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White flowers finish last: pollen-foraging bumble bees show biased learning in a floral color polymorphism

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Abstract Pollinator-driven selection is thought to drive much of the extraordinary diversity of flowering plants. Plants that produce floral traits preferred by particular pollinators are more likely to receive conspecific pollen and to evolve further adaptations to those pollinators that enhance pollination and ultimately generate floral diversity. Two mechanisms in particular, sensory bias and learning, are thought to explain how pollinator preference can contribute to divergence and speciation in flowering plants. While the preferences of pollinators, such as bees, flies, and birds, are frequently implicated in patterns of floral trait evolution, the role of learning in generating reproductive isolation and trait divergence for different floral types within plant populations is not well understood. Floral color polymorphism in particular provides an excellent opportunity to examine how pollinator behavior and learning might maintain the different floral morphs. In this study we asked if bumble bees showed innate preferences for different color morphs of the pollen-only plant *Solanum tridynamum*, whether bees formed preferences for the morphs with which they had experience collecting pollen from, and the strength of those learned preferences. Using an absolute conditioning protocol, we gave bees experience collecting pollen from a color polymorphic plant species that offered only pollen rewards. Despite initially-naïve bees showing no apparent innate bias toward human-white versus human-purple flower morphs, we did find evidence of a bias in learning. Specifically, bees learned strong preferences for purple corollas, but learned only weak preferences for hypochromic (human-white) corollas. We discuss how our results might explain patterns of floral display evolution, particularly as they relate to color polymorphisms. Additionally,

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we propose that the ease with which floral visual traits are learned—i.e., biases in learning—can influence the evolution of floral color as a signal to pollinators.

Keywords Bumble bee · Pollen reward · Learning · Color polymorphism · Biases in learning · Preference

Introduction

Pollinator-mediated selection on floral traits is held to be a common process driving the evolution and diversity of flowering plants (Johnson 2006; Van der Niet and Johnson 2012). As a consequence of adaptation to pollinators, plants often exhibit suites of floral traits that closely match the traits of their pollinators (Anderson et al. 2009; Newman et al. 2014). Accordingly, shifts in floral traits, such as floral form, scent, or color, are often associated with pollinator shifts and with reproductive isolation in plants (Briscoe Runquist and Moeller 2014; Van der Niet et al. 2014). Two mechanisms underlying pollinator behavior in particular are thought to contribute to plant evolution: innate bias and learning. Some innate biases are thought to have evolved due to pollinator adaptation to flowers (termed adaptive preference); other biases evolved in a context separate from foraging on flowers (i.e.; pre-existing sensory bias; Schaefer and Ruxton 2010; Schiestl and Johnson 2013). Behaviors formed through associative learning are particularly intriguing because they are thought to contribute to rapid diversification of floral cues and rewards (Gegear and Burns 2007).

Learning is ubiquitous in animals and has been studied in a variety of pollinators (e.g., Fukushi 1989; Lunau 1992; Kelber and Pfaff 1997; Weiss 1997; Hurly and Healy 2002; Chittka and Thomson 2005). Pollinators learn to associate a variety of floral cues, including color, scent, texture, heat and iridescence, with floral rewards, such as nectar and pollen (Muth et al. 2016). Because learned behaviors are often durable, quickly formed (Giurfa 2007), and may be exhibited in response to evolutionarily novel stimuli, learning has the potential to rapidly alter floral preferences. In fact, pollinators typically exhibit strong preferences for species with which they have had experience (e.g., Weiss 1997; Chittka et al. 1999; Russell et al. 2015). These behavioral changes increase pollen transfer between conspecific plants, while reducing receipt of pollen from heterospecific plants (Gegear and Laverty 1995; Hopkins and Rausher 2012). Learning could thus contribute to reproductive isolation between flowering plant species and evolutionary divergence (Gegear and Burns 2007; Schiestl and Johnson 2013). Accordingly, patterns of floral trait evolution are thought to reflect learned pollinator preferences in part (Schiestl and Johnson 2013).

In this study, we focus on how learning might shape variation in floral traits within plant populations. Plants often exhibit intraspecific variation in their floral display traits—floral color polymorphisms are a common example (Weiss 1995; Rausher 2008). Color polymorphisms in plant populations often arise from single loss-of-function mutation, resulting in a species with both highly pigmented and hypochromic (often human-white) flower morphs (e.g., Fig. 1) (Rausher 2008; Malerba and Nattero 2012). These simple shifts in floral visual display traits are thought to favor the formation of reproductive barriers between flower morphs, and thus divergent evolution, as a consequence of strong pollinator visual preferences (Gegear and Burns 2007). Learned preferences for floral visual traits

