated forms of quercetin were responsible for UV-absorption in petals. Quercetin is a UV-absorbing flavonol and is present in *A. anserina*'s vegetative tissue (Kombal and Glasl 1995; Proestos et al. 2008).

2.2.2 Phenotypic variation in the field

We measured UV-absorbing area, petal area, and UV proportion (Fig. 1a) on 4 -22 flowers in each of 13 populations from California, Oregon, Washington, Michigan, Pennsylvania, and New York (Fig. 2, Table A1). In each population, we collected a single flower every 2+ meters along transects. Similar field collection of a related clonal plant, *Fragaria vesca* subsp. *bracteata*, yielded genetically distinct individuals (Li et al. 2012). In total, we collected and scored 218 flowers between June 13 and July 4, 2011. Populations from Michigan, Pennsylvania, New York, and eastern Washington are referred to as *A. anserina* (n=4) while populations along the Pacific coast of the Northwestern USA are referred to as *A. pacifica* (n=9) based on distinctions proposed by Rousi (1965) (Table A1).

For the majority of populations, we took UV photographs of fresh petal tissue on the day of flower collection. For three populations in the Great Lakes Region (MI, PA, NY; Table A1), flowers were pressed for one to three days and photographed dry. Pressing flowers in this manner does not influence UV proportion (paired *t*-test; t=-1.39 P=0.2, df=11, M. Koski, unpublished), however petal area and UV-absorbing area decrease ~40% upon drying (petal area, t=16.4, P<0.001, df=11; UV-absorbing area, t=12.6 P<0.001, df=11). Thus, petal area and UV-absorbing area from the Great Lakes populations were not compared to the remaining populations in analyses discussed below.

2.2.3 UV photography and measurement of UVP

We chose two to three random petals from each flower and flattened them under UV-transmitting glass on a white paper background. We photographed petals with a Nikon D40x (Nikon ®) converted to Broad Spectrum (Lifepixel, Mulkiteo, WA), with a 28mm Series E Lens (Nikon ®)

equipped with a reverse-mounted 2" Baader U-Filter (Baader Planetarium, Mammendorf, Germany). The conversion to broad spectrum allows the camera sensor to collect UV and infrared wavelengths, while the UV filter removes visible and infrared wavelengths but transmits UV light between 320 and 380 nm with peak transmission at 340 nm. We illuminated the image field with a UV lamp (ML-49, UVP©) with peak UV emission at 365nm. All photographs included a standard scale for measurements of area in mm^2.

We measured UV proportion following Yoshioka et al. (2005). We removed the backgrounds of photographs using Adobe Photoshop CS5, and obtained color channels (red, blue and green) in ImageJ (Rasband 2012). Using the red channel, we thresholded the UV-absorbing area of each petal and measured it in mm^2. Using the blue channel we measured petal area in a similar fashion. UV proportion was calculated as the UV-absorbing area/petal area (Fig. 1a). The averages of UV-absorbing area, petal area, and UV proportion for replicate petals of each flower were used in analyses.

2.2.4 Plant material for broad sense heritability and trait correlations

We collected plantlets of five to 10 genotypes from each of eight populations (CA3, CA4, OR1, OR3, WA2, MI, PA and NY; Table A1) along transects every 2+ meters in 2011, and transplanted them into a soil mix of Fafard #4 (Concord Fafard Inc., Agawam, MA) and sand (2:1) in 266 mL pots in a greenhouse at the University of Pittsburgh. We produced clones from 68 genotypes across all populations (mean= 3.8 clones per genotype, n=257) by vegetative propagation in September in the same pot conditions as parental plants. We randomly arranged clones in the greenhouse where conditions were 12.8°C/10°C (day/night). Clones received 122±33 mg of 13:13:13 N:P:K fertilizer (Nutricote Total Controlled Release Fertilizer with Micronutrients ™ Type 100) in October 2011.

We subjected clones from the Great Lakes populations (MI, PA, and NY) to a winter

treatment of darkness and 4.4°C for 22 days in a growth chamber. Plants from the Pacific coast do not experience such a harsh winter and were therefore kept in the greenhouse at 10°C/4.4°C (day/night) with no supplemental lighting for 22 days. After the winter treatment, all plants experienced 15.5°C/10°C (day/night) in the greenhouse and received an additional 122±33 mg of fertilizer.

2.2.5 Phenotypic variation in the greenhouse

On each greenhouse-grown clone, we collected two flowers on the first day of anthesis. We photographed petals in a similar manner to flowers in the field. However, we placed a UV-absorbing standard the image field, and illuminated petals with two UV mini lamps (UVP©) positioned at a constant distance at 45 degree angles. We measured UV proportion in ImageJ as above. For 14 clones (5%) we only photographed petals from one flower and overall we analyzed UV proportion for 500 flowers.

On the first flower of each clone we measured reflectance of a single petal at the base and the apex (Fig. 1a-c). We took spectral measurements with an Ocean Optics USB4000 spectrometer with a UV-NIR DH-2000-BAL deuterium-tungsten light source (Ocean Optics, Dunedin, FL). With CLR v. 1.05 (Montgomerie 2008), the reflectance spectra were binned into 1nm measurements. UV ($R_{300\text{-}400}$) and green ($R_{510\text{-}600}$) reflectance are relevant to the majority of insect visual systems (Peitsch, 1992, Briscoe & Chittka, 2001), so we calculated brightness ($R_{300\text{-}700}$), UV chroma ($R_{300\text{-}400}/R_{300\text{-}700}$), and green chroma ($R_{510\text{-}600}/R_{300\text{-}700}$) at the petal base and apex (Fig. 1c) with CLR v. 1.05 (Montgomerie 2008). We measured UV reflectance as $R_{300\text{-}400}$ (Fig. 1c).

On the first day that each clone flowered, we collected the most recently expanded terminal leaflet with forceps. From this tissue sample we extracted total phenolics following Day (1993).

Specifically, we lightly boiled samples for nine minutes in 10 ml of acidified methanol (Methanol:HCl:H_20, 90:5:5, v:v:v), filtered the extract through 0.9 μm screen, and brought it back to 10 mL with acidified methanol. After extraction, we dried the tissue sample at 40°C for 48 hours and weighed it to the nearest 0.1 mg.

To estimate the concentration of UV-absorbing compounds in the vegetative tissue, we created a standard curve for quercetin (SigmaAldrich, Q4951), a common flavonoid present in leaf tissue of *A. anserina* (sensu lato), and the main component of UV-absorbing portion of the petals in various *Potentilla* spp. closely related to *Argentina* spp. (Harborne and Nash 1984). Four 96-well plates containing extracts from tissue samples and two replicates of eight quercetin standards were measured for absorbance at 360nm on an Epoch microplate spectrometer (BioTek, Winooski, VT). We relativized the quercetin concentration of each sample by the dry weight of the leaf tissue to produce a standardized measurement of UV-absorbing compounds in units of mg/mg (quercetin/leaf tissue).

2.2.6 Statistical analysis

Phenotypic variation in the field: To test for population-level variation for UV proportion and its components (UV-absorbing area, petal area) we used ANOVA with population as a random effect. We first assessed population variation for UV-absorbing area, petal area, and UV proportion for *A. pacifica* populations alone and for UV proportion for *A. anserina* populations alone. Then, we assessed population variation for UV proportion for the species aggregate (*A. anserina* and *A. pacifica* populations). UV proportion was arcsine transformed and petal area and UV-absorbing area were ln transformed to improve normality prior to analysis. To partition the proportion of variation in UV proportion among taxa, populations, and flowers, we used a nested ANOVA. Statistics were performed in SAS v. 9.3 (SAS Institute Inc., Cary, NC, USA).

Phenotypic variation in the greenhouse: To characterize phenotypic variation in the greenhouse we calculated the mean and coefficient of variation (CV) for all traits measured for each taxon separately. We compared the mean and CV of traits between taxa using genotypic mean trait values. We used ANOVA with taxon, and population nested within taxon, as random effects to compare means. UV proportion was arcsine transformed, UV chroma and UV reflectance (apex and base) and the concentration of UV-absorbing compounds were ln + 1 transformed. The remaining traits were ln transformed prior to analysis to improve normality. To determine if the degree of phenotypic variation within *A. pacifica* and *A. anserina* differed we used a two-tailed *F* test for differences between CVs (Zar 1998).

Finally, to determine the degree to which population mean UV proportion in the field predicted that in the greenhouse, we correlated mean population UV proportion from the field and the greenhouse using Pearson-product moment correlation. We scored UV proportion in both contexts for eight populations across both taxa, and genotypic mean UV proportion values were used to generate greenhouse population means.

Broad-sense heritability: We estimated the broad-sense heritability of UV-absorbing area, petal area UV proportion, floral spectral traits (Fig. 1c), and concentration of foliar UV-absorbing compounds, for *A. anserina* and *A. pacifica* separately using the intraclass correlation coefficient. This estimate of clonal repeatability is an upper bound to heritability (in the broad sense) as maternal effects can contribute to variation (Lessells and Boag 1987; Falconer and Mackay 1996). However, mothers of clones were grown in a common environment for at least ten weeks prior to cloning so maternal environmental effects should be minimized. To increase accuracy of repeatability, UV-absorbing area, petal area, and UV proportion for two flowers per clone were

used as clone-level measures (Falconer and Mackay 1996). For all other traits, only one measure per clone was made.

We calculated the intraclass correlation coefficient and 95% confidence intervals (CI) for the point estimates with the ICC package in 'R' (Wolak et al. 2012) using the "THD" option which calculates CIs according to Thomas and Hulquist (1978). This method is appropriate for unbalanced designs and provides adequate estimates of the CI at all levels of intraclass correlation coefficient (Donner and Wells 1986). Heritability of a trait was significant if CIs did not overlap zero. We consider this assessment of significance to be conservative since the THD method calculates larger CIs than similar methods (Donner and Wells 1986). For the estimate of UV-absorbing compound concentration, there was a significant effect of the microplate tray (n=4), thus, we removed the effect of tray using ANOVA and calculated the intraclass correlation coefficient of the residuals. UV proportion was arcsine transformed, UV chroma and UV reflectance (apex and base) and UV-absorbing compound concentration were ln + 1 transformed. The remaining traits were ln transformed prior to analysis to ensure normality. We estimated broad-sense heritabilities for *A. pacifica* and *A. anserina* separately after the removal of the population effect on each trait with ANOVA.

Broad-sense genetic correlations: We estimated genetic correlations among traits for *A. pacifica* and *A. anserina* separately using Pearson product-moment correlation coefficients of genotypic mean trait values after the removal of the population effect with ANOVA (Ashman 1999; Klinkhamer and van der Veen-van Wijk 1999) using PROC CORR in SAS. Genotypic means were calculated from the phenotypic values of all clones for a given genotype. To determine whether pattern traits were associated with spectral traits we estimated the correlation of UV-absorbing area and UV proportion with a) UV reflectance and UV chroma, b) brightness, and c)

green chroma (Fig. 1c). To determine whether there was within-flower spatial association for spectral qualities we estimated correlations between the base and apex for UV chroma, UV reflectance, brightness, and green chroma (Fig. 1c). Finally, to answer whether floral UV pattern was correlated with foliar UV-absorbing compounds we correlated UV-absorbing area and UV proportion with the concentration of foliar UV-absorbing compounds. UV proportion was arcsine transformed, UV chroma and UV reflectance (apex and base) were ln + 1 transformed, and the remaining traits were ln transformed prior to analysis to improve normality. We used Bonferroni correction for multiple comparisons to evaluate the table-wide significance of correlation coefficients (Rice 1989), however we discuss the implications of correlations that were significant at the $P<0.05$ level before this correction when they relate directly to the questions posed.

2.3 RESULTS

2.3.1 Phenotypic variation in the field

UV proportion varied quantitatively in the field (Fig. 2). We found significant variation among populations for UV proportion for *A. pacifica*, but not for *A. anserina* (Table 1, Fig 3). Across populations, the UV proportion range was larger for *A. pacifica* (0.34 to 0.99) than *A. anserina* (0.44 to 0.73) (Fig. 3). UV proportion variation within populations was high for *A. pacifica* and *A. anserina* (Table 1, Fig. 3). On average, UV proportion variation within *A. pacifica* populations was ~47% greater than that within *A. anserina* populations (Fig. 3, *A. pacifica*, average CV=0.21, n=9; *A. anserina*, average CV=0.11, n=4, Table A1). For the species-aggregate ~21% of the variation in UVP was among taxa, 26% was among populations, and ~53% was among flowers within populations (Table 1). UV-absorbing area and petal area varied significantly among *A. pacifica* populations (Table 1).

2.3.2 Phenotypic variation in the greenhouse

Genotypic mean UV proportion in the greenhouse ranged from 0.53 to 0.96 for *A. pacifica* and 0.35 to 0.56 for *A. anserina*. The variation in UV proportion was similar between taxa ($CV_{pacifica}$=0.14, $CV_{anserina}$=0.13, Table 2). Across taxa, population mean UV proportion measured in the field was highly positively correlated with that in the greenhouse (r= 0.82, P<0.0001, n=8; Tables A1 and A2a). The concentration of UV-absorbing compounds in the foliage was, on average, 33% higher in *A. pacifica* than *A. anserina* ($F_{1,6.6}$=11.1, P= 0.01, Table 2) but the variation was the same between taxa (CV=0.14). Brightness, UV reflectance, and UV chroma from the petal apex were 18%, 140% and 120% higher in *A. anserina*, respectively ($F_{1,6.86}$=36.1, P< 0.001; $F_{1,6.1}$=10.5, P= 0.02, $F_{1,6.1}$=28.1, P= 0.002, respectively; Table 2), but UV reflectance and UV chroma were more variable in *A. pacifica* than *A. anserina* (UV reflectance: $CV_{pacifica}$=0.37, $CV_{anserina}$=0.17; UV chroma: $CV_{pacifica}$=0.34, $CV_{anserina}$=0.12; Table 2). Conversely, green chroma at the petal apex was 5% higher in *A. pacifica* ($F_{1,6.2}$=28.29, P= 0.02), however the variance was similar between taxa ($CV_{pacifica}$=0.02, $CV_{anserina}$=0.01). Green chroma at the base of petals was 2% higher in *A. anserina* ($F_{1,6.5}$=11.5, P= 0.01), but the mean and variance for the remaining spectral traits at the petal base were similar between taxa (Table 2).

2.3.3 Broad-sense heritability

UV-absorbing area and UV proportion were significantly heritable in both taxa, with UV proportion having particularly high heritability ($H^2_{pacifica}$= 0.86, $H^2_{anserina}$= 0.85; Table 3). In general, spectral properties of flowers had lower heritabilities (range: -0.08-0.20; Table 3) than UV pattern, and none of the spectral traits were significantly heritable in *A. anserina* (Table 3).

Conversely, brightness, UV chroma, UV reflectance and green chroma at the apex of petals were significantly heritable in *A. pacifica* (0.17-0.20; Table 3). Spectral properties measured at the base of petals were not significantly heritable in either taxa (Table 3). Heritability for foliar concentrations of UV-absorbing compounds in *A. pacifica* and *A. anserina* were similar but only significant in *A. pacifica* ($H^2_{pacifica}$= 0.17, $H^2_{anserina}$= 0.16; Table 3).

2.3.4 Broad-sense genetic correlations

UV pattern and quantitative measures of spectral traits: UV proportion was negatively correlated with both UV chroma and UV reflectance in *A. pacifica* (r = -0.54, P<0.0001; r = -0.49, P<0.01, n=46, respectively). Thus, as expected from the photographs of floral UV pattern, in *A. pacifica,* flowers with higher UV proportion reflected less UV from the apex of petals. Conversely, in *A. anserina* (Table 3) neither correlation existed, most likely due to low variation for UV reflectance and chroma (Table 2). UV proportion was positively correlated with green chroma at the apex of petals in *A. pacifica* before Bonferroni correction (r = 0.38, P <0.01, n=46) but not in *A. anserina* (Table 3). Neither UV-absorbing area nor UV proportion were correlated with any other spectral properties at the petal base or apex (Table 3).

Spatial covariance in spectral traits: Spectral correlations between the base and the apex of petals were not significant for UV reflectance and chroma, nor any other spectral trait in *A. pacifica* (Table 3). In *A. anserina,* however, prior to Bonferroni correction, UV chroma and brightness were positively correlated spatially (both r=0.49, P=0.02).

2.4 DISCUSSION

We report for the first time in any wild species that UV floral pattern displays extensive

quantitative variation, and is heritable, and thus potentially able to respond to selection. Consideration of color at two regions of petals revealed both positive correlation, and possible dissociation between regions, indicating taxon-specific variation in the evolutionary potential of overall flower color. Finally, the lack of positive correlation between foliar UV-absorbing compounds and floral UV proportion suggests that UV flower pattern could respond to selection independently from the vegetative organs. We discuss our results in the context of the potential for evolution of floral traits in the UV spectrum.

2.4.1 Variation in UV pattern

Floral UV pattern was highly variable for the *A. anserina* species aggregate, ranging from the presence of a small UV-absorbing bulls-eye (UV proportion of 0.35) to the absence of a bulls-eye all together (i.e., nearly complete UV-absorption: UV proportion of 0.99). Spectrophotometric measurements verified the photographic measures of UV proportion—UV proportion and UV-absorbing area correlated more strongly with UV spectral properties (UV reflectance and chroma) than with other spectral parameters (brightness or green chroma). Interestingly, the majority of variation for UV proportion was within populations. This study reveals that a population considered monomorphic for flower color in the human-visible spectrum can be very phenotypically diverse in the UV spectrum, and thus from the perspective of flower-visiting insects. Although intraspecific variation has been observed in *Potentilla argyrophylla* and *P. eriocarpa* (a UV bulls-eye morph and a uniformly UV-absorbing morph; Naruhashi and Ikeda 1999), *Nemophila menziesii* (Hydrophyllaceae) (Cruden 1979), and *Mimulus guttatus* (Phyrmaceae) (S. Bodbyl Roels, pers. comm), our results emphasize quantitative variation not only for floral UV pattern but its association with other spectral properties, which have been underappreciated. This study joins the most rigorous documentation of intraspecific variation for

UV proportion—that seen in *Brassica rapa* (Brassicaceae) where UV proportion ranged from 0.25 to 0.71 among different cultivars (Yoshioka et al. 2005). While this range is high, it lacks genotypes with nearly complete UV absorption, whereas in the *A. anserina* aggregate, some individuals lack UV pattern all together (Fig. 2), begging the questions of how and why variation in pattern is maintained. Exploration of the potential biotic and abiotic selective agents that may maintain UV pattern variation in the *A. anserina* aggregate is under study.

We can only speculate as to the evolutionary causes of the observed differences in mean and variance of UV proportion between the two taxa (Table 2). *Argentina anserina* is visited mainly by solitary bees and syrphid flies (M. Koski, pers. obs) but the predominant pollinators could differ geographically. If different pollinator types have different preferences for floral UV traits, then this could lead to the observed differences in UV phenotype . In addition, abiotic conditions of the two subspecies (e.g. exposure to UV irradiance, cold) may also contribute to variation noted between the taxa. However, genetic drift can also lead to differences in genetic and phenotypic variation among populations or taxa (Lande 1976).

2.4.2 Broad-sense heritability of UV pattern and spectral properties

Broad-sense heritabilities for UV-absorbing area, petal area, and UV proportion were all significant (Table 2), with UV proportion having particularly high heritability (≥0.85) relative to the average heritability for several floral traits (i.e., 0.39; Ashman and Majetic 2006). Population mean UV proportion in the field was tightly correlated with that observed in the greenhouse. Taken together, these findings suggest that variation in the field was unlikely due to a plastic response to environmental variation, but rather underlying genetic differentiation. Syaffarudin et al. (2006) also estimated high broad-sense heritability for UV proportion in two generations of controlled crosses for *Brassica rapa,* and suggested that many genes may influence UV proportion,

but that dominance variation was high.

We also documented lower but significant heritability for additional floral spectral parameters (UV reflectance, UV chroma, brightness and green chroma) in *A. pacifica* (Table 3), adding to the limited data on genetic underpinnings of spectrophotometrically-assessed flower color properties (chroma; Hodges et al. 2002; hue; Epperson and Clegg 1988, Hopkins and Rausher 2011). Despite being lower than pattern components, the heritability of these color parameters is well within the range of heritabililty for floral traits (i.e., -0.2-1.0; Ashman and Majetic 2006). This quantitative spectral variation could be functionally important since pollinator and non-pollinator agents can impose selection on brightness in *Lobelia siphilitica* (Caruso et al. 2010). However, since observed heritabilities for spectral properties were low in the current study system, selection would have to be strong to contribute to their evolution. A number of studies have also found a genetic basis for discrete flower color morphs scored by eye (Menéndez et al. 1997; Eujayl et al.1998), or by measuring absorbance of extracted floral pigments (e.g; Bradshaw and Schemske 2002). Unfortunately, the variety of methods utilized for measuring flower color and the variety of spectral parameters extracted from reflectance spectra preclude rigorous comparisons of heritabilities across taxa. Thus, there is a need to standardize the characterization of flower color to progress our understanding of its variation and evolution.

2.4.3 Trait correlations within flowers, and between flowers and leaves

UV chroma and brightness were correlated within flowers in *A. anserina*, suggesting that some spectral parameters may be spatially constrained across petals. For example, if there was selection for increased UV reflectance at the apex, the response would be petal-wide, i.e., increase at the base as well. This correlation may pose a constraint to the evolution of UV reflectance if maintaining some level of UV absorbance at petal bases is adaptive (e.g. for pollination; Thompson

1972). This assumption, however, should be taken with caution since heritability of UV chroma and reflectance were nonsignificant in *A. anserina.* Our finding of spatial correlation for color in *A. anserina* joins Hodges et al. (2002) who found a positive correlation between chroma of the base and spur petals in a cross between *Aquilegia formosa* and *Aquilegia pubescens* (r=0.63). In *A. pacifica*, however, we did not detect spectral correlations between the apex and base of petals. Thus, in this taxon, color of the petal base and apex may evolve independently. The differences in covariance between the species suggest that there may be ways to uncouple color traits across petals, and future work should be aimed at understanding how this occurs, both developmentally and genetically.

We did not detect a significant correlation between UV pattern components (UV proportion or UV-absorbing area) and foliar UV-absorbing compound concentration, suggesting independence of UV-absorbing compounds in the vegetative and floral tissue. That is, if UV proportion is dependent upon floral concentrations of UV-absorbing compounds, then UV proportion could change (either plastically or as a response to selection) without a concomitant a shift in foliar biochemical properties (or vice versa). This result implies that if selection acts on flower color pattern in this system, it may only be direct rather than indirect through selection on vegetative biochemistry as has been suggested for discrete color morphs in other studies (Warren and Mackenzie 2001; Coberly and Rausher 2003). However, the lack of positive correlation could reflect a number of developmental or experimental factors. First, UV proportion may not positively covary with the concentration of UV-absorbing compounds in petals. That is, flowers with larger UV proportion may have a more even distribution of UV-absorbing compounds than flowers with lower UV proportion, as opposed to higher concentrations. Second, spatial variation in gene expression or post-transcriptional regulation in petal tissue may give rise to variation in UV proportion as this type of regulation underlies color pattern variation in other taxa (*Anthirrinum*,

Almeida et al. 1989; *Petunia*, Jorgenson 1995; *Ipomoea*, Durbin et al. 2000). If variation in fine-scale regulation underlies UV proportion variation, rather than gross plant-wide differences in chemical profiles (e.g. Warren and Mackenzie 2001; Coberly and Rausher 2003), we should not expect a correlation between phenolic concentration in leaves and flowers and thus between foliar UV-absorbing compounds and UV proportion. Moreover, gene mutation in the anthocyanin pathway can influence phenolic production in both leaves and flowers similarly in some species, but only in flowers only for other species (Mooney et al. 1995). Finally, phenolic profiles of flowers and vegetative tissue can be different in other systems (Williams et al. 1996). Thus, our measure of UV-absorbing compounds may have included multiple phenolic compounds that may not be present in flowers, obscuring the correlation with the causative compound of UV pattern. Further studies on the development of UV proportion are required to pinpoint the causative factor for the dissociation between UV proportion and foliar UV-absorbing compounds.

2.4.4 Conclusions

Our study in two taxa of the *A. anserina* aggregate highlights the quantitative nature of flower color—in pattern and spectral qualities—especially those in the UV spectrum, and begs for more extensive studies of this nature in other species. If the broad-sense heritability for UV proportion estimated herein reflects narrow sense heritability, then UV proportion has the capacity to respond to selection, and thus the functional significance of floral UV patterning requires closer attention. However, spatial covariation for spectral properties existed within flowers in only one taxa , and flower pattern was not associated with foliar concentrations of UV-absorbing compounds in either, suggesting that UV proportion and other flower spectral qualities could evolve independently within the flower and from vegetative biochemical properties.

Table 2-1: Variation for components of UV pattern (UV-absorbing area, petal area, UV proportion) measured from field-collected plants of *Argentina pacifica, A. anserina,* and both taxa combined (*Argentina* aggregate)

		a) Population effect		b) Percent variance		
Taxon	Trait	*F*	df	Among taxa	Among populations	Within populations
A. pacifica	UV-absorbing area	7.5*	8, 162	.	25.6	74.4
	Petal area	9.7*	8, 162	.	31.5	68.5
	UV proportion	10.2*	8, 162	.	32.7	67.3
A. anserina[t]	UV proportion	2.4	3, 43	.	11.6	88.4
Argentina aggregate	UV proportion	11.5*	12, 205	20.9	26.0	53.1

Note- *F* and df values are from ANOVA with population as a random effect (a). Tests for a population effect were performed for each taxon separately and then combined (*Argentina* aggregate). [t]UV-absorbing area and petal area were not included in analyses because flowers from three of four *A. anserina* populations were analyzed dry (see methods for details).* $P<0.0001$

Table 2-2: Mean (± SD) and coefficient of variation for concentration of foliar UV-absorbing compounds, components of UV pattern (UV-absorbing area, petal area, UV proportion) and spectral traits (brightness, UV reflectance, UV chroma and green chroma) measured at the petal apex and base measured on greenhouse-grown plants of *Argentina pacifica* and *A. anserina*.

	Mean±SD			CV		
Trait	*A. pacifica*	*A. anserina*	*P*	*A. pacifica*	*A. anserina*	*P*
UV-absorbing compound concentration	0.008±0.001	0.006±0.001	0.01	0.14	0.14	0.42
UV Pattern Components						
UV-absorbing area	78.4±19	27.6±6.5	<0.001	0.24	0.23	0.35
Petal area	99.5±22	60.6±8.6	0.01	0.23	0.14	0.008
UV Proportion	0.80±0.11	0.45±0.06	0.003	0.14	0.13	0.22
Spectral traits: apex						
Brightness	110.8±9.5	130.5±9.7	<0.001	0.09	0.07	0.26
UV reflectance	5.84±2.2	14.0±2.4	0.02	0.37	0.17	<0.001
UV chroma	0.052±0.018	0.11±0.01	0.002	0.34	0.12	<0.001
Green chroma	0.43±0.007	0.41±0.005	0.002	0.02	0.01	0.19
Spectral traits: base						
Brightness	109.4±6.7	110.8±9.2	0.96	0.06	0.08	0.95
UV reflectance	0.8±0.31	0.67±0.16	0.37	0.39	0.24	0.08
UV chroma	0.007±0.003	0.006±0.002	0.33	0.40	0.33	0.16
Green chroma	0.445±0.004	0.45±0.003	0.004	0.01	0.01	0.19

Note- Concentration of vegetative UV-absorbing compounds is in mg quercetin/mg leaf tissue. Components of floral UV pattern are in mm^2. Genotypic means of clones were used for the calculation of summary statistics. *P*-values are from ANOVAs for differences between means, and *F*-tests for differences between CVs.

Table 2-3: Broad-sense heritability (diagonal) and trait correlations (off-diagonal) for the concentration of foliar UV-absorbing compounds (UVAC), UV pattern components; UV-absorbing area (UVA) petal area (PA) and UV proportion (UVP); and spectral properties (brightness, UV reflectance, UV chroma and green chroma) at the petal apex and base for *Argentina pacifica* (a) and *A. anserina* (b).

		UV pattern components				Spectral properties: petal apex				Spectral properties: petal base			
		UVAC	UVA	PA	UVP	Brightness	UV Reflectance	UV Chroma	Green Chroma	Brightness	UV Reflectance	UV Chroma	Green Chroma
UV pattern components	UVAC	0.17*	0.28	0.38**	0.01	-0.04	0.07	0.05	-0.04	0.18	-0.17	-0.22	0.17
	UVA		0.43*	0.75***	0.59***	-0.03	0.07	0.08	-0.04	-0.01	-0.04	-0.09	0.16
	PA			0.32*	-0.06	0.12	0.41**	0.47**	-0.34*	-0.02	0.12	0.07	0.19
	UVP				0.86*	-0.23	-0.49**	-0.54***	0.38**	-0.01	-0.17	-0.15	-0.03
Spectral properties: apex	Brightness					0.18*	0.45**	0.22	0.11	0.02	0.02	0.02	0.02
	UV Reflectance						0.18*	0.87***	-0.55***	0.06	-0.11	-0.16	0.29*
	UV Chroma							0.20*	-0.73***	0.09	0.04	-0.04	0.28
	Green Chroma								0.17*	0.11	-0.29*	-0.25	0.21
Spectral properties: base	Brightness									0.02	-0.17	-0.38**	0.46**
	UV Reflectance										-0.04	0.96***	-0.55***
	UV Chroma											0	-0.65***
	Green Chroma												0.03

(b) *Argentina anserina*

UV pattern components	UVAC	0.16	0.37	0.42*	0.2	0.37	0.23	0.02	0.12	0.38	-0.43*	-0.48*	-0.05
	UVA		0.74*	0.88***	0.86***	0.32	0.04	-0.08	0.07	0.27	-0.19	-0.26	-0.2
	PA			0.42*	0.51*	0.4	0.12	-0.01	0.09	0.28	-0.28	-0.33*	-0.17
	UVP				0.85*	0.08	-0.11	-0.15	0.05	0.14	-0.05	-0.1	-0.18
Spectral properties: apex	Brightness					0	0.82***	0.52*	-0.29	0.49*	-0.09	-0.05	0.03
	UV Reflectance						0.05	0.86***	-0.64**	0.45*	0.34	0.2	0.23
	UV Chroma							0.02	-0.91***	0.12	0.55**	0.49*	0.4
	Green Chroma								0.11	0.07	-0.64**	-0.62**	-0.33

Spectral properties: base	Brightness	0.06	-0.05	-0.36	-0.01
	UV Reflectance		-0.08	0.94***	0.05
	UV Chroma			0.15	0.06
	Green Chroma				0.09

Note- Values for *A. pacifica* are right of diagonal while those for *A. anserina* are left. Heritability values are on the diagonal (top, *A. pacifica*; bottom, *A. anserina*) and noted with an asterisk if significant. For correlations, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. All correlations with $P<0.001$ remained significant after Bonferonni correction for multiple comparisons.

