2017

"Hummingbird" floral traits interact synergistically to discourage visitation by bumble bee foragers

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"Hummingbird" floral traits interact synergistically to discourage visitation by bumble bee foragers

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Abstract. Pollination syndromes are suites of floral traits presumed to reflect adaptations to attract and utilize a "primary" type of animal pollinator. However, syndrome traits may also function to deter "secondary" flower visitors that reduce plant fitness through their foraging activities. Here we use the hummingbird-pollinated plant species *Mimulus cardinalis* as a model to investigate the potential deterrent effects of classic bird syndrome traits on bumble bee foragers. To establish that *M. cardinalis* flowers elicit an avoidance response in bees, we assessed the choice behavior of individual foragers on a mixed experimental array of *M. cardinalis* and its bee-pollinated sister species *M. lewisii*. As expected, bees showed a strong preference against *M. cardinalis* flowers (only 22% of total bee visits were to *M. cardinalis*), but surprisingly also showed a high degree of individual specialization (95.2% of total plant transitions were between conspecifics). To determine *M. cardinalis* floral traits that discourage bee visitation, we then assessed foraging responses of individuals to *M. cardinalis*-like and *M. lewisii*-like floral models differing in color, orientation, reward, and combinations thereof. Across experiments, *M. cardinalis*-like trait combinations consistently produced a higher degree of flower avoidance behavior and individual specialization than expected based on bee responses to each trait in isolation. We then conducted a series of flower discrimination experiments to assess the ability of bees to utilize traits and trait combinations associated with each species. Relative to *M. lewisii*-like alternatives, *M. cardinalis*-like traits alone had a minimal effect on bee foraging proficiency but together increased the time bees spent searching for rewarding flowers from 1.49 to 2.65 s per visit. Collectively, our results show that *M. cardinalis* flowers impose foraging costs on bumble bees sufficient to discourage visitation and remarkably, generate such costs through synergistic color-orientation and color-reward trait interactions. Floral syndromes therefore represent complex adaptations to multiple pollinator groups, rather than simply the primary pollinator.

Key words: bumble bee; complex adaptation; floral specialization; Mimulus; *multi-sensory floral signal; multisensory integration; plant–pollinator interactions; pollination syndrome; secondary pollinator.*

INTRODUCTION

Floral diversity has long been attributed to differences between pollinator species in their foraging strategies (Grant 1949, Sapir and Armbruster 2010, Van der Niet et al. 2014). A major requirement for pollinators to initiate and maintain floral divergence is that they show a high degree of selectivity when exploiting floral resources (Grant 1994). Such floral selectivity is thought to be reflected in pollination syndromes (Faegri and Van Der Pijl 1979), which are convergent suites of covarying floral traits associated with pollination by specific types of animals (e.g., bees, birds, moths). It is widely assumed that syndromes are adaptations to attract and utilize the most effective specific or "primary" pollinator type (Stebbins 1970, Fenster et al. 2004). But, many flowers

displaying classic syndrome traits are also visited and pollinated by animals that do not conform to the pollination syndrome concept (Waser et al. 1996, Rosas-Guerrero et al. 2014). These "secondary" pollinators have the potential to confer significant reproductive costs to plants through competitive interactions with primary pollinators, (Levin and Anderson 1970, Possingham 1992), increased pollen transfer to heterospecific plants (Waser 1986), and inefficient pollen pickup/deposition at flowers (Castellanos et al. 2003). Consequently, floral syndromes are also likely to contain traits to discourage secondary pollinators from visiting flowers or force individuals to adopt a foraging strategy that increases pollen transfer efficiency, such as flower constancy (Goulson 1999). Such "negative" trait adaptations (i.e., traits that function in deterrence rather than attraction) are expected to play a particularly important role in the evolutionary shift from one syndrome to another by maintaining floral integrity when new (primary) and ancestral (secondary) pollinators co-occur (Faegri and Van Der Pijl 1979, Fenster et al. 2004, Thomson and Wilson 2008).