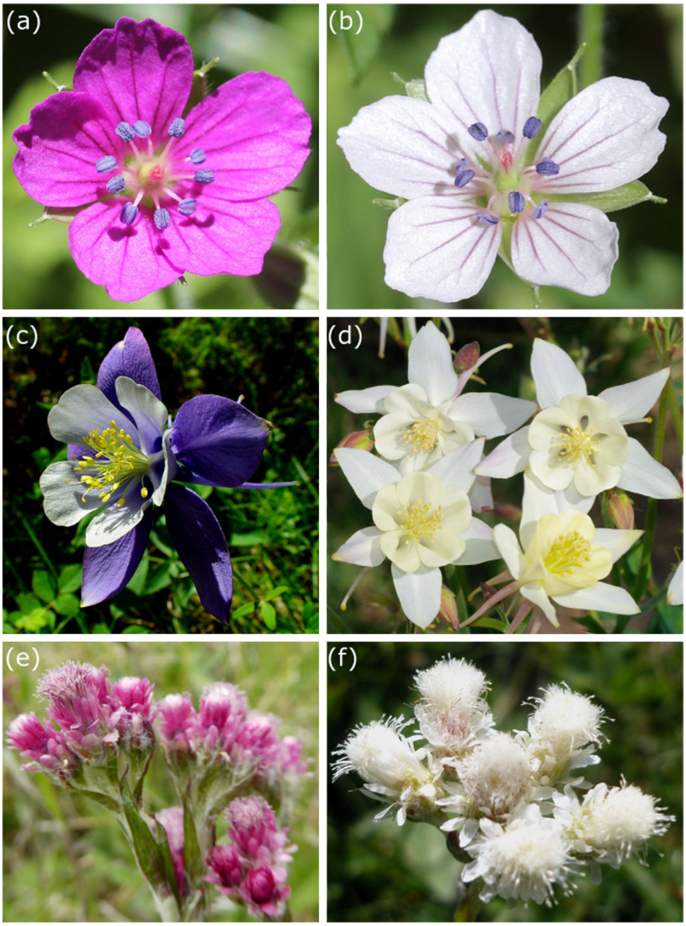

Fig. 1 Many color polymorphic species exhibit both highly pigmented and hypochromic flower morphs, as in **a**, **b** *Geranium thunbergii*, **c**, **d** *Aquilegia coerulea*, and **e**, **f** *Antennaria dioica*. *G. thunbergii* offers pollen and nectar rewards and is pollinated by *Hymenoptera* (especially bees), but also some *Diptera* and *Lepidoptera* (Kandori 2002). *A. coerulea* offers pollen and nectar rewards and is pollinated by *Hymenoptera* (bees) and *Lepidoptera* (hawkmoths) (Brunet 2009). *A. dioica* offers a nectar reward and is pollinated by a variety of *Coleoptera*, *Diptera*, *Hymenoptera*, and *Lepidoptera* (Willis and Burkill 1903). Photographs: **a**, **b** Alpsdake, **c** Josef F. Stuefer, **d** Alvesgaspar, **e** Biodehio, **f** Isidre blanc. Licensing: **a**, **b**, **d** CC BY-SA 3.0 CC, **c** CC BY 2.0, **e**, **f** BY-SA 4.0

have been studied in taxonomically diverse pollinators, including bees, flies, and birds (Lunau and Maier 1995). However, how learning might affect the maintenance of genetic floral color polymorphisms is still poorly understood. Pollinators learn some floral features more easily than others (e.g., Hempel de Ibarra and Giurfa 2003; Guerrieri et al. 2005); one might expect that the strength of resulting preferences or the speed with which these preferences are formed would therefore vary among flower morphs. Accordingly, pollinators should transfer more conspecific pollen between strongly preferred flower morphs and show less fidelity to weakly preferred morphs (Gegear and Burns 2007). Differences in the efficacy of learning could thus have immediate and profound fitness consequences for novel floral forms.

While a number of studies have investigated pollinator preferences for particular morphs (e.g., Waser and Price 1981; Epperson and Clegg 1987; Bradshaw and Schemske 2003), the role of learning in formation of these preferences is unclear. In this study we asked whether pollen-foraging bumble bees (*Bombus impatiens*) expressed innate and learned preferences for a pigmented versus a hypochromic (human-white) floral morph. We examined the degree to which floral experience affected preferences for each morph and additionally explored the role of specific flower parts, corolla versus anther, in mediating differences in learning performance. We studied the pollen-only species, *Solanum tridynamum*. Populations of *S. tridynamum* exhibit a naturally-occurring color polymorphism (J. Wiens pers. comm.) composed of a pigmented, human-purple morph and a hypochromic, nearly human-white morph (Fig. 2). Here we will refer to the human-purple morph as purple and the human-white morph as white. Both morphs conceal their pollen rewards within the anthers, which release their pollen when vibrated ('sonicated') by bees. A previous study demonstrated that bumble bees given experience collecting pollen from *S. tridynamum* rapidly formed durable preferences for the species over other pollen-only species (Russell et al. 2015). That study employed only the more common

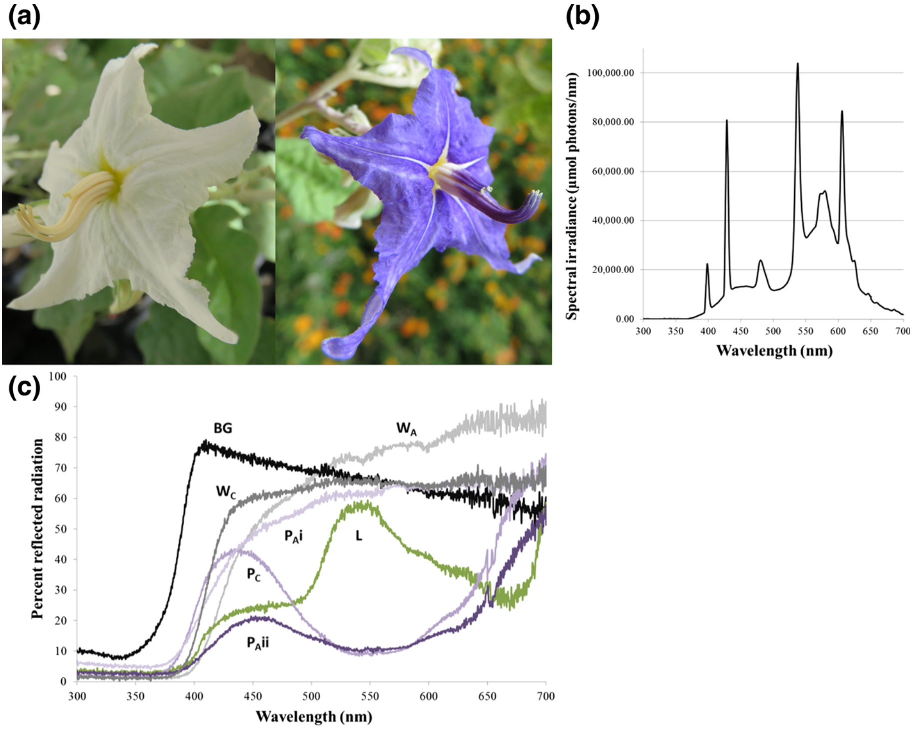

Fig. 2 **a** Human-white and human-purple forms of color polymorphic *S. tridynamum*. **b** The irradiance of the florescent lights illuminating the foraging arena. **c** The reflectance spectra of the foraging arena background (BG) and the color polymorphic *S. tridynamum*: purple morph corolla (P_C), proximal (P_Ai) and distal ($P_A$ii) areas of anthers, white morph corolla (W_C) and anthers (P_A), and leaf (L). Spectra of the corollas of all species were made from the peripheral tissue and not the yellowish central tissue

purple morph; here we used both morphs to examine patterns of innate and learned preference.