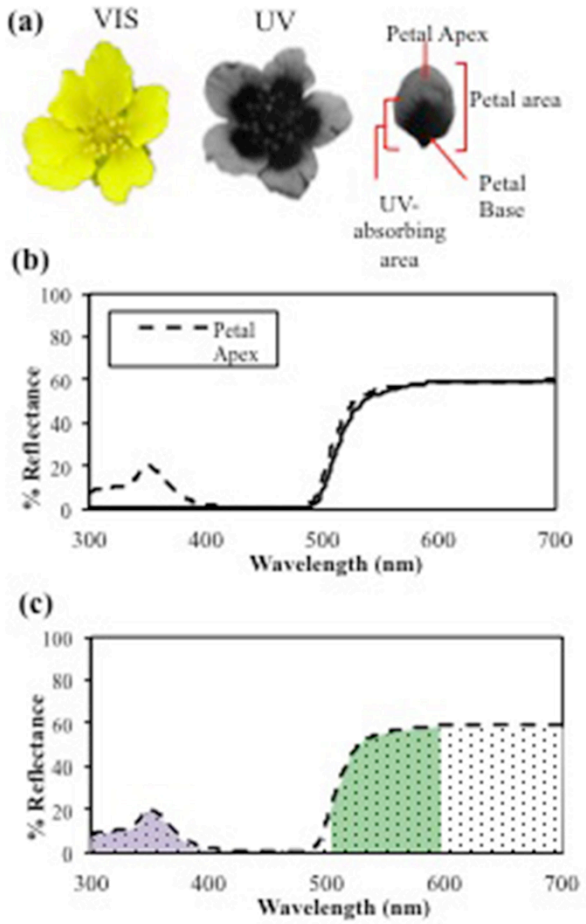


Figure 2-1: (a) *Argentina pacifica* flower in the human-visible (VIS) and ultraviolet (UV) spectrum. Components of floral pattern, UV-absorbing area , and petal area, are depicted on a single petal. UV proportion was measured as UV-absorbing area/petal area. (b) An exemplary floral reflectance spectrum from the petal apex and base of an *A. pacifica* flower. The petal apex reflects in the UV spectrum (300-400 nm) while the base does not. (c) Spectral traits scored at the petal apex and b were: brightness (dotted area), UV reflectance (purple area), UV chroma (purple/dotted), and green chroma (green/dotted).

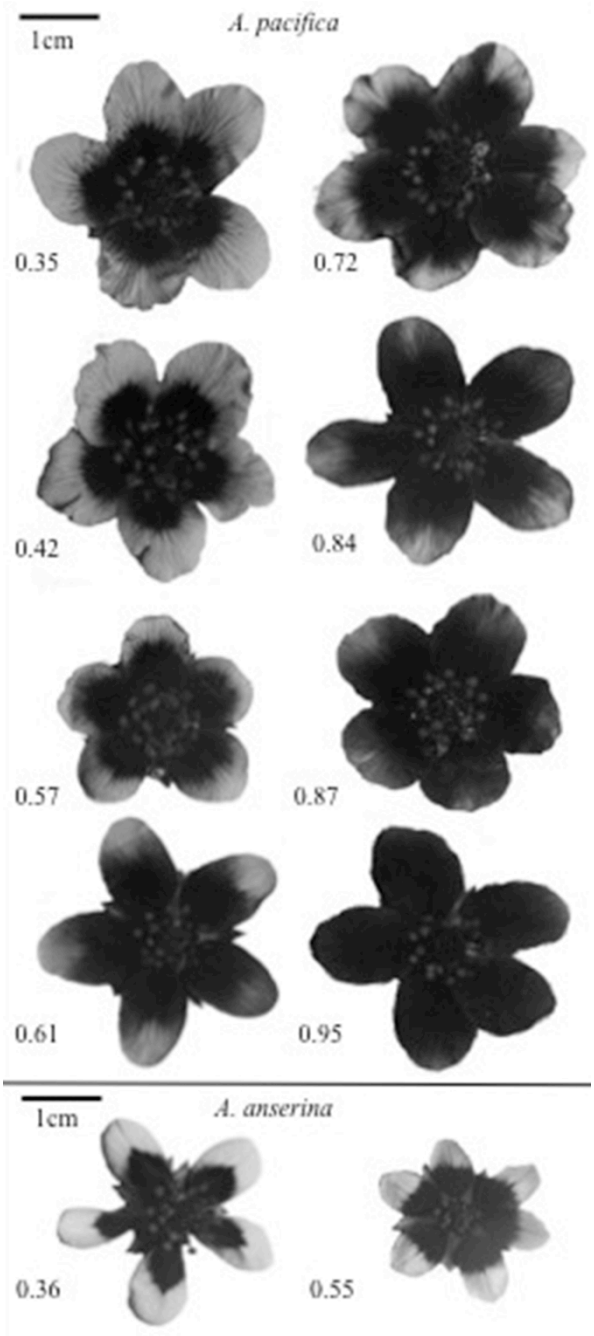


Figure 2-2: Representative UV images of flowers of *Argentina anserina* and *A. pacifica* displaying different UV proportion. Associated UV proportion values are indicated next to each flower.

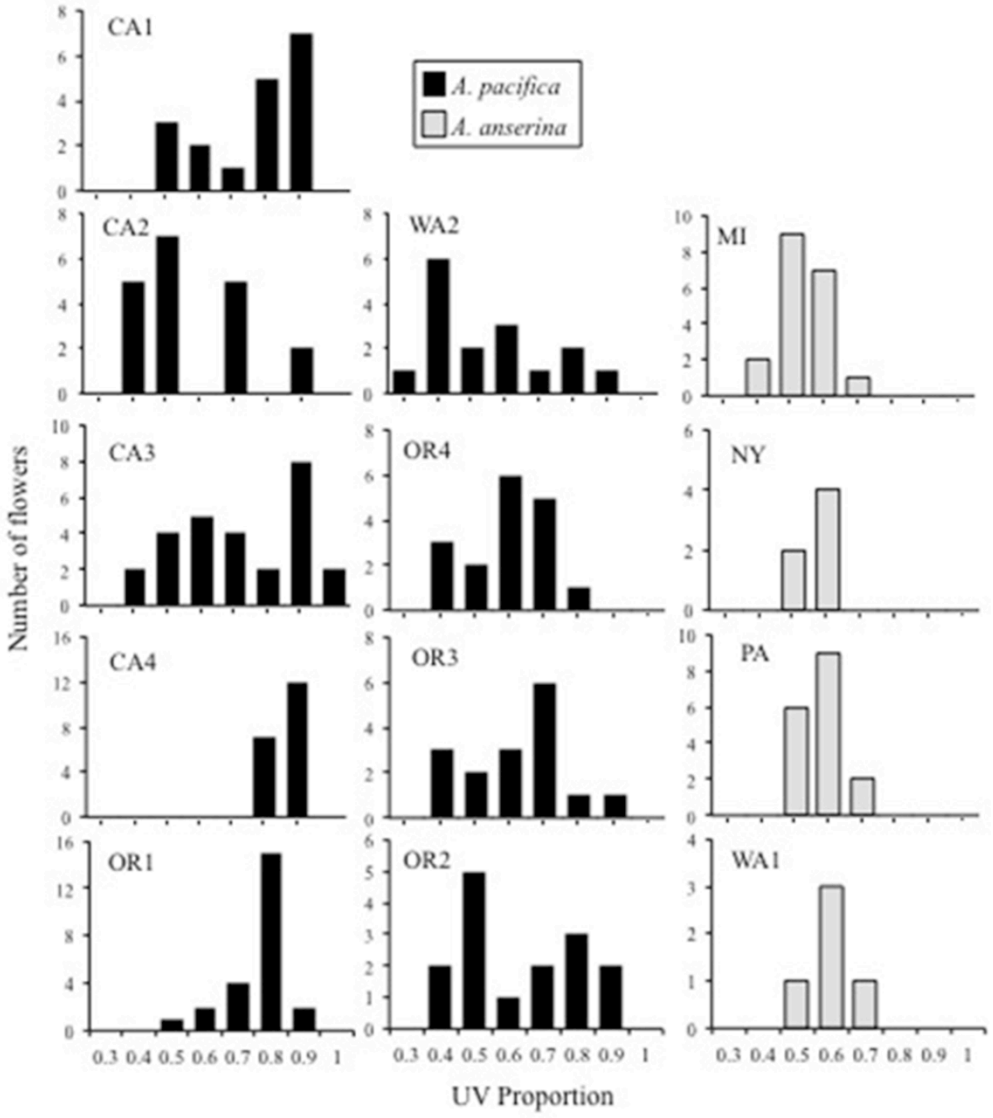


Figure 2-3: Frequency distribution of UV proportion measured in the field from nine *Argentina pacifica* populations (black), and four *A. anserina* populations (gray). See Table A1 for summary statistics and locations for each population.

3.0 DISSECTING POLLINATOR RESPONSES TO A UBIQUITOUS ULTRAVIOLET FLORAL PATTERN IN THE WILD

Koski, M. H. and T.-L. Ashman. 2014. *Functional Ecology* 28: 868-877.

3.1 INTRODUCTION

Circular 'bullseye' color patterns on flowers can attract pollinators (Kulger 1930; Free 1970; Lehrer *et al.* 1995; Johnson & Dafni, 1998; however see Manning 1956) and aid in their proximate orientation to the center of a flower once they arrive (Free 1970; Johnson & Dafni 1998; Manning 1956; Dinkel & Lunau 2001), that is, act as nectar guides. Most insects are UV-perceptive (Briscoe & Chittka 2001) and bullseye floral patterns in the UV spectrum—invisible to the naked human eye—are pervasive among angiosperms (e.g., Horovitz & Cohen 1972; Guldberg & Atsatt 1975). A particular pattern whereby petal bases absorb UV while the apices reflect UV, is found in many systems (e.g., Thompson *et al.* 1972; Horovitz & Cohen 1972; Guldberg & Atsatt 1975; Naruhashi & Ikeda 1999; Gronquist *et al.* 2001). Since UV reflection is relevant to most insect visual systems, the assertion that this pattern functions as a nectar guide for pollinators has been widely held (Thompson *et al.* 1972; Eisner *et al.* 1973; Guldberg & Attsat 1975; Utech & Kawano 1975). Despite this, and the fact that UV reflectance may be as relevant to insect visual systems as human-visible color (Kevan *et al.* 2001), most studies have explored the effects of such patterns that are clearly visible to humans on pollinator visitation and behavior. There is a need to test whether UV reflection and/or pattern on petals increases floral conspicuousness and whether pattern does indeed aid in pollinator orientation.

The UV bullseye on flowers manifests from UV-absorptive petal bases and UV-reflective petal apices. UV-absorption at the central part of flowers creates a "gradient of centripetally

increasing spectral purity" and it is suggested this can enhance pollinator foraging efficiency (Lunau 1992). Some support for the nectar guide function of the UV bullseye comes from laboratory experiments where UV-absorptive regions associated with nectar rewards on false flowers elicited a foraging response in *Apis mellifera* (Daumer 1956). Further, Lunau and Wacht (1994) showed that the syrphid fly, *Eristalis tenax,* extended its proboscis over areas of purely green/yellow reflection, but the presence of UV reflection inhibited this behavior, suggesting that UV-absorption may be important for the elicitation of foraging behavior. In the field various bee species (*Xylocopa* spp., *Centris* spp., *Gaesischia exul*, *Megachile* sp., *Trigonia* spp.) were observed landing on the UV-absorptive banner petals of asymmetric flowered *Caesalpinia eriostachys* and *Parkinsonia aculeata* (Fabaceae) (Jones & Buchmann 1974), and both UV and human-visible petal markings on *Delphinium nelsonii* flowers influenced bumblebee preference and behavior (Waser & Price 1985). In none of these study species, however, did the flower possess the classic UV bullseye pattern of actinomorphic flowers, and there are limitations in extending generalities from laboratory studies to field conditions with regard to the function of floral UV pattern as a nectar guide. Behaviors elicited by UV reflection and absorption in lab-reared insects on artificial flowers may not hold for all flower-visiting taxa, and thus the use of naturally occurring, non-naïve insects and natural flowers can shed light on the function of varying floral patterns in a natural pollination community. For example, *Argentina anserina*, the focus of this study, is only very rarely visited by honeybees or bumblebees (M. Koski, personal observation), thus these insects for which we have the most behavioral data are unlikely to be the most important taxa to influence evolution of floral traits in this system. While learning can affect the preference of pollinators for certain floral phenotypes (e.g., Laverty 1980), examining the behaviors of experienced individuals can provide a 'real-world' picture of how varying floral phenotypes affect insect behavior and consequently, plant reproductive fitness.

More recent field experiments have shown that elimination of UV-reflection from petals can decrease visitation by various bee species; *Apis mellifera scutellata* (Johnson & Andersson 2002; Welsford & Johnson 2012), *Lipotriches* spp. (Peter & Johnson 2008; Welsford & Johnson 2012), *Bombus* spp. (Rae & Vamosi 2012), and *Patellapis* sp. (Welsford & Johnson 2012). Male individuals of the bee fly, *Megapalpus capensis*, show preference for more complex patterns of UV reflective petal spots on *Gorteria diffusa* (de Jager & Ellis 2012). However, UV reflection did not influence visitation from the syrphid flies, *Allograpta* spp. or the bee, *Hylaeus matamoko* (Campbell *et al.* 2010). In most of these manipulative experiments, UV-reflection was uniformly reduced across petals, eliminating any naturally-occurring spatial variation of UV reflection. As a result it remains unclear whether elimination of UV-reflection or the elimination of pattern itself reduces the conspicuousness of flowers. Examining this distinction can provide insight into the evolution of floral UV traits. For instance, it is held that UV reflection can increase the conspicuousness of flowers, however since the majority of flowers that reflect UV also have a bull's eye pattern, there may also be an advantage to maintaining some degree of UV absorbance to achieve floral color contrast (e.g., Lunau 1992; Lunau & Wacht 1994). A study that compares visitation between UV-patterned flowers and those that either uniformly absorb *or* reflect UV would help to clarify this issue. Which feature is most important for mediating pollinator visitation and orientation behavior has yet to be determined for any species with UV pattern despite the fact that UV reflective, bull's eye, and uniformly UV absorbing petals are all phenotypes that exist in nature (e.g., Riesberg & Schilling 1985; Naruhashi & Ikeda 1992; Koski, unpublished). Further, whether the UV absorbing base of petals, the most common UV flower pattern, is preferred by pollinators relative to the reverse pattern (e.g., UV-reflective petal bases and –absorbing apices) has not been tested in the field.

Different flower color preference among taxa that pollinate the same species can explain

the maintenance of flower color variation in the human-visible spectrum (e.g., Streisfeld & Kohn 2007). While most important flower-visiting insects (Hymenoptera, Diptera, Lepidoptera) have visual acuity in the UV spectrum (Briscoe & Chittka 2001), sensitivities can vary among taxa. For example, the wavelength of peak UV sensitivity varies slightly among hymenopterans (Peitsch *et al.*1992), and some dipterans possess accessory UV pigments that may heighten their UV sensitivity relative to most hymenopterans (Warrant & Nilsson 1996; Briscoe & Chittka 2001). Thus, UV features on flowers may be more important to some taxa than others, and differential responses to varying intensity of UV or varying UV patterns on petals could be taxon-specific. Such variation may be important for generalist pollinated plants with UV pattern variation. Indeed, both discrete and quantitative variation in the presence or size of the UV bull's eye are known not only in *Argentina anserina* (Koski & Ashman, 2013), but in other systems as well (Cruden 1972; Naruhashi & Ikeda 1999).

Here, we manipulate UV floral properties on the petals of *Argentina anserina* L. (Rosaceae), a widespread, generalist-pollinated plant whose uniformly yellow flowers have a bullseye in the UV spectrum (Koski & Ashman 2013). We use field experiments to compare attraction rates (number of approach, landing, and foraging visits per flower per hour), foraging rate (number of foraging visits per flower per hour), foraging behavior (likelihood of foraging), and orientation behavior (likelihood of orienting to the center of the flower) of small bees and syrphid flies to flowers with petals that possess a UV bullseye pattern versus those with (a) no UV reflection/no pattern (Fig. 1a), (b) full UV reflection/no pattern (Fig. 1b) and (c) an inverted pattern of UV-reflection (UV-absorption at the apex, reflection at the base; Fig. 1c). We address the following questions: 1) Does elimination of UV reflection and/or the elimination of the bullseye pattern reduce pollinator attraction or foraging rate and/or retard foraging and orientation behavior? 2) Is the common pattern (UV-absorptive flower center) preferred, or would the inverse pattern

also increase pollinator attraction or foraging rate, and enhance foraging and orientation behavior? 3) Do bees and flies respond similarly to different UV patterns? We discuss our results in the context of the potential adaptive function of the UV bullseye and consequences for species with variation in UV floral phenotypes.

3.2 METHODS

3.2.1 Study system

Argentina anserina (formerly *Potentilla anserina*) is a self-incompatible, hermaphroditic, stoloniferous herb that inhabits moist areas in Europe and North America (Rousi 1965). Its flowers are predominantly visited by small bees and syrphid flies (Koski pers. obs.) but bumblebees have also been observed (Miyanishi *et al.* 1991). While flowers appear uniformly yellow to humans, the apices of petals are UV-reflective while the bases are UV-absorptive and classified as 'UV-green' to bees (Gumbert *et al.* 1999; Arnold *et al.* 2010). The area of floral UV absorption relative to flower area (hereafter, UV Proportion) is variable for *A. anserina* (0.30-0.99), and in the Great Lakes Region, the area for the current study, it ranges from 0.43 to 0.73 in the field (Koski & Ashman 2013).

3.2.2 Study area and plant material

Pollinator observations at arrays of manipulated *A. anserina* flowers took place at Pymatuning Laboratory of Ecology (PLE) in Northwestern Pennsylvania (PLE; 41° 38' 35.14" N 80° 25' 32.10" W). *Argentina anserina* did not occur in the immediate vicinity of the arrays, and the only known species nearby the arrays with a UV bullseye was *Ranunculus acris* (Ranunculaceae) which grew in roadside ditches ~0.5 miles from the arrays (M. Koski, Pers. Obs.). One closely related,

yellow-flowered species, *Potentilla canadensis*, was flowering in very low abundance near the arrays and its petals were completely UV-absorbing (M. Koski, unpublished). Dominant flowering species at the site included *Fragaria virginiana*, and *Rubus allegheniensis* (both with actinomorphic, white, uniformly UV-absorbing flowers), *Securigera varia*, and *Lotus sp.* (both with zygomorphic, uniformly UV-absorbing flowers) (Koski, unpublished). *Argentina anserina* flowers used in artificial arrays were collected from two populations (41° 54' 24.07" N 80° 48' 15.05" W and 41° 51' 09.57" N 80° 33' 31.65" W) on the shore of Lake Erie in Northeastern Ohio, USA, and transported in a cooler to PLE. Flower size was not different between the populations (diam., mean±SE; 15.9±0.55mm vs. 16.4±0.82mm; t= -0.54, P=0.59), and collections from these were pooled into a stock 'population' from which we randomly allocated flowers to the following experiments.

3.2.3 Floral manipulation

Three array types were created: UV absorbing (Fig. 1a), UV reflecting (Fig. 1b) and inverse UV bullseye (Fig. 1c). In each array there were two flowers in each of three categories; two control groups (O and T) with the 'wild type' phenotype of a UV bullseye, and a test group with a novel floral phenotype (absorbing [A], reflecting [R], or inverse [I]; Figs 1a-c). One control flower accounted for olfactory changes (O) while the other controlled for both olfactory and tactile changes (T) made to the test flowers.

To achieve the absorbing test flower (A), we spread a mixture of Parsol MXC and Parsol 1789 sunscreens dissolved into duck preen gland fat (Marryat Real Duck Grease, Switzerland) (hereafter, sunscreen mixture) onto the upper side of petals with a small paintbrush (Johnson & Andersson 2002; Peter & Johnson 2008). To control for scent of the sunscreen mixture, the O flower received this same mixture on the underside of petals. To control for both scent and petal

texture the T flower received duck fat on the top and the sunscreen mixture on the bottom of petals. Duck fat alone did not greatly alter the spectral properties of the petals when applied to their upper side (Fig. 1a, Fig S1a,b).

To achieve complete UV reflection on petals of the reflecting test flower (R), the upper surface of petals was painted with yellow UV-reflective paint (UV Yellow, Fish Vision UV Lure Paint) (Fig. 1b). Similar UV-reflective paints were used to manipulate eye-spot phenotypes on butterfly wings (Prudic *et al.* 2011). We applied paint to the underside of petals on the O flower to control for scent. On the T flower, we painted only the UV-reflective apices of upper surface of petals to control for scent and petal texture. The paint was scented (Koski pers. obs.) and had only a slightly higher reflectance than naturally occurring tips of flowers (Fig. S1a, d, e).

For the inverse test flower (I) we painted the base of petals with yellow UV-reflective paint and the apex of petals with the sunscreen mixture. This effectively inverted the bullseye pattern in the UV (Fig. 1c). The O flowers received UV reflective paint and the sunscreen mixture on the underside of the petals. The T flowers received paint on the UV-reflective apices of petals and sunscreen on the UV-absorptive bases of petals (recreating the 'wild type' bullseye phenotype). For all manipulations, care was taken to avoid spreading paint or sunscreen over nectaries and anthers. We measured spectral reflectance at the petal base and apex for all of the controls and test flowers with an Ocean Optics USB4000 spectrometer with a UV-NIR DH-2000-BAL deuterium-tungsten light source (see Appendix S1 in Supporting Information).

3.2.4 Array set up and pollinator observations

We recorded pollinator responses to flowers in arrays from May 17 to June 18, 2012. Circular arrays consisted of six flowers in total—two flowers of each type (O, T controls and an A, R or I test, as appropriate) arranged alternately (Fig. 2). Flowers were placed in water-filled

microcentrifuge tube 'aquapics' attached to stakes to achieve natural flower height (Fig. 2). Observation periods of single arrays ranged from 45 min to 2 hrs. At half hour intervals, the flowers were rotated to a different location in the circular array. If a flower wilted before the observation period was completed, we replaced it with a fresh flower of the same treatment type, and to control for the presence of a fresh flower in one treatment group, we replaced a single random flower from each of the other treatments with a fresh flower. Two arrays were placed ~7m apart and were observed simultaneously by different observers. Halfway through each observation period, flowers from one array were swapped with those from the other array to reduce spatial and observer bias. We alternated array types between morning (900 to 1200 hrs) and afternoon (1200 to 1600 hrs). In total, we observed 12 arrays with A test flowers ('absorbing arrays'; 22.25 hrs of observation), 12 with R test flowers ('reflecting arrays'; 21.75 hrs) and 10 with I test flowers ('inverse arrays'; 19 hrs).

We recorded all insects that a) approached, b) landed on, or c) landed on and foraged at a flower. Approach visits were scored if a pollinator hovered over, but did not make contact with the flower. Landing visits consisted of those in which pollinators landed on a petal (regardless of orientation) but did not forage for nectar or pollen. Visits were scored as foraging when insects clearly sought pollen and/or nectar. In general, bees foraged by pivoting over the gynoecium and androecium, or orienting their bodies horizontally at the base of the androecioum and moving in circles around it (e.g., Chagnon *et al.* 1993), while flies positioned themselves over the gynoecium and androecium, or on the petal and probed at nectaries at the base of the petals (Fig. 2). Activity of flower-visitors was high, making it difficult to determine whether a pollinator was making its first visit to the array, so we recorded all visits including successive visits by the same insect to a different flower in the array. However, if a pollinator left a given flower and quickly revisited it, then this was considered a single visit. Both first and subsequent visits by a pollinator reflect

preferences of the insect for a floral phenotype, and it is not uncommon to consider both in datasets of pollinator behavior (e.g., Schemske & Bradshaw 1999). We note that multiple visits from the same insect are not independent data points as a result of learned or inherent behavioral differences between individuals. We believe however, that the data are representative of the choices of many insects as insect activity was very high at arrays and on natural flowers surrounding arrays (>200 insects in a 10x10m area during a given array; Koski, personal observation) and experiments were conducted over a period greater than one month. Thus, data are unlikely biased towards only a few individual insects.

For visits in which insects landed on flowers, we recorded orientation behavior as a) oriented to the center of the flower (either by landing on the center or landing on the edge and walking to the center), or b) did not orient to the center of the flower (e.g., walked across petal without walking to the center). Across all array types, 98.5% of visits were by bees and syrphid flies, and these were recorded separately so that we could determine whether response to flowers differed between these broad groups. We were unable to identify visitors to a more detailed taxonomic distinction than 'bee' and 'fly' due to high rates of visitation. Members of the fly group included syrphid flies (Syrphidae) from two genera; *Episyrphus* and *Sphaerophoria*. Peak visual sensitivity in the UV spectrum is known in at least seven dipteran species, including one syrphid (*Eristalis tenax;* Horridge *et al.* 1975). Bee visitors to arrays were from four families; Apidae (*Epioloides* sp., *Holcopasites* sp.), Andrenidae (*Callopsis coloradensis, Callopsis* sp., *Perdita* sp.), Megachilidae (*Stelis* sp.) and Halictidae (*Lasioglossum* spp.). The majority of Apidae species whose visual systems have been characterized are UV-sensitive, as are all Andrenidae and Megachilidae, but UV-sensitivity is not yet known for Halictidae species (Peitsch *et al.* 1992). Other rare visitors (Lepidoptera, ant, large fly, small muscid fly, ladybug, weevils) did not contact reproductive parts and were not considered further. Similar types of insects have been observed to

visit flowers in natural populations in the Great Lakes area (Koski, personal observation).

For each replicate array we characterized four responses to each floral phenotype for bees and flies separately as follows. We calculated total attraction rate to each flower type (visits/flw/hr) using all types of visits recorded for each group (approach without landing, land without foraging, and foraging). This metric represents the degree to which insects are attracted, from a distance, to a given flower type. We then assessed foraging rate to each flower type (foraging visits/flw/hr) using only the visits in which insects foraged. This metric categorizes a 'legitimate' visit that is the best proxy for the pollination success of a flower. We then scored foraging behavior as the proportion of total visits that led to foraging (hereafter 'proportion foraging'). Finally, we scored orientation behavior as the proportion of landing visits (landed and landed/foraged) that led to centering (hereafter 'proportion centering').

3.2.5 Statistical analyses

For each array type, we analyzed attraction rate and foraging rate using mixed-model ANOVAs (SAS, Proc MIXED; SAS v. 9.3, SAS Institute, Cary, NC) with flower treatment, pollinator type and flower x pollinator as fixed effects, and replicate and all interactions with replicate as random effects. We used planned contrasts to compare rates between the controls O and T (which have the same visual phenotype), and between T and a given test (A, R, or I) flower (different phenotypes) (e.g., Meléndez-Ackerman & Campbell 1998). When the two controls were not significantly different, it indicates that there was no effect of the paints/carriers on behaviors to the phenotypically identical flowers, and they were pooled and compared to the test flower. If O and T were different, this indicated that manipulation to the upper side of petals influenced flower-visitors, and thus, the comparison between T and the test flower offered the best evaluation of the sole effect of the flowers' visual phenotype. We present both the results of the pooled contrast and

contrasts between the T flower and a given test (A, R, or I). Attraction rate was ln transformed for the reflecting array, and attraction rate and foraging rate were ln + 1 transformed for the absorbing and inverse arrays to satisfy the assumption of normality.

To test the effect of flower treatment on foraging behavior (proportion of total visits that resulted in foraging) in the absorbing and inverse arrays we used mixed-model ANOVAs (SAS, Proc MIXED) with flower, pollinator, and their interaction as fixed effects, and replicate and all interactions with replicate as random effects. In the reflecting array, however, data were negatively skewed and could not be transformed to meet the assumption of normality, so we used a generalized linear mixed model (SAS, Proc GLIMMIX) with a binomial distribution and a Logit link function. When modeled with a binomial distribution the Generalized χ^2/DF value was closer to one (1.12) than when modeled with a Poisson distribution (0.11), indicating that the binomial distribution was a better fit for the data (Schabenberger 2005).

To test the effect of floral manipulations on the orienting behavior we analyzed proportion of centering visits using a generalized linear mixed model as described above with a binomial distribution. For each array, the χ^2/DF value was closer to one when using a binomial distribution (0.84-1.23) than when using a Poisson distribution (0.02-0.04).

