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NATURAL HISTORY NOTE

Intraspecific floral color variation as perceived by pollinators and non-pollinators: evidence for pollinator-imposed constraints?

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Abstract

Pollinator-mediated selection is expected to constrain floral color variation within plant populations, yet populations with high color variation (at least in human visual space) are common in nature. To explore this paradox, we collected floral reflectance spectra for 34 populations of 14 putatively bee-pollinated plant species of north-central New Mexico, USA, and translated them into three different animal visual spaces. We asked, (1) is intrapopulation variation in flower color constrained to be lower than the discrimination threshold of the putative dominant pollinators? And, (2) is perceived intrapopulation variation in flowers higher for non-pollinating animals than it is for the presumed dominant pollinator group? We found evidence consistent with some pollinator-imposed constraints on floral color in our species, with the majority (70.6%) of populations having > 95% of flower–flower or flower-centroid comparisons (where the centroid represents the mean phenotype) estimated to be visually indiscriminable to bees. We also found that perceived floral color variation was significantly greater for two non-pollinating groups—birds and humans—than for bees. Our results suggest that a large portion of human-perceived floral color variation within populations persists because it is effectively invisible to pollinators. In this scenario, human-perceived color may evolve neutrally (via drift) or via indirect selection on correlated characters such as herbivore- or drought-resistance, consistent with previous studies identifying non-pollinator agents of selection on flower color.

Keywords Plant–pollinator interactions · Signalling · Reflectance spectra · Bee vision · Natural selection · Color polymorphism

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Introduction

Floral color is an important trait that pollinators use to select the flowers they visit (Fenster et al. 2004; Schiestl and Johnson 2013; Dyer et al. 2012), and thus pollinators can act as selective agents on flower color (Rausher 2008; Waser and Price 1981; Renoult et al. 2013). Because different animal clades possess different color-sensitive receptors and cognitive mechanisms, the way each pollinator group perceives color is unique (Renoult et al. 2017). As a result, we often find that flowers are colored in ways that exploit the color vision of their primary pollinators (Shrestha et al. 2013; Dyer et al. 2012).

Determinants of intraspecific floral color variation have long been of interest to biologists (Rausher 2008; Warren and Mackenzie 2001). If a population is pollinated by a single group of pollinators, such as bees, we expect intrapopulation variation in floral color (as perceived by that group) to be low in response to pollinator-driven selection (Fenster et al. 2004; Rausher 2008; Waser and Price 1983). That is, when alternate colors arise through mutation, they should be selected against, as pollinators tend to visit the most common color (Smithson 2001; Eckhart et al. 2006), perhaps because animals tend to find it easier to remember more common types of their food (Gegear and Laverty 2001). Despite this expectation, plant species with high intrapopulation color variation (at least as perceived by humans) occur regularly in nature. Numerous studies have investigated such high variation (Table 1; reviewed in Rausher 2008; Warren and Mackenzie 2001). However, very few of these studies have actually quantified floral variation in pollinator visual spaces (for exceptions, see: Ortiz et al. 2015; Campbell et al. 2012; Keasar et al. 2016; Garcia et al. 2018), leading to questions about both the relevance and maintenance of such color variation.

Even when analyses of spectra find that two flowers occupy different positions in a pollinator's color space, the distinction between them may not be perceptible to the pollinator (Dyer and Chittka 2004). All visual organisms have visual thresholds, defined by the minimum difference between two colors that is discriminable (e.g., Dyer and Chittka 2004; Olsson et al. 2017; Wyszecki and Stiles 2000; see "Methods"). The existence of these thresholds suggests that there may be effectively invisible intrapopulation color variation that escapes direct selection from pollinators. We propose that flower colors might be evolutionarily constrained such that they vary only up to a certain threshold, which remains imperceptible to their dominant pollinators (Fig. 1). Thus, a fundamental unanswered question is (1): is intrapopulation variation in flower color constrained to be lower than the perceptual threshold of the dominant pollinators?

Because non-pollinating animals typically do not exert selection on flowers (with the exception of herbivores, see Table 1), we do not expect them to select against rare alternate colors that stand out in their visual space, as was described above for pollinating species. Recurrent mutation, genetic drift and/or indirect selection via genetic correlations might act to diversify the appearance of flowers to these non-pollinator species, perhaps with little counteracting selection constraining the variation. We thus ask the novel question (2): is perceived intrapopulation variation in flower color higher for non-pollinating animals than it is for the dominant pollinator group?

To answer these questions in one geographic region, we measured the spectral reflectance of multiple individuals within one to three populations of each of 14 native plant species, representing seven plant families of putatively bee-pollinated flowers (see "Methods") in north-central New Mexico. We modelled these spectra in bee, human,

Table 1 Cases of intrapopulation floral color variation for which pollinator visitation and/or selection has been investigated