Methods

Outline of experiment

We conducted an experiment with five experience treatments (one no-experience treatment and four morph-experience treatments). The no-experience treatment involved an assessment of responses by flower-naïve bees to four types of flowers. In that treatment, flower-naïve bees were presented with equal numbers of the two naturally-occurring color morphs of *S. tridynamum* (purple and white) as well as two mosaic morphs. Mosaic morphs consisted of corollas from one natural morph and anthers from the other natural morph (purple corolla/white anther and white corolla/purple anther morphs). All flowers were manipulated such that pollen could not be collected from them. In the remaining four treatments, flower-naïve bees were given experience with flowers in a training phase; specifically, they were allowed to collect pollen from 1 of the 4 morphs. In a subsequent test phase, they were allowed to forage in an array of four unrewarding morphs identical to that in which bees in the no-experience treatment foraged. The no-experience treatment evaluates innate preference in terms of what morphs bees first visited, and also the overall preference resulting beyond what was observed in first visits. The four morph-experience treatments evaluate how a bee's early experience in collecting pollen from one morph affects its preference among morphs subsequently. The use of mosaic morphs allowed us to determine which flower part affected preference. Details of system and protocol follow.

Bees

We used 52 workers from 3 colonies of *Bombus impatiens* in experiments conducted between May 2015 and September 2015. Colonies were purchased from Koppert Biological Systems (Howell, MI, USA). We used approximately equal numbers of bees from each colony and for each treatment. We allowed bees to forage daily for sucrose and pollen in arenas constructed of plywood (L × W × H 82 × 60 × 60 cm). The arenas had clear acrylic ceilings and were lit from above by 40 W 60 Hz fluorescent lights (Lithonia Lighting). The colony was kept dark while lights in the arenas were set to a 14:10 light:dark cycle. Colonies had access to ad libitum 2 M sucrose solution and pulverized honeybee-collected pollen (Koppert Biological Systems, MI, USA) within the foraging arena. Sucrose solution was dispensed via braided cotton wicks (6 inch Braided Cotton Rolls, Richmond Dental) that extended into 40 dram vials (BioQuip Products, Inc., USA). Pollen was presented using custom-made feeders (Russell and Papaj 2016) consisting of chenille fibers, glued to the inside walls of 40 dram vials (BioQuip Products, Inc., USA).

Plants and floral morphs

In behavioral assays, we used freshly clipped flowers from 13 *S. tridynamum* plants raised in a university greenhouse. Eight plants produced white morph flowers and five plants produced purple morph flowers. Ten *S. tridynamum* plants were provided by a local museum (Arizona-Sonora Desert Museum, Tucson, AZ) and a further three plants were raised from seeds. Plants were fertilized weekly (Miracle Gro, NPK = 15-30-15) and grown under natural light in a portable field cage (1.8 × 1.8 × 1.8 m, BioQuip Products, Inc., USA). Approximately 1444 flowers were used in experiments.

Each of four floral morphs used in our experiment was constructed from two freshly clipped flowers (see Russell et al. 2015). One flower had its anthers excised where the filament joined with the corolla (leaving the "corolla"). The other flower had its perianth mostly removed, leaving a circle of corolla tissue to which the stamens, including their anthers, were joined. This circle of tissue was hot-glued into the center of the flower that had had its anthers removed. Four morphs were produced in this way (Fig. 3). Two were mosaic morphs of contrasting color combinations: white morph anthers glued to purple morph corolla (mosaic 1: P_CW_A) and purple morph anthers glued to white morph corolla (mosaic 2: W_CP_A). The other two were sham morphs of the naturally-occurring color combination: white morph anthers glued to white morph corolla (white sham: W_CW_A) and purple morph anthers glued to purple morph corolla (purple sham: P_CP_A). We did not observe any wilting or browning in these morphs. Control assays comparing sham and intact purple flowers confirmed that cutting and gluing the tissue in this way did not affect bee behavior (see Russell et al. 2015).

In test assays, we manipulated receipt of a pollen reward using glue. To create unrewarding flowers, drops of glue (Elmer's Glue All, Elmer's Products, Inc.) were applied to

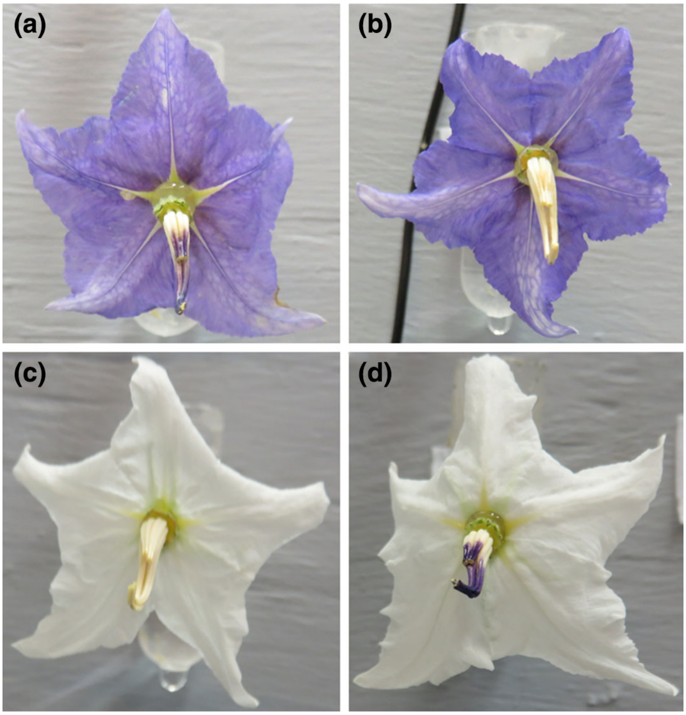

Fig. 3 Sham and mosaic *S. tridynamum* flowers used in the study. **a** Purple morph anthers glued to purple morph corolla (purple sham: P_CP_A), **b** white morph anthers glued to purple morph corolla (mosaic 1: P_CW_A), **c** white morph anthers glued to white morph corolla (white sham: W_CW_A), **d** purple morph anthers glued to white morph corolla (mosaic 2: W_CP_A)

the tip of each poricidal anther with a clean toothpick and allowed to dry for 5 min. The glue sealed the anther pore, preventing release of pollen.