3.3 RESULTS

3.3.1 Absorbing arrays

We observed 930 bee visits and 319 fly visits across the 12 replicates of absorbing arrays, and foraging behavior was recorded for all 1249 visits. Of the 1004 visits in which pollinators landed on flowers, we scored orientation behavior for 987 visits. Flower type significantly influenced

attraction rate (Table 1a, Fig. 3a). Attraction rate to the fully absorbing (A) test flowers was, on average, ~13% lower than to control flowers (O and T) with a bullseye, which were not different from each other (O vs. T; Table 1a, Fig. 3a). This significant difference persisted in the comparison of the T with the A flower, attraction rate to the absorbing flower was ~10% lower (Table 1a, Fig. 3a). Foraging rate was influenced by flower type but the difference was between the controls (Table 1a, Fig. 3d), and thus bull's eye flowers did not elicit a higher rate of foraging than the fully absorbing flowers. There was no flower by pollinator type interaction (Table 1a) effect on either attraction or foraging rate, indicating that the response to UV manipulation was similar between bees and flies.

While bees were more likely to forage during a visit than flies (Table 1a), the likelihood of foraging visits was not different between flower types (Table 1a; Fig. 3g). That is, pollinators were equally likely to forage on a bullseye control flowers (O or T) as they were on absorbing (A) flowers. There was no flower by pollinator type effect on proportion foraging (Table 1a). Again, bees were more likely to orient to center than flies once they landed on flowers (Table 1), but orientation behavior was not influenced by flower type (Table 1a; Fig. 3j).

3.3.2 Reflecting arrays

Across the 12 replicates of reflecting arrays we observed 830 bee visits and 484 fly visits (n=1314). Foraging behavior was scored for all but one visit (n=1313) and orientation behavior was scored for 1009 of the 1020 visits in which pollinators landed. Overall, flower type influenced attraction rate (Table 1b). For both pollinator groups uniform UV reflection and elimination of pattern (R flower) decreased attraction rates significantly (~18%) relative to the controls (O and T) which did not elicit different attraction rates (Table 1b; Fig. 3b). A significant reduction to the fully reflective flower (~11%) still exists when comparing only the T and the R flower (Table 1b).

Despite the fact that attraction rate was influenced by flower type, the rate of foraging visits was only marginally influenced (F=2.66, P=0.09, Table 1).

Flower type did not influenced the likelihood of pollinator foraging (Table 1b; Fig. 3h) or orientation of either pollinator group (Table 1b; Fig. 3k) but bees and flies differed in these behaviors (Table 1b).

3.3.3 Inverse arrays

We observed 1077 bee and 372 fly visits across the ten replicate inverse arrays and foraging behavior was scored for every visit (n=1449). Orientation behavior was scored for all visits in which pollinators landed on the flower (n=1093). Floral manipulation affected attraction rate and foraging rate (Table 1c, Fig. 3c, f). The 'normal' bullseye pattern controls had the highest attraction rate (~16% higher than inverse) and did not differ from one another (Table 1c). Comparing attraction rates of the I to the T only, there was a 12% reduction for the inverse flower type. Foraging rates were lower in the inverse treatment when controls were grouped (~21%) and when only comparing the T and I flower (~15%) (Table 1c). Bees and flies responded similarly to the floral manipulations (Table 1c).

Flower type influenced the proportion of foraging visits but the difference was between the controls (O vs T, Table 1c, Fig. 3i) rather than the tactile control and the test flower (T vs. I, Table 1c), indicating that the inverse UV pattern did not affect the probability of foraging. Flower type did not affect the likelihood of pollinators orienting to the center of flowers, but bees and flies differed in this behavior (Table 1c).

3.4 DISCUSSION

We show experimentally that the presence of the floral UV bullseye pattern increases the

conspicuousness of flowers to small bees and syrphid flies, but not their likelihood of foraging or orienting. Our findings are important contributions to the understanding of the role of UV reflection and pattern in mediating plant-pollinator interactions for three primary reasons. First, the presence of the UV bullseye did not increase the likelihood of insect foraging nor their ability to orient to the center of flowers, calling into question its function as a nectar guide at close range. Second, in other studies UV-reflection alone is shown to increase insect visitation, but we found that an increased area of UV reflection on petals led to a decrease in insect attraction relative to flowers with patterned petals, and we attribute this to the elimination of pattern. Third, we confirm that the most common UV floral pattern—UV-absorbing petal bases and reflecting tips—was more conspicuous to bees and flies than the inverse pattern, and increased the foraging rate, but did not affect the likelihood of insect foraging or orienting to the flowers' center. We suggest that this specific UV pattern functions to increase floral apparency from a distance but may not necessarily act as a proximate pollinator orientation guide as long proposed.

3.4.1 Is the UV bullseye a nectar guide?

While many studies describe UV absorption at petal bases as a nectar guide (Thompson *et al.* 1972; Eisner *et al.* 1973; Guldberg & Attsat 1975; Utech & Kawano 1975) we found that UV-absorbing petal bases had no effect on bee or fly orientation to floral rewards or their likelihood of foraging despite high power to detect these effects afforded by the large number of visitors observed and replication of each array type. In contrast, Jones and Buchmann (1974) observed various taxa of bees orienting to the UV-absorptive petal of two species (*Caesalpinia eriostachys* and *Parkinsonia aculeata*). Two factors differ between our study system and those of Jones and Buchmann which could contribute to the disparity between our findings: 1) Flowers of *Argentina anserina* are radially symmetric, and 2) Floral rewards (pollen and nectar) are not concealed.

Conversely, the species studied by Jones and Buchmann have irregular flowers with concealed nectar. If these differences are causal, then together, the two studies bolster the assertion that nectar guides are more important in irregular flowers than symmetric ones because nectaries of the former are more difficult for pollinators to locate (Manning 1956). We suggest that, in our system, pollen and/or scent cues (Lunau 2000; Pernal & Currie 2002: Ashman *et al.* 2005) may alone be effective orientation guides. Our study joins Kulger (1930) who found that bumblebees were equally as likely to locate rewards on flowers with and without central nectar guides. We therefore caution against assuming that UV patterns on petals function as orientation cues in all systems. However we acknowledge that handling time was not assessed in our study and others have shown that handling time can be reduced by the presence of nectar guides (Waser & Price 1983; Leonard & Papaj 2011).

Interestingly, lab and field studies have shown that the presence of a 'target' or bullseye can increase the ability of pollinators to orient to the center of a flower or flower mimic (Free 1970; Johnson & Dafni 1998; Manning 1956; Dinkel & Lunau 2001). A behavioral explanation for why we did not find this is that experienced flower-visitors may be accustomed to landing on the center of radially symmetric flowers regardless of the presence of a target. It is possible that the presence of the bullseye pattern influences learning behavior in early life, but fails to function as a nectar guide for an experienced pollinator. However, Leonard and Papaj (2011) showed that linear markings on flower petals increased the ability of *Bombus impatiens* to discover nectar both immediately (inherently) and after experience with foraging. The artificial flowers used by Leonard and Papaj were about three times larger than the natural flowers of *A. anserina* however. 'Nectar guides' may be more likely to orient pollinators in larger-flowered systems, and studies that consider the effect of flower size on the magnitude of 'nectar guide' effectiveness would help to address this proposed idea.

3.4.2 UV reflectance or pattern: which mediates visitation?

Recent studies show that floral UV-reflectance mediates plant-pollinator interactions. Namely, elimination of UV-reflectance from petals reduced visitation rate by various bees (Johnson & Andersson 2002; Rae & Vamosi 2012; Welsford & Johnson 2012) and reduced visitation due to loss of UV-reflectance can reduce reproductive fitness (Peter & Johnson 2008). In the present study, elimination of UV-reflectance and pattern from petals in the absorbing array did indeed reduce attraction rates (Fig 3a), but surprisingly, eliminating pattern by increasing the UV-reflective area on petals had the same effect (Fig. 3b). Thus, our results suggest that contrast in the UV spectrum on petals (UV pattern) may be more important in mediating insect attraction than UV reflection alone. Our work corroborates experiments by Hertz (1931) which suggest that more 'broken' patterns are preferred to less 'broken' patterns, and Kulger (1930) who found that the presence of nectar guides influences conspicuousness of flowers at a distance, but not insect orientation behavior once in contact with the flower.

Examining only foraging rate (i.e., legitimate visits), the elimination of the bullseye did not reduce visitation in the absorbing array suggesting that, despite the fact that bullseye flowers were more conspicuous, they did not experience increased functional visitation. When pattern was eliminated such that the petals were uniformly UV-reflecting, the bullseye flower tended to receive higher foraging rates (Fig. 3e). Interestingly, despite the non-significant overall effect of flower (P=0.09), pairwise comparisons show that the entirely reflecting flower experienced a marginally significant reduction in foraging rate (T vs R, P=0.07, O and T vs. R, P=0.03; Fig. 3e). Thus, considering the results from the absorbing and reflecting arrays together, we can cautiously speculate that the presence of UV absorption (uniform or bullseye) could be effective at eliciting foraging behavior, corroborating Daumer (1956), and Lunau and Wacht (1994). However, this

assertion needs to be substantiated with data from arrays that simultaneously compare pollinator responses to UV-reflecting and -absorbing flower types.

3.4.3 Pervasiveness of the UV bullseye flower pattern

The UV bullseye pattern is common among angiosperms and not restricted to any particular plant family (e.g., Asteraceae, Thompson *et al*., 1972; Skogin 1977; Brassicaceae, Horovitz & Cohen, 1972; Rosaceae, Naruhashi & Ikeda 1999). The rate of foraging, a metric that only included the 'legitimate' visits in which insects made contact with reproductive parts of flowers, was only influenced by inverse flower type, in which the common bullseye pattern was reversed. Thus, our results suggest that there may be a fitness advantage for individuals with the common UV bullseye relative to the inverse pattern via increased pollinator foraging, however, phylogenetically-controlled tests are required to show that this phenotype is an example of convergent evolution in response to pollinator preference. Lunau (1992) suggests that a UV-absorptive flower centers can aid in recognition, orientation, and landing abilities of bees and flies. We show that UV-absorptive flower centers may indeed be important for floral apparency as a result of either inherent preference for floral UV bullseye patterns or a familiarity with these in nature (floral constancy). A possible explanation for reduced attraction rates to the inversed bullseye flowers may derive from its lower contrast from the vegetative background. UV-reflection from green vegetation is low (generally <5%; Caldwell *et al.* 1983), so flowers with UV-reflective petal apices may be more apparent to insects. Reduced contrast from the background for the inverse flower may have resulted in a visually smaller flower than those with the common UV bullseye.

We observed behaviors of wild insects and do not know their level of experience. Given that the pollinators were not necessarily naïve, we can not address whether the behavior we

recorded was innate or learned. However, others have shown that naïve bees show a preference for patterned flower mimics as opposed to non-patterned flower mimics (Free 1970; Lehrer *et al*. 1995; Leonard & Papaj 2012). In the field, visitation to both mimic and real flowers is increased by the presence of pattern (bees; Hansen *et al.* 2011; flies; Johnson & Dafni 1998). Pollinators in the present study may have been experienced with other flowers that possess the more common UV bullseye pattern because *Ranunculus acris,* which has a yellow, UV bullseye flower, did grow in the area. The dominant flowering plants within the immediate vicinity of arrays, however, did not possess the UV bullseye pattern (see Methods). If pollinators foraged locally, then they would not have been exposed to flowers with a UV bullseye pattern. Further studies using naïve insects will help to determine whether preference for the bull's eye pattern is innate or learned for these insects.

3.4.4 Implications for naturally-occurring variation in UV pattern

This study was aimed at understanding the function of UV pattern experimentally, but it has implications for species with naturally-occurring intraspecific variation for UV pattern. For example, populations can be polymorphic for the presence or absence of the UV bullseye (Naruhashi & Ikeda 1999; Cruden 1972) or can display quantitative variation in its size (Koski & Ashman 2013), and this variation can be heritable (Yoshioka *et al*., 2005; Syafaruddin *et al*., 2006; Koski and Ashman, 2013). If the pollination dynamics observed in the present study are representative of natural conditions in *A. anserina* and other systems, then individuals that lack the UV bullseye may attract fewer insects than those with UV pattern. However, this may not necessarily lead to significant differences in foraging rates (Fig. 3d), which correlate positively with female and male fitness (e.g., Galen 1989; Ashman 2000). This lack of difference could explain the maintenance of extensive variation for the size of the bullseye in many populations of

A. anserina, including fully UV-absorbing (Koski and Ashman 2013). However, our results indicate that flowers with an aberrant bullseye (inverse) would experience reduced fitness (Fig. 3f), and interestingly this phenotype is not known to exist in *A. anserina,* or, to our knowledge, any other species.

Variation for UV pattern exists in many species (e.g., Rieseberg & Schilling 1985; Naruhashi & Ikeda 1992; Koski & Ashman 2013) and our study shows that pollinators respond to this variation. However, understanding the direct fitness consequences of this variation requires further study. We suggest that factors other than pollinators should also be considered. For example, UV-absorbing compounds in petals that give rise to UV pattern could protect against abiotic stress (cold/heat. Rivero *et al*., 2001; UV radiation, Jansen *et al*.1998) or florivore damage (Gronquist *et al*. 2001). Conflicting selection pressures of these types may maintain variation in pattern (e.g., Frey 2004). Selection analyses that utilize natural variation are much needed to deepen our understanding of the functional significance of UV floral patterns.

Table 3-1: Results from linear models testing the effect of flower treatment (O, T, test [A, I, R]), pollinator type (Poll) and their interaction on attraction rate ((approaches+lands+forages)/flowers/hour)), foraging rate (forages/flowers/hour) proportion of foraging visits, and proportion of centering visits in each array type ((a) absorbing, (b) reflecting, (c) inverse). Planned contrasts to test for differences between controls and test flowers (A, I, R) versus controls were used when the effect of flower was significant.

Source of variation and flower contrasts			Attraction Rate	Foraging Rate	% Foraging Visits	% Centering Visits
	num. df	den. df	*F*-value	*F*-value	*F*-value	*F*-value
(a) Absorbing						
Flower	2	20-22	6.91**	4.53*	1.67	0.26
O vs *T*	1	22	1.44	6.89*	-	-
T vs *A*	1	22	5.98*	0	-	-
O+*T* vs *A*	1	22	12.37**	-	-	-
Poll	1	11	13.84**	17.69**	13.03**	14.53**
Flw x Poll	2	20-22	0.17	1.14	1.6	0.2
(b) Reflecting						
Flower	2	18-22	6.57**	2.66	0.16	0.22
O vs *T*	1	22	1.83	-	-	-
T vs *R*	1	22	5.15*	-	-	-
O+*T* vs *R*	1	22	11.56**	-	-	-
Poll	1	11	4.66†	6.57*	8.09*	14.30**
Flw x Poll	2	18-22	0.6	1.61	0.78	0.01
(c) Inverse						
Flower	2	17-18	9.74**	9.16**	3.96*	1.03
O vs *T*	1	18	1.06	3.74	5.93*	-
T vs *I*	1	18	10.24**	5.48*	0	-
O+*T* vs *I*	1	18	18.41***	14.59**	-	-
Poll	1	9	7.16*	9.05*	1.46	21.33**
Flw x Poll	2	17-18	2.75	3.07	0.51	1.71

† $P<0.06$ *$P<0.05$, **$P<0.01$, ***$P<0.001$

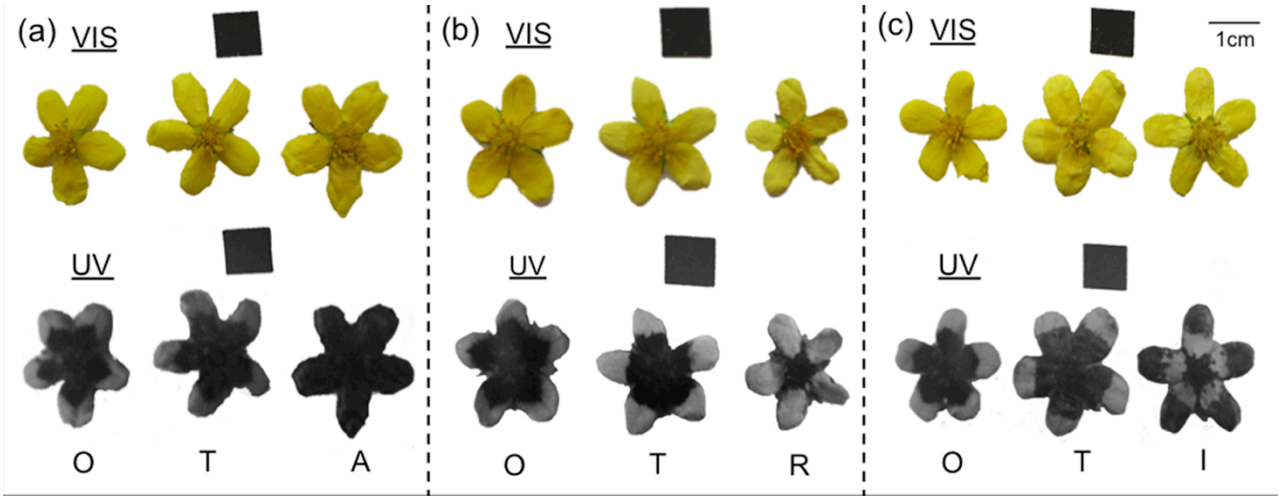


Figure 3-1: Flower types in (a) absorbing, (b) reflecting, and (c) inverse arrays in the human-visible (VIS) and ultraviolet (UV) spectrum. Olfactory and tactile control flowers (O and T) in each array type had a UV bullseye phenotype, while test flowers (A, R, I) differed in UV pattern between array types. A UV-absorbing black standard is included in each photo.

Figure 3-2: A An example of an array in which cut flowers were placed in microcentrifuge tubes elevated on florist sticks (top). A syrphid fly (bottom right) and solitary bee (bottom right) foraging on flowers of *Argentina anserina* in experimental arrays.

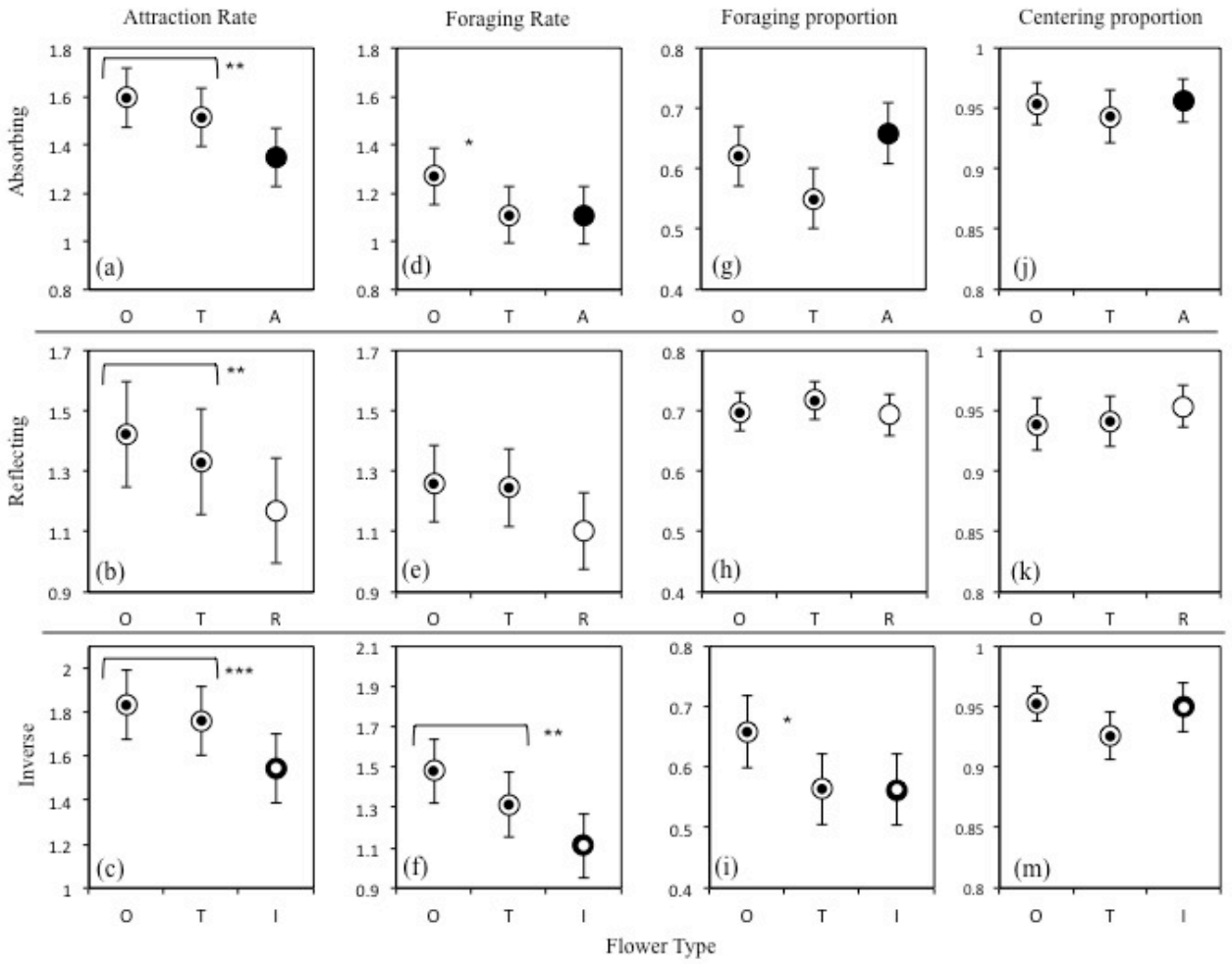


Figure 3-3: The effect of UV pattern on bee and fly attraction rate, foraging rate, likelihood of foraging (foraging proportion) and likelihood of orienting to center (centering proportion) to olfactory and tactile control flowers (O and T) and test flowers (A, R, I) in three array types (absorbing, reflecting, inverse). Brackets indicate that the controls were not different and were therefore pooled and compared to the test flower (Table 1). least squares means +/- SE from linear models using ln or ln+1 transformed (visitation rate) or raw data (foraging proportion) are plotted, and *P*-values are derived from planned contrasts. In cases in which a generalized linear mixed model was used, LSmeans and SEs were back transformed and plotted for graphical purposes (h, j, k, m). Data points denote the floral phenotype where black represents UV-absorption and white represents UV-reflection. Note that scales on the x-axes vary. **P*<0.05, ***P*<0.01, ****P*<0.0001.

4.0 AN ALTITUDINAL CLINE IN UV FLORAL PATTERN CORRESPONDS WITH A BEHAVIORAL CHANGE OF A GENERALIST POLLINATOR ASSEMBLAGE

Koski, M. H. and T.-L. Ashman. 2015. *Ecology*. *in press.*

4.1 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 (Gomez et al. 2009a). 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. 2010), whether pattern affects pollination success (e.g., pollen receipt) has received less attention. Notable exceptions include Medel et al. (2008)

who showed that selection on petal spots depends on the visitation preferences of pollinators (insects versus hummingbirds), and Hansen et al. (2010) 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 ultraviolet (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 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 multi-trait approach that incorporates UV reflectance and pattern.

Changes in altitude can create conditions for clinal variation in plant-pollinator interactions a number of ways (Galen 1989, Campbell 1997, Totland 2001, Malo and Boanza 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 2004). 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 on 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: (a) does the size of the bullseye display an altitudinal cline? (b) does the dominant pollinator type visiting *A. anserina* change with altitude? (c) does floral preference or visitation behavior of pollinators vary with size of the bullseye, and/or altitude? (d) does increased flower visitation lead to increased pollen export and receipt, and (e) does the direction and/or strength of the relationship between bullseye size and pollen receipt change with altitude?

4.2 METHODS

4.2.1 Study System

Argentina anserina (Rosaceae) is a perennial herb, widely distributed in temperate regions (Rousi 1965). In the Colorado Rocky Mountains it grows from ~2100 to ~3650 meters above sea level (hereafter, MASL), and flowers from June to early August. Populations at low altitude flower ~1-2 wks earlier than those at high altitude, but there is substantial flowering overlap (M. H. Koski, pers. obs.). Flowers are radially symmetrical, are borne singly on pedicels, and have numerous (30-40) uni-ovulate pistils. They live one to three days (M. H. Koski, pers. obs.), 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, pers. obs.). 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 (Koski pers. obs.), 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).

4.2.2 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 >1000m (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], % 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 two meter

intervals. Sample size ranged from 2 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 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 1nm intervals and calculated total brightness as reflectance between 300-700nm, and UV chroma as $R_{300\text{-}400}/R_{300\text{-}700}$ 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 et al. 2007). When we detected significant spatial autocorrelation, we determined the best fit correlation function (exponential, Gaussian, or spherical; Dormann et al. 2007) by comparing 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 1993) using the 'nlme' package (Pinheiro et al. 2015).

4.2.3 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 [38.51464N, -106.99503E] and L2 [38.36533N -107.19703E]), and two higher altitude populations (3004, 3295 MASL; hereafter H1 [38.11335N -106.93162E

] and H2 [38.02602N -107.16904E]) 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). Collections took place between 10:00 and 17:00 on 2-5 days from June 9 - July 7 at L1 and L2, and June 29 – July 7 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, pers. obs.), and hereafter are referred to as pollinators. We performed a Spearman's rank correlation between the index and altitude.

4.2.4 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% to 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 dissolved in duck preen gland fat 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 paints. 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 roughly 15cm 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 9-12 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 (SAS PROC GLIMMIX) 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 x 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.

4.2.5 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 0-4 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/pistil 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 0-5 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, however it was not significant, so we report only the results of linear regression.

4.2.6 Relationship 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 (Kearns and Inouye 1993). We enumerated conspecific pollen grains per stigma under a Zeiss Fluorescent microscope. 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 (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, thus 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 (Galen and Newport 1987, Dudley 1996, Caruso 2000, Parachnowitz 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 (PROC GLM). Because petal UV chroma and brightness were not measured in L2, we performed two separate ANCOVAs: (1) with all traits from L1, H1, and H2 and (2) all populations but no data on UV chroma and brightness.

4.3 RESULTS

4.3.1 Floral traits, pollinator assemblage, and altitude

UV bullseye size varied from 34 to 77% of the flower and population mean UVP increased with altitude ($UVP=1.5e^{-4} \times MASL + 6.6E^{-3}$, $R^2=0.83$, $P<0.001$, N=12; Fig. 1). In contrast, variation in

other floral traits (petal size, brightness and UV chroma) was not associated with altitude (*b* range -1.46E^{-5} to 5.8E^{-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 was exponential (AIC_{exp}= -36.7; AIC_{Gauss}= -34.8; AIC_{sphere}= -35.9). Inclusion of spatial autocorrelation in the regression model, however, did not reduce the influence of altitude on bullseye size (UVP = 1.5E^{-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, Table 2a).

4.3.2 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 (Poll. × Alt.: *P* <0.0001, Table 1a; Fig. 2a; Table 2a). Specifically, at low altitude, visits by Hymenoptera were more than eight-times that 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 (Poll: *P*<0.0001). Visitation preference did not depend on pollinator type (Bullseye size × Poll: *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 × Alt: *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 altitudes (Fig. 2b). Bullseye size affected pollinator behavior (Bullseye size × Behavior *P*=0.015, Table 1a), but the strength of the effect depended on altitude (Bullseye size × Behavior × Alt.; *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 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 × Poll. × Behav. × Alt., 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.

4.3.3 Relationship between visit number and pollen receipt and export

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

4.3.4 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). And, this result can be extended to 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 ($\beta'_{\text{bullseye}} = -0.75$; $P<0.0001$; Table 2; Fig 4a), but in H2 larger bullseyes tended to receive more pollen ($\beta'_{\text{bullseye}} = 0.134$, $P=0.08$; Table 2; Fig. 4d). However, no relationship was seen in L2 or H1 (Table 2; Figs. 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 ($\beta'_{\text{UVchroma}} = -0.38$, $P< 0.05$) but not in H1 or H2 (Table 2).

4.4 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 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 *Argentina anserina*, clinal variation in the floral UV bullseye results, at least in part, from changes in floral 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 but a 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 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 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, Gomez et al. 2009a, 2009b), 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, Gomez et al. 2009a,

2009b). 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, the 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 2012) 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 (Koski, pers. obs.), 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.

Table 4-1: (a) Fixed effects from generalized mixed-model testing the effects of altitude (low vs. high), bullseye size (small [~25% absorbing] vs. large [~80% absorbing]), pollinator type (hymenopteran or dipteran), behavior (approach, forage), and their interactions on the number of visits to *Argentina anserina* flowers in floral arrays. (b) Pre-planned contrast between bullseye sizes for foraging visits only within altitudes, and (c) random effects included in the model.

	$F_{1,320}$	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 x Alt.	3.7	0.056
Poll. x Alt.	50	<0.0001
Behavior x Alt.	2.49	0.116
Bullseye x Poll.	0.85	0.356
Bullseye x Behavior	6.01	0.015
Behavior x Poll.	3.18	0.075
Bullseye x Poll. x Alt.	0.13	0.723
Bullseye x Behavior x Alt.	5.91	0.016
Bullseye x Poll. x Behavior	3.61	0.058
Poll. x Behavior. x Alt.	1.32	0.251
Bullseye x Poll. x Behav. x Alt.	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	Z	P
Population (Altitude)	0.078	0.28
Array (Population)	2.46	0.007

† $F_{1, 3.53}$, Alt.= Altitude, Bullseye =bullseye size, Poll. = Pollinator type, Behav. = Behavior

Table 4-2: (a) Altitude (meters above sea level) and the proportion of dipteran pollinators visiting *Argentina anserina* flowers in the four focal populations. (b) The relationship between floral traits and pollen receipt in measured as standardized regression coefficients (β'_i) . In L2, brightness and UV chroma were not measured ('NA'). Sample sizes are provided for each population.