Plant species	Color variants (human hues)	Pollinators	Do color variants have differential fitness?†	Do color variants have differential pollinator visitation?	Do pollinators impose selection?‡	Other traits associated with color	Non-pollinator agents of selection	References
Aquilegia caerulea	Blue/White	Bees, Hawkmoths	Yes	Yes	Yes	Unknown		Miller (1981)
Clarkia gracilis	Spotted/Non	Bees	Yes	Yes	Unknown	Unknown		Jones (1996)
Clarkia xantiana	Spotted/Non	Bees	Unknown	Yes	Unknown	Unknown		Eckhart et al. (2006)
Claytonia virginica	Red, various shades	Bees	Yes	Yes	Yes	Herbivore, pathogen resistance	Herbivores, Pathogens	Frey (2004)
Delphinium nelsonii	Blue/White	*Bombus*, Hummingbirds	Yes	Yes	Yes	Unknown		Waser and Price (1981, 1983)
Gentiana leucomelaena	Blue/White	*Apis*, Flies, Ants	Unknown	Yes	Unknown	Flower lifespan, pollen number, ovule number		Mu et al. (2011)
Gentiana lutea	Yellow/Orange	Mostly *Bombus*	Yes	Yes	Yes	Herbivore resistance	Herbivores	Veiga et al. (2015)
Geranium nepalense	Pink/White	Bees, Flies	Yes	Yes	Unknown	Flower temperature		Tang et al. (2016)
Hydrophyllum appendiculatum	Blue/White	*Apis mellifera*	Yes	No	No	Flower number		Wolfe (1993)
Ipomoea purpurea	White/Purple	Bees	Yes	Yes	No	Flower size		Rausher and Fry (1993), Fry and Rausher (1997)
Linanthus parryae	Blue/White	Beetles	Yes	No	No	Drought tolerance	Climatic factors	Schemske and Bierzychudek (2001, 2007)
Linaria canadensis	Purple/Blue	Bees	Unknown	Unknown	Unknown	Flower size		Wolfe and Sellers (1997)
Linum pubescens	Yellow/Purple	Flies	No	Unknown	Unknown	Flower size		Wolfe (2001)

Table 1 (continued)

Plant species	Color variants (human hues)	Pollinators	Do color variants have differential fitness?[†]	Do color variants have differential pollinator visitation?	Do pollinators impose selection?[‡]	Other traits associated with color	Non-pollinator agents of selection	References
Lobelia siphilitica	Blue to Purple	*Bombus*	Yes	Yes	Yes	Herbivore resistance	Herbivores	Caruso et al. (2010)
Lobularia maritima	Purple/White	Ants, Flies	Yes	Yes	Yes	Flower number		Gomez (2000)
*Lysimachia arvensis**	Red/Blue	Bees	Yes	Yes	Yes	Flower size, pollen number, ovule number	Climatic Factors	Ortiz et al. (2015)
Malva moschata	Red/White	Bees	No	Yes	Unknown	Anther number		Frey et al. (2011)
Phlox pilosa	Pink/White	Butterflies	Unknown	Unknown	Unknown	Unknown		Levin and Kerster (1967)
Platystemon californicus	Yellow/White	Bees, Wind	Unknown	Yes	No	Unknown		Hannan (1981)
Raphanus raphanistrum	Yellow/White	Bees	Yes	Yes	Yes	Unknown		Stanton et al. (1989)
Raphanus sativus	Yellow/White/Pink	Bees	Yes	Yes	Yes	Herbivore resistance	Herbivores	Irwin and Strauss (2005), Irwin et al. (2003)
*Silybum marianum**	Purple/White	Bees, Wasps, Beetles	Unknown	Yes	No	Unknown		Keasar et al. (2016)
*Wahlenbergia albomarginata**	Pale Blue/White	Bees	Unknown	No	No	Unknown		Campbell et al. (2012)

Studies involving introduced plants (and therefore novel, non-coevolved plant–pollinator interactions), studies on horticultural varieties, and studies on rewardless (food deceptive) species have been excluded. Of the 23 systems included, only three have been examined via visual modelling within the relevant pollinator visual space (noted with *). Color variants are defined here as flowers that differ in their color appearance to at least one animal species, and include cases of both discrete and continuous variation. This table was inspired by Table 1 of Rausher (2008), but scoring is independent and more recent research has been added

[†] ”Yes” or “No” requires measurement of whole-plant fitness; “Unknown” if only of a subcomponent of fitness (e.g., seeds per fruit) was measured

[‡] ”Yes” requires evidence beyond the presence of both differential fitness and differential pollinator visitation, e.g. demonstration that differential fitness disappears under a pollen supplementation treatment, or demonstration via paternity analyses of differences in male siring success