Experimental protocol

All trials took place in a foraging arena (L × W × H, 82 cm × 60 cm × 60 cm) painted gray on floor and sides. For both training and testing, freshly clipped flowers were horizontally displayed (their natural orientation) on custom-built water tubes (see Russell et al. 2015), to prevent desiccation. The water tubes were Velcro mounted on the arena wall, facing the flight chamber's nest entrance. Flowers were arranged on the board in a Cartesian grid with each water tube spaced 7 cm apart in the horizontal and vertical axes of the grid. Fresh flowers were used at the start of every trial and for every bee. Flowers were never reused across training and testing, or across bees. We systematically alternated the five treatments in time to control for effects of day and time of day on behavior.

Our experiment consisted of five experience treatments (one no-experience treatment and four morph-experience treatments). In the no-experience treatment, we assayed innate preference in terms of first visits to the four floral morphs as well as preference beyond the first visits. We presented flower-naïve bees with an array composed of two flowers of each of the four morphs, all of which were unrewarding. Flowers were arranged in a 3 × 3 grid without a central flower (eight total targets). Targets of different types were assigned to positions such that all position–target–type combinations were equally represented across all trials and no single type of target appeared more than once in a row or column within a given array. To initiate testing, a single flower-naïve individual was introduced into the arena. We recorded landings made by the test bee on the various morphs, a landing being defined as the bee touching the flower with at least three of its legs simultaneously. Landings consisted of two types: landings with sonication buzzes and landings without sonication buzzes. Sonication buzzes were identified by their distinctive sound and occurred only after a bee had landed. Bees were allowed to make up to 20 landings with sonication buzzes after which the trial was terminated.

The four morph-experience treatments evaluated the degree to which preference for a floral morph was shaped by collection of pollen. We used an absolute conditioning (S+) protocol (Giurfa 2007). Bees were individually trained and tested. No pre-training of any kind was used. Each conditioning treatment corresponded to training on one of the four morphs described above. To initiate training, a vertical array of nine flowers of a single flower morph in a 3 × 3 grid was set up in the arena. One to four flower-naïve individuals were then introduced into the arena simultaneously. When a bee landed on a flower in a training array, the other bees were quickly removed from the arena by catching them with vials and returned to the colony. While these bees were being captured, the test bee continued to visit flowers and did not exhibit signs of being threatened by our activity, such as aggressive behavior or attempts to escape from the arena. Bees in these treatments were allowed to collect pollen for 20 flower visits in a single training trial. We used the occurrence of sonication buzzes as a proxy for pollen collection, because sonication is the only behavior these bees use to extract pollen from the flower's poricidal anthers. It is a consistent and reliable indicator of pollen collection. By this criterion, all bees in all conditioning treatments successfully collected pollen in 20 visits.

After the training trial, the test subject was labeled with a unique color combination of acrylic paint and returned to the colony. When the test subject emerged from the nest, always within 20–40 min after training, it was allowed to forage in a test array identical to that used in the no-experience treatment (two unrewarding flowers of each of the four morphs; see above). Bees were allowed to make up to 20 landings with sonication buzzes after which the test trial was terminated. A testing trial was occasionally terminated before 20 visits were made, if the bee did not forage on the array for a period of 5 min. We used all landings, with or without sonication buzzes, to estimate preference, and to understand how pollen rewards affected preference.

During training and testing, bees made repeated visits to the same flowers within an array. Although floral pollen would have diminished in flowers over time as a bee collected it during training, no bees appeared to deplete individual flowers of their pollen. To confirm that bees visited multiple flowers in a given trial, both training and testing, we counted the number of unique flowers landed upon in the trial. All bees in all treatments, both in training (if any) and testing, visited multiple flowers. We included all visits (including revisits) in our analyses.

To facilitate recording of behavior, video for all tests was captured at 30fps with a high-definition digital camcorder (Canon VIXIA HF R400) positioned in front of the array. Audio was input to the camcorder using an external microphone (33-3013 Lavaliere Microphone, RadioShack) attached to the center of floral arrays. A Zoom H2 Handy Recorder (ZOOM Corporation) was used to amplify and verify sonication buzzes in training and test trials.

Data analyses

All behavioral data from the experiment were analyzed using R v.3.2.0 (R Development Core Team 2010). We used all landings on various flower morphs to estimate preference.

We used an exact multinomial test (EM) to analyze whether naïve bees had a preference for any of the four flower morphs with their first landing choice that was different from the expected (25 %; i.e., random expectation for an assay with four choices). We report the likelihood ratio via the xmulti() function in the XNomial package (Engels 2015). In addition we ran Chi-square (χ^2) tests via the chisq.test() function in R to analyze whether naïve bees had a preference for the purple sham morph over others and/or whether they preferred the white sham morph over others, with their first landing choice. Lastly we used a χ^2 test to analyze whether naïve bees had a preference for mosaic flowers (P_CW_A and W_CP_A pooled) versus pure sham flowers (P_CP_A and W_CW_A pooled).

To analyze whether the proportion of landings by initially flower-naïve bees differed between mosaic flowers (P_CW_A and W_CP_A pooled) and pure sham flowers (P_CP_A and W_CW_A pooled), we ran paired *t*-tests via the aov() function in R.

We used a hierarchical Bayesian model (BayesPref package) designed for multinomial count data to analyze differences in preference across the four morphs (a detailed description of this analysis can be found in Fordyce et al. 2011; Forister and Scholl 2012). MCMC runs were conducted for 40,000 generations with the first 10,000 generations discarded as burn-in for all analyses. Using the ‘plot’ diagnostic tool, MCMC samples were examined to confirm even sampling of the posteriors.

We utilized pairwise comparisons of posterior probabilities (i.e., ‘PP’) to identify significant differences among estimates of preference for each of the four morphs (BayesPref package). When preference for a particular morph is greater than preference for another morph in more than 95 % of the sampled MCMC steps, preference estimates are considered to be significantly different (Fordyce et al. 2011). Posterior probabilities can be interpreted similarly to $P - \alpha$ (where $\alpha = 0.05$) in a frequentist approach. Because pairwise comparisons give values for both choice A over B and choice B over A (values that are complementary: A over B is equal to 1 − [B over A]), we report only the smaller value. We use a Bayesian approach (rather than MANOVAs or GLMERs, for instance), because to our knowledge it is the only statistical approach that reports pairwise differences and simultaneously does not suffer from inflated type I/II error rates when analyzing differences between categories that (a) are not independent, (b) lack moderate correlation between dependent variables, (c) have outliers, (d) and do not have homogeneity of variances.