	Population			
	L1	L2	H1	H2
(a)				
Altitude (m)	2337	2617	3005	3295
Proportion Diptera	0.11	0.34	0.66	0.66
(b)				
Floral Trait	β'_i	β'_i	β'_i	β'_i
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
N	60-76	67	68	66

†P=0.06-0.08; *P<0.05; **P<0.0001

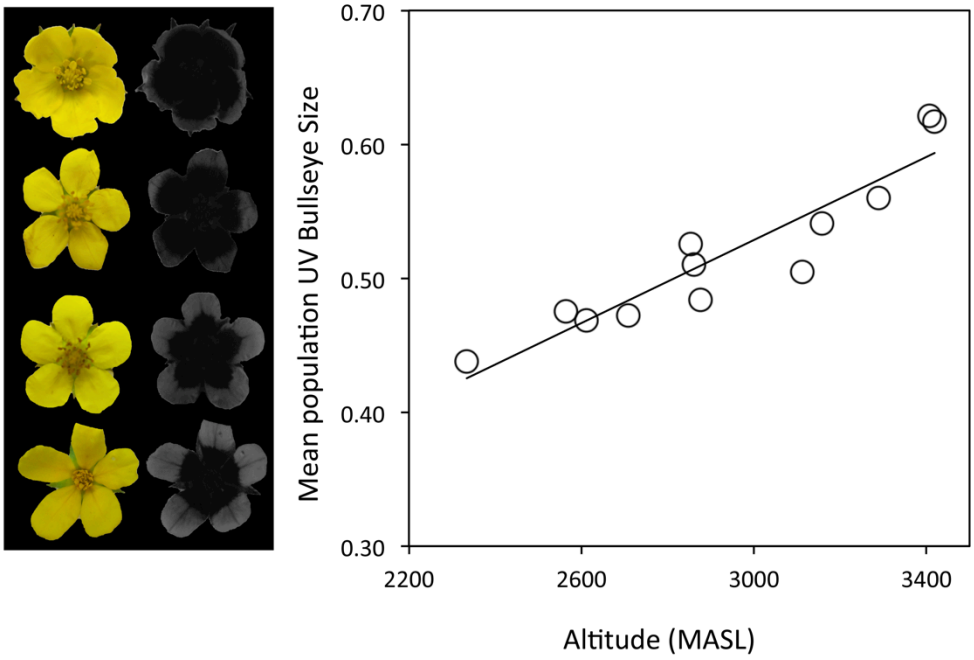


Figure 4-1: Mean size of the UV bullseye (the proportion of the petal that absorbs UV) in *Argentina anserina* increases with altitude (meters above sea level [MASL]) across 12 populations in Colorado, USA (bullseye size =1.5e-4 × MASL + 6.6e^{-3}, P<0.0001, R^2=0.83). Representative flowers in the human-visible (yellow) and UV (grayscale) spectrum are provided on the y-axis to show the variation in bullseye size ranging from 0.35-0.62.

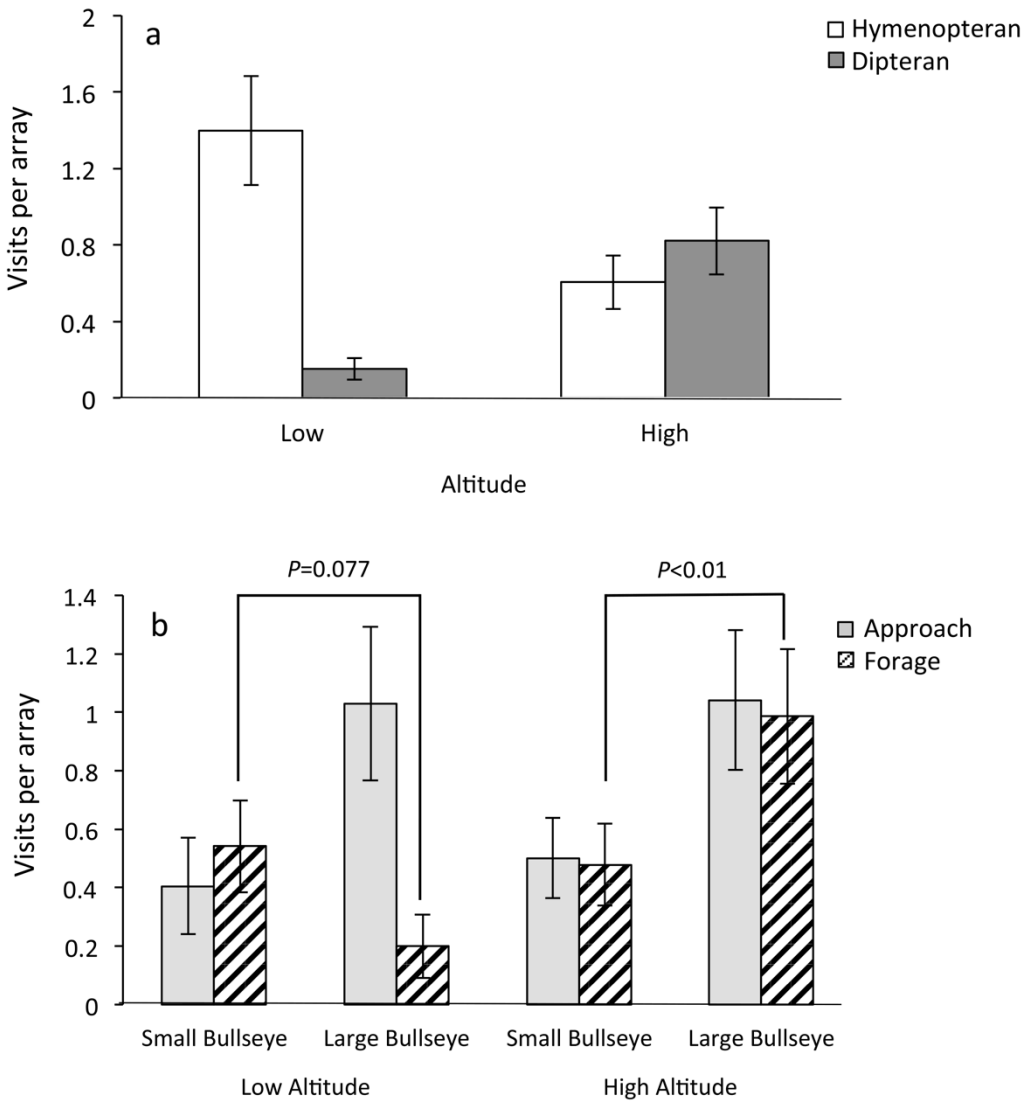


Figure 4-2: (a) The number of visits to *Argentina anserina* flowers per floral array by hymenopterans (white bars) and dipterans (black bars) at low and high altitude populations, and (b) the number of approaches (gray) and forage visits (striped) to flowers with small bullseyes (~25% absorbing) and 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 denoted compare the number of foraging visits between flower types within an altitude class in (b).

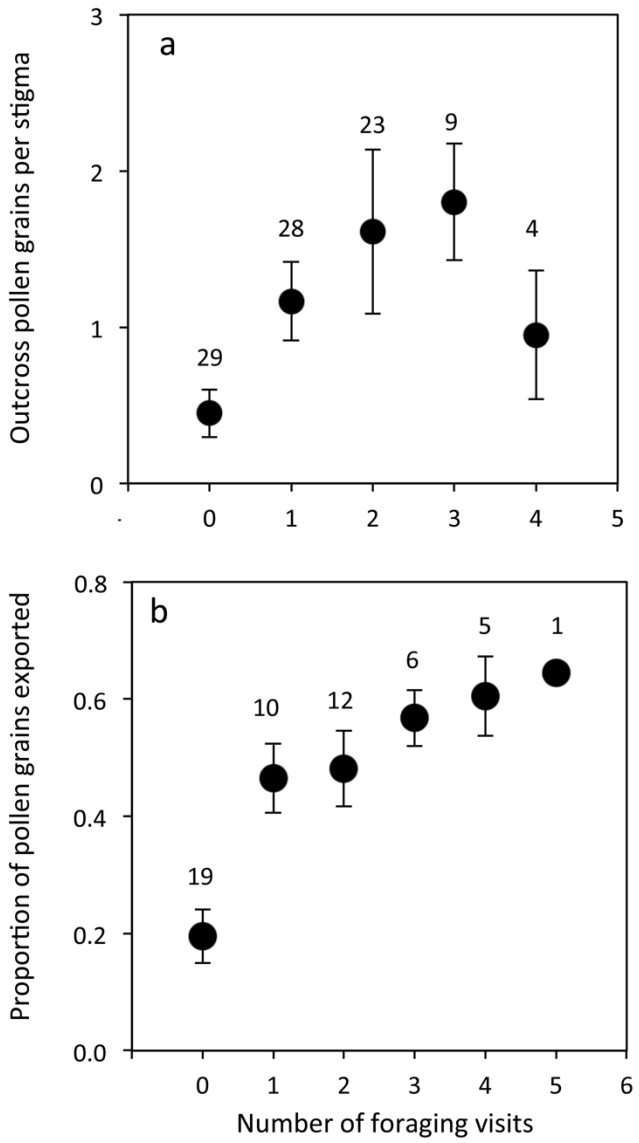


Figure 4-3: The association between the number of foraging visits by pollinators and (a) outcross pollen receipt per style, and (b) the proportion of pollen grains removed from *Argentina anserina* flowers. Values are means ±1 SE and sample sizes are given above each point.

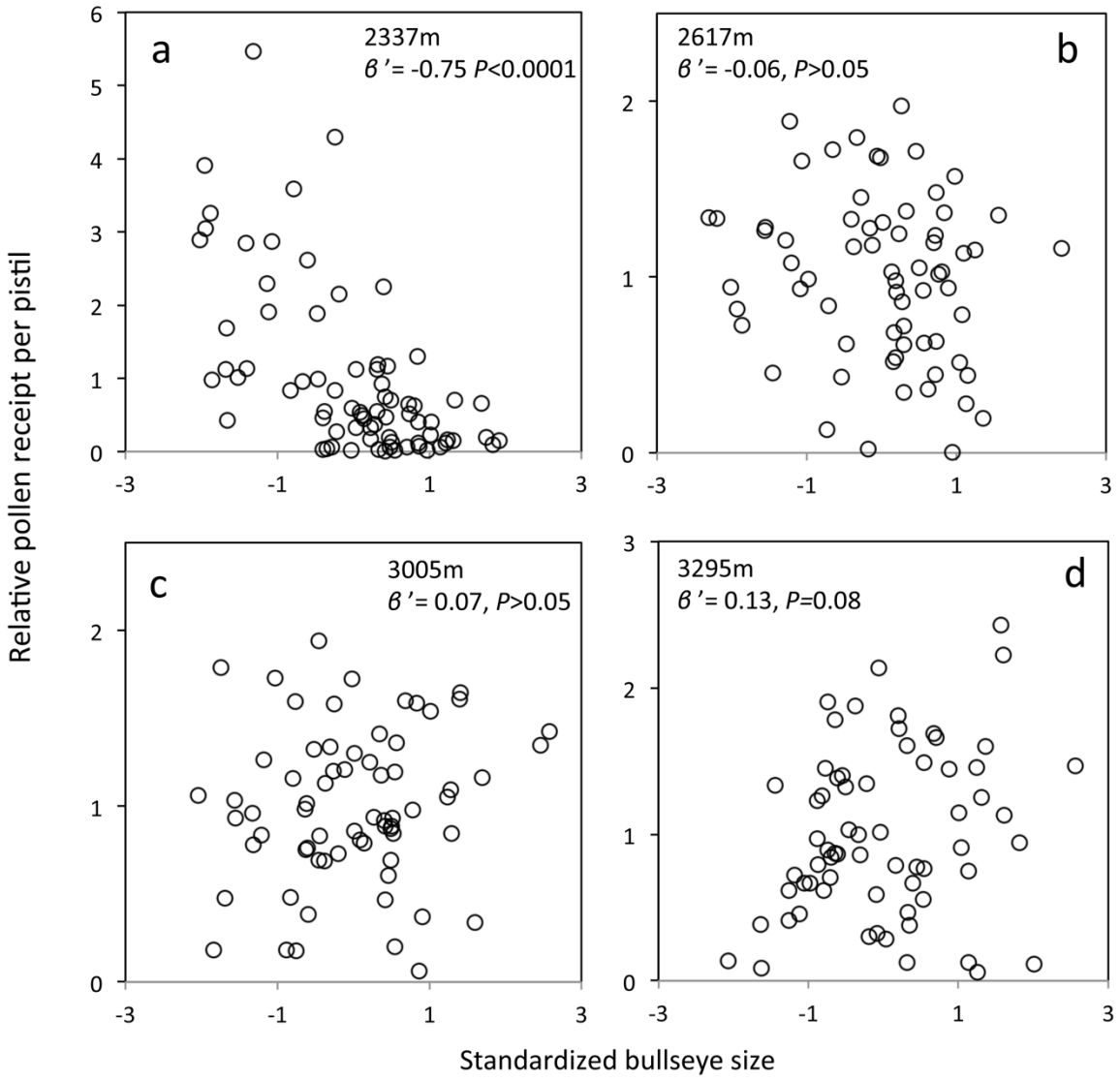


Figure 4-4: Standardized pollen receipt per pistil plotted against UV bullseye size (standardized UVP; the proportion of the petal that absorbs UV) in four populations of *Argentina anserina* with varying altitude. Panels (a), (b), (c), and (d) correspond with L1, L2, H1 and H2, respectively. The partial regression coefficient for bullseye size (UVP) and *P*-value for each is reported in each panel. For full regression model results see Table 2.

5.0 FLORAL PIGMENTATION PATTERNS PROVIDE AN EXAMPLE OF GLOGER'S RULE IN PLANTS

Koski, M. H. and T.-L. Ashman. 2015. *Nature Plants*. doi: 10.1038/nplants.2014.7.

5.1 INTRODUCTION

A major goal of ecology is to identify principles that unify our understanding of patterns of biodiversity. A list of ecogeographic rules have been formulated to explain positional or environmental variation in morphology or life history in terrestrial and aquatic systems across the globe (Gaston et al. 2008). Yet, their applicability to both animal and plant kingdoms, and their mechanistic drivers, often remain open questions (Gaston et al. 2008). Gloger's rule and its corollaries state that endothermic animals in more equatorial regions will have darker pigmentation than those in more polar regions (Burtt 1981; Caro 2005). Increased pigmentation towards equatorial latitudes in animals including humans (Jablonski and Chaplin 2010), neotropical and old world primates (Santana et al. 2012; Hamada et al. 2008), and house mouse (Lai et al. 2008) can be seen as a manifestation of this rule. This pattern is predicted to derive from protective functions afforded by greater pigmentation against abiotic or biotic stressors that increase with decreasing latitude, such as heat, humidity, predation and ultraviolet irradiance (Burtt 1981; Caro 2005; Millien et al. 2006). We extend tests of Gloger's rule and the underlying protective hypothesis to flowering plants. We focus on a common floral pattern—a UV 'bullseye' where petal bases are UV-absorbing while petal tips reflect UV (Guldberg and Atsatt 1975; Harborne and Nash 1984) (Fig. 1a). This floral pattern exists in at least 36 families of angiosperms and is known to result from variation in UV absorbing pigments (Harborne and Nash 1984) or in some cases, variation in

cell shape (Gorton and Vogelmann et al. 1996).

While diversity in flower color and pattern has been traditionally ascribed to divergent pollinator-mediated selection (Rausher 2008), more recent work has implicated abiotic factors (e.g., heat, drought, and UV irradiance) as selective agents as well (Whittall and Strauss 2006). Abiotic selection is often thought to act indirectly on flower color via pleiotropic effects of genes that mediate stress tolerance (Whittall and Strauss 2006). However, the micro-environment of the flower can vary with petal pigmentation, so abiotic factors such as ambient UV irradiance could instead impose selection directly. Specifically, flowers with more UV reflectance, either due to larger reflective petal areas or higher intensity of UV reflection, will have floral environments with higher UV irradiance that can adversely affect the viability of gametes produced within them (Fig. 2). So in contrast to conventional wisdom that the UV bullseye floral pattern functions to enhance pollinator's distance perception of flowers (Koski and Ashman 2014) and/or orientation to floral rewards, we propose that flowers with larger bullseyes (larger areas that absorb UV; Fig. 2b) may also protect pollen from UV damage post anthesis (Torabinejad et al. 1998; Koti 2005) and thus bullseye size is under selection mediated by UV irradiance. Since UV irradiance is higher at lower latitudes (Herman 2010), we predict that UV-mediated selection will contribute to latitudinal clines in the bullseye size, such that bullseyes will increase with increasing proximity to the Equator, supporting Gloger's rule.

5.2 METHODS AND RESULTS

We combined field-collected phenotypic data from 34 populations of silverweed cinquefoil, *Argentina anserina* (Rosaceae), a native plant distributed in temperate zones of both hemispheres. Sampling represented latitudinal transects in four regions, three in the northern hemisphere (Pacific

Coast USA, Rocky Mountains USA, Great Lakes USA) and one in the southern hemisphere (New Zealand). Silverweed cinquefoil has bowl-shaped flowers that appear uniformly yellow to the unaided human eye, but have a UV-absorbing bullseye (Fig. 1a, 2a) that is due to the presence of flavonol glycosides at the petal bases (Harborne & Nash 1984). We measured floral bullseye size by scoring the relative area of petals that absorb UV (UV proportion) (Yoshioka et al. 2005; Koski and Ashman 2013) on 456 flowers by digital analyses of UV photographs. We determined whether mean population bullseye size is larger closer to the Equator after accounting for variation explained by regional transect and altitude. We then addressed whether UV irradiance predicts variation in bullseye size after accounting for other potential climatic agents of selection (temperature, precipitation) and region. Latitude explained 39% of the variation in bullseye size after accounting for region and altitude ($F_{1,22}$=28.4, P<0.0001, Table 1a). Within each of the four geographic regions, bullseye size increased with increasing vicinity to the Equator (Fig. 1b), that is, a larger proportion of the petal area was pigmented with UV-absorbing compounds at lower latitudes, which is in line with Gloger's rule. The relationship between bullseye size and latitude did not differ among regions (region x latitude $F_{3,22}$=1.87, P=0.16; Table 1a; Fig. 1b) while the relationship between bullseye size and altitude did (region x altitude, $F_{3,22}$=5.21, P<0.01; Table 1a), suggesting that latitude was a more consistent predictor of bullseye size variation than was altitude. Three bioclimatic variables that can covary with latitude (UV-B irradiance, temperature, precipitation) together explained 33% of the variation in bullseye size after accounting for region (Table 1b), but UV irradiance was the only significant bioclimatic predictor ($F_{1,19}$=11.82, P<0.01, Table 1b), explaining 24% of the variation. The slope of the relationship between UV-B irradiance and bullseye size was similarly positive in each region (region x UV-B Irrad., $F_{2,19}$=1.76, P=0.20; Table 1b; Fig. 1c). Together, these results demonstrate that geographic variation in a UV flower pigmentation pattern follows Gloger's rule, and that UV irradiance is the most important climatic

factor underlying the global pattern.

We tested the prediction that UV irradiance favors individuals with larger bullseyes by measuring phenotypic selection on bullseye size using pollen viability as a fitness parameter under experimentally modified levels of ambient UV (absent, present, Fig. S1). Four to six flowers per plant were harvested from 71 plants with varying bullseye size that were grown in a glasshouse for several years. From each plant, half of the flowers were exposed to UV irradiance and half were protected from UV irradiance. After exposure, we scored *in vitro* pollen germination as a component of male fitness in selection analyses. In the absence of UV irradiance directional selection favored the smallest floral bullseyes (Fig. 3a). Conversely, exposure to UV favored individuals with intermediate sized bullseyes (Fig. 3b). Analysis of covariance showed that while the directional component of selection did not differ between UV treatments (treatment x bullseye size, $F_{1,126}$=2.40, P=0.12; Table 2), the nonlinear component did (treatment x bullseye size^2, $F_{1,126}$=3.80, P=0.05; Table 2). An explicit test of the location of the trait optimum, Mitchell-Olds Shaw Test (Oksanen et al. 2013), confirmed that the fitness function was unimodal in the presence of UV (P=0.04, optimum at -0.12 SD units; Fig. 3b), but not in the absence of UV (P=0.98; optimum at -1.55 SD units, near the minimum of -1.62; Fig. 3a). Thus, both parametric and non-parametric (Schluter 1988) analyses show that UV exposure favored flowers with greater pigmentation (optimal bullseye size increased by 1.43 SD units; Fig. 3, Fig. S2). Given that the experimental levels of UV-B were only 43% of what flowers typically experience at noon in July under natural conditions, even larger bullseyes might be favored in nature, if all else is equal.

To verify that the bullseye is a target of selection (Conner et al. 2003) by UV irradiance, we recorded the effect of varying bullseye size on pollen viability using artificial flowers. We created flowers that had one of three discrete bullseye sizes (small, medium, large; Fig. 4) but were otherwise phenotypically identical in overall size and in human-visible color. Anthers from

glasshouse-grown plants were placed in each type of artificial flower and we exposed the flowers to UV-present and UV-absent environments as above. Viability of pollen was scored *in vitro* after exposure. Overall, the presence of UV reduced pollen viability by ~12% (Treatment, $F_{1,21}$ =8.74, P<0.01), but the effect of UV treatment depended on bullseye size (treatment x bullseye size, $F_{2,41}$ =3.23, P=0.05; Fig. 4). In the absence of UV irradiance, pollen viability was unaffected by flower type (all pairwise comparisons, P=1.0; Fig. 4). However, in the presence of UV irradiance, pollen from the small bullseye flower had the lowest germination rate (28% lower than large bullseye with UV present, P=0.02, Fig. 4), whereas pollen from the largest bullseye had a germination rate equivalent to that seen in flowers in the absence of UV (P>0.99; Fig. 4). From this we conclude that bullseye size modifies the floral UV environment, with larger bullseyes providing greater protection of pollen from UV exposure than small ones (as in Fig. 2). The combination of the repeated geographic pattern and the results from experimental manipulations of both the UV environment and the bullseye trait strongly support the prediction that UV irradiance imposes selection on the size of the UV bullseye in a manner consistent with Gloger's rule.

5.3 DISCUSSION

All tests of Gloger's rule to date have been with animals, and the majority of studies have not fully explored the mechanistic underpinnings of the rule. This study extends Gloger's rule by showing that it applies to a floral phenotype in plants, and identifies UV irradiance as an agent of natural selection that drives latitudinal variation. Interestingly, UV irradiance is likely the main driver of darker human skin pigmentation towards equatorial regions (Jablonski 2000; Jablonski and Chalpin 2010) may drive latitudinal trends of darker eye masks towards the Equator in Neotropical primates (Santana et al. 2012), and may account for overall pigmentation differences in Old-World

Macaques (Hamada et al. 2008). Our result that latitude explains 39% of bullseye variation after the effects of region and altitude are accounted for, is not as strong as the latitudinal association found for human skin reflectance values (r=0.83-0.97 depending on wavelength [Jablonski 2000]), but is more on par with the association in Thai macaques (R^2= 0.32-0.74 depending on body location [Hamada et al. 2008]), and is much stronger than that seen in house mouse (3.3% of variation in coat color [Lai et al. 2012]), though we should note that these comparisons combine traits measured in different manners. Latitudinal clines in color may be common in flowers. For instance, a recent study found that the proportional representation of dark blue to lighter red flowers in scarlet pimpernel increased at lower latitudes (Arista et al. 2013), a pattern of increased darkness also consistent with Gloger's rule, although not noted in the original paper. Moreover, flowers with exposed anthers may have evolved greater resistance to UV-B pollen damage than those with structures that protect anthers (Zhang et al. 2014), highlighting the role of UV as a driver of floral evolution. For flowers with exposed pollen, our data indicate that the evolution of increased UV-absorbing pigmentation in petals is another avenue for protecting pollen from UV-B. We have shown that UV irradiance can exert selection on optimal bullseye size, but we caution that tests should additionally determine whether changes in pollinator assemblage with latitude (Dupont et al. 2009) could also play a role in floral pigmentation variation. The shape of selection via other fitness parameters (e.g., seed production), and the potential cost of producing highly pigmented flowers (as suggested in Fig. 2a) may also factor into the optimal bullseye size at a given location.

These results add a novel dimension to the accumulating evidence for the action of non-pollinator forces in the diversification of floral traits by underscoring UV irradiance as an important climatic agent. Past ozone depletion has led to increased UV irradiance, especially at mid-latitudes and polar regions (Herman 2010), and future changes in climate and ozone levels will continue to alter UV exposure in terrestrial systems (Ballaré et al. 2011). The current study

provides insight into how floral color phenotypes may respond adaptively to global change. These changes, however, may counter preferences of pollinators. For instance, while an increase in UV irradiance favors greater floral UV absorption, potentially leading to loss of UV pattern, the opposite—greater UV reflection and/or the presence of UV pattern—increases pollinator attraction to flowers of silverweed cinquefoil and other species (Johnson and Andersson 2002; Koski and Ashman 2014). Thus, global pigmentation responses may generate a mismatch between pollinator preferences and floral phenotypes, further complicating the health of an important ecosystem service (Costanza et al. 1997). Tests of Gloger's rule for floral color variation among taxa in a phylogenetically-controlled manner, as well as color variation among flowering plant communities or among plant organs, will be important for testing the extent to which this animal rule applies to plants.

Table 5-1: General linear ANOVAs testing the effects of (a) latitude and altitude and (b) bioclimatic variables on mean population bullseye size. Both models first accounted for regional variation.

(a)

Effect	Df	Type I SS	MS	*F*	*P*
Region	3, 22	0.59	0.20	63.30	**<0.0001**
Altitude	1, 22	0.00	0.00	1.38	0.25
Latitude	1,22	0.09	0.09	28.35	**<0.0001**
Region x Altitude	3,22	0.05	0.02	5.21	**<.01**
Region x Latitude	3,22	0.02	0.01	1.87	0.16

(b)

Effect	Df	Type I SS	MS	*F*	*P*
Region	3,19	0.59	0.20	48.89	**<0.0001**
Temperature	1,19	0.02	0.02	3.81	0.07
Precipitation	1,19	0.01	0.01	1.38	0.25
UV-B Irradiance	1,19	0.05	0.05	13.45	**<0.01**
Region x Temp.	3,19	0.05	0.02	4.27	**0.02**
Region x Precip.	3,19	0.01	0.00	0.79	0.51
Region x UV-B Irrad.	3,19	0.01	0.01	1.76	0.2

Table 5-2: Fixed effects from ANCOVA testing for the effect of UV exposure (Treatment) on directional (bullseye size) and quadratic (bullseye size^2) selection on bullseye size. Random effects of population, date of exposure, and their interactions with treatment were included in the model, were nonsignificant (0.36<*P*<0.94) and are not shown.

Effect	$F_{1, 126}$	*P*
Treatment	2.71	0.10
UV bullseye size	1.11	0.30
UV bullseye size^2	5.91	**0.02**
Teatment x bullseye size	2.4	0.12
Treatment x bullseye size^2	3.8	**0.05**

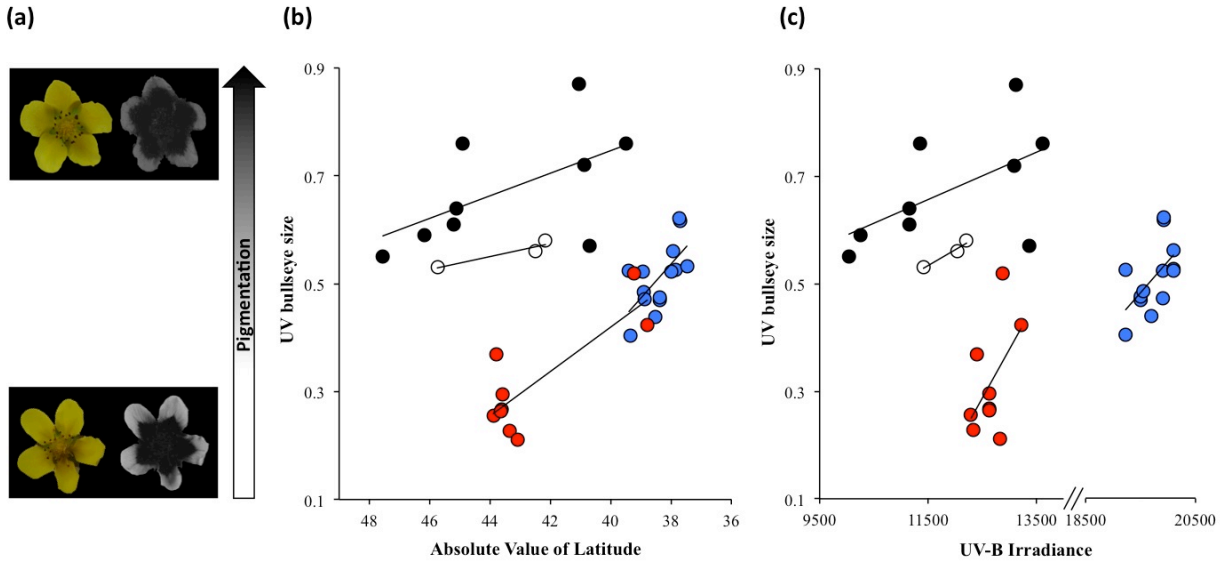


Figure 5-1: Size of the floral UV bullseye increases with proximity to the Equator in silverweed cinquefoil (*Argentina anserina*). (a) Representative images of *Argentina anserina* flowers in visible (color) and UV (grayscale) spectra displaying a range of UV bullseyes sizes in New Zealand. In UV images, darker areas of flowers absorb UV, while lighter areas reflect UV. (b) UV bullseye size (*x*-axis) was measured as the UV-absorbing proportion of petal area, and proximity to the Equator is the absolute value of latitude (*y*-axis). The *y*-axis is reversed such that populations on the left are at high latitudes and those on the right are at lower latitudes. (c) UV-B irradiance experienced during the flowering season (J/m^2/day) predicts variation in bullseye size (see Table 1b), and the relationship between bullseye size and UV-B irradiance is positive in all regions. In (b) and (c) colors represent region (black=Pacific Coast; white=Great Lakes; red=New Zealand; blue=Rocky Mountains).