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In this study, we investigated the potential role of pollinator deterrence in evolutionary shifts from bee pollination (melittophily) to bird pollination (ornithophily). Bee-to-bird transitions, which are frequent and phylogenetically widespread in angiosperms, are often accompanied by a shift to flowers displaying classic bird syndrome traits. These include red/orange coloration, a narrow corolla tube, reflexed petals, horizontal/semi-pendant orientation, and copious amounts of dilute nectar (Beardsley et al. 2003, Thomson and Wilson 2008). There is good evidence that bumble bees avoid flowers displaying bird syndrome traits when other floral options are available (Sutherland and Vickery 1996, Schemske and Bradshaw 1999, Fenster et al. 2004), but the mechanisms underlying such avoidance behavior in bees remain unclear. In some cases, bees may not be able to physically access nectar rewards and therefore do not recognize flowers as a potential source of food (Grant 1994). It has also been hypothesized that bees do not have the visual capacity to distinguish red coloration of bird flowers from background foliage and thus cannot "see" them (Raven 1972, Crepet 1984, Charlesworth and Charlesworth 2000, Bradshaw and Schemske 2003, Fenster et al. 2004, Rausher 2008, Shrestha et al. 2013, Bergamo et al. 2016). However, a number of bumble bee species have been shown to detect, exploit, and even preferentially visit red flowers under field and laboratory conditions (Chittka and Waser 1997, Irwin and Brody 1999, Mayfield et al. 2001, Gegear and Burns 2007, Forrest and Thomson 2009, Martinez-Harms et al. 2010), suggesting that the behavioral avoidance of bird flowers by bees is mediated through some other mechanism.

One possibility is that bird syndrome traits substantially increase the cost of visitation relative to flowers of other available plant species. In this view, bees adopt flower avoidance as a foraging strategy to maximize their rate of energetic gain. Several characteristics of the classic bird pollination syndrome have the potential to reduce bee foraging efficiency and thus serve an "anti-bee" instead of, or in addition to, a "pro-bird" function (Castellanos et al. 2004). For instance, the reflexed lower petal typical of bird flowers may increase the amount of time required for bees to extract nectar (Castellanos et al. 2004, Zung et al. 2015). In addition, signaling traits of bird flowers such as red coloration, horizontal orientation, and reduced display size may increase foraging costs to bees by decreasing the speed and accuracy of foraging decisions (Rodriguez-Girones and Santamaria 2004, Burns and Dyer 2008, Rodriguez-Girones et al. 2015), and potentially interact with one another to further increase the magnitude of such costs (Gegear and Laverty 2005, Raguso and Willis 2005, Gegear and Burns 2007, Campbell 2009, Leonard and Masek 2014). Nectar traits of bird flowers such as high volume and low concentration may also function to deter bee visitors by reducing sugar intake rates (Heinrich 1975, Harder 1986, Cnaani et al. 2006) or increasing the amount of energy expended to produce honey in the colony (Bolten and

Feinsinger 1978). However, few studies to date have experimentally manipulated bird syndrome flowers to test if these traits, or combinations thereof, impose foraging costs on bees sufficient to act as a behavioral deterrent.