To analyze the effect of experience on corolla or anther morph preference we used binomial generalized linear mixed effect models (GLMERs), specifying type II Wald chisquare (χ^2) tests via the Anova() function in the car package (Fox 2015). For these models we included 'BeeID' as a random factor and visits as repeated measures within BeeID and the response variable 'morph choice' (white or purple corolla; white or purple anther) and the factor 'treatment' (Naïve, P_CP_A, P_CW_A, W_CW_A, W_CP_A). GLMERs were carried out using the glmer() function in the lme4 package (Bates et al. 2015). In cases of significant effects, we ran Tukey's post hoc test using the glht() function in the multcomp package (Hothorn et al. 2015) to determine which pairs were significant.

For all GLMERs, maximal models were run first. For each analysis, we performed two rounds of backward elimination (as described in Fox 2015). We checked first whether any interaction terms should be eliminated from the model and then whether any main effects should be removed. We used the anova() function in R to examine significance for each of these effects relative to the full model.

To analyze potential interactions between corolla and anther morphs, we used mixed multinomial logit models (MMNLs). We included 'BeeID' as a random factor and the fixed factors 'treatment' (P_CP_A, P_CW_A, W_CW_A, W_CP_A, and Naïve), 'anther choice' (purple or white), and 'corolla choice' (purple or white). We also ran MMNLs for each treatment separately, to examine interactions within a treatment. MMNLs were carried out using the mlogit() function in the mlogit package (Henningsen and Toomet 2011; Croissant 2012).

Reflectance and irradiance spectra and bee color space

We characterized the color of flower morphs, leaves, and the background against which the flowers were presented in experiments, using reflectance and irradiance spectra. Each reflectance spectrum consists of the mean of five measurements. Each measurement was taken from a different leaf or flower (or part of the foraging arena). Reflectance spectra for all samples were measured using an UV–VIS spectrophotometer (Ocean Optics USB2000) with tungsten-deuterium light source (Ocean Optics DH2000) and a fluoropolymer white standard (USRS-99-010 Spectralon; Labsphere, NH, USA). An RPH reflectance probe (Ocean Optics) was held at constant height and angle above the samples using a holder that shielded the probe from extraneous light. Reflectance measurements were taken using a 5 ms integration time in the same session.

Irradiance within the flight arena was measured at the center of the foraging array using a P600 UV/VIS optical fiber (Ocean Optics), a CC-3-UV-T cosine-corrected (180 degrees) irradiance probe (Ocean Optics), and a tungsten-deuterium calibration light source (Ocean Optics DH2000). Irradiance measurements were taken using a 50 ms integration time.

To characterize what bees perceived, we used our reflectance and irradiance measurements to plot color morphs within a color space for *B. impatiens*. The color space diagram (i.e., color hexagon) and table were made in accordance with Chittka (1992), using data on receptor spectral sensitivities for *B. impatiens* from Skorupski & Chittka (2010). Chromatic contrast was measured as the Euclidean distance between stimuli in the color hexagon (Chittka 1992). Achromatic contrast was measured as the green contrast

Table 1 Visual contrast between flower structures and background stimuli

Background stimulus	Chromatic contrast					Achromatic contrast				
	Purple corolla	Purple anther prox.	Purple anther distal	White corolla	White anther	Purple corolla	Purple anther prox.	Purple anther distal	White corolla	White anther
Arena	0.137	0.220	0.074	0.283	0.369	0.318	0.031	0.344	0.013	0.009
Leaf	0.425	0.101	0.226	0.117	0.210	0.254	0.064	0.288	0.081	0.102
Purple corolla	-	0.291	0.123	0.350	0.424	-	0.298	0.047	0.309	0.323
White corolla	0.350	0.087	0.222	-	0.108	0.309	0.018	0.337	-	0.022

between stimuli (Giurfa and Vorobyev 1997). We used the arena wall on which the flowers were displayed as the background stimulus for the color hexagon and the irradiance of the overhead arena lights in calculations of receptor excitation values. For visual contrast measurements presented in Table 1, we additionally used *S. tridynamum* leaves, corolla, and anthers as the background stimulus.

Results

Initially-flower-naïve bees did not express preferences for one flower morph over another

In the no-experience treatment, there was no significant difference in the number of flower-naïve bees that made their first landing on any of the four flower morphs, compared to the expected number of first landings (EM: $P = 0.859$; % bees that made their first landing on P_CP_A: 16.7; P_CW_A: 33.3; $W_CW_A = 22.2$; $W_CP_A = 27.8$, $N = 18$). In particular, bees did not innately prefer the purple sham morph over others; neither did they prefer the white sham morph over others (χ^2-tests: P_CP_A versus P_CW_A: $\chi^2 = 1$, $P = 0.317$; P_CP_A versus W_CW_A: $\chi^2 = 0.1429$, $P = 0.706$; P_CP_A versus W_CP_A: $\chi^2 = 0.5$, $P = 0.480$; W_CW_A versus P_CW_A: $\chi^2 = 0.4$, $P = 0.527$; W_CW_A versus W_CP_A: $\chi^2 = 0.0909$, $P = 0.763$). Bees tended to make more first landings on the mosaic morphs than on the sham morphs, but the difference did not approach statistical significance (χ^2-test: $\chi^2 = 0.889$, $P = 0.346$; % bees that made their first landing on shams: 38.9; or on mosaics: 61.1, $N = 18$).