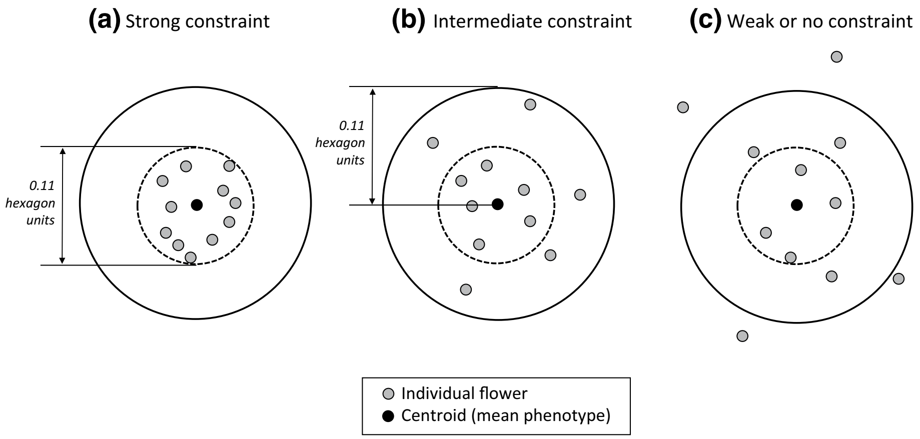

Fig. 1 Hypothetical patterns of flower color variation in relation to bee discrimination thresholds. The same region within the bee hexagon colorspace (Chittka 1992) is depicted in each panel. The position of each gray dot represents the subjective color (hue and saturation) of an individual flower, while the black dot is the population centroid (geometric center), and thus the mean phenotype. Circles represent the application of bee color-discrimination thresholds (0.11 hexagon units, Dyer 2006) to flower–flower (dashed circle) or flower-centroid (solid circle) comparisons, respectively. Three scenarios are shown reflecting different potential levels of bee-related evolutionary constraint. **a** At least 95% of flower–flower pairwise distances are less than threshold (i.e. within a *diameter* of 0.11 hexagon units); consistent with strong bee-related evolutionary constraint. **b** At least 95% of flower-centroid pairwise distances are less than the threshold (i.e. within a *radius* of 0.11); consistent with intermediate evolutionary constraint. **c** More than 5% of flower-centroid distances are greater than the threshold; consistent with weak or absent evolutionary constraint

and bird visual spaces and compared perceived floral color variation for pollinators (bees) with that perceived by the two groups of non-pollinators. Because these questions were inspired by our investigations of *Sphaeralcea polychroma* [Malvaceae] (LaDuke 1985), a highly variable species in human visual space (Fig. 2), we included several congeners of *S. polychroma* that are less variable in human visual space.

Fig. 2 Human-perceived floral color variation in *Sphaeralcea polychroma* (Malvaceae). Each flower was collected from a different individual plant, collected within a 100 m radius within a single population at the Sevilleta National Wildlife Refuge, New Mexico, USA (population SpPo6_1, Table 1). Note that much of the variation in this species is invisible to bees as 61% of the pairwise comparisons between these flowers fell below the 0.11 hexagon unit visual threshold (Fig. 3a), even though every flower–flower comparison was discriminable to humans (Online Appendix 2). (Color figure online)

Methods

Flower collection and spectrophotometry

We collected individual flowers from 14 native New Mexican species, blooming in the fall of 2017 (Table 2). Based on documented visitation by bees (see Table 2) and a lack of documented visitation by other pollinator groups, we assume these species are primarily bee-pollinated, though we recognize that visitation rates do not always indicate pollination quantity or quality at the level of individual pollinator species (King et al. 2013). For the family Asteraceae, an inflorescence is morphologically integrated to function as a single flower, and therefore we treat their inflorescences as "flowers" and their ray flowers as "petals" hereafter. Species were chosen from among those widely flowering during the field season, and included multiple *Sphaeralcea* species to act as a comparison to the distinctly variable (in human vision) *S. polychroma*. For each species, we collected a single flower from each of 15 different individual plants chosen randomly within each of 1–3 different populations (see Table 2), with the exception of one population of *Geranium caespitosum* (GeCa3_2), for which only 14 individuals were collected. We thus sampled a total of 34 populations (see Table 2) and 509 individuals across all species. From each plant, a flower was chosen randomly from among the recently opened, unfaded flowers and collected for spectral analysis. While changes in floral age and pollination status can sometimes trigger coloration changes, this phenomenon is considered uncommon among all angiosperm species (reviewed in Ruxton and Schaefer 2016) and was not observed in any of the species we sampled.

We transported flowers to the lab in an ice-cooled cooler to ensure freshness and used spectrophotometry to quantify spectral reflectance within 3 h of collection. Our sampling design of one spectrum per flower was informed by Dalrymple et al. (2015), which indicated that flower color can be quite precisely estimated with a single measurement. Floral reflectance was measured using an AvaSpec 2048 spectrophotometer, a bifurcated coaxial fiber optic reflectance probe (Avantes FCR-7uv200-2-ME), and an AvaLight-XE xenon light source (Avantes BV, Apeldoom, The Netherlands). Prior to reading the samples, the spectrophotometer was calibrated relative to a white standard PFTE tile (Avantes WS-2). Reflectance was measured with the probe held perpendicular to and 8.0 mm from the petal, with consistent distance enforced through a small nail connected to the probe. While there is discussion in the literature about the optimal angle (45° vs. 90°) to measure floral reflectance (Chittka and Kevan 2005; White et al. 2015), in practice, color components (hue or saturation) within bee visual space calculated at these two angles are highly correlated (see Appendix S1 of Gray et al. 2018). For the majority of species, petals within a flower were identical and one petal was chosen randomly for analysis; but for *Penstemon ambiguus*, the lower central corolla lobe was chosen; for *Psorothamnus scoparus*, the keel petal was chosen. Spectral measurements were then taken either 1 cm from the base for longer petals (e.g. *Helianthus petiolaris*), or 1/3 of the distance from the base to the tip for smaller petals (e.g. *Macaranthera tanecitifolia*). This standardized procedure ensured that petal regions with similar characteristics (e.g. UV-reflecting vs. non-UV-reflecting) were measured across individuals within a species. Spectral processing and visual modelling were carried out using the R package 'pavo' (Maia et al. 2019). We first trimmed the spectra to 300–700 nm, and then set spurious negative reflectance values to zero using the *procspec* command. All spectra are plotted in Online Appendix 1.