Here we test for "anti-bumble bee" floral traits in hummingbird-pollinated *Mimulus cardinalis*. *M. cardinalis* and its bee-pollinated sister species *M. lewisii* represent an example of pollinator-mediated macroevolution, with floral phenotypes, phylogenetic data, and principle pollinators supporting an evolutionary shift from bumble bee to hummingbird pollination (Vickery 1995, Bradshaw et al. 1998, Beardsley et al. 2003). In contrast to the typical bird syndrome flowers of *M. cardinalis*, bee-pollinated *M. lewisii* flowers have lavender-pink coloration, upright flower orientation, and small volumes of concentrated nectar (Fig. 1A). Observational field studies have shown that several bumble bee species can access nectar of *M. cardinalis* flowers and in doing so act as pollinators, but selectively avoid them when *M. lewisii* flowers are also available (Vickery 1978, 1990, Sutherland and Vickery 1996, Schemske and Bradshaw 1999). To confirm captive bumble bees also exhibit avoidance responses to *M. cardinalis* flowers, we quantified the type and degree of foraging selectivity shown by individual
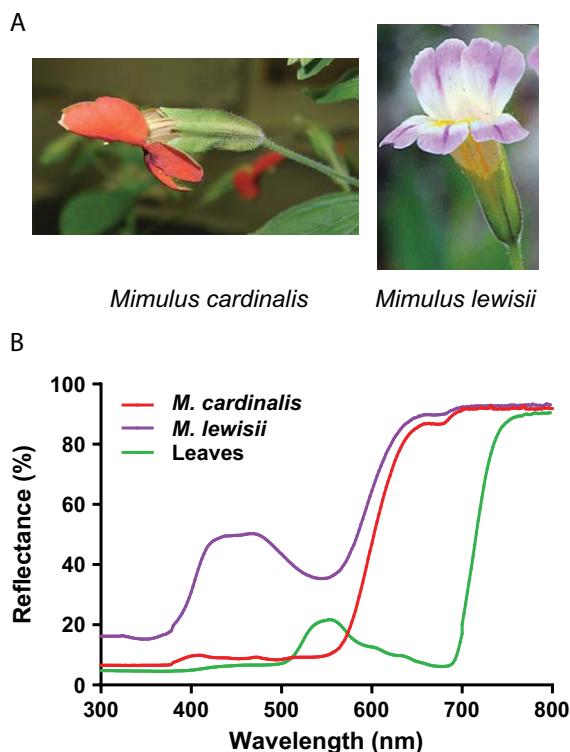

FIG. 1. (A) Flowers of hummingbird-pollinated *Mimulus cardinalis* and its bumble bee-pollinated sister species *M. lewisii* with (B) corresponding spectral reflectance curves (B). Values at each wavelength represent an average of three plants. Photos: R. J. Gegear.

bees on an experimental array containing a mixture of *M. cardinalis* and *M. lewisii* plants. As expected, bees preferentially avoided *M. cardinalis* flowers, but also showed a strong species repetition bias (i.e., individuals moved sequentially between plants of the same species). We then conducted a series of trait manipulation experiments with floral models to determine whether color, orientation, and reward characteristics of *M. cardinalis* function alone or together to produce avoidance behavior and individual specialization in bees. Finally, we conducted a series of flower discrimination experiments to determine if the utilization of *M. cardinalis* floral traits alone or in combination reduces bee foraging efficiency. Collectively, our findings indicate that *M. cardinalis* traits interact synergistically to increase foraging costs to bees, thereby making flower avoidance an economic decision strategy.

MATERIALS AND METHODS

Plants

We established greenhouse populations of *M. cardinalis* and the Sierra Nevada race of *M. lewisii* (Fig. 1A) from seeds obtained through Thompson and Morgan (Jackson, New Jersey, USA) and Seeds Trust High Altitude Gardens (Hailey, Idaho, USA), respectively. Plants were housed under controlled light (14 h light:10 h dark) and temperature (15–22°C). Spectral reflectance of *M. cardinalis* and *M. lewisii* flowers and leaves were measured from 200–800 nm with a Perkin-Elmer Lambda19 UV/Vis/NIR Spectrophotometer (London, Ontario, Canada; see Fig. 1B). For experiments, plants were transferred from the main greenhouse area into an adjacent room with a bumble bee colony.

Bumble bees

Bombus impatiens (subgenus *Pyrobombus*) colonies were obtained from Biobest Biological Systems Canada (Leamington, Ontario, Canada). *B. impatiens* foragers readily collect nectar from *M. lewisii* and *M. cardinalis* flowers under greenhouse conditions and act as pollinators while doing so (R. J. Gegear, *unpublished data*). *B impatiens* has also been used previously to study floral adaption in this pollination system (Owen and Bradshaw 2011). Based on phylogenetic, morphological, and behavioral traits, *B. impatiens* is classified in the same functional group as other bumble bee pollinators (*B. huntii*, *B. centralis*, and *B. vosnesenskii*) known to visit *M. lewisii and M. cardinalis* flowers throughout their native range (Vickery 1978, Schemske and Bradshaw 1999, Byers et al. 2014).