Because bees in the no-experience treatment foraged exclusively on unrewarding flowers, we might expect their overall preference to reflect the pattern shown in first landings. This was indeed the case. These bees showed no significant landing preference for any 1 of the 4 morphs over another (Fig. 4; differences in pairwise posterior probabilities: P_CP_A versus P_CW_A, PP = 0.2718; P_CP_A versus W_CW_A, PP = 0.2381; P_CP_A versus W_CP_A, PP = 0.3548; P_CW_A versus W_CW_A, PP = 0.0927; P_CW_A versus W_CP_A, PP = 0.4036; W_CP_A versus W_CW_A, PP = 0.1269). In particular, they did not prefer the purple sham morph over others; neither did they prefer the white sham morph over others (Fig. 4). In fact, initially flower-naïve bees showed a significant landing preference for the

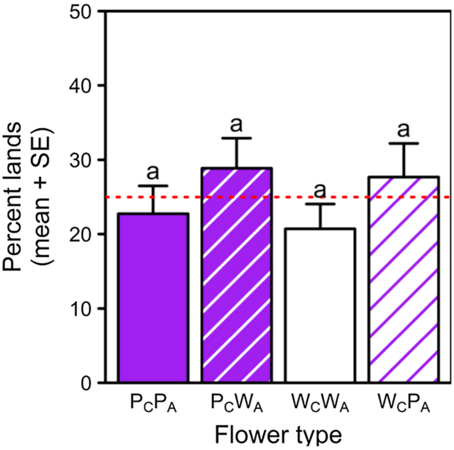

Fig. 4 Floral preference for initially-naïve bees visiting mixed arrays in the no-experience treatment. $N = 18$ bees. The *hatching color* indicates the anther morph color and the *background color* indicates the corolla morph color. A *solid fill* indicates that the anther and corolla are from the same color morph. *Letters* indicate pairwise differences at posterior probabilities <0.05. *Dashed line* at 25 % indicates random expectation for an assay with four choices. (Color figure online)

mosaic morphs (P_CW_A and W_CP_A pooled) over the sham morphs (P_CP_A and W_CW_A pooled) (paired *t* test: $t_{17} = -2.9619$, $P < 0.009$; mean % landing choice for mosaic flowers ± SE: 56.5 ± 2.2). In other words, flower-naïve bees foraging on unrewarding flowers showed a landing preference for morphs of *S. tridynamum* that do not, to our knowledge, occur in nature.

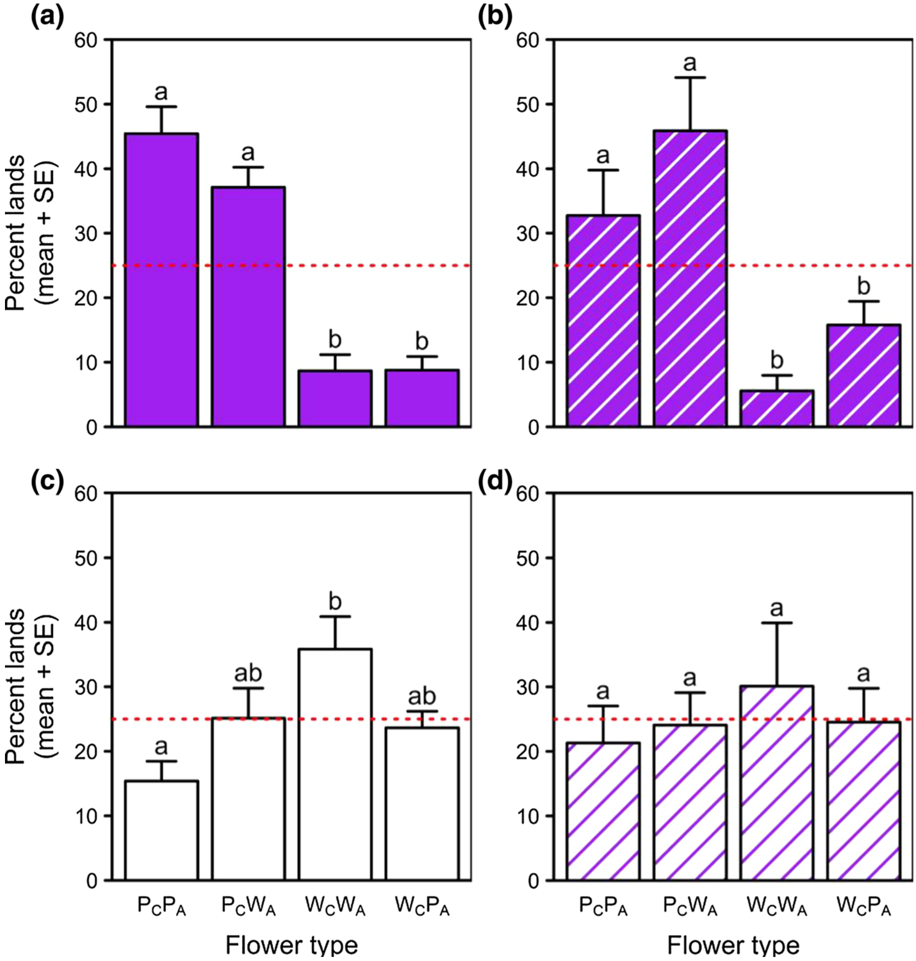

Fig. 5 **a** Floral preference for bees experienced on **a** P_CP_A, **b** P_CW_A, **c** W_CW_A, and **d** W_CP_A. $N = 9$ for each treatment, aside from $N = 7$ for bees given experience on W_CW_A. The *hatching color* indicates the anther morph color the bee experienced and the *background color* indicates the corolla morph color that the bee experienced during training. A *solid fill* indicates that the anther and corolla combination used in training were from the same color morph. *Letters* indicate pairwise differences at posterior probabilities <0.05 within a morph-experience treatment. *Dashed line* at 25 % indicates random expectation for an assay with four choices. (Color figure online)

Bees expressed stronger learned preferences when given experience with flowers with purple corollas

Bees given experience on flower morphs with purple corollas and then tested in the mixed array after 20–40 min expressed strong, significant landing preferences for morphs with purple corollas relative to morphs with white corollas (Fig. 5a, b). The anther-corolla combination of experience did not significantly affect landing preferences (Fig. 5a, b; exact differences in pairwise posterior probabilities are found in Table S1, S2).

In contrast, bees given experience on flowers with white corollas showed weak landing preferences for flowers with white corollas in the test condition (Fig. 5c, d). Specifically, bees given experience on W_CP_A exhibited no significant preference and bees given experience W_CW_A exhibited a small, but significant preference for W_CW_A flowers relative to P_CP_A flowers, but to neither of the other two combinations (Fig. 5c, d; exact differences in pairwise posterior probabilities are found in Table S3, S4).