Table 2 Plant populations sampled in this study (New Mexico, USA), including localities, floral characteristics, and bee visitation information

Species	Population code	Latitude (°N)	Longitude (°W)	Family	Human Hue	Bee Hue[a]	Bee visitation citations	Bee visitors
Baileya multiradiata	BaMu_1	34.403	− 106.672	Asteraceae	Yellow	Green	Cockerell (1906), Lowe and Foltz-Sweat (2017)	*Agapostemon, Diadasia, Halictus, Melissodes, Megachile*
	BaMu_2	35.556	− 106.809					
	BaMu_3	33.774	− 106.904					
Fallugia paradoxa	FaPa_1	35.556	− 106.809	Rosaceae	White	Blue–Green	Buchmann (1985), Cockerell (1906)	*Bombus, Perdita, Melecta*
Geranium caespitosum	GeCa3_1	35.180	− 106.391	Geraniaceae	Reddish Purple	Blue	Hessing (1988)	*Apis, Bombus*
	GeCa3_2	35.256	− 106.406					
	GeCa3_3	35.162	− 106.295					
Glandularia bipinnatifida	GlBi2_1	34.269	− 106.670	Verbenaceae	Pink to Purple	Blue–Green to Blue	Cockerell (1906), KCP pers. obs.	*Agapostemon*
	GlBi2_2	35.034	− 106.354					
	GlBi2_3	35.162	− 106.295					
Helianthus petiolaris	HePe_1	33.774	− 106.904	Asteraceae	Yellow	Green to UV-Green	Hurd et al. (1980)	*Andrena, Apis, Bombus, Dufourea, Hesperapis, Megachile, Melissodes,* others
	HePe_2	35.034	− 106.354					
	HePe_3	33.734	− 106.977					
Macrantherea tanecitifolia	MaTa2_1	34.403	− 106.672	Asteraceae	Light purple	Blue to Blue–Green	KCP pers. obs.	Unidentified Halictidae
	MaTa2_2	35.034	− 106.354					
	MaTa2_3	33.774	− 106.904					

Table 2 (continued)

Species	Population code	Latitude (°N)	Longitude (°W)	Family	Human Hue	Bee Hue[a]	Bee visitation citations	Bee visitors
Nama hispidum	NaHi_1	34.403	– 106.673	Boraginaceae	Light Purple	Blue-UV	Tyrl et al. (1984)	*Conanthalictus, Specodosoma*
Penstemon ambiguus	PeAm_1	34.404	– 106.673	Plantaginaceae	White	Blue–Green	KCP pers. obs.	Unidentified Anthophila
	PeAm_2	35.034	– 106.354					
Psorothamnus scoparus	PsSc6_1	33.163	– 107.220	Fabaceae	Dark Purple	UV-Blue to Blue	Rozen and Rozen (1986)	*Caupolicana, Martinapis*
	PsSc6_2	35.556	– 106.809					
	PsSc6_3	33.774	– 106.904					
Schoenocrambe linearifolia	ScLi2_1	35.034	– 106.354	Brassicaceae	Light Purple	Blue–Green	Lewis and Schupp (2014)	*Lasioglossum, Halictus*
Sphaeralcea angustifolia	SpAn_1	35.142	– 106.683	Malvaceae	Orange	UV-Blue to UV	Pendery and Rumbaugh (1993)	*Diadasia, Macrotera*
	SpAn_1	35.034	– 106.354					
Sphaeralcea hastulata	SpHa_1	34.403	– 106.672	Malvaceae	Orange	UV-Blue to UV	Pendery and Rumbaugh (1993)	*Diadasia, Macrotera*
	SpHa_2	33.774	– 106.904					
	SpHa_3	33.774	– 106.904					
Sphaeralcea incana	SpIn2_1	35.556	– 106.809	Malvaceae	Orange	UV-Blue to UV	Pendery and Rumbaugh (1993)	*Diadasia, Macrotera*
	SpIn2_2	33.163	– 107.220					
	SpIn2_3	33.774	– 106.904					
Sphaeralcea polychroma	SpPo6_1	34.403	– 106.672	Malvaceae	White, pink, red, to violet	UV-Blue to UV	Pendery and Rumbaugh (1993)	*Diadasia, Macrotera*
	SpPo6_2	33.734	– 106.977					
	SpPo6_3	33.163	– 107.220					

[a] Determined from visual modeling based on our spectral data (see "Methods")

Conversion of floral spectral data into visual spaces

We estimated the subjective perception of floral signals using models of color vision appropriate for the viewers of interest. Namely, we used the color hexagon model for bees (Chittka 1992), the receptor-noise limited model for birds (Vorobyev and Osorio 1998), and the CIELab model for humans (Wyszecki and Stiles 2000). Though they vary in their underlying assumptions, each of these models allows colors to be represented as points in a space delimited by the number and sensitivity of photoreceptors, while accounting for factors such as the structure of viewing backgrounds and signals, veiling and incident light, and more species-specific features of visual processing and perception (Maia and White 2018; Kemp et al. 2015). Crucially for the questions at hand, the distances between points in these spaces can be interpreted as measures of the subjective difference between colors, with values less than a behaviorally-validated 'threshold' of discrimination (which may vary by species and/or model; see "Discussion") likely to be indiscriminable to a given viewer. In the color hexagon, where hue is indicated by the radial angle and saturation is indicated by the distance from the (0, 0) origin, psychophysical testing of bumblebee and honeybee behavior under ideal laboratory conditions suggests colors separated by a Euclidean distance of 0.11 'hexagon units' are indiscriminable without aversive differential conditioning (Dyer 2006; Dyer and Neumeyer 2005; Dyer and Chittka 2004). That is, bees are unable to discriminate between two colors separated by this distance unless they are first trained with rewarding and aversive colored stimuli that are simultaneously presented under optimal (laboratory) viewing conditions. Since, on average, this training is unlikely to represent the experience of bees in complex natural settings, we take the value of 0.11 hexagon units as a conservative threshold of discrimination for our purposes. In the receptor-noise limited and CIELab models, color distances are expressed as weighted Euclidean distances (ΔS and ΔE, respectively), with values of 1.0 for diurnal birds, and 2.3 for humans, taken to (conservatively) delimit the threshold below which colors are expected to be indiscriminable under ecologically relevant conditions (reviewed in Olsson et al. 2017; Wyszecki and Stiles 2000).