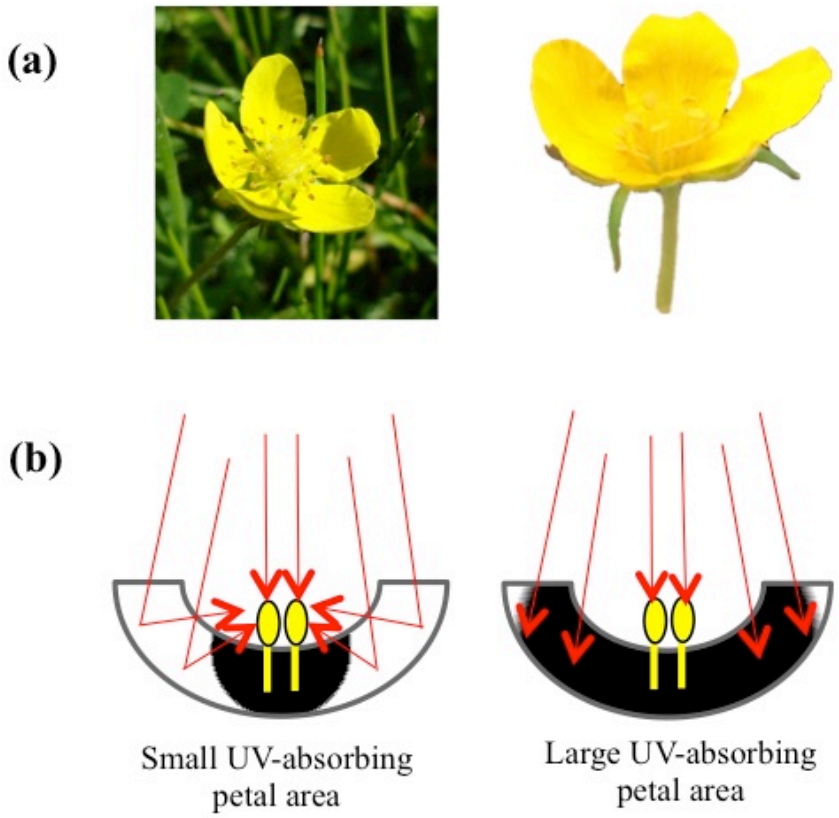


Figure 5-2: Hypothesis for how variation in the UV bullseye influences floral microenvironment. (a) In situ (left) and cross-sectional view (right) of the bowl-shaped architecture of an Argentina anserina flower. (b) Schematic of the hypothesized effect of UV-absorbing bullseye size on the reflectance of UV-light within a flower. A flower with a smaller UV-absorbing petal area (left) reflects UV light from petal tips onto pollen-bearing anthers (yellow structures), whereas a flower with a larger area of UV-absorption (right) absorbs UV light across a larger area, reducing diffuse reflection and thus, UV-exposure of pollen.

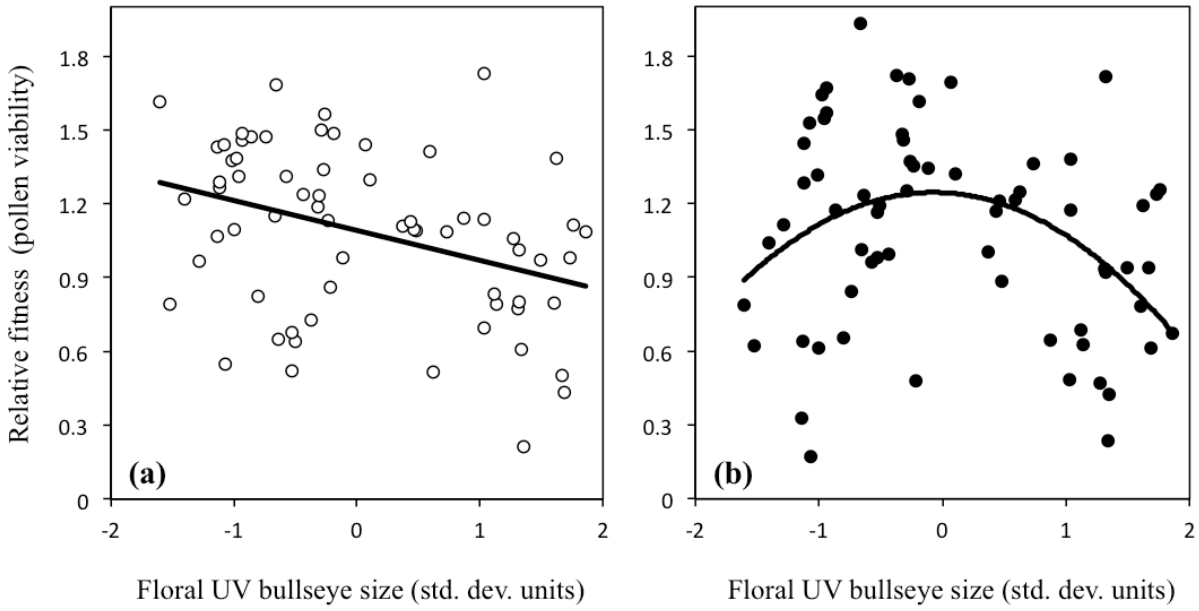


Figure 5-3: Optimal UV bullseye size increases in the presence of ambient UV. Pollen viability (relative) as a function of UV bullseye size (standard deviation units) in the absence (a) and presence (b) of experimental UV exposure. In the absence of UV, directional selection was observed (pollen viability= -0.12 × bullseye + 1.1; R^2=0.13; *P*=0.002). In the presence of UV, stabilizing selection was observed (pollen viability= -0.15 × bullseye^2 – 0.02 × bullseye + 1.2; R^2=0.14; *P*=0.009]).

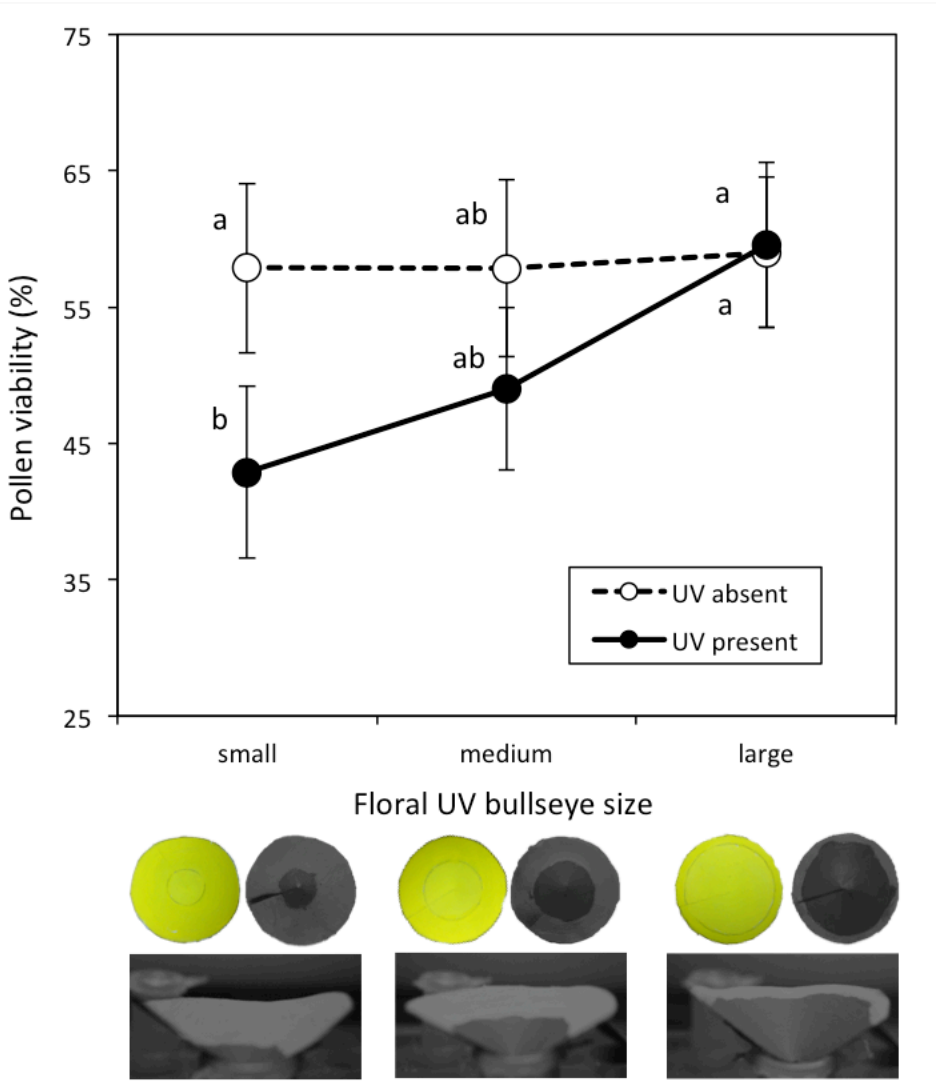


Figure 5-4: UV bullseye size is a target of selection via UV irradiance. Mean percent viability of pollen (±1 SE) placed in artificial flowers with small, medium, or large UV bullseyes in the presence (filled circle, solid line) or absence of UV (open circle, dashed line). Means that do not share a common letter are significantly different at *P*<0.05 as determined by Tukey-Kramer Post-hoc tests. Top-down human-visible and UV images (above), and cross-sectional UV-images (below) of artificial flowers are shown on the *x*-axis (darker areas are UV-absorbing and lighter areas are UV-reflecting).

6.0 MACROEVOLUTIONARY PATTERNS OF FLOWER COLOR TRAITS AN ASSOCIATIONS WITH BIOGEOGRAPHY IN THE SPECIOSE CINQUEFOIL GROUP

6.1 INTRODUCTION

Elucidating the extent to which ecological factors, phylogenetic relatedness, or trait correlations contribute to phenotypic variation among species provides insight into whether traits may be adaptive. Floral traits have served as an important model for our understanding of trait evolution in a phylogenetic context (e.g., Whitall and Hodges 2007; Smith 2010). Flower color and color patterns, in particular, are important mediators of plant-pollinator interactions. Thus, it is not surprising that most studies that evaluate macroevolutionary patterns in flower color look to pollination as the most likely ecological process that may drive such variation (Smith et al. 2008; Rausher 2010; van der Neit & Johnson 2012; Muchhala et al. 2014; Grossenbacher & Stanton 2014). While pollinators are likely strong contributors to floral diversification, there is increasing evidence that, at a microevolutionary level, abiotic factors can contribute to variation in flower color (Arista et al. 2013) and pigmentation pattern (Koski & Ashman 2015). To date however, whether abiotic factors may contribute to diversification in floral traits using phylogenenetically informed analyses has been little considered.

Most support for that abiotic affects on flower color suggests that selection acts pleiotropically on genes that confer floral pigmentation (Reviewed by Rausher 2008). In particular, products of the flavonoid biosynthetic pathway that impart protection against UV, temperature, and/or drought stress, also underlie petal pigmentation (Rausher 2008; Wessinger and Rausher 2012). Thus abiotic selection could affect flower color evolution in concert with pollination. Additionally, recent evidence suggests that abiotic factors may act directly on flower color pattern,

because light reflected from flowers may affect the floral environment where pollen and ovules are housed (Koski & Ashman 2015). In particular, larger areas of UV absorbance on petals can protect pollen from UV damage, leading to covariance of floral color pattern and UV incidence in *Argentina anserina* (e.g., latitudinal variation driven by UV irradiance [Koski & Ashman 2015], and altitude [Koski & Ashman 2015]). Addressing whether such patterns are observed on a macroevolutionary scale will be important to assess generalizability of the adaptive nature of UV floral pattern.

Flower color and pattern variation among taxa may also be structured by evolutionary history. That is, phylogenetic signal (closely related species are more phenotypically similar than distantly related ones), rather than extrinsic ecological factors, may shape variation. However, most studies find low phylogenetic signal for flower color in closely related species (Smith et al. 2008; Muchala and Smith 2014; Gomez et al. 2015), and in diverse flowering communities (McEwen & Vamosi 2010; LeCroy et al., in prep) suggesting that selection may strongly influence floral color traits. Furthermore, floral pattern traits appear to be variable among closely related taxa (Reisberg and Schilling 1985; Naruhashi and Ikeda 2002), yet, these studies have not evaluated whether pigmentation pattern displays signatures of phylogenetic signal.

Phylogenetic trait correlations may reflect genetic constraint to evolution, or correlated selection on suites of traits. Observations of multiple diverse floras suggest that visibly yellow flowers are more likely to be UV-reflective and possess UV pigmentation pattern than white flowers (Guldberg and Atsatt 1975, Inouye and Pyke 1998, Dyer 1996). A relationship between yellow flowers and UV reflective patterns could reflect a biochemical constraint. In the genus *Potentilla*, for example, carotenoid pigments that confer yellow coloration are also UV reflective (Harborne and Nash 1984). Conversely, many colorless flavonoids (appearing white) or blue-red anthocyanins, are UV-absorbing (Harborne and Nash 1984). Observation of such a correlation

however, in the absence of data on floral biochemistry, could also reflect correlational selection for both yellow pigmentation and UV reflective pattern (e.g, by pollinators; Papiorek et al. 2015). While such correlations have been casually observed, a test of their validity will benefit from a phylogenetically-informed assessment of correlation.

Here, we assess the contributions of phylogenetic relatedness, trait correlations, and abiotic factors to variation in UV flower color pattern, and human-visible flower color in the cinquefoil genus, *Potentilla,* and closely related genera (*Ivesia*, *Horkelia*). *Potentilla* is a speciose, geographically widespread genus. Species have yellow, white, or pink-red flowers, and there is particularly striking variation among taxa in the extent of floral UV pigmentation (Harborne and Nash 1984; Naruhashi and Ikeda 1999). To address factors that underlie macroevolutionary patterns of UV pattern and human-visible flower color in *Potentilla* we constructed a molecular phylogeny of 177 species, characterized floral traits, and assessed bioclimatic variables of species' habitats. We specifically asked, (a) is there phylogenetic signal for human-visible flower color, insect-perceived flower color (including UV reflection) and, UV pattern? (b) Is the evolution of UV pigmentation and human-visible flower correlated? (c) Do biogeography and associated abiotic factors shape color and UV pattern variation across taxa?

6.2 METHODS

6.2.1 System

Potentilla is widespread, speciose genus with a largely temperate and arctic distribution. Conservative estimates suggest that there may be from 300-430 species in the group. Recent molecular phylogenies have placed the genera *Horkelia* and *Ivesia* (Western North American) within *Potentilla* (Dobes and Paule 2010; Topel et al. 2011). Flowers are five-merous, radially symmetrical, and can be white, pink-red, or yellow in color. UV reflective pattern is noted from

many taxa, as well as uniform UV absorption (Harborne and Nash 1984; Naruhashi and Ikeda 1999,). UV absorption is manifested via UV-absorbing flavonoid compounds (e.g. quercetin) while yellow pigmentation is due to carotenoids, and red pigmentation is underlain by cyanidins (Harborne and Nash 1984). Of the taxa for which pollination has been assessed, flowers are generalist-pollinated by a variety of solitary bees, and flies of several genera (Koski & Ashman 2014; in review)

6.2.2 Phylogeny

Plant material: We obtained material from the Royal Botanic Garden Edinburgh Herbarium (E), Carnegie Mellon Museum of Natural History Herbarium (CM), Rancho Santa Ana Botanic Garden Herbarium (RSA), Harvard University Herbaria (A), University and Jepson Herbaria (UC & JEPS), and Rocky Mountain Herbarium (RM). Additionally, we utilized published sequence data from GenBank. For accession information including herbarium voucher, collector, location, and/or Genbank reference, refer to Appendix 1.

DNA Extraction: We extracted total genomic DNA from 1-2mg of dried herbarium leaf tissue using the CTAB method following Doyle and Doyle (1987). DNA preprations were NaoAc + ETOH precipitated as an additional washing step and then resuspended in elution buffer and stored at 0C prior to PCR.

PCR and DNA Sequencing: We sequenced two nuclear (ITS, ETS) and one chloroplast (*trnL-F*) marker. We obtained the full ITS region using the ITS-1 (Urbatsch et al. 2000) and ITS4 (White et al. 1990) . For some taxa (25%), we used internal primers ITS2 (White et al. 1990) and ITS3b (Baum et al. 1994) to obtain the full length ITS region. The ETS region was sequenced using

ETS1 and IGS6 primers (Oh and Potter 2005). The partial *trn*L-F region was sequences using trnL^{UAA}3' and trnF^{GAA} primers (Taberlet et al. 1991). PCR reactions consisted of 1X PCR buffer (Qiagen 10x buffer with $MgCl_2$), 100100 µM of each dNTP, 0.5 µM of each forward and reverse primer, 1.5 units of *Taq* polymerase and 1 µl of genomic DNA in a 25 µl reaction, and used the following amplification protocol: 2.5 minutes at 95°C, followed by 35 cycles of : 95°C for 30 seconds, annealing temperature for 30 seconds, and 72°C for 60 seconds, and a final extension at 72°C for eight minutes. Annealing temperatures were 50°, 54° and 51°C for ITS, ETS, and trnFL reactions, respectively.

PCR products were confirmed on a 1% agrose gel. Amplified PCR products were purified using Exo-SAP(Affymetrix/USB, Cleveland, OH), and were sequenced on an ABI 3730XL DNA analyzer (Applied Biosystems/Life technologies, Carlsbad, CA). Consensus sequences of forward and reverse reads were constructed using Sequencer 5.3 (Gene Codes Corp., Ann Arbor, MI, USA). In few cases, one direction yielded a low-quality read, and thus, only one direction was used.

Phylogenetic reconstruction:

ITS, ETS and *trn*FL sequences were aligned separately using ClustalW and gaps were manually edited in MEGA v. 5.2.2. There were three sites of ambiguous alignment in the *trn*L-F matrix which were deleted (272-313, 388-406, 518-541). We verified the best nucleotide substitution model for each gene using jModelTest2 (Guindon and Gascuel 2003). To test for congruence of tree topologies from the three genes, we implemented the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999) in PAUP v. 4.0b10. We created three separate Bayesian maximum credibility trees with BEAST (Drummond et al. 2012) that included the taxa for which all three regions were available (n=155). For all trees we used a lognormal relaxed clock,

GTR+G+I substitution model, and a speciation birth-death process. Maximum credibility trees(MCC) were created in TreeAnnotator v. 1.8.1 with 2000 burnin trees. The SH test indicated that the nuclear ITS phylogeny was the best tree (-lnL=20514.5) and that it was congruent with the nuclear ETS tree (P=0.28) as expected. The topology derived from the chloroplast *trn*FL gene however, was incongruent (P<0.05). We identified five instances of toplogical incongruence that lead to this result (see Results).

We proceeded with concatenation of all three regions for generation of the backbone tree for use in comparative analyses. The final sequence matrix included 183 taxa. We constructed the phylogeny with BEAST (v. 1.8.1). The three genes were modeled with a GTR+G+I substitution model, a lognormal relaxed clock, and we used a birth-death process of speciation. The MCMC chain was 10,000,000 generations and parameters were logged every 1000. We time-calibrated the phylogeny by setting the node of the Argentina clade (*P. anserina, P. anserinoides, P. lignosa, P. fulgens, P. lineata, P. microphylla, P. stenophylla, P. festiva, P. peduncularis,* and *P. cardotiana, P. leuconota*) to 21.2-32MYA based on previously published estimates of divergence time made from a fossil-calibrated phylogeny (Dobes and Paule 2010). We generated an ultrametric MCC tree with TreeAnnotator v 1.8.1 from 10000 trees after discarding 2000 burnin trees.

6.2.3 Phenotypic and bioclimatic data

Floral phenotypes:

We measured UV proportion (UVP; the proportion of the petal that absorbs UV) from herbarium specimens from CM, RM, E, RSA, UC, JEPS, A, and Kew Herbarium (K). The minimum requirement for a flower to be measureable was the presence of a single adaxially-facing petal. When multiple petals on a flower met this requirement, all were measured and then averaged. When a single plant had multiple available flowers, they were measured and averaged

for a plant-level mean. When two separate plants on a herbarium sheet had [a] measureable flower(s), these were treated as separate specimens.

Flowers were photographed in the UV spectrum, and we measured UVP following Koski & Ashman (2013). We scored UV floral pattern first quantitatively as a proportion (UVP, 0-1), and second as a discrete binomial character. Specifically, we scored 'UV reflecting' flowers as having a detectable amount of the petal with UV reflection (UVP< 0.9) an 'UV absorbing' as UVP ≥ 0.9. Human visible flower color was scored as white, yellow, or red (including 'pink' flowered species). We ascertained color from descriptions on herbarium sheets, and online descriptions (e.g., efloras.org), or images (e.g., Calflora.org). Three species were polymorphic, possessing either cream white or yellow flowers (*Potentilla recta*, *Drymocallis glandulosa*, and *D. lactea* var. *austiniae*). We scored these as 'yellow' because they have the ability to produce yellow pigments. Finally, we scored 'insect visible' flower color by combining data on UV reflection and human visible flower color (flowers were yellow, UV yellow, white, UV white, red, or UV red). All flowers with UVP≤0.5 were considered to be mostly UV reflecting (e.g., a yellow flower with UVP=0.3 was considered 'UV yellow'), whereas flowers with UVP >0.5 were considered UV absorbing (a yellow flower with UVP=0.88 is 'yellow'). While some taxa have color patterns in the human-visible spectrum (e.g., *P. thurberi*), we could not reliably score this phenotype from online images or descriptions. Additionally, quantitative estimates of flower color via reflectance spectrometry was unreliable on herbarium samples.

We did not collect phenotypic data for five species included in the molecular phylogeny (*I. arizonica* var. *saxosa*, *H. fusca* var. *pseudocapitata*, *P. humifusa*, *P. neumaniana*, *P. parvifolia*, and *S. parviflora*). These were removed from the phylogeny for comparative analyses. For one species, *P. peduncularis*, we analyzed UVP from an image published by Naruhashi and Ikeda (1999). For *P. anserinoides*, we used five samples collected from the field (Koski and Ashman 2015), and for

P. anserina, we analyzed two dried flowers from greenhouse-grown plants (Koski, unpublished).

Bioclimatic data:

Using the Global Biodiversity Information Facility (GBIF.org), we gathered georeferenced plant localities based on herbarium records or observations. For additional georeferenced collections localities, we searched the following herbarium databases; SEINnet, PNW Herbarium, Tropicos, Royal Botanic Gardens Edinburgh and University of Vienna Herbarium. We evaluated georeferenced locations for accuracy (e.g., collections made out of the natural or naturalized range, like those in botanic gardens or in oceans, were eliminated), and eliminated duplicate datapoints using Microsoft Access. For all legitimate accessions, we extracted bioclimatic data using Worldclim in DIVA-GIS (Hijmans et al. 2005). Additionally, we extracted information on UV irradiance from UV layers in DIVA-GIS. The BIOCLIM and UV variables were averaged for each species.

6.2.4 Phylogenetic signal

We estimated phylogenetic signal for UVP using Blomberg's K (Blomberg et al. 2003) where $K<1$ suggests that more related species resemble each other less than expected under a Brownian motion model of evolution, and $K>1$ indicates they are more similar. We calculated K with the 'phylosig' function (ape package [Paradis et al. 2004]) and tested whether it is significantly different than one (Brownian motion) by randomizing trait values across the tree 500 times. We repeated this test on 200 random posterior trees to account for phylogenetic uncertainty. We then used 'phylosignal' function (picante [Kembel et al. 2010]) to test whether signal was significantly greater than zero (i.e., phylogenetic relatedness explains none of the variation). We set the number of randomizations to 500, and this was repeated on all 200 trees.

To estimate phylogenetic signal for discretely characterized human visible and insect perceived flower color, and UV reflection , we estimated Pagel's λ (Pagel 1999) using the fitDiscrete function in the geiger package (Harmon et al. 2008). Pagel's λ ranges between zero and one, with a value of one indicating trait evolution consistent with Brownian motion. We estimated Pagel's λ using maximum likelihood optimization on 200 posterior trees. Then, we modeled trait evolution on the 200 trees with lambda set to 0 (star phylogeny). Finally we modeled traits on 200 trees with λ set to 1. We determined whether optimized λ was significantly greater than zero and significantly different than one using log likelihood ratio tests implemented in R.

6.2.5 Correlated trait evolution

We assessed correlated evolution between human-visible flower color and UV pattern in two ways. First, we considered human-visible flower color as binary (yellow vs. nonyellow), and the UV reflection as present (UVP < 0.90) or absent (UVP > 0.90) to evaluate whether the UV reflection and yellow flowers showed a signature of correlated evolution. We grouped white and red flowers because previous studies suggest that yellow flowers are more likely than white or red to reflect UV (Guldberg and Atsatt 1974; Harborne and Nash 1984; Dyer 1996). We used Pagel's test of correlated evolution for binary characters (Pagel 1994) with the 'fitPagel' function in the 'phytools' package (Revell 2012) which compares model fit values of dependent or independent transition matrices for two binary traits.

Second, to determine whether the evolution of yellow flowers and smaller UVP (i.e., larger UV reflective areas) were associated, we used a phylogenetic ANOVA (Garland et al. 1993) using the 'phylANOVA' function in R's phytools package (Revell 2012). Specifically, quantitative estimates of UVP was modeled as a function of discrete human-visible flower (white, yellow, red). Holm-Bonferroni adjusted post-hoc tests were used to assess pairwise differences among flower

color groups (yellow, white, red). All analyses were performed on 200 random posterior trees to account for phylogenetic uncertainty.

6.2.6 Abiotic and biogeographic associations with floral phenotype

We evaluated whether UVP was associated with mean latitude and altitude of species occurrences using phylogenetic least squares regression (PGLS) (Grafen 1989). We modeled species mean UVP as a function of mean latitude and mean altitude of species occurences with a Brownian motion phylogenetic correlation structure (‘corBrownian, ape) of the MCC tree, with ‘gls’ in the ‘nlme’ package (Pinheiro et al. 2015). We then ran the same model but with an Ornstein-Ulenbeck correlation structure (Martins and Hansens 1997) using the corMartins function in ape. An O-U model assumes a stabilizing selection parameter (alpha) constrains variation. We compared AIC values to determine the best model, but present results of both.

We then evaluated whether UVP was affected by temperature, precipitation, and UV irradiance (see Koski & Ashman 2015) using PGLS. We modeled UVP first as a function of average annual bioclimatic variables (BIO1, BIO12, Annual UV), and then as a function of bioclimatic variables experienced during the putative growing season assumed to be the ‘warmest quarter’ of the year (i.e., when flowers are likely to be open; BIO10, BIO18, Highest Quarter UV). We again compared AIC values of BM and OU models. For all PGLS models, we tested for multicollinearity among predictor variables using variance inflation factors (‘vif’ in R).

To assess whether the presence of UV reflection, human-visible flower color, and insect perceived flower color were associated with distinct biogeographic or bioclimatic parameters we used a series of phylogenetic ANOVAs. We modeled latitude, altitude, and bioclimatic variables each separately as a function of discretely scored color parameters with a phylogenetic correlation structure of the MCC tree.

6.3 RESULTS

6.3.1 Phylogeny

The final character matrix consisted of 177 species and 1920 bp (ITS=792, ETS=544, trnLF=584). Sixty-seven species included had not yet been placed in a molecular phylogenetic construct (Erikkson et al. 2003; Dobeš and Paule 2010; Töpel et al. 2011; Töpel et al. 2012; Faghir et al. 2014). Our phylogeny was, for the most part, topologically congruent with others published (Dobeš & Paule 2010; Töpel et al. 2011). *Potentilla* was largely monophyletic and included the monophyletic *Horkelia*/*Ivesia* clade (p=1), a similar finding to previous studies (Dobeš & Paule 2010; Töpel et al. 2011; Fig. 3). Previous studies found *P. biennis* (Dobeš & Paule 2010) and *P. norvegica* (Töpel et al. 2011) to be within the *Horkelia*/*Ivesia* clade, however our analysis revealed that three additional *Potentilla* species were within *Horkelia*/*Ivesia* (*P. rivalis*, *P. newberryi*, and *P. intermedia*), and, with marginal support (p=0.94), that these *Potentilla* are monophyletic and are sister to the *Horkelia*/*Ivesia* clade. A number of *Potentilla* that are morphologically similar to *Argentina* grouped with *Argentina* (p=1), as expected based on previous phylogenies (Dobeš & Paule 2010) (Fig. 3). Additionally, *Potentilla drummondii* grouped with the monophyletic *Drymocallis* clade (p=1; Fig. 3).

Within *Potentilla* there was support (p=0.97) for the previously described Alba clade (Fig. 3) which consists largely of white to pink-petaled species with a European distribution (Topel et al. 2011). The previously-described Fragarioides clade was also recovered (pp=1; Fig. 3), but included two species that were within the Alba clade (*P. articulata*, *P. biflora*) in Topel et al.'s (2011) phylogeny. The Reptans clade described by Topel et al. (2011) and Dobes and Paule (2010) was also recovered (p=1; Fig. 3). The largest and least molecularly diverged group which included *Potentilla* from North America, Asia, Europe was consistent with other studies (p=0.93). Our combined nuclear-chloroplast phylogeny resolved a clade not previously described that included *P.*

stolonifera, *P. dickinsii, P. elatior*, and two species not included in previous phylogenies, *P. riparia* and *P. centigrana*. Additionally, Dobes and Paule's (2010) chloroplast phylogeny placed *P. alba* and *P. elatior* as sisters, however we *P. alba* to be sister *to P. sterilis* (p=1), and *P. elatior* to be sister to *P. riparia* (p=1). The MCC tree topology, and tree file for two hundred random posterior trees with which downstream comparative analyses utilized are provided in Appendix 1, and Appendix 2, respectively.