Learned preferences were mediated by the corolla, but not the anther

To examine the role of corolla and anther in learned preferences, we use the same datasets used above (results from all five treatments' test condition), but we pooled data across morphs to estimate corolla morph preference and anther morph preference. Overall there were differences among treatments in their percent landing response to morphs with a purple corolla (Fig. 6a; GLMER overall effect: Type II Wald χ^2 test: $\chi^2 = 32.501$, $df = 4$, $P < 0.0001$). Specifically, treatments where bees were trained to morphs with a purple corolla showed a significant landing preference in the test condition for either morph with

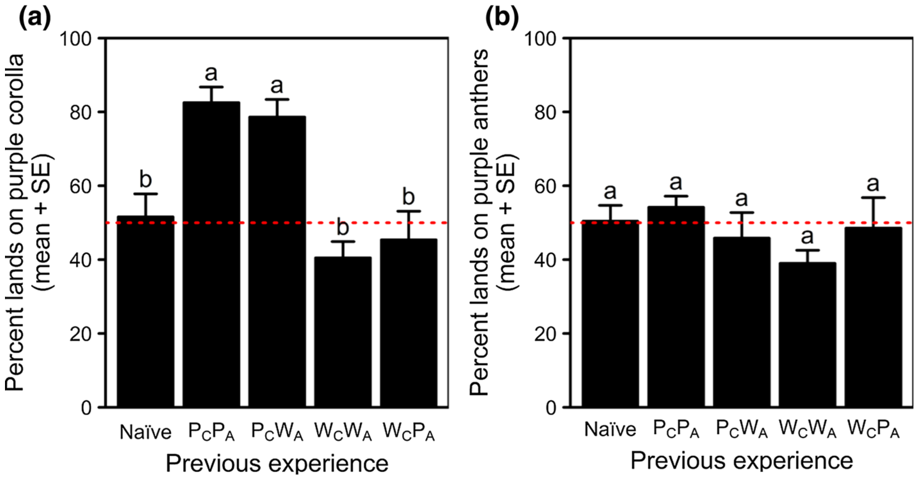

Fig. 6 We use a combined dataset, presented previously in Figs. 4 and 5, but here we analyze corolla morph choice independently of anther morph choice. **a** Corolla morph and **b** anther morph preference, for initially-naïve and experienced bees visiting mixed arrays. $N = 9$ for each morph-experience treatment, aside from $N = 7$ for bees given experience on W_CW_A. $N = 18$ bees for the no-experience (naïve) treatment. Differences in *letters above bars* indicate significant pairwise differences at $P < 0.05$ according to a Tukey's post hoc test. *Dashed line* at 50 % indicates random expectation for an assay with two color choices (purple vs. white): consequently, *bar* values >50 % indicate a preference for purple, while *bar* values <50 % indicate a preference for white

purple corolla; treatments where bees were trained to morphs with a white corolla however showed no significant landing preference (according to Tukey's post hoc tests; Fig. 6a). Overall there were no differences among treatments in percent landing response to morphs with a purple anther (Fig. 6b; GLMER overall effect: Type II Wald χ^2 test: $\chi^2 = 5.4494$, $df = 4$, $P = 0.2442$). Likewise, bees in none of the morph-experience treatments showed significant landing preferences in the test condition for the anther morph of experience (according to Tukey's post hoc tests; Fig. 6b).

Anther and corolla generally did not interact to affect preference

Across the five treatments, there was a significant effect of corolla morph, but not anther morph, on floral landing preference in the test condition (MMNL: corolla morph effect: $t = 2.8457$, $P < 0.0045$; anther morph effect: $t = 0.2823$, $P = 0.7777$). In addition there was a trend for an overall interaction between anther and corolla morph on landing preference in the test condition which was not significant (MMNL; anther morph × corolla morph effect: coefficient estimate = −0.4797, $t = -1.6467$, $P = 0.0996$). Specifically, there was a tendency for bees to prefer mosaic morphs over sham morphs. This effect was larger with respect to morphs with white anthers than morphs with purple anthers.

Furthermore, using MMNLs to examine each treatment independently, we found no significant interaction between effects of anther and corolla morph on landing preference in the test condition of any treatment. However, for bees in the no-experience treatment, as well as bees that had been given experience with P_CW_A flowers, there was a trend for an interaction in the test condition; as above, there was a tendency for bees to prefer mosaic morphs over sham morphs (MMNL: naïve treatment: coefficient estimate = −0.43507, $t = -1.8172$, $P = 0.0692$; P_CW_A training morph treatment: coefficient estimate = −0.72249, $t = -1.6563$, $P = 0.0977$).

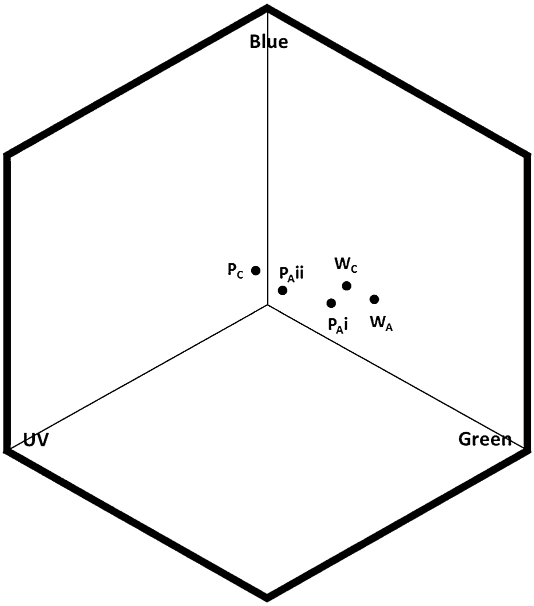

Fig. 7 The loci in *B. impatiens* color space of color polymorphic *S. tridynamum*: purple morph corolla (P_C), proximal (P_Ai) and distal ($P_A$ii) areas of anthers, white morph corolla (W_C) and anthers (P_A). Spectra of the corollas of all species were made from the peripheral tissue and not the yellowish central tissue

Spectra and color space

The two pure morphs exhibit similar chromatic contrast from the arena background and should be readily discriminable (Figs. 2, 7). The white morph however exhibits much less achromatic contrast from the arena background, compared to the purple morph; against a more natural background, a *S. tridynamum* leaf, these patterns change little (Table 1). Against the *S. tridynamum* leaf the purple morph, however, exhibits even greater chromatic contrast, while the white morph exhibits much less chromatic contrast (Table 1).