With respect to model parameters, we drew on the receptor sensitivities of *Apis mellifera* (Peitsch et al. 1992) as a representative bee pollinator, since the hexagon model is well validated in this species (detailed above) and the sensitivities of photopigments underlying trichromatic vision are highly conserved across the Hymenoptera (Briscoe and Chittka 2001). For birds, we used the visual phenotype of an average violet-sensitive avian viewer for receptor-noise modelling, given that receptor sensitivities are similarly conserved across birds (Bennett and Théry 2007; Hart 2001). Of course the derived discrimination thresholds should be viewed as a current best-estimate for these broad groups, which may be refined in light of further knowledge of the realized abilities of viewers (see "Discussion"). We specified a relative receptor density of 1:2:2:4 (ultraviolet: short: medium: long wavelength receptors), used a signal-to-noise ratio yielding a Weber fraction of 0.1, and assumed that noise is proportional to the Weber fraction and independent of the magnitude of receptor stimulation (Vorobyev and Osorio 1998). Finally, we used the CIE 10° color matching functions for CIELab modelling. In all cases we normalized receptor stimulation against a leaf-green visual background derived from 30 leaves of *Lomandra longifolia* (whose reflectance profile is typical of 'green' vegetation; plotted in Online Appendix 1), and assumed a D65 'standard daylight' illuminant.

Statistical analysis

> Is intrapopulation variation in flower color constrained to be lower than the perceptual threshold of the presumed dominant pollinators (bees)?

If a population of flowers is constrained such that all variation is lower than the bee visual threshold, then a population should have no flower–flower pairs separated by more than 0.11 hexagon units (Fig. 1a). We thus compared all pairwise distances between flowers in each population sampled. However, analyses of average pairwise distance between flowers might exaggerate the variation perceived by pollinators, if pollinators instead evaluate a given flower based on its similarity to an ideal mean flower (i.e., use a search image) as opposed to all flowers in the population, including extremes (Fig. 1b). Thus, we conducted an alternative analysis of pairwise distances between individual flowers and the population centroid (geometric center, thus the mean phenotype), with each population's centroid calculated by averaging the x, y coordinates of all 15 members of the population in the bee hexagon.

We then evaluated whether the data are consistent with strong constraints (Fig. 1a), moderate constraints (Fig. 1b), or weak or no constraints (Fig. 1c) by assessing whether—analogous to the conventional $P = 0.05$—95% of the observed distances fall within a given discrimination threshold. Thus, for example, the data for a given population would be judged consistent with moderate constraints imposed by bees if $< 5\%$ of flower-centroid distances were > 0.11 hexagon units (corresponding to the model depicted in Fig. 1b).

> Is perceived intrapopulation variation in flower color greater for non-pollinating animals (humans and birds) than it is for the presumed dominant pollinator group (bees)?

We chose humans and birds as our representative non-pollinator groups because visual models with behaviourally-validated discrimination thresholds exist for both (see above), and neither are known to pollinate any of the plant species in our dataset (see references cited in Table 2). To test whether perceived floral color variation is higher for non-pollinators than for bees, we first performed calculations of flower–flower and flower-centroid distances using human and avian visual models, as detailed above; note that centroids in receptor-noise limited space were calculated via the population-wise averaging of floral reflectance spectra prior to modelling, for convenience. We then calculated the fraction of comparisons (flower–flower, or flower-centroid) within a population that exceeded the respective discrimination threshold in each of the models and then compared them (bee vs. human; bee vs. bird) via Wilcoxon signed-rank tests, implemented in R (R Core Team 2017).

Results

Floral color variation in relation to bee discrimination thresholds

Across all intrapopulation flower–flower comparisons, 89.8% were estimated to be indiscriminable to bees without differential conditioning (< 0.11 hexagon units), and only 10.2% were broadly discriminable (> 0.11 units, Fig. 3a). For flower-centroid comparisons, 96.9%