Incongruence between chloroplast and nuclear topologies was caused by five instances. *Potentilla algida* grouped with the Alba clade in the ITS phylogeny but was within the large *Potentilla* group in the ETS and *trn*LF phylogeny. *Potentilla oweriniana* was sister to *Fragaria vesca* in the *trn*LF phylogeny but was within the Alba clade in the ETS and ITS phylogenies. *Potentilla drummondii* was sister to *P. millefolia* in the *trn*LF phylogeny however grouped with the *Drymocallis* clade in both ITS and ETS phylogenies. *Potentilla biennis* was within the Ivesioid clade in the ITS and ETS phylogenies however was within the Fragarioides clade in the trnLF phylogeny. Finally, *P. norvegica*, *P. rivalis*, and *P. intermedia*, and *P. newberryi*, were were in the *Potentilla* clade in the trnLF phylogeny, however were within the Ivesioid clade in the ITS and ETS phylogenies.

6.3.2 Floral phenotypes

We estimated UVP and petal area on 533 specimens. One to 10 samples per taxon (mean=3) were sampled. Species average UV proportion ranged continuously between 0 (fully UV reflective petals) and 1 (fully UV absorptive petals) (Fig. 1). Eighty species possessed UV pattern (UVP < 0.9), while 97 were uniformly UV absorbing (UVP > 0.9). Species with UV-reflection on petals were observed in *Potentilla*, *Ivesia*, *Horkelia*, and *Duchesnea*, but not *Drymocallis, Dasiphora,* or *Sibbaldia*. Within *Potentilla* the Alba clade was the only one in which no species

displayed UV reflection. The majority (84%) of UVP variation was among, rather than within, taxa (ANOVA; $F_{175,357}$=10.33, P<0.0001; 80% when taxa with one sample were removed from the dataset, $F_{134,349}$=10.14, P<0.0001).

The majority of species have yellow flowers in the human-visible spectrum (n=141). Thirty species have white flowers while 7 have red flowers (restricted to *Potentilla* species). UV reflection was seen on flowers of all human-visible colors. 'Insect perceived' color fell into all possible categories; 5 species were red, 28 were white, 96 were yellow, 2 were UV-red, 2 were UV-white, and 44 were UV-yellow.

6.3.3 Biogeographic parameters

We obtained 374,829 georeferenced datapoints for 177 species (mean=2117.7). The range of points obtained per taxon was wide (1 - 80,979), reflecting the fact that some species are known from very restricted ranges (e.g., *P. deorum* endemic to Mt. Olympus, Greece) whereas others have a global distribution (e.g., *A. anserina*). The average latitude of species' range spanned from tropical zone (21.07N; Mexican endemic, *P. ranunculoides*) to the arctic zone (76.49N; circumpolar, *P. pulchella*), but most taxa are of temperate distribution (mean=43.04N). Average altitude ranged between 32-4395 meters above sea level with an average of (mean= 1714.9 meters).

6.3.4 Phylogenetic signal

Phylogenetic signal for UVP as measured by Blomberg's K (0.13), was significantly greater than zero, but significantly lower than that expected under Brownian motion (i.e., K=1) (Fig. 2; Fig. 3). The presence of UV reflection displayed phylogenetic signal as measured by Pagel's λ (0.63), which was significantly greater than zero, but not from one (Fig. 2; Fig. 4).

Measured with Pagel's λ, phylogenetic signal for human-visible flower color (yellow, white, red), 'insect color', were all greater than zero. Human perceived flower color displayed signal significantly lower than expected under Brownian motion (i.e., λ=1), while λ for insect color was not different than 1 (Fig. 2).

6.3.5 Phylogenetic Trait correlations

Considering phylogenetic relatedness, UVP did not differ among taxa with yellow, white, or red flowers (Phylogenetic ANOVA, F=7.4, P=0.28). All Holm-Bonferroni posthoc tests of pairwise comparisons among color groups were not significant (P>0.05). When considering flower color as binary (yellow vs. non-yellow), and UV pattern as binary (reflective if UVP < 0.90), we detected correlated evolution between yellow and UV reflection (log likelihood independent model= -211.2, log likelihood dependent model= -200.7; log likelihood ratio test, P<0.001).

6.3.6 Phylogenetically-controlled geographic and abiotic associations:

UVP was larger in species growing at higher latitude and altitude under an OU model of evolution (latitude, P=0.01; altitude P=0.001; Table 1), which provided a significantly better fit than a BM model (AIC 112 vs. 126). Under a BM model, UVP was not affected by either parameter (both P>0.26; Table 1).

UVP increased with cooler mean annual temperatures (P<0.0001), and tended to increase with higher annual UV irradiance (P=0.07) under an OU model of evolution (Table 1). The OU model fit significantly better than a BM model (AIC 112 vs. 125), under which, UVP increased significantly as a function of cooler temperatures (P=0.015) and higher UV irradiance (P=0.04) (Table 1).

Considering bioclimatic parameters experienced only during the putative growing season,

an OU model again fit significantly better than a BM model (AIC 113 vs. 130). Under the OU model, UVP increased as a function of cooler temperatures (P<0.0001), but rainfall and UV irradiance did not affect UVP (both P>0.34) (Table 1). Under a BM model, no parameters predicted UVP (all P>0.1). Variance inflation factors among predictors in all PGLS models fell below 1.6, thus models did not violate assumptions.

Species with UV reflection on flowers and those that were uniformly UV absorbing did not grow at different latitude or altitude (phylogenetic ANOVAs, both P>0.3). Species with UV reflection experienced higher annual temperatures (F=10.8, P=0.03), and marginally higher growing season temperature (F=7.9, P=0.07). Species with different human-visible flower color did not grow at different latitude or altitude, or experience different bioclimatic factors (F range = 0.07-3.5, P range = 0.5- 0.84). Similarly, species with different insect-visible flower color were not geographically or bioclimatically disparate (F range= 0.8-6.0, P range = 0.15-0.88).

6.4 DISCUSSION

This work provides insight into factors that shape floral color and pattern diversity in *Potentilla*. First, we detected phylogenetic structuring of all flower color and pattern parameters considered, but the extent of UV pigmentation showed the greatest evolutionary lability. Second, the evolution of yellow flowers and the presence of UV reflection are correlated, suggesting nonindependence of human-visible color and UV pattern phenotypes. Finally, quantitative estimates of UV pattern, but not human-visible color, 'insect-perceived' color, was influenced by geography and abiotic variables. In particular, UV pigmentation increased with latitude and altitude due to association with cooler temperatures and UV irradiance, respectively. The strongest effect of temperature on UV pigmentation variation among taxa, however differs from findings of previous studies showing that UV-mediated selection underlies intraspecific UV pigmentation

diversity. This suggests that processes shaping intraspecific diversity may not underlie macroevolutionary patterns of UV pigmentation variation.

The presence of UV reflection from petals displayed phylogenetic signal similar to expectations under Brownian motion, whereas quantitative estimates of UV pigmentation (UVP) showed significantly positive, but reduced phylogenetic signal. Human-visible and insect-perceived color displayed similar levels of phylogenetic signal, but signal for human-visible flower color was significantly less than expected under Brownian motion (Fig. 2). Overall, results suggest that quantitative estimates of floral pattern may be more labile than other discretely-measured floral color parameters. We caution however that this may be due to differences in the quantitative and qualitative nature of the traits, or the differences in the metrics used to measure phylogenetic signal (Blomberg's K vs. Pagel's λ). It is difficult to generalize broad patterns of phylogenetic signal for flower color since the measurement of color is highly varied among studies. However, the estimates for flower color in *Potentilla* tend to be higher than other studies that scored flower color discretely (lambda=0 in Erysimum; Gomez et al. 2015) or quantitatively (Vamosi and McEwen 2008; Muchhala and Smith 2014; LeCroy et al. unpublished). The high phylogenetic signal noted in *Potentilla* is likely due to the fact that white flowers are restricted to the Alba and *Horkelia*/*Ivesia* clade within *Potentilla*, *Sibbaldia* and *Drymocallis* (Fig. 4). Overall, the finding of significant signal for flower color and UV pattern parameters in *Potentilla* suggest that it is important to consider phylogenetic relatedness when evaluating ecological associations with these phenotypes.

This work provides evidence that there is an association between the evolution of UV reflection and yellow flowers, an association that, until now, had only been noted in diverse floras without phylogenetic insight (Guldberg and Atsatt 1974; Dyer 1996). We must caution that we grouped red and white flowers as 'non-yellow' in this analysis because previous studies suggest

that yellow flowers are more likely to reflect UV than white or red flowers (Harborne and Nash 1984; Dyer 1996). Nonetheless, our analysis supports correlated evolution of UV reflection and yellow pigmentation. This may be due to biochemical constraint or correlated selection. For instance, in the absence of physical coloration provided by epidermal petal cell shape, yellow carotenoids may be important for reflecting UV, posing a constraint to the evolution of UV reflection through trait correlation. Indeed, only two of the thirty identified white flowers reflected UV (Fig. 4). Interestingly, three of the pink-red flowered species (cyanidin-pigmented [Harborne and Nash 1984]) displayed UV reflection. The biochemical or physical mechanism by which this occurs warrants further study. An alternate hypothesis for the association between yellow and UV reflection and patterning is pollinator-mediated correlated natural selection. Yellow, UV-absorbing flowers appear 'green' to pollinators and may thus not be easily distinguished from a vegetative background. UV reflecting yellow flowers however may increase signal reception by pollinators. Data on the color of ground cover in *Potentilla* habitats could help to address this hypothesis.

UV pigmentation was higher at higher latitudes, a result that opposed expectations based on Gloger's Rule and observed in *A. anserina* (Koski and Ashman 2015). However, UV pigmentation increased with altitude, a result that was observed in the Colorado Rocky Mountains for *A. anserina* (Koski and Ashman in review). In *A. anserina* the altitudinal increase in UVP was associated with both a change in pollinator assemblage (increased dipteran aboundance), pollinator behaviors (pollinators preferred larger bullseyes), and higher UV irradiance. The specific biotic and abiotic factor(s) that contribute to latitudinal and altitudinal increases in UVP at a macroevolutionary scale should be evaluated. We found marginal support that UVP increased with UV irradiance when controlling for phylogenetic relatedness (Table 1), however temperature appears to be the most influential abiotic factor measured in this study. This association is logical given that temperatures decrease with increasing latitude and altitude. We suggest that the effect of

temperature may be due to a) pleiotropic effects of selection by low temperature, or b) pollination context associated with temperature. First, UV-absorbing pigments like flavonoids can impart resistance to low temperature stress. Second, fly pollination increases with increasing latitude (Elberling and Oleson 1999; Dupont et al. 2009) and altitude (Kearns 1992), and thus, pollinators, rather than direct effects of abiotic factors per se, could underlie the associations seen here. Indeed changes in pollinator community assembly and behaviors can affect spatial UVP variation within taxa (Koski & Ashman in review).

Taken together, our results bring to the fore the potential importance of UV patterning because it, but not other color traits, associated with biogeography and bioclimatic variables. Functional tests addressing whether greater UV pigmentation yields higher fitness in cooler temperatures, and data on pollinator assemblages will greatly enhance our understanding of other factors that may contribute to widespread geographic patterns of flower color and UV pattern variation in *Potentilla*.

Table 6-1: Results of PGLS analyses assessing the effect of latitude and altitude (Geographic), annual temperature, precipitation and UV exposure (Bioclimatic [Annual]), and bioclimatic variables experienced during the warmest quarter of the year (Bioclimatic [Growing season]).

Model Type	Correlation structure	Parameter	Effect	*P*	Model AIC	alpha
Geographic	Brownian	Latitude	-0.13	0.26		
		Altitude	-0.015	0.56	126.6	NA
	Ornstein-Uhlenbeck	Latitude	**0.31**	**0.013**		
		Altitude	**0.09**	**0.001**	112.1*	1
Bioclimatic (Annual)	Brownian	Annual Temp.	**-0.23**	**0.015**		
		Annual Precip.	0.004	0.94		
		Annual UV	**0.11**	**0.041**	125.5	NA
	Ornstein-Uhlenbeck	Annual Temp.	**-0.39**	**0.0002**		
		Annual Precip.	0.04	0.42		
		Annual UV	0.11	0.07	112.1*	1
Bioclimatic (Growing Season)	Brownian	Seasonal Temp.	-0.12	0.1		
		Seasonal Precip.	-0.015	0.62		
		Seasonal UV	0.09	0.17	130.2	NA
	Ornstein-Uhlenbeck	Seasonal Temp.	**-0.29**	**0.0001**		
		Seasonal Precip.	-0.02	0.42		
		Seasonal UV	0.07	0.34	113.8*	1

Effects in bold are significant at $P<0.05$, and the best fit model based on AIC is indicated with by '*'

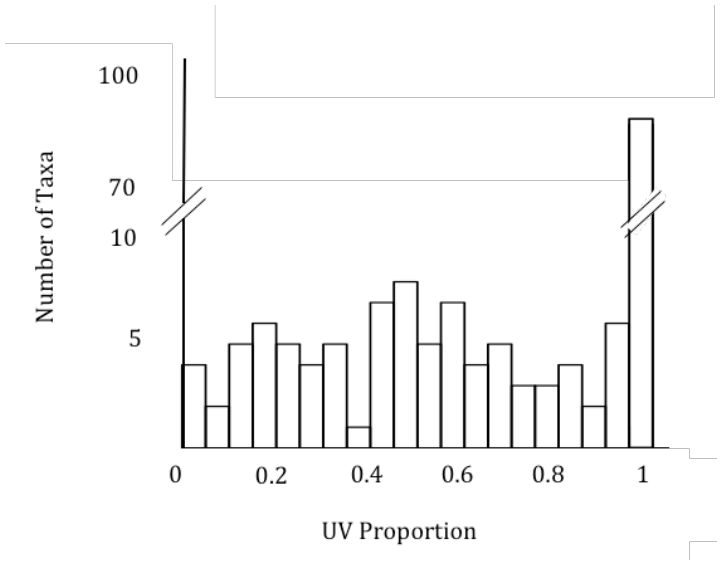


Figure 6-1: Frequency distribution of UV proportion (the relative area of UV absorption on petals) for 177 *Potentilla* and other closely related genera (see text). In analyses in which UV reflection was considered a binary trait, any taxon with average UV proportion less than 0.90 was considered to possess UV reflection.

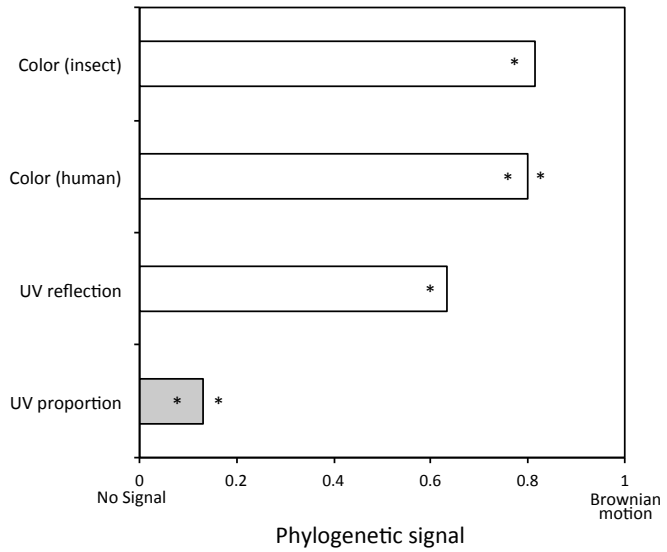


Figure 6-2: Phylogenetic signal for flower color and color pattern parameters in *Potentilla* and closely related genera. Phylogenetic signal was measured with Pagel's λ for discrete characters (open bars), and Blomberg's K for quantitatively estimated UV proportion (gray bar). Asterisks to the left of the bar's apex indicate that λ or K was greater than zero, while asterisks on the right of the bar's apex indicate that signal was significantly lower than 1.

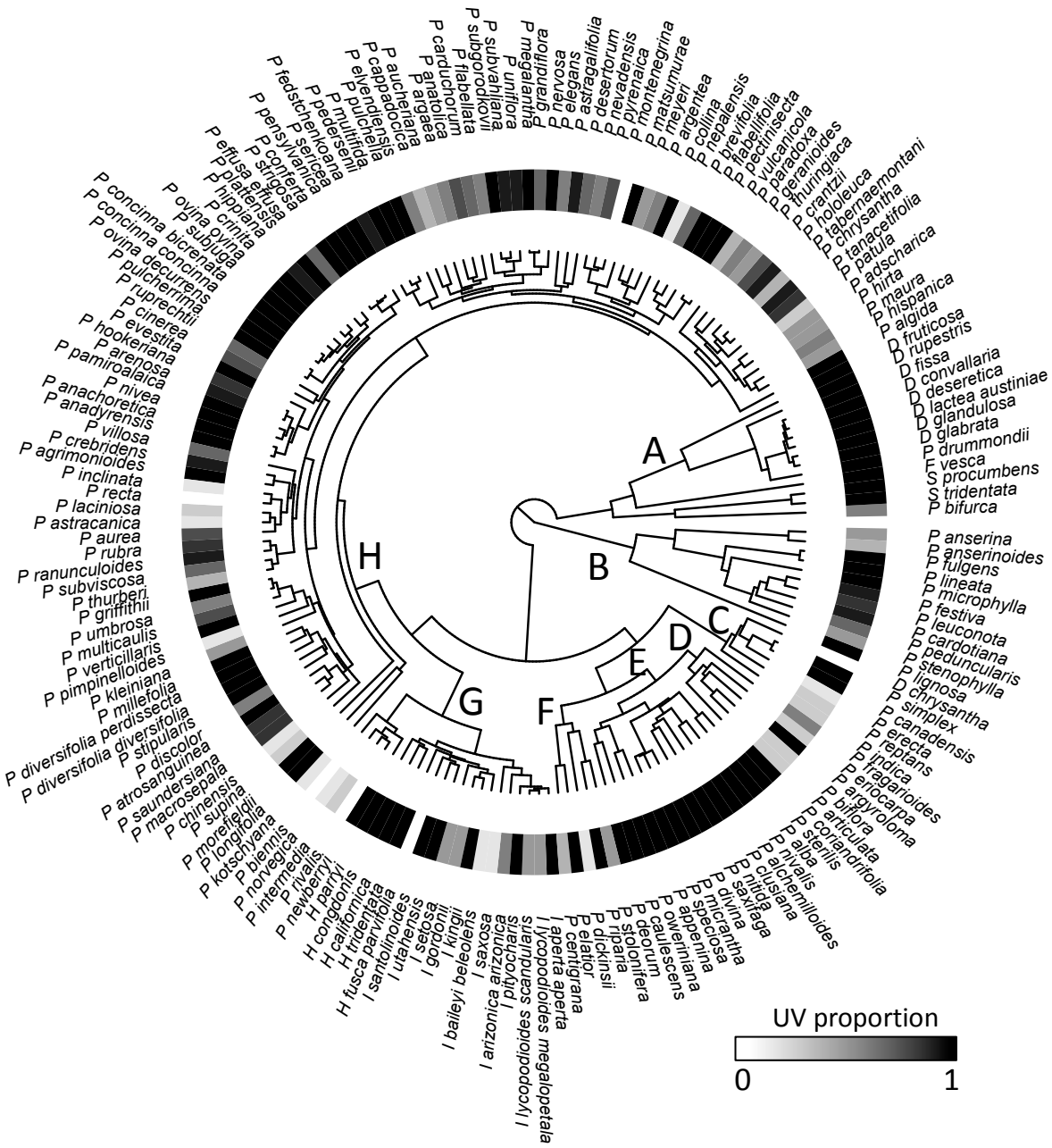


Figure 6-3: UV proportion mapped continuously to the Bayesian maximum clade credibility phylogram of *Potentilla*. We indicated some of the major clades with high support (posterior probabilities >0.95) with A-H. A= *Drymocallis,* B=*Argentina*, C=Reptans, D= Fragarioides, E=Alba, F=newly resolved clade; G=Horkelia/Ivesia clade (note, posterior =0.94); H= *Potentilla*.

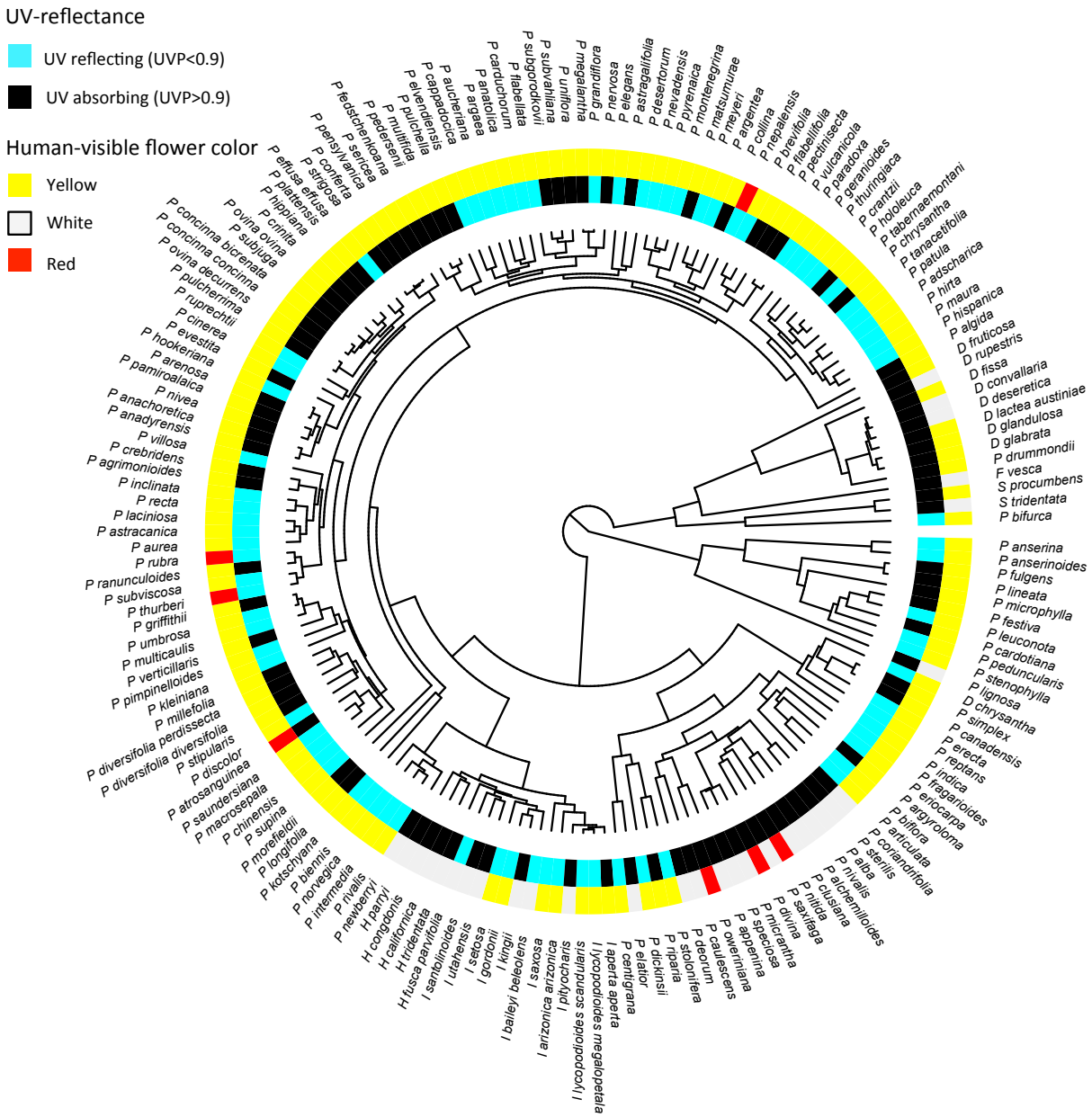


Figure 6-4: The presence (cyan) or absence (black) of UV reflectance, and human-visible flower color (yellow, white, red) mapped to the Bayesian maximum clade credibility phylogram of *Potentilla*.

APPENDIX A

ADDITIONAL TABLES (CHAPTER 2)

Table 7-1: Mean (±SD) and coefficient of variation (CV) for UV pattern components; UV-absorbing area, petal area, and UV proportion, measured on field-collected plants from nine *Argentina pacifica* populations and four *A. anserina* populations.

				UV pattern components					
				UV-absorbing area (mm^2)		Petal area (mm^2)		UV proportion	
Population	Latitude (N)	Longitude (W)	# Flowers	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
A. pacifica									
CA1	39.4903	123.795	18	67.9 ± 27.5	0.40	87.7 ± 24.4	0.28	0.76 ± 0.15	0.20
CA2	40.6830	124.225	18	82.8 ± 21.6	0.26	145.9 ± 23.7	0.16	0.57 ± 0.16	0.28
CA3	40.8606	124.094	27	77.8 ± 27.2	0.35	109.7 ± 30.2	0.28	0.72 ± 0.19	0.26
CA4	41.0611	124.146	19	97.9± 19.3	0.20	112.8 ± 21.5	0.19	0.87 ± 0.05	0.06
OR1	44.9075	124.029	25	94.3 ± 19.7	0.21	125.1 ± 23.2	0.19	0.76 ± 0.10	0.13
OR2	45.1033	123.983	16	79.2 ± 21.6	0.27	112.9 ± 16.2	0.13	0.64 ± 0.15	0.24
OR3	45.1928	123.955	16	69.8 ± 30.9	0.44	112.7 ± 28.7	0.25	0.61 ± 0.14	0.23
OR4	46.1622	123.924	17	76.9 ± 19.7	0.26	129.4 ± 24.4	0.19	0.59 ± 0.11	0.18
WA2	47.5492	123.044	16	53.0 ± 21.0	0.40	98.4 ± 26.1	0.27	0.55 ± 0.18	0.33
A. anserina									
MI*	45.7462	84.899	19	17.8 ± 5.9	0.33	34.2 ± 10.8	0.32	0.53 ± 0.06	0.12
PA*	42.1619	80.081	17	10.0 ± 2.9	0.29	17.0 ± 3.9	0.23	0.58 ± 0.07	0.13
WA1	47.4230	118.0	5	34.4 ± 11.6	0.34	58.9 ± 19.4	0.33	0.58 ± 0.05	0.09
NY*	42.4850	79.359	6	10.8 ± 2.0	0.19	19.7 ± 4.4	0.22	0.56 ± 0.05	0.09

Note- * Indicates that traits were measured on dried flowers.

Table 7-2: Population mean (±SD), and the coefficient of variation (CV) for (a) the concentration of foliar UV-absorbing compounds, and UV pattern components (UV-absorbing area, petal area, UV proportion); (b) spectral properties (brightness, UV reflectance, UV chroma, green chroma) of the petal apex and (c) petal base for five populations of *Argentina pacific* and three populations of *A. anserina* measured on greenhouse-grown clones of multiple genotypes.