For all experiments, a gated mesh tunnel was attached to one side of the colony box so that we could control forager traffic entering the greenhouse room. At least two colonies were used per experiment. All newly emerged workers within a colony were marked for identification with different color combinations of acrylic paint. When not being tested, foragers were allowed to freely collect 30% sucrose solution from plastic feeders located inside the colony. Colonies were directly supplied with pollen ad libitum to facilitate nectar foraging during experiments. All foragers were flower naïve prior to experiments. For Experiments 2 and 3 (laboratory experiments), colonies were connected to an indoor flight cage (325 × 240 × 221 cm) with a gated-tunnel constructed from wire mesh. The cage was illuminated by two Ultra SunTM 6500K (ZooMed Laboratories, San Luis Obispo, USA) and two Sylvania GRO-LUX fluorescent lights (Wilmington, Massachusetts, USA).

Experiment 1: Do bumble bee foragers avoid M. cardinalis flowers?

The goal of this experiment was to quantify the type and degree of foraging selectivity shown by bees in response to natural floral trait differences between *M. cardinalis* and *M. lewisii*. We first established that bees had the capacity to harvest nectar from both species by allowing a small group of marked bees (two to four) to leave the colony and successively forage on a "pure" array of each species. Each array contained 50 potted plants with 5–10 flowers in bloom, which were replaced after 1 h of foraging activity to ensure that nectar rewards were always available to bees. We allowed bees to forage on each species for 2 hours per day over a 2-d period (4 h total foraging time). Bees were monitored throughout the 2-h period to make sure that they gained similar levels of experience on flowers of each species prior to testing. On the day immediately following this pre-exposure period, we recorded the first 25 plant visits of individual bees on an experimental array containing 50 plants (approximately 400 flowers) of each species, which were intermixed in a checkerboard pattern with 20 cm separating adjacent plants. In this way, bees always encountered both species as they moved among plants. We consider a bee to have made a plant visit if it landed on a flower and entered the corolla tube to obtain nectar reward. A total of 10 bees from three colonies were tested in this manner. Plants were replaced between bees to ensure that all flowers contained nectar rewards.

Following Gegear and Laverty (2005), we analyzed the plant visit sequence for each bee to generate two indices of foraging selectivity. First, we tested for a species preference by comparing the proportion of visits to *M. cardinalis* plants (species preference) to the proportion expected based on random species selection. Second, we tested for a species repetition bias by comparing the proportion of foraging moves between conspecific plants to the proportion expected based on a random selection sequence. Given that the two species were equally abundant and available to bees, the expected proportion of overall visits to each species is 0.5. Together, these measures of floral selectivity provide a robust indicator of the degree to which bee foraging choices would affect pollen flow among plants, and therefore plant fitness.

Experiment 2: Are bumble bee responses to M. cardinalis flowers mediated by a single trait or trait combination?