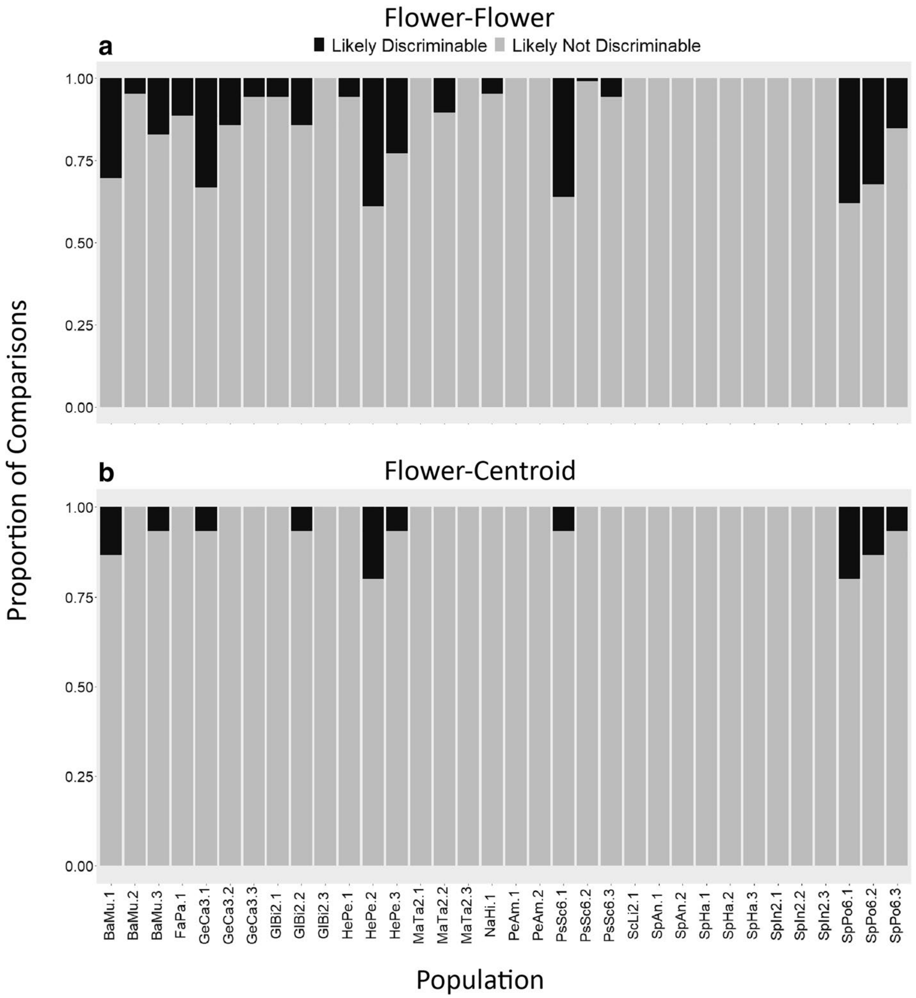

Fig. 3 Bee visual space: the proportions of **a** distances between flower–flower pairs or **b** distances between flowers and their population centroid, in relation to bee discrimination thresholds. Grey represents pairwise comparisons that are likely indiscriminable (< 0.11 hexagon units), while black represents pairwise comparisons that are likely discriminable (> 0.11 hexagon units). Data represent 34 populations of 14 New Mexico plant species (see Table 2). Equivalent figures showing distances in relation to human and bird discrimination thresholds are available in Online Appendices 2 and 3, respectively

of all comparisons were estimated to be indiscriminable (< 0.11 hexagon units), with only 3.1% discriminable (> 0.11 units, Fig. 3b).

On a population basis, 50.0% of populations (17 of 34) examined had > 95% of flower–flower distances fall below 0.11 hexagon units, thus meeting our criterion for "strong constraints" (see Fig. 3a). Another 20.6% of populations (7 of 34) had > 95% of measured flowers within 0.11 hexagon units of the centroid, thus meeting our criterion for "moderate constraints" (Fig. 3b). The remaining 29.4% of populations (10 of 34) had ≥ 5% of measured flowers at least 0.11 or more hexagon units from the centroid, suggesting that