Results summary

Summing up, the results show that initially-naïve bees exhibited no landing preference for any one morph either on their first landing (when bees were truly flower-naïve) or after foraging in an array where no flowers were rewarding. These bees did not prefer the morphs that occur in nature. In fact, after searching in an array of all-unrewarding flowers, the bees showed an overall landing preference for the mosaic morphs, preferring patterned-over unpatterned (sham) morphs.

Despite the apparent absence of an innate preference, bees showed a bias in terms of how experience shaped preference: experience with the purple morph had a greater effect on preference than experience with the white morph. Learned preferences, and the bias in learning, were mediated by corolla identity and not anther identity. Finally, bees did not adopt a stronger preference for the specific anther-corolla combination with which they had been given experience.

Discussion

Pollinator preference can reflect innate dispositions (including sensory bias and adaptive innate preference) as well as effects of experience. Both innate and learned responses can shape the evolution of the floral display (Schiestl and Johnson 2013). The results presented here suggest that the evolution of floral color signals may conceivably reflect how readily particular colors are learned. While we did not find evidence of innate bias in color responses for naïve bees, we did find a bias in the outcome of experience. Specifically, bees learned strong preferences for a purple morph of *S. tridynamum*, but learned only weak ones for a co-occurring white (hypochromic) morph. To our knowledge, this is the first demonstration that pollinators show a bias in learning particular color morphs within a naturally-occurring floral color polymorphism. It is likewise the first evidence that pollinators can learn a preference for a color morph in the context of pollen collection; virtually all studies of pollinator behavior and floral color polymorphism involve nectar as the floral reward.

Floral color polymorphisms involving hypochromic morphs are common, perhaps because such morphs are relatively readily generated by loss-of-function mutations in genes for floral pigment production (Rausher 2008; Wessinger and Rausher 2012). These morphs, which are generally human-white in color, are often found in low frequencies in color polymorphic populations (Table S5). Our findings here suggest that biases against learning the color of these morphs might curtail the success of such morphs and perhaps even contribute to the low frequencies in which they occur. To further address these possibilities, it would be interesting to examine preference learning in more pollinator species for more plant species that exhibit color polymorphisms involving hypochromic morphs.

Why might bees have difficulty forming a preference for hypochromic morphs? One possibility is that biased learning reflects a difference in reward quality between the two morphs. In our study, pollen was the sole reward. If the white morph offered less or poorer pollen, we would expect learning of flowers with white anthers to be less effective than learning of flowers with purple anthers. This was not the case. For example, bees learned purple corollas paired with white anthers just as well as they learned purple corollas paired with purple anthers, which we would not expect if anthers of different colors offered rewards of different value. Biased learning was more likely due to differences in the distinctiveness of each morph's floral visual cues. Mapped onto bee color space (Chittka 1992), the two morphs exhibit similar chromatic contrast from the arena background and should be readily discriminable (Fig. 7; Table 1). However, compared to the pigmented morph, the hypochromic morph exhibits very little achromatic green contrast with the arena background. Furthermore, we would expect differences in more natural conditions: when we examined achromatic and chromatic contrast against a *S. tridynamum* leaf, we found that while achromatic contrast changes little, the purple morph exhibited much greater chromatic contrast, while the white morph exhibited even less chromatic contrast (Fig. 7; Table 1). As a consequence, we expect that the hypochromic *S. tridynamum* morph is likely even less discriminable in real-world foraging conditions. Although bees use achromatic contrast to both detect and learn floral colors, achromatic contrast is much more important in the latter task (Dyer and Spaethe 2008), which could explain why bees in our naïve preference test showed equal preference for the four flower types.

While bees did not innately prefer any of the morphs, initially-naïve bees foraging on unrewarding flowers surprisingly showed a preference for the mosaic morphs, the equivalent of which is not found in nature, over the naturally-occurring morphs. The mosaic morphs show greater contrast in color between corolla and anther, which might explain the preference. A number of studies have demonstrated that foraging bees exhibit innate preferences for artificial flowers with strong center-surround ('bulls-eye') color contrast and furthermore, are able to learn such patterns when paired with a nectar or pollen reward (Lunau et al. 1996; Hempel de Ibarra et al. 2001; Muth et al. 2016).

Plants frequently evolve floral display traits that cause flowers and floral rewards to be more attractive or easier to locate for pollinators (Lunau 1991; Leonard and Papaj 2011; Hempel de Ibarra et al. 2015). Given that initially-naïve bees exhibited significant preferences for patterned (mosaic) flowers in our study, we might expect to see similarly-patterned flowers in nature. That we have not seen these patterned (mosaic-like) morphs invites speculation as to why. Possibly, anthocyanin pigmentation in *S. tridynamum*'s anther and corolla is controlled by a single pathway and mutations in the function or regulation of the pathway necessarily involve its complete disruption throughout both floral structures. Alternatively, mutations giving rise to patterned morphs occur, but selection may not favor a contrasting pattern for various reasons. For example, a cryptic anther might reduce attack by a florivore. In addition, bees in our study did not adopt a significantly stronger preference for the specific anther-corolla combination with which they had been given experience, even for the mosaics. As such, there may be less selection for a visually-contrasting anther than the overall results of the naïve test might imply.

In conclusion, pollen-foraging bees can learn preferences for flower morphs of a particular plant species. Furthermore, the strength of these preferences is dependent on the corolla morph that the bee was conditioned to. Although both sensory biases and learning undoubtedly contribute to floral trait evolution generally, our results suggest learning may influence the evolution of floral color polymorphisms. In addition, biases in learning may be a key, yet often overlooked, mechanism driving the evolution of floral display traits generally (ten Cate and Rowe 2007) and the maintenance of floral color polymorphisms specifically. We predict that much as innate preferences are often conserved within a pollinator group (e.g., Lunau and Maier 1995), biases in learning may also be conserved within a given pollinator functional group. We suggest that future experiments should test this prediction directly by examining and comparing the learning performance of bees (not just well-studied honey- and bumble bee species) and other important pollinator taxa, such as flies and butterflies, on hypochromic and pigmented floral morphs.

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