(a)				UV pattern components					
		UV-absorbing compounds		UV-absorbing area		Petal area		UV proportion	
Population	Genotypes	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
A. pacifica									
CA3	10	0.09±0.01	0.12	98.7±14	0.14	118±20	0.17	0.84±0.08	0.10
CA4	9	0.07±0.01	0.13	66.0±4	0.06	72±5	0.06	0.92±0.03	0.03
OR1	8	0.08±0.01	0.15	84.6±20	0.24	111±22	0.20	0.77±0.06	0.07
OR3	10	0.08±0.006	0.07	81.5±10	0.12	109±9	0.08	0.75±0.08	0.11
WA2	9	0.09±0.01	0.14	58.9±13	0.21	85±9	0.11	0.69±0.12	0.17
A. anserina									
PA	8	0.06±0.008	0.14	25.1±4	0.17	59±6	0.10	0.42±0.05	0.11
NY	5	0.06±0.008	0.14	26.3±3	0.13	57±5	0.09	0.46±0.04	0.09
MI	9	0.06±0.009	0.15	30.1±9	0.29	64±11	0.17	0.46±0.07	0.15

(b)

		Spectral properties: petal apex							
		Brightness		UV reflectance		UV chroma		Green chroma	
Population	Genotypes	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
A. pacifica									
CA3	10	110±9.0	0.08	5.1±1.5	0.30	0.05±0.010	0.26	0.43±0.005	0.012
CA4	9	104±9.3	0.09	2.8±0.93	0.34	0.03±0.008	0.31	0.44±0.006	0.015
OR1	8	111±8.2	0.07	6.9±1.7	0.25	0.06±0.011	0.18	0.42±0.002	0.005
OR3	10	114±9.0	0.08	7.2±1.47	0.20	0.06±0.012	0.19	0.42±0.005	0.011
WA2	9	115±9.5	0.08	7.3±0.99	0.14	0.06±0.007	0.12	0.42±0.004	0.009
A. anserina									
PA	8	127±7.4	0.06	13.1±2.2	0.17	0.10±0.012	0.12	0.41±0.005	0.011
NY	5	131±7.8	0.06	15.1±2.6	0.18	0.11±0.016	0.14	0.40±0.007	0.018
MI	9	133±12.2	0.09	14.2±2.26	0.16	0.10±0.011	0.10	0.41±0.004	0.010

(c)

		Spectral properties: petal base							
		Brightness		UV reflectance		UV chroma		Green chroma	
Population	Genotypes	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
A. pacifica									
CA3	10	111±3.6	0.03	0.77±0.11	0.15	0.007±0.001	0.20	0.45±0.004	0.008
CA4	9	108±9.7	0.09	0.75±0.36	0.49	0.007±0.004	0.57	0.44±0.006	0.014
OR1	8	109±6.7	0.06	0.58±0.16	0.27	0.005±0.001	0.26	0.45±0.002	0.005
OR3	10	110±5.4	0.05	0.82±0.30	0.36	0.007±0.002	0.29	0.45±0.002	0.005
WA2	9	109±7.9	0.07	1.05±0.35	0.33	0.010±0.003	0.34	0.44±0.003	0.007
A. anserina									
PA	8	111±7.9	0.07	0.58±0.14	0.24	0.005±0.002	0.30	0.45±0.002	0.005
NY	5	103±8.3	0.08	0.72±0.22	0.30	0.007±0.002	0.31	0.45±0.002	0.005
MI	9	113±12.2	0.11	0.81±0.26	0.32	0.006±0.002	0.27	0.45±0.007	0.016

APPENDIX B

ADDITIONAL FIGURES (CHAPTER 3)

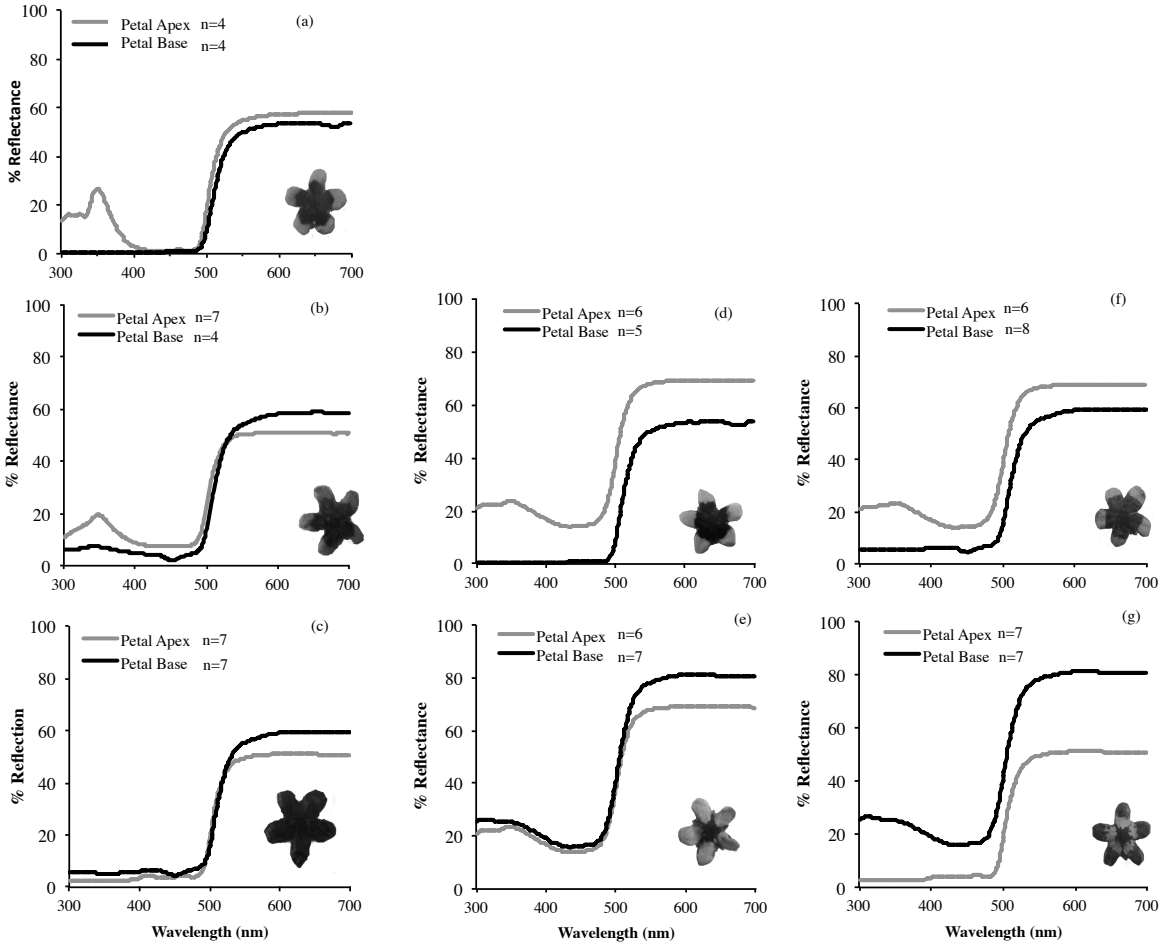


Figure S1 (Figure 7-1): Reflectance spectra of olfactory, tactile, and test flowers. Average reflectance spectra of petal bases (black) and petal apices (gray) of olfactory control flowers for each array (a), tactile controls in the absorbing, reflecting and inverse arrays (b,d,f respectively), and test flowers in the absorbing reflecting, and inverse arrays (c, e, g, respectively). The number of samples measured for each is given in the legend of graphs. Ultraviolet flower images correspond with each reflectance spectrum.

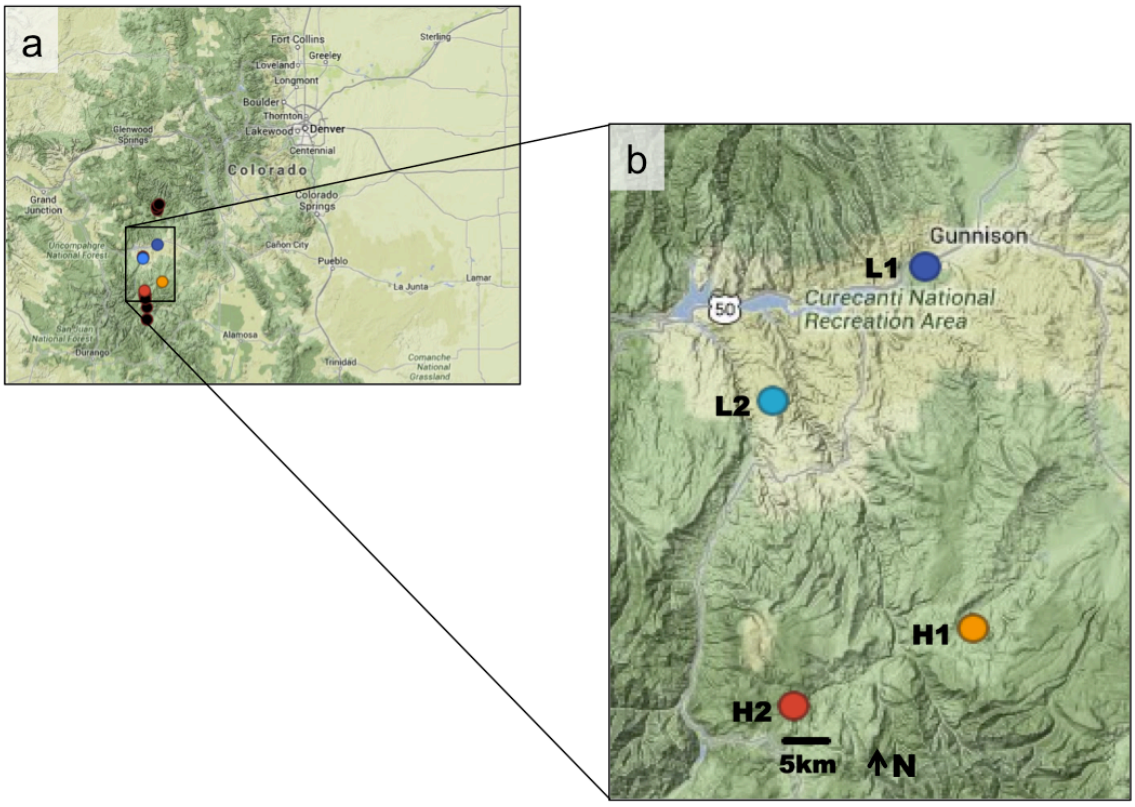
APPENDIX C

ADDITIONAL FIGURES AND TABLES (CHAPTER 4)

Table 7-3: Location, altitude, ecological features, and mean and variance of floral traits for each site

	Site											
	MWL	**BRMP3**	**BRMP2**	**PLK**	**NCC**	**KP**	**MLT**	**TCT2**	**TCT1**	**CD**	**RL**	**FR**
Latitude	38.515	38.384	38.365	38.881	37.838	38.945	38.914	37.980	37.979	37.933	37.714	37.709
Longitude	-106.995	-107.191	-107.197	-106.998	-107.141	-106.974	-107.003	-107.168	-107.157	-107.164	-107.140	-107.141
MASL	2334	2564	2611	2709	2854	2861	2876	3113	3157	3289	3408	3419
% Full Sun	1.00	0.93	0.97	.	0.89	0.91	0.92	0.91	0.74	0.86	0.99	0.92
% Soil Moisture (w:w)	0.17	0.22	0.29	0.39	0.55	0.30	0.07	.	0.38	0.19	0.16	0.19
No. flowers photographed	5	10	9	6	10	15	2	7	5	7	7	15
No. flowers color measured	5	10	9	6	10	13	2	7	5	7	7	10
UVP	0.44	0.48	0.47	0.47	0.53	0.51	0.48	0.50	0.54	0.56	0.62	0.62
CV UVP	0.12	0.12	0.21	0.18	0.21	0.11	0.17	0.17	0.10	0.21	0.07	0.11
Mean petal area (mm^2)	42.89	25.56	27.57	22.88	40.35	34.18	32.17	22.17	33.15	31.30	37.96	33.34
CV petal brightness	0.21	0.42	0.31	0.18	0.23	0.28	0.40	0.19	0.26	0.27	0.22	0.19
Mean petal brightness	109.99	110.51	113.16	104.88	118.46	94.59	109.82	95.55	102.96	91.37	111.04	114.29
CV Brightness (*R*300-*R*700)	0.07	0.10	0.08	0.12	0.08	0.11	0.01	0.10	0.08	0.08	0.08	0.12
Mean UV chroma (%)	0.10	0.09	0.10	0.10	0.12	0.09	0.11	0.11	0.08	0.09	0.08	0.09
CV UV chroma	0.12	0.07	0.09	0.13	0.17	0.07	0.22	0.31	0.09	0.14	0.17	0.22

MASL= meters above sea level; UVP = UV proportion, the proportion of the petal that absorbs UV; brightness = reflectance between 300 and 700nm;
UV chroma = reflectance between 300-400 divided by brightness. Percent soil moisture was measured as (soil wet weight - soil dry weight) / soil wet weight.



Appendix B (Figure 7-2): (a) Map of Colorado, with the *Argentina anserina* populations sampled denoted by points. (b) The four focal populations within which we measured the relationship between pollen receipt and floral traits, pollinator preference, and characterized pollinator community composition for *A. anserina* are highlighted and labeled.

Table 7-4: Pearson product moment correlations among floral traits in four *Argentina anserina* populations in which the floral trait-pollen receipt relationship was measured. In L1 and L2, the number of open flowers was invariable and therefore correlations were not measured for this trait (denoted by '−'). In L1, brightness and UV chroma were not measured (denoted by '.')

	Population			
	L1	L2	H1	H2
UVP vs. petal size	0.04	-0.21	-0.12	0.01
UVP vs. brightness	-0.10	.	0.01	-0.31*
UVP vs. UV chroma	-0.12	.	-0.14	-0.25*
UVP vs. open flowers	−	−	0.07	0.01
Petal size vs. brightness	**0.46****	.	**0.42****	0.29*
Petal size vs. UV chroma	**0.48****	.	0.31**	0.30*
Petal size vs. open flowers	−	.	-0.13	0.05
Brightness vs. UV chroma	**0.38****	.	**0.48****	0.29*
Brightness vs. open flowers	−	.	-0.04	0.05
UV chroma vs. open flowers	−	−	0.03	-0.07
N	60-76	68	66	67

*$P<0.05$, **$P<0.01$, bold= significant after Bonferroni correction. 'L' refers to low altitude and 'H' refers to high altitude. UVP= UV proportion, the proportion of the petal that absorbs UV; brightness= reflectance between 300 and 700nm; UV chroma= reflectance between 300-400nm divided by brightness.

APPENDIX D

ADDITIONAL INFORMATION (CHAPTER 5)

Floral traits and bioclimatic variables

To quantify floral UV bullseye size we scored UV proportion (the relative area of petal UV absorption) from UV photographs of field-collected flowers (see Koski and Ashman 2013 for detail). Collections were made by walking linear transects through the entire extent of populations and collecting a flower every 2+ meters. We scored UV proportion for 456 plants from 34 pristine natural populations from the Pacific Coast (Koski and Ashman 2013) (n=9), the Rocky Mountains (n=13), the Great Lakes (Koski and Ashman 2013) (n=3), and New Zealand (n=9) between June 2011 and January 2013 (Supplementary Table 1). We scored 2-27 flowers per population ($\overline{x}$=13.4) depending on population size and proportion flowering. On all, or a subset of flowers in 28 populations (Supplementary Data Table 1), we recorded spectral reflectance at the petal apex and base using a UV-VIS spectrophotometer (Ocean Optics USB4000 or Jaz spectrophotometer, Dunedin, FL, USA), and calculated UV chroma ($R_{300\text{-}400}/R_{300\text{-}700}$) using CLR software [version 1.05, R. Montgomerie]. Only UV chroma at the petal tip was correlated with UV proportion, but neither tip nor base chroma were associated with latitude (Supplement 3). For this reason we only pursued subsequent laboratory experiments to examine the effects of UV proportion on pollen viability.

Using WorldClim[31] (2.5x2.5min resolution) and DIVA-GIS (v. 7.5), we obtained mean annual temperature (BIO 1) and precipitation (BIO 12) for each population. From gIUV (Beckmann et al. 2014) we obtained UV-B exposure from the UVB5 layer (Sum of monthly mean UV-B during highest quarter). This GIS layer maps UV-B measurements in 15 arc-minute

steps globally, taken from the Ozone Monitoring Instrument aboard the NASA EOS Aura Spacecraft from 2004-2013 (Beckmann et al. 2014).

Test of Gloger's rule and abiotic associations with UV bullseye size

We used the absolute value of latitude to represent a population's distance from the Equator. We used analysis of covariance (ANCOVA) to model average population UV bullseye size as a function of the categorical variable of region, the continuous terms of altitude and latitude, and the interactions between region and the continuous terms.

To determine which bioclimatic variable(s) predicted bullseye size, we modeled mean population bullseye size as a function of region, temperature, precipitation, UV-B, and the interaction between region and bioclimatic variables using ANCOVA. Both ANCOVAs were performed with PROC GLM in SAS [SAS 9.3; SAS Institute Inc., 2011].

UV-mediated phenotypic selection on bullseye size

Plant material

To assess selection across a range of bullseye sizes we sampled individuals from a stock population of silverweed cinquefoil grown in the glasshouse for several years composed of plants originally collected from 14 sites across the three North American transects. Three weeks before flowering, plants received ~122mg of slow-release fertilizer (Nutricote). During experiments, plants grew in a growth chamber with 11hr days at 15.5°C/10°C (day/night). We measured bullseye size on 3-4 clones per individual in a previous study (Koski and Ashman 2013), or took measurements directly on the flowers during the experiment.

UV exposure and pollen germination

From 71 plants we removed flowers, and placed them in water-filled microcentrifuge tubes in a 'UV chamber' illuminated with full-spectrum lighting. The first flower from each plant was randomly assigned to a UV absent or present treatment (Fig. S1). Flowers were haphazardly arranged and exposed to 6 hrs light/12 hrs dark/6 hrs light to mimic diurnal rhythms. After exposure, we left pollen to germinate *in vitro* and scored germination, blinded. See Supplement 2 for lighting, UV treatment, and pollen germination details.

The second flower from each plant received the opposite treatment of the first flower. We scored germination on 4-6 flowers per plant and calculated mean pollen viability for each treatment-by-individual. We removed five pollen-sterile individuals (<0.05% germination regardless of treatment) from the dataset prior to analysis.

Phenotypic selection analyses

Pollen viability was used as a component of male fitness. We calculated relative pollen viability ($viability_x$/mean viability) in each treatment and standardized bullseye size (Z-score). We regressed fitness on bullseye size and then on bullseye size and its squared term to test for directional and stabilizing or disruptive selection, respectively (Lande and Arnold 1983). To assess whether selection differed between treatments we used ANCOVA with fixed effects of Treatment, UV bullseye size, UV bullseye size^2, and Treatment x Trait interaction terms. A significant interaction between treatment and bullseye size or its squared term indicates significant differences in directional or stabilizing selection between the treatments, respectively. We used population of origin, the average date of flowering for each individual within each treatment (Z-score), and their interactions with treatment as random effects to account for any

population-level variation in pollen viability, and temporal variation in UV exposure as bulbs aged. Date is analogous to a random blocking factor that can be used in selection analyses (e.g. Etterson 2004). The between-within option (DDFM=BETWITHIN) was used to estimate the denominator degrees of freedom since this is recommended for designs with many random effects and unbalanced data [UCLA: Statistical Consulting Group; http://www.ats.ucla.edu/stat/sas/notes2/]. Next, we examined nonparametric relationships between relative fitness and bullseye size using cubic splines with λ values that minimized GCV scores (Schluter 1988), ($\lambda_{UV\ absent}$=10, $\lambda_{UV\ present}$= -2; Fig. S2). Finally, we tested whether fitness optima in each treatment was at an intermediate value of bullseye size using the Mitchell-Olds Shaw Test (MOStest) in R's vegan package (Oksanen et al. 2013). This tests whether the relationship between a dependent and independent variable is unimodal, or simply increasing/decreasing quadratic (null expectation), by testing whether the peak is at the minimum or maximum of the independent variable (trait value).

UV bullseye as a target of selection

To determine if bullseye size is a target of UV-mediated selection, we tested UV's effect on pollen placed in artificial flowers with varying bullseye size. We constructed conical paper flowers (Rite in the Rain, Tacoma, WA) painted with yellow UV-reflective (Koski and Ashman 2014) and UV-absorbing paints ('UV Yellow', Reel Wings Decoy Company inc. and, '509 Sunny Yellow', Plaid Enterprises, Inc., Norcross, GA). Three bullseye sizes spanning natural variation were created; small (~20% UV proportion), medium (~50% UV proportion) and large (~80% UV proportion). Artificial flowers were otherwise identical (Fig. 4).

Twenty-two individuals from the stock population were pollen donors for the experiment.

From each, we removed six dehisced anthers from a single flower, and placed each anther onto a microscope cover slip placed inside one of six artificial flowers (two small, two medium, two large). One set of three flowers (small, medium, large) received a UV present treatment and the other a UV absent treatment. Pollen was germinated *in vitro* and germination scored.

To test the effects of bullseye size, UV treatment, and their interaction on pollen viability, we used a mixed general linear model (SAS PROC MIXED) with treatment, bullseye size, and their interaction as fixed effects. Pollen donor identity and its interactions with fixed effects were random effects. We assessed pairwise differences between bullseye size-UV treatment combinations using Tukey-Kramer post-hoc adjusted *P*-values.

Table 7-5: Region, location (latitude and longitude), altitude, sample size, mean bullseye size (UV proportion, i.e., the proportional area of the petals that absorb UV), UV chroma ($R_{300\text{-}400}/R_{300\text{-}700}$) at the base and apex of petals, and bioclimatic variables (mean annual temperature, annual precipitation, mean UV-B incidence during the highest UV quarter) for each population surveyed.

REGION*	POPULATION NAME	LATITUDE (dd)	LONGITUDE (dd)	ALTITUDE (Ft. above sea level)	NUMBER FLOWERS SCORED, UVP	MEAN FLORAL UV BULLSEYE SIZE (UVP)	NUMBER FLOWERS SCORED, UV CHROMA	MEAN UV CHROMA, PETAL APEX	MEAN UV CHROMA, PETAL BASE	MEAN UV-B IRRAD. DURING HIGHEST QUARTER (J/m^2/day)	ANNUAL MEAN TEMP. (°C)	ANNUAL RAINFALL (mm)
CO	BRMP2	38.365	-107.197	8566	9	0.469	9	0.095	0.016	19507.793	2.513	397
CO	BRMP3	38.384	-107.191	8412	10	0.475	10	0.104	0.010	19507.793	2.592	395
CO	CBP	39.346	-105.931	10095	17	0.403	-	-	-	19230.762	-0.213	558
CO	CD	37.933	-107.164	10791	7	0.560	7	0.088	0.009	20096.854	-1.400	846
CO	FR	37.709	-107.141	11217	15	0.617	10	0.095	0.005	19923.320	-0.408	812
CO	KP	38.945	-106.974	9386	15	0.522	13	0.091	0.014	19909.539	0.013	547
CO	MCR	39.392	-105.829	9429	15	0.524	-	-	-	19230.762	1.200	469
CO	MLT	38.914	-107.003	9436	2	0.484	2	0.112	0.014	19543.607	0.196	557
CO	MWL	38.515	-106.995	7657	5	0.438	5	0.102	0.003	19694.789	3.050	314
CO	NCC	37.838	-107.141	9364	10	0.526	10	0.122	0.006	20096.854	0.246	656
CO	PLK	38.881	-106.998	8888	6	0.472	7	0.102	0.018	19909.539	0.788	534
CO	RL	37.714	-107.140	11181	7	0.622	7	0.083	0.011	19923.320	-0.408	812
CO	TCT	37.980	-107.163	10285	12	0.523	12	0.095	0.008	20096.854	-0.804	759.5
GL	PG	42.485	-79.359	581	6	0.560	5	0.115	0.007	12042.152	8.858	1014
GL	PI	42.162	-80.081	576	17	0.580	8	0.104	0.005	12209.407	9.283	1036
GL	WSP	45.746	-84.899	587	19	0.530	9	0.105	0.009	11415.283	6.104	780
NZ	CWL	-43.605	171.058	2241	20	0.267	19	0.130	0.010	12636.238	8.283	1233
NZ	LCR	-43.335	171.589	1893	5	0.227	5	0.125	0.010	12334.619	9.004	1027
NZ	LE	-43.628	171.099	2188	11	0.264	11	0.132	0.011	12636.238	8.317	1143
NZ	LP	-43.092	171.783	1985	6	0.211	6	0.114	0.011	12832.785	7.838	2213
NZ	ML	-43.577	171.183	2044	16	0.295	16	0.134	0.011	12636.238	8.604	1166
NZ	RH	-43.889	172.237	0	17	0.255	17	0.108	0.004	12292.781	11.725	644
NZ	ROS	-38.786	177.130	1519	8	0.423	8	0.102	0.011	13212.818	10.763	2102
NZ	TUT	-39.212	176.890	568	12	0.519	13	0.135	0.011	12866.742	13.050	1396
NZ	TYP	-43.798	172.373	417	13	0.369	4	0.092	0.010	12407.956	11.771	637
PAC	AM	40.861	-124.094	8	27	0.720	10	0.046	0.007	13091.971	11.871	1116
PAC	CL	39.490	-123.795	28	18	0.760	-	-	-	13611.770	11.342	1044
PAC	FL	45.193	-123.955	11	16	0.610	10	0.063	0.007	11155.389	10.425	2265
PAC	HB	40.683	-124.225	5	18	0.570	-	-	-	13358.356	11.604	1231

PAC	HC	45.103	-123.983	13	15	0.640	-	-	-	11155.389	10.175	2422
PAC	HH	47.549	-123.044	10	17	0.550	10	0.063	0.010	10041.581	9.779	1578
PAC	SAL	44.908	-124.029	10	25	0.760	8	0.062	0.005	11354.788	10.500	2234
PAC	SR	46.162	-123.924	13	18	0.590	-	-	-	10249.276	10.458	1944
PAC	TRN	41.061	-124.146	34	22	0.870	10	0.026	0.007	13124.070	11.225	1384

*CO=Colorado Rockies USA; GL=Great Lakes; NZ=New Zealand; PAC=Pacific Coast USA

"-" indicates missing data

Supplement 2: Materials and methods for UV lighting, treatment groups (UV present or absent), an *in vitro* pollen germination.

UV lighting and treatments

The lighting chamber was illuminated by four equally spaced UV-transmitting lights (26W Repti Glo 10.0 UVB, Rolf C. Hagen Corp., Mansfield, MA) and a single broad spectrum light to provide non-UV wavelengths (25W Daylight Basking Spot, Rolf. C. Hagen Corp.). It was constructed of PVC pipe wrapped in black plastic to eliminate external light. Due to discontinuation of UV bulbs used in the first experiment with natural flowers, we used the available replacement bulbs (26W UVB 150, Rolf C. Hagen Corp.) during the second experiment with artificial flowers.

To block UV in the UV absent treatment, we placed a 4x4in piece of UV-blocking film (Rosco no. 3114) 1 inch over the flower, effectively blocking UV wavelengths from reaching the flower but transmitting light at other wavelengths (Fig. S1). To control for the filter, we placed Saran Wrap^{tm} 1 inch above flowers in the UV present treatment, which transmitted light in the UV spectrum (Fig. S1). We confirmed the efficacy of treatments by measuring the absolute irradiance at the flower level in each experimental treatment with a Jaz spectrometer (Ocean Optics, Dunedin, FL; Fig. S1). To compare the experimentally created UV environment to that experienced in nature, we integrated W/m^2 between 300 and 315nm in the experimental chamber and at noon in a natural population in Colorado at 2700m above sea level. Temperature of the floral environment under the plastic filters was not different (UV absent vs. UV present: 25.4±0.29 vs. 25.7±0.32°C; t=-0.79, P>0.40). In the chamber, flowers were haphazardly arranged and exposed to 6 hrs light/12 hrs dark/6 hrs light to mimic diurnal rhythms under field

conditions.

In vitro pollen germination

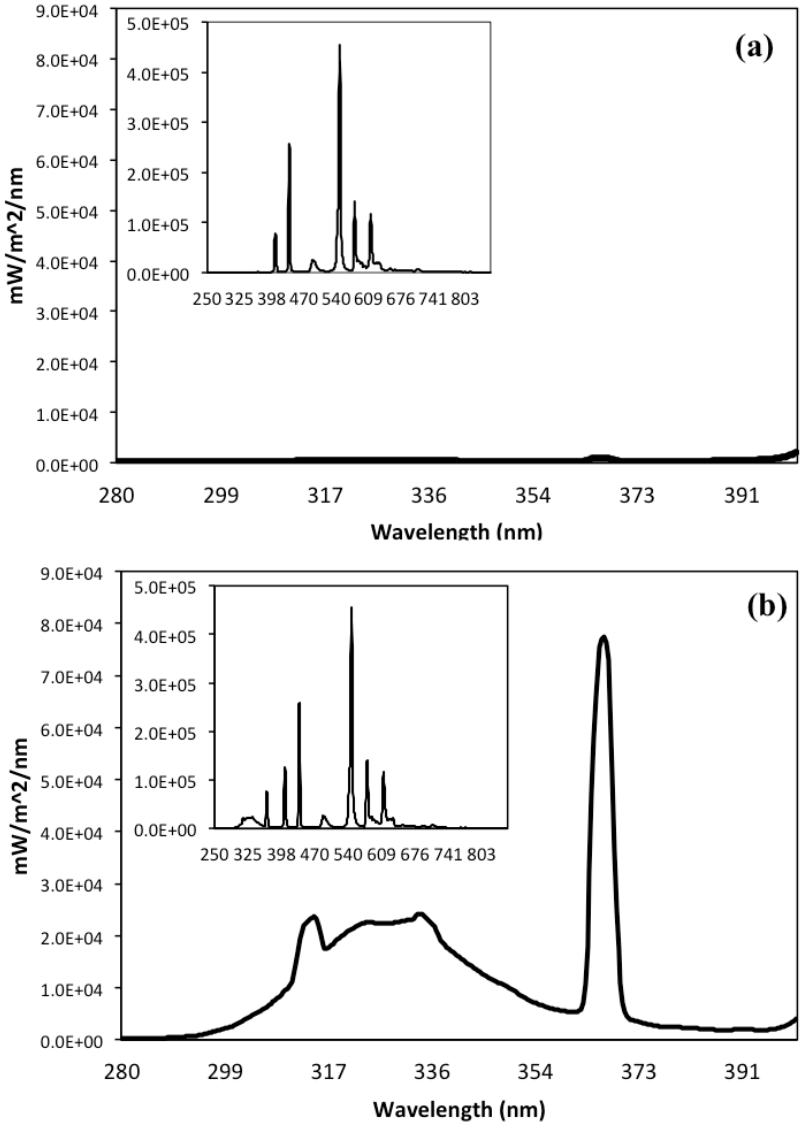
After exposure, we removed a dehisced anther from each flower and spread pollen in a drop of 10% sucrose Brewbaker-Kwack solution (Torabinejad et al. 1998) on a microscope coverslip. This was placed in a petri dish with a piece of moist filter paper and pollen was allowed to germinate under light for 18 hours. After germination, we added a drop of Farmer's fixative (EtOH:Acetic Acid; 3:1) to each sample to arrest germination (Torabinejad et al. 1998). The cover slip was then inverted onto a microscope slide and placed in a refrigerator at 4°C until pollen germination was scored. Within two days of slide preparation we scored a minimum of 200 randomly selected pollen grains per sample as either ungerminated or germinated using light microscopy. Grains were considered germinated when they had a pollen tube that was at least as long as the pollen grain (Torabinejad et al. 1998). Proportion pollen viability per flower was calculated as germinated grains/(germinated + ungerminated grains).

Supplement 3: Examination of UV chroma's correlations with UV bullseye size, and association with latitude.