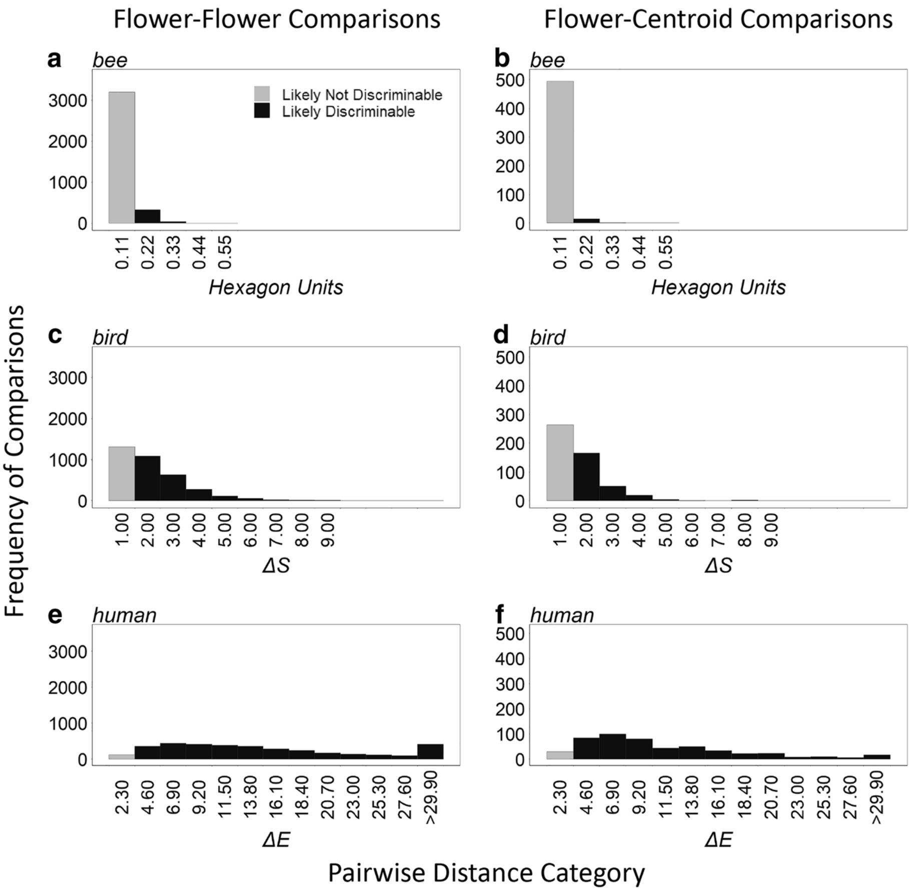

Fig. 4 Comparisons of visually-modelled color variation in pollinator and non-pollinator visual spaces for 34 populations of 14 New Mexico plant species. Histograms show the distributions of within-population flower–flower (**a**, **c**, **e**) and flower-centroid (**b**, **d**, **f**) distances. Grey bars represent the counts of pairwise comparisons that are likely indiscriminable. Black bars represent the counts of comparisons that are likely discriminable, in increasing multiples of units that we best understand to be the minimum discriminable distance for each species. For bees (**a**, **b**), the majority of comparisons are indiscriminable, while for birds (**c**, **d**) and humans (**e**, **f**) the majority of comparisons are discriminable

constraints were weak or absent (Fig. 3c). Thus, we found evidence consistent with pollinator-imposed constraints on floral color in the majority (70.6%) of our populations.

Floral color variation in pollinator and non-pollinator visual spaces

In avian visual space, 37.0% of all intrapopulation flower–flower comparisons were estimated to be indiscriminable, while the remaining 63.0% were likely discriminable (Fig. 4c). For flower-centroid comparisons, 51.9% were estimated to be indiscriminable, while the remaining 48.1% were likely discriminable (Fig. 4d).

In human visual space, 3.4% of all intrapopulation flower–flower comparisons were estimated to be indiscriminable (<2.3 CIELab units), while 96.6% were likely discriminable

(Fig. 4e). For flower-centroid comparisons, 5.9% of comparisons were indiscriminable, while the remaining 94.1% were likely discriminable (Fig. 4f).

Intrapopulation floral color variation was significantly more likely to be discriminable for birds than bees in both flower–flower ($P<0.0001$, $W=1082.5$; $N=34$ populations) and flower-centroid ($P<0.0001$; $W=1082$; $N=34$ populations) comparisons. Similarly, intrapopulation floral color variation was significantly more likely to be discriminable for humans than bees (flower–flower comparisons, $P<0.0001$, $W=1156$, $N=34$ populations; flower-centroid comparisons, $P<0.0001$, $W=1156$, $N=34$ populations).

Discussion

Implications for the evolution of floral color variation

Our data suggest that the majority (70.6%) of populations surveyed have levels of intrapopulation color variation that are consistent with either moderate or strong constraints imposed by their presumed main pollinators, bees. This is likely because bees show higher visitation rates to (and thus increase fitness of) individuals with flowers that are not visually distinct (Smithson 2001), with correspondingly lower visitation rates to visually distinct individuals. Such pollinator-generated selection might apply to all populations, even those that are not pollen limited, as increased visitation rates should increase male fitness even when female fitness is unaffected (Stanton et al. 1989).

Many populations, however, did contain appreciable numbers of pairwise comparisons that were estimated to be visually distinct to bees (Fig. 3). Further work is required to determine the roles that bees play in possibly selecting against these outliers. Bees may still visit these variants, if they are not too visually distinct. Alternately, bees may not notice (and thus may not visit) some color variants (Dyer and Chittka 2004; Smithson 2001; Papiorek et al. 2013), but do these variants actually have lower fecundity because of their color, and if so, how do their underlying alleles persist in these populations?

The presence of pollinator-discriminable floral color variants, both in species in our dataset and in three species from the literature (see Ortiz et al. 2015; Campbell et al. 2012; Keasar et al. 2016), suggests that if pollinators do generate constraints on floral color variation (Dyer et al. 2012), other factors may moderate the level of constraint. Because pollinator preferences tend to be context-dependent (Hersch and Roy 2007), color variants could persist as pollinator preferences change over the course of years or during the season. It is also possible that we overestimate the importance of pollinators in constraining variation in some environments. Receiving fewer visits may have no effects on female fitness if visitation rates are high overall (Smithson 2001), which we might expect in environments like the southwestern US deserts where droughts can produce years with few available floral resources. Finally, floral color variation that is detectable to pollinators may ultimately be maintained by factors other than pollinators. Floral color is often pleiotropic (Schoen et al. 1984; Rausher 2008) and can covary with traits such as herbivore resistance (Irwin et al. 2003; Strauss et al. 2004) and drought resistance (Vaidya et al. 2018; Schemske and Bierzychudek 2001; Warren and Mackenzie 2001), which are expected to be under selection in many populations.

Perhaps stronger evidence consistent with the hypothesis that pollinators constrain floral color variation is our finding that, for the plant species studied, little variation is perceived by bees relative to the extreme variation perceived by animals that play no role in their pollination (Fig. 4). This finding is consistent with the idea that flowers adapt to the vision of their pollinators (Schiestl and Johnson 2013), as none of the plant species in our study are pollinated by birds or humans. We note that human vision may be useful in a wide variety of contexts as a "non-pollinator" visual system, as primates have rarely been responsible for the direct pollination of flowers (Sussman and Raven 1978), except in recent cases of domesticated plants.