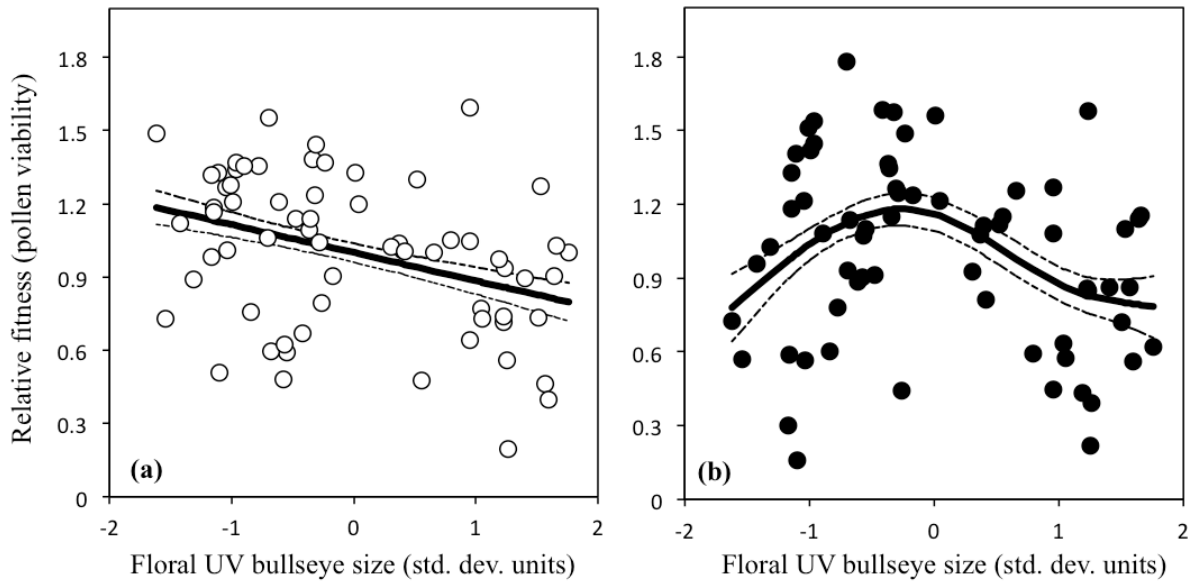
To explore whether spatial patterns in the intensity of UV absorption adhered to predictions of Gloger's rule, we associated UV chroma measured at the petal base and apex with UV bullseye size, and modeled each chroma value as a function of region, altitude, latitude, and the region x altitude and region x latitude interactions.

There was no correlation between mean population UV bullseye size and basal UV chroma (r = -0.27, P= 0.17), and all flowers are strongly UV-absorbing at the base of petals.

Larger UV bullseyes tended to have lower UV chroma at petal tips (r= -0.77, P<0.001). Basal petal UV chroma did not vary among regions ($F_{3,16}$=1.9, P=0.17) and did not covary with latitude ($F_{1,16}$=2.1, P=0.17). However we detected a significant interaction between region and latitude ($F_{3,16}$=3.9, P=0.03), suggesting that latitude did not consistently predict variation across regions. In addition, apical petal UV chroma varied significantly among regions ($F_{3,16}$=32.2, P<0.0001) but no other factors explained variation (e.g., Latitude, $F_{1,16}$=2.1, P=0.17). Since the intensity of UV absorption did not show latitudinal variation, but UV bullseye size did, this suggests that the primary trait responding to ecological changes with latitude is UV bullseye size.



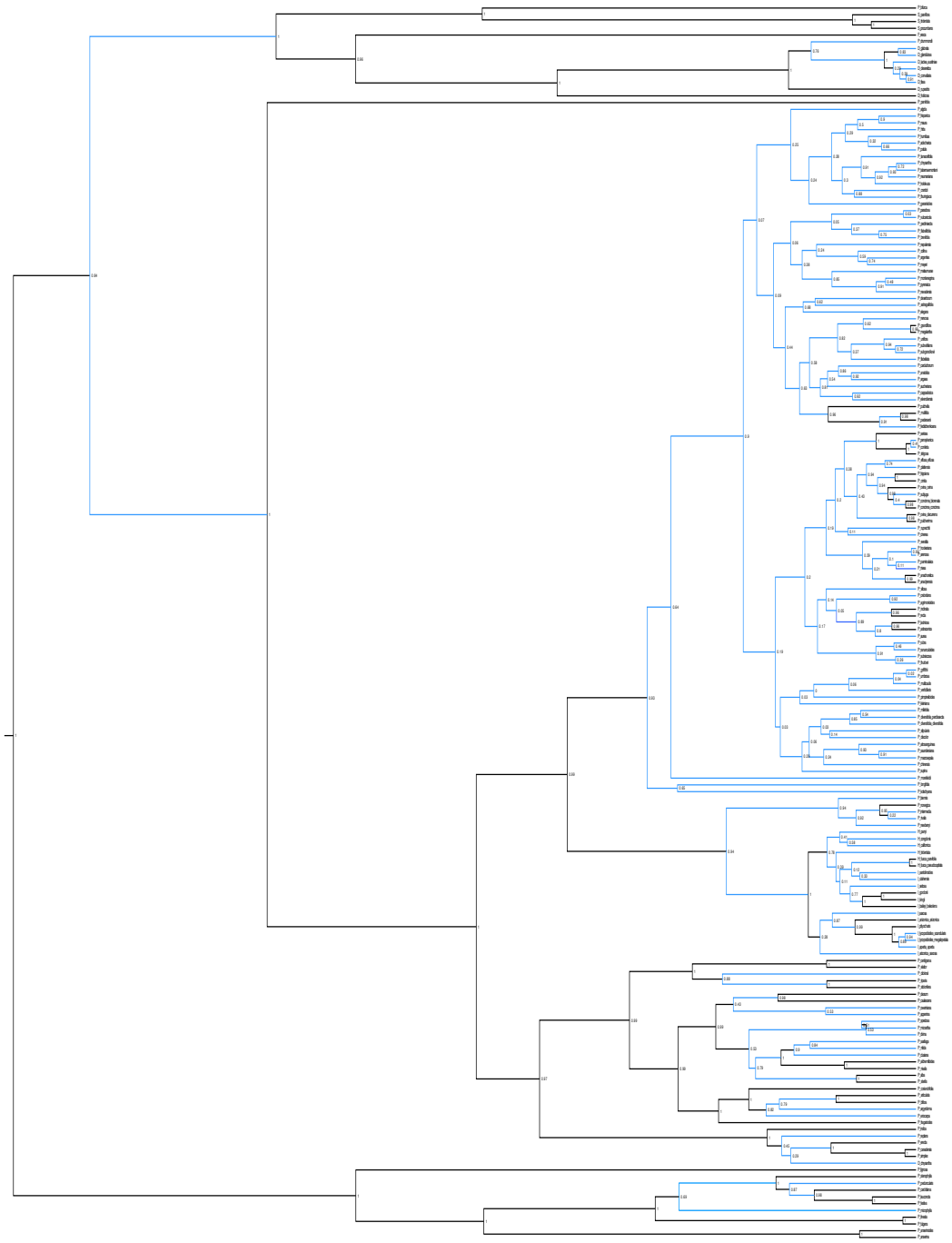
Extended Data figure 1 (Figure 7-3): Absolute irradiance (mW/m^2/nm) in the UV spectrum (280-400nm) in the UV absent (a) and UV present (b) treatments used for the selection experiments. Inset graphs display irradiance from 250-850nm for each treatment.



Extended Data figure 2 (Figure. 7-4): Cubic splines (Schluter 1988) of pollen viability (relative) as a function of UV bullseye size (standard deviation units) in the absence (a) and presence (b) of experimental UV. Solid lines are cubic splines and dashed lines are 95% bootstrap confidence intervals.

APPENDIX E

ADDITIONAL INFORMATION (CHAPTER 5)



Appendix 1 (Figure 7-5): Bayesian 50% maximum clade credibility tree for 177 accessions of *Potentilla* and closely related genera. Posterior probabilities are associated with each note. Branches associated with nodes of ≥0.95 support are black while those with support ≤0.95 are blue.

Table 7-6: Accession data for leaf tissue samples used for DNA extraction.

Species	Herbarium	Collector	Country	Year	Genbank ITS	Genbank ETS	Genbank *trn*L-F
Argentina anserina							
ITS		T. Eriksson 644			U90788.1		
ETS		Eriksson T. TE#153				FN421405.1	
trnL-F	HEID 805505						GQ384662.1
Dasiphora fruticosa							
ITS					GU444027.1		
ETS		T. Eriksson 806				FJ422355.1	
trnL-F		MO 04930363					GQ384680.1
Drymocallis convallaria							
ITS, ETS, trnL-F	UC1728144	Noel & Patricia Holmgren	USA (ID)	1988			
Drymocallis deseretica							
ITS, ETS, trnL-F	UC1583253	Noel & Patricia Holmgren	USA (UT)	1984			
Drymocallis fissa							
ITS, ETS, trnL-F	CM496153	F. H. Utech 92-904	USA (CO)	1992			
Drymocallis glabrata							
ITS, ETS, trnL-F	UC1583221	N. & P. Holmgren & S. Keller	USA (UT)	1984			
Drymocallis lactea var. austiniae							
ITS, ETS	JEPS80322	Vernon H. Oswald & Lowell Ahart	USA (CA)	1996			
Drymocallis rupestris							
ITS, ETS		M. Lundberg 6			FJ356163	FJ422359.1	
trnL-F	HEID 806644						
Duchesnea chrysantha							GQ384650.1
ITS, ETS, trnL-F	CM508074	Koji Yonekura	Japan	2002			
Duchesnea indica							
ITS		T. Eriksson s.n.			U90792.1		
trnL-F		Eriksson s.n.					AJ512242.1
Fragaria vesca							
ITS, ETS, trnL-F		Eriksson & Smedmark 43			AJ511771.1	FJ422362.1	AJ512232.1
Horkelia californica var. elata							
ITS, ETS, trnL-F	JEPS93802	V. H. Oswald & L. Ahart	USA (CA)	1998			
Horkelia congdonis							
ITS, ETS, trnL-F	UC1787123	Michael Honer	USA (CA)	2002			
Horkelia fusca var. parviflora							
ITS, ETS, trnL-F	CM322771	M. J. Williams et al	USA (NV)	1983			
Horkelia fusca var. pseudocapitata							
ITS	UC1561186	Ginger V. King	USA (OR)	1987			

ITS, ETS	CM485378	F. H. Utech 90-871	USA (CA)	1990			
trnL-F							GQ384732.1
Ivesia aperta var. aperta							
ITS, ETS	UC1559690	Arnold Tiehm & Jan Nachlinger	USA (NV)	1984			GQ384744.1
trnL-F							
Ivesia arizonica var. arizonica							
ITS, ETS, trnL-F	RSA508466	Tiehm & Nachlinger 9156	USA (NV)	1984			
Ivesia arizonica var. saxosa							
ITS, ETS, trnL-F	UC1559755	A. Tiehm & M. Williams	USA (NV)	1982			
Ivesia baileyi var. beneolens							
ITS, ETS, trnL-F	RSA508468	Tiehm & Ertter 9029	USA (NV)	1984			
Ivesia gordonii							
ITS, ETS	CM473284	B. Moseley	USA (ID)	1990			
trnL-F							GQ384725.1
Ivesia kingii							
ITS, ETS, trnL-F		J.L. Reveal et al. #4782			FN430787	FN421377	FN561735
Ivesia lycopodioides subsp. megalopetala							
ITS, ETS	RSA131949	Quibell 6509	USA (CA)	1957			
Ivesia lycopodioides subsp. scandularis							
ITS, trnL-F	RSA663921	Holmgren & Holmgren 11022	USA (CA)	1985			
Ivesia pityocharis							
ITS, ETS, trnL-F	UC1728514	Arnold Tiehm	USA (NV)	1997			
Ivesia santolinoides							
ITS, ETS	CM265071	Thorn & DeDecker	USA (CA)	1969			
trnL-F							GQ384743.1
Ivesia saxosa							
ITS, ETS	UC1559752	Jim Morefield	USA (CA)	1984			
trnL-F							GQ384742.1
Ivesia setosa							
ITS, ETS, trnL-F	CM480028	A. Tiehm 12076	USA (NV)	1994			
Ivesia utahensis							
ITS, ETS	CM361607	Holmgren & Holmgren	US (UT)	1984			
trnL-F							GQ384738.1
Potentilla adscharica							
ITS, ETS, trnL-F	E00409739	Davis & Hedge D29539	Turkey	1957			
Potentilla agrimonioides							
ITS, ETS, trnL-F	E00663810	Elias, Shelter & Murray 7131	Russia	1984			
Potentilla algida							
ITS**, ETS, trnL-F	RSA376319	Sojak	Kyrgyzstan	1979			
Potentilla alba							
ITS, ETS		Topel M. MA122			FN430774	FN421355.1	
trnL-F	HEID 805513						GQ384664.1
Potentill alchemilloides							

ITS, ETS, trnL-F		A. Anderberg & A.-L. Anderberg 26			FJ356168	FJ422367	FJ422297
Potentilla anachoretica							
ITS, ETS, trnL-F	E00663806	Korobkov 77-213	Russia	1977			
Potentilla anadyrensis							
ITS, ETS, trnL-F	E00663807	Petrovski	Russia	1982			
Potentilla anatolica							
ITS, ETS, trnL-F	E00409724	Davis 45494	Turkey	1966			
Potentilla anserinoides							
ITS, ETS, trnL-F		Koski	New Zealand	2012			
Potentilla apennina var. appenina							
ITS, ETS	E00128311	Akeroyd et al. 4200	Italy	1983			
Potentilla arenosa							
ITS, ETS, trnL-F	Arnold	Deyl & Sojak	Russia	1976			
Potentilla argaea							
ITS, ETS, trnL-F	E00409678	Lamond 4760	Azerbaijan	1971			
Potentilla argentea							
ITS, ETS, trnL-F		Topel M. MA 143			FN430808.1	FN421387.1	FN561750
Potentilla argyroloma							
ITS, ETS	E00205426	Klein 9223	Iran	1978			
Potentilla articulata							
ITS, ETS, trnL-F	KGB 324				FN555611.1	FN421410.1	FN666414
Potentilla astracanica							
ITS, ETS, trnL-F	CM250465	Markova	Bulgaria	1971			
Potentilla astragalifolia							
ITS, ETS, trnL-F	RSA307559	Elias et al. 4781	Russia	1978			
Potentilla atrosanguinea							
ITS, ETS, trnL-F		Topel M. MA125			FN430778	FN421372	FN556398
Potentilla aucheriana							
ITS, ETS, trnL-F	E00409752	Soják	Iran	1977			
Potentilla aurea							
ITS, ETS	CM359702	Pistarino	Italy	1989			
trnL-F	HEID 805691						GQ384667.1
Potentilla biennis							
ITS, ETS	RM601177	Dorn 6284	USA (WY)	1995			
trnL-F							GQ384775.1
Potentilla biflora							
ITS, trnL-F		Eriksson T. TE#207			FN430826		FN561749
Potentilla bifurca (=*Sibbaldianthae bifurca*)							
ITS, ETS, trnL-F	Gray	Ho et al. 1716	China	1996			
Potentilla brevifolia							
ITS, ETS	RM579088	Evert 18257	USA (WY)	1989			
trnL-F							GQ384748.1
Potentilla canadensis							

ITS, ETS, trnL-F	CM376697	W. A. Zanol 970	USA (PA)	1992			
Potentilla cappadocica							
ITS, ETS, trnL-F	E00409794	C. Tobey 982	Turkey	1965			
Potentilla cardotiana							
ITS, ETS, trnL-F	Gray	Heng et al. 28068	China	2005			
Potentilla carduchorum							
ITS, trnL-F	E00081576	Davis & Polunin 24592	Turkey	1954			
Potentilla caulescens							
ITS, ETS, trnL-F		Topel M. MA 133			FN430819.1	FN421379.1	FN556399
Potentilla centigrana							
ITS, ETS, trnL-F	CM349338	Kurosawa & Iketsu	Japan	1988			
Potentilla chinensis							
ITS, ETS, trnL-F	CM349851	Zhenhai	China	1988			
Potentilla chrysantha							
ITS, ETS, trnL-F		Topel M. MA142			FN430803	FN421385	FN556400
Potentilla cinerea							
ITS, ETS, trnL-F	E00128298	Gardner & Gardner 1982	Spain	1982			
Potentilla clusiana							
ITS, ETS, trnL-F		Antonelli A. AA 353			FN430812.1	FN421403.1	FN556401.1
Potentilla collina							
ITS, ETS	Gray	Barta 2001-121	Austria	2001			
trnL-F	HEID 806679						GQ384674.1
Potentilla concinna var. concinna							
ITS, ETS, trnL-F	RM396676	R. Williams	USA (WY)	1987			
Potentilla conferta							
ITS, ETS, trnL-F	Arnold	Tsvelev et al.	Kazakhstan	1955			
Potentilla coriandrifolia							
ITS, ETS, trnL-F	Arnold	Boufford et al. 29124	China	1998			
Potentilla crantzii							
ITS, trnL-F		Eriksson T. TE 703			FN555609		FN556402
Potentilla crebridens							
ITS, ETS, trnL-F		Eriksen B. BE 569-4			FN430811	FN421356	FN561731
Potentilla crinita							
ITS, ETS, trnL-F	RSA 462447	Gustafson 2461	USA (NM)	1982			
Potentilla deorum							
ITS, ETS, trnL-F	E00663821	Archibald 369	Greece	1964			
Potentilla desertorum							
ETS	RSA376320	Sojak	Kyrgyzstan	1979			
trnL-F	HEID 806610						GQ384643.1
Potentilla dickinsii							
ITS, ETS, trnL-F		Topel M. MA123			FN430775.1	FN421402.1	FN561727.1
Potentilla discolor							
ITS	PS1079MT01				FJ980389.1		

ETS		Topel M. MA141				FN421396.1	
trnL-F	CM311461	Guilin expedition 70092	China	1984			
Potentilla diversifolia var. diversifolia							
ITS, ETS, trnL-F	RM729240	Kinter 301	USA (WY)	1994			
Potentilla diversifolia var. perdissecta							
ITS, ETS	RM584476	W. Fertig	USA (WY)	1990			
Potentilla drummondii							
ITS, ETS		Eriksson T. BGE#1			FN430776.1	FN421357.1	
trnL-F							GQ384753.1
Potentilla effusa var. effusa							
ITS, ETS	RM563123	B.E. Nelson	USA (WY	1983			
trnL-F							GQ384762.1
Potentilla elatior							
ITS, ETS	E00409317	Sojak	Russia	1983			
Potentilla elegans							
ITS, ETS		Eriksen B. 1440 1			FN430779	FN421358	
Potentilla elvendensis							
ITS, ETS, trnL-F	E00201630	Rechinger 47328B	Iran	1974			
Potentilla erecta							
ITS, trnL-F		Topel M. MA124			FN430780.1		FN556405.1
Potentilla eriocarpa							
ITS, ETS, trnL-F	Arnold	Boufford et al. 29172	China	1998			
Potentilla evestita							
ITS, ETS, trnL-F	RSA376313	Sojak	Kazakhstan	1979			
Potentilla fedstchenkoana							
ITS, ETS, trnL-F	RSA376316	Sojak	Uzbekistan	1979			
Potentilla festiva							
ITS, ETS, trnL-F	Arnold	1984 Sino-American Bot. Exp. 869	China	1984			
Potentilla flabellata							
ITS, ETS, trnL-F	RSA376317	Sojak	Tajikistan	1981			
Potentilla flabellifolia							
ITS, ETS, trnL-F		Topel M. MA 164			FN430810	FN421392	FN556406
Potentilla fragarioides							
ITS, ETS, trnL-F	Gray	Heng et al. 28716	China	2005			
Potentilla fulgens							
ITS, ETS, trnL-F	Arnold	Boufford et al. 28386	China	1998			
Potentilla geranioides							
ITS, ETS, trnL-F	E00409804	Davis 10181	Lebanon	1945			
Potentilla glandulosa var. reflexa							
ITS, ETS, trnL-F	JEPS96069	Vernon H. Oswald & Lowell Ahart	USA (CA)	1996			
Potentilla grandiflora							
ITS, ETS		Topel M. MA 149			FN430806	FN421400	
trnL-F	HEID 805511						GQ384663.1

Potentilla griffithii							
ITS, ETS, trnL-F	Arnold	Zhen-Ju 114043	China	1981			
Potentilla hippiana							
ITS, ETS		Eriksson T. BGE#2			FN430801	FN421359	
trnL-F		Eriksson T. TE Bot. Gard. Edinb.					FN556409
Potentilla hirta							
ITS, ETS, trnL-F	RSA460710	Germand & Ledoux 1974	France	1974			
Potentilla hispanica							
ITS, ETS, trnL-F	RSA532516	Podlech 47594	Morroco	1989			
Potentilla hololeuca							
ITS, ETS, trnL-F	RSA376303	Sojak	Kazakhstan	1979			
Potentilla hookeriana							
ITS, ETS	CM457218	W. J. Cody & J. B. McCanse 2156	Canada (NT)	1949			
trnL-F							GQ384747.1
Potentilla humifusa							
ITS, ETS, trnL-F	E00409788	Davis 43796A	Turkey	1966			
Potentilla incinata							
ITS, ETS	E00409461	Ekim 783	Turkey	1971			
trnL-F	HEID 805340						GQ384658.1
Potentilla intermedia							
ITS, ETS, trnL-F	E00663778	Cantell	Finland	1937			
Potentilla kleiniana							
ITS, ETS, trnL-F	CM295009	Seto	Japan	1982			
Potentilla kotschyana							
ITS, ETS, trnL-F	E00409661	Davis & Hedge 26865	Turkey	1957			
Potentilla laciniosa							
ITS, ETS, trnL-F	E00201631	Hewer 3839	Iran	1976			
Potentilla leuconota							
ITS, ETS, trnL-F	CM274520	Bartholomew et al. 973	China	1980			
Potentilla lignosa (=Tylosperma lignosa)							
ITS, ETS	E00409304	Archibald 8045	Turkey	1986			
trnL-F	W 1990-6892						GQ384793.1
Potentilla lineata							
ITS, ETS	Arnold	Boufford et al. 30823	China	2004			
Potentilla longifolia							
ITS, ETS	RSA544969	Skvortsov	Russia	1989			
trnL-F	MO 04263053						GQ384706.1
Potentilla macrosepala							
ITS, ETS, trnL-F	Arnold	Bartholomew et al. 653	China	1984			
Potentilla matsumurae							
ITS	CM263168	Onogi	Japan	1979			
Potentilla maura							
ITS, ETS, trnL-F	E0063802	Courtney 19	Morocco	1981			

Potentilla megalantha							
ITS, ETS, trnL-F	CM382796	Deguchi	Japan	1986			
Potentilla meyeri							
ITS*, ETS, trnL-F	E00409447	Görk, Hartvig & Strid 24029	Turkey	1984			
Potentilla micrantha							
ITS, trnL-F		Eriksson T. TE#149			FN430823		FN561746
Potentilla microphylla							
ITS, ETS, trnL-F		Topel M. MA 144			FN430809	FN421388	FN556412
Potentilla millefolia							
ITS, ETS	JEPS 81657	L.R. Heckard & L. C. & R. Ornduff	USA (CA)	1969			
trnL-F							GQ384765.1
Potentilla montenegrina							
ITS, ETS		Eriksson T. BGE#3			FN430782	FN421361	
trnL-F		Eriksson T. TE Bot. Gard. Edinb.					FN556413
Potentilla morefieldii							
ITS, ETS	JEPS44268	W.L. Jepson	USA (CA)	1917			
trnL-F							GQ384750.1
Potentilla multicaulis							
ITS	CM283809	Ze-Ying	China	1980			
trnL-F	MO 05329310						GQ384691.1
Potentilla multifida							
ITS, ETS, trnL-F		Erikssen T. TE705			FN430818	FN421374	FN561734
Potentilla nepalensis							
ITS, ETS, trnL-F		Topel M. MA163			FN430821	FN421390	FN561743
Potentilla nervosa							
ITS, ETS, trnL-F	Arnold	Sojak	Kyrgyzstan	1979			
Potentilla neumaniana							
ITS, ETS, trnL-F		Eriksson T. BT#1			FN666607	FN421370	FN556414
Potentilla nevadensis							
ITS, ETS	E00663775	Stocken 238.63	Spain	1963			
trnL-F	HEID 806627						GQ384647.1
Potentilla newberryi							
ITS, ETS	UC1587099	Schoolcraft et al.	USA (NV)	1991			
trnL-F	MO 05690792						GQ384710.1
Potentilla nitida							
ITS, ETS		Eriksson T. TE825			FN430795	FN421375	
trnL-F	HEID 806879						GQ384679.1
Potentilla nivalis							
ITS, ETS, trnL-F	E00663773	Harrold 553	Spain	1978			
Potentilla nivea							
ITS, ETS, trnL-F		Eriksen B. 1672:1			FN430816	FN421371	FN561729
Potentilla norvegica							
ITS, ETS, trnL-F		Eriksen B. BE 1567:1			FN430817	FN421362	FN561730

Potentilla ovina var. decurrens							
ITS, ETS	RM627230	Hartman	USA (WY)	1994			
trnL-F							GQ384767.1
Potentilla ovina var. ovina							
ITS, ETS, trnL-F	RM609206	Evert 6645	USA (WY)	1984			
Potentilla oweriniana							
ITS, ETS, trnL-F	E00409404	Davis & Hedge 30397	Turkey	1957			
Potentilla pamiroalaica							
ITS, ETS, trnL-F	RSA418933	Sojak	Kazakhstan	1987			
Potentilla paradoxa							
ITS, ETS, trnL-F	RM521586	R. L. McGregor	USA (KS)	1980			
Potentilla parvifolia							
ITS, ETS	CM366160	Z. Quing-sheng	China	1989			
Potentilla patula							
ITS, trnL-F	E00500251	Sukhorukov 112	Russia	2011			
Potentilla pectinisecta							
ITS, ETS, trnL-F	RM763737	B.E. Nelson	USA (WY)	2001			
Potentilla pedersenii							
ITS, ETS, trnL-F		Eriksen B. 05-24			FN430799	FN421404	FN556415
Potentilla peduncularis var. peduncularis							
ITS, ETS, trnL-F		Topel M. MA173			FN430820	FN421389	FN561742
Potentilla pensylvanica							
ITS, ETS	RM705795	Nelson 31917	USA (WY)	1994			
trnL-F							GQ384774.1
Potentilla pimpinelloides							
ITS, ETS, trnL-F		Topel M. MA139			FN430793	FN421384	FN556417
Potentilla plattensis							
ITS, ETS	RM585069	Fertig 2439	USA (WY)	1990			
trnL-F							GQ384768.1
Potentilla pyrenaica							
ITS, ETS	E00128293	Gardner & Gardner 760	Spain	1980			
trnL-F	HEID 806643						GQ384649.1
Potentilla pulchella							
ITS, ETS, trnL-F	E00663796	Halliday H560	Norway	1965			
Potentilla pulcherrima							
ITS, ETS	RM585044	Fertig 3447	USA (WY)	1990			
trnL-F							
Potentilla ranunculoides							
ITS, trnL-F	CM224183	Antipovitch	Mexico	1928			
Potentilla recta							
ITS, ETS, trnL-F		Eriksson T. BT#2			FN430784	FN421393	FN556419
Potentilla reptans							
ITS, ETS, trnL-F		Topel M. MA131			FN430815	FN421368	FN561728

Potentilla riparia							
ITS, ETS	CM294202	Murata et al.	Japan	1976			
Potentilla rivalis							
ITS, ETS	CM457492	Krivda	Canada (MB)	1956			
trnL-F							
Potentilla rubra							
ITS, ETS, trnL-F	CM224184	Antipovitch	Mexico	1927			
Potentilla ruprechtii							
ITS, ETS, trnL-F	E00409744	Sojak	Russia	1983			
Potentilla saundersiana							
ITS, ETS, trnL-F	CM283802	Zhen-Ju	China	1981			
Potentilla saxifrag							
ITS, ETS, trnL-F	E00663795	Charpin 13914	France	1977			
Potentilla sericea							
ITS, ETS, trnL-F	Arnold	Nabrob 857	Mongolia	1926			
Potentilla simplex							
ITS, ETS	CM457495	S. Brisson & C. Hamel 12,277	Canada (QC)	1967			
trnL-F	MO 05171738						GQ384717.1
Potentilla speciosa							
ITS, ETS, trnL-F	RSA352162	Archibald 6847	Turkey	1985			
Potentilla stenophylla							
ITS		Eriksson & Vretblad TE763			AJ511780		
ETS		Eriksson T. GBT#1				FN421381	
Potentilla sterilis							
ITS, ETS, trnL-F		Eriksson T. TE734			FN555612	FN421376	FN561732
Potentilla stipularis							
ITS	E00663784	Argent	Greenland	1962			
Potentilla strigosa							
ITS, ETS, trnL-F	Arnold	See photo- in Russian	Russia	1949			
Potentillla stolonifera							
ITS, ETS, trnL-F		Eriksen B. 1382:1			FN430814	FN421363	FN556420
Potentilla subgorodkovii							
ITS, ETS, trnL-F	RM521873	Lackschewitz 10549,	USA (MO)	1983			
Potentilla subjuga							
ITS, ETS	CM298016	Siplivinsky	USA (CO)	1982			
trnL-F							GQ384776.1
Potentilla subvahliana							
ITS, ETS, trnL-F		Eriksen B. 931-3-05			FN430783	FN421364	FN556421
Potentilla supina							
ITS, ETS	E00409603	Hewitt 272	Turkey	1970			
trnL-F	HEID 806484						GQ384641
Potentilla tabernaemontani							
ITS, ETS		Eriksson T. SG#1			FN555608	FN421365	

trnL-F		Eriksson T. Spont. GBG					FN556466
Potentilla tanacetifolia							
ITS, ETS, trnL-F		Eriksson T. ex. Leipzig-98			FN430797	FN421366	FN556422
Potentilla thurberi							
ITS, ETS, trnL-F		Topel M. MA138			FN430792	FN421383	FN561740
Potentilla thurangiaca							
ITS, ETS, trnL-F		Topel M. MA119			FN430777	FN421406	FN556423
Potentilla umbrosa							
trnL-F		HEID 806401					GQ384633.1
Potentilla uniflora							
ITS, ETS, trnL-F		Eriksen B. 271-4-05			FN430785	FN421367	FN556425
Potentilla verticillaris							
ITS, ETS, trnL-F	RSA376301	Sojak	Mongolia	1965			
Potentilla villosa							
ITS, ETS, trnL-F		Topel M. MA127			FN430786	FN421369	FN556426
Sibbaldia procumbens							
ITS, ETS		M. Lundberg 4			FJ356174	FJ422374	
Sibbaldiopsis tridenta							
ITS, ETS, trnL-F	CM524448	B. L. Isaac & C. F. Chuey 21231	Canada (NL)	2011			

* ITS1-2 only, **ITS3-4 only

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