Implications for the study of flower color

Our results highlight that large differences exist in perceived floral color variation across different visual systems. While the human visual system has frequently been used to identify species of flowers to investigate for maintenance-of-variation questions (Table 1), it is not a reliable guide to what species appear variable to relevant selective agents (Renoult et al. 2017). There are scenarios where our perception of flowers can inform how bees see flowers; for example, *Sphaeralcea polychroma* is variable in both bee and human vision (though less so in the former than the latter, see Online Appendix 2). However, we can also encounter false positives. For instance, *Glandularia bipinnatifida* appears variable in human vision, but bees can see very little of that variation, with 92.6% of flower–flower pairs and 97.7% of flower-centroid pairs effectively indiscriminable to bees. Perhaps most importantly, we may not recognize relevant variation, e.g. *H. petiolaris* and *Baileya multiradiata* were distinctly variable in bee visual space but relatively invariant to humans. The mismatch between human and other visual systems affects other research areas, and has been highlighted especially in studies of plumage-based avian sexual signalling (Cuthill et al. 1999; Eaton 2005; Endler and Mielke 2005).

Explicit modeling of the color-distance between flowers in pollinator visual spaces is critical as investigators think about which questions to ask about floral color and in which systems to pursue them. For instance, in cases where a population appears variable to human observers, but much of that variation exists below discrimination thresholds for pollinators, questions about how pollinators shape floral color would be less fruitful research avenues than investigations of alternative biotic (e.g., herbivores, Irwin et al. 2003; Strauss et al. 2004) or abiotic factors (e.g., drought, Vaidya et al. 2018) as agents of selection on color. In contrast, studies on variation in cryptically colorful species (to humans) like *H. petiolaris* might reasonably focus on pollinator visitation as a selective force, as individual-level color variation is clearly visible to bees. By measuring floral color variation in pollinator spaces, we can ask better questions about the origin and maintenance of intraspecific variation in plant traits.

Overall, our results suggest the possibility that species in which the maintenance of intrapopulation floral color variation have been studied (e.g., Table 1) could be a biased subset from nature, one that may emphasize color variation that is striking to human investigators but is potentially irrelevant to pollinators. In support of this idea, we note several patterns from this literature. First, in several species, we do not have evidence of clear fitness differences associated with flower color (39% of the 23 species in Table 1 are scored as "unknown" or "no" for fitness differences), though this pattern could reflect a lack of study effort on this particular question. Second, in species where color variants *do* have differential fitness (14 systems in Table 1), it is not uncommon to lack direct evidence that the selection is pollinator-mediated (5 or 36% of these species), even when it is specifically evaluated. Third, even when differential pollinator visitation has been documented, it may arise not because of color itself but because color covaries (through pleiotropy or linkage) with other morphological traits that affect visitation, such as flower size (e.g. Mu et al. 2011; Gomez 2000). Such associations between color and other floral traits are common, occurring in at least 61% of the species in Table 1, and a recent study has further indicated that color and scent can be tightly integrated across species (Kantsa et al. 2017), suggesting the possibility that they may be linked within species as well. Thus, differential pollinator visitation is not a foolproof indication that pollinators can visually distinguish floral color variants. A final pattern is that non-pollinator agents of selection have been documented for 26% of the species in Table 1, a percentage perhaps not that different in magnitude from that with documented pollinator-mediated selection (39%). This is perhaps the most surprising pattern, given long-held assumptions that pollinators are the relevant selective agents affecting flower color (Fenster et al. 2004). All these patterns are consistent with the idea that in some fraction of plant species, human-perceived intraspecific floral color variation may be invisible to pollinators and thus may escape pollinator-mediated direct selection.

Caveats

Both visual spaces and discrimination thresholds may vary within animal groups, and may also vary with environmental conditions and context (De Ibarra et al. 2014; Dyer 2012; Garcia et al. 2017; Olsson et al. 2017). Given that little of this variation has been explored, our approach necessarily treats the groups we have examined (bees, birds, humans) as monolithic with regard to their color perception. More generally, the models themselves will develop along with advances in our understanding of receiver visual processing, such as, for example, the recent finding that luminance is not a dimension of colour perception in bees as it is in humans (Ng et al. 2018). Such models are also incapable of capturing the breadth of sensory processes that may be at play in shaping constraints on floral evolution, including signal detection, learning, categorisation, and selective attention, which present fruitful avenues for future inquiry. We thus consider our results on pollinator-imposed constraints to be preliminary. However, we note that visual models for bees, birds and humans, and their respective discrimination thresholds, are among the most well-developed and rigorously tested in existence (reviewed in De Ibarra et al. 2014; Fairchild 2013; Olsson et al. 2017; Kelber et al. 2003), and we argue that for our dataset, the observed stark contrasts in levels of floral color variation perceived by pollinators versus non-pollinators are unlikely to disappear with future refinements.

Conclusion

A role for bees in constraining floral color variation in species they pollinate has often been hypothesized (Fenster et al. 2004; Dyer et al. 2012; Papiorek et al. 2013) but is rarely tested. Our data on 14 putatively bee-pollinated species show that most intrapopulation color variation does not exceed bee discrimination thresholds, but exceeds non-pollinator discrimination thresholds, and are thus consistent with this hypothesis. Our results suggest that a large portion of human-perceived floral color variation within populations persists because it is effectively invisible to pollinators, and may evolve neutrally (via drift) or via indirect selection on correlated characters such as herbivore- or drought-resistance.

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Data Availability The datasets generated and analysed during the current study are available from the Open Science Framework repository at https://doi.org/10.17605/osf.io/nxj7m.

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