Due to the logistical difficulty of performing trait manipulation experiments with real plants, we used floral models to determine the separate and combined effects of color, orientation, and reward differences between *M. lewisii* and *M. cardinalis* on the foraging choices of bees. These traits were selected for study because they are thought to play a critical role in the ethological separation of *M. lewisii* and *M. cardinalis* (Bradshaw et al. 1998) and shifts from melittophily to ornithophily in many other plant taxa (Thomson and Wilson 2008). The basic design of floral models (hereafter referred to as "flowers") consisted of a *M. lewisii*-shaped paper "corolla" (Fig. 2A, left side) fixed around the entrance of a 1.5-mL polypropylene microcentrifuge tube (Fisher Scientific, Waltham, Massachusetts, USA) with the cap removed. Flowers were held 3 cm above the surface of the array with a 6 cm section of green pipe cleaner that was bent such that they had either an "upright" (*M. lewisii*-like)
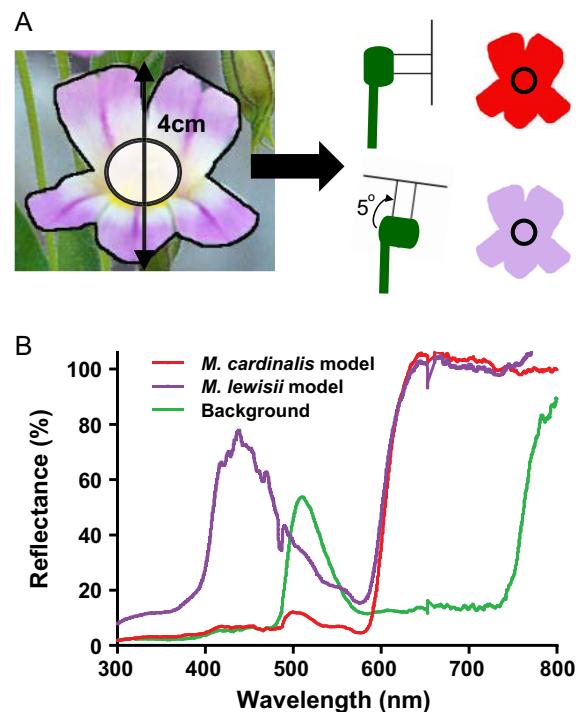

FIG. 2. *Mimulus cardinalis*-like and *M. lewisii*-like floral models and associated traits. (A) Each model flower consisted of a *M. lewisii*-shaped paper corolla (left side) fixed to a polypropylene microcentrifuge tube, which was then oriented in either a horizontal (*M. cardinalis*-like, middle upper) or an upright (*M. lewisii*-like, middle lower) position. Flower color of each species was transferred from digital images of petals to blank corolla outlines using photo editing software (right side). (B) Spectral reflectance curves of *M. cardinalis*-like and *M. lewisii*-like paper corollas and the digital print of background foliage used to cover floral arrays. Values at each wavelength represent an average of three corollas and locations on the background print.

or "horizontal" (*M. cardinalis*-like) orientation (Fig. 2A, middle). Flower colors of each species were replicated in Adobe Photoshop (San Jose, California, USA) by first using the "eyedropper" tool to select colors from digital floral images and then using the "fill" tool to transfer the color to an outline of the corolla (Fig. 2A, right side). Colored corolla images were then printed on both sides of a sheet of laser photo paper with a Xerox Docucolor 12 (Norwalk, Connecticut, USA) color laser printer and fixed to microcentrifuge tubes of a similar color. Fig. 2B shows the spectral reflectance measurements for *M. cardinalis*-colored and *M. lewisii*-colored corollas from 200–800 nm, which were taken with an Ocean Optics (Dunedin, Florida, USA) USB 4000 hand-held spectrophotometer and processed with SpectraSuite (Ocean Optics, Dunedin, Florida, USA) software (hereafter referred to as "red" and "lavender" coloration, respectively). Floral rewards of each species were replicated by placing either 1 μL of 30% sucrose solution (*M. lewisii*-like) or 3 μL of 10% (*M. cardinalis*-like) sucrose solution (mass/mass) at the bottom of the microcentrifuge tube with a micropipette. These values fall well within the range of nectar rewards reported for each species (Vickery 1995, Sutherland and Vickery 1996, Bradshaw et al. 1998, Bradshaw and Schemske 2003) and other genera with melittophilous-ornithophilous species pairs (Fenster et al. 2006, Guzman and Wilson 2012).

Floral arrays were created by embedding pipe cleaner "stems" (Fig. 2A, middle) in a horizontal sheet of Styrofoam (1.4 m × 1 m × 0.03 m thick) that was covered with a digital image of natural *M. lewisii* and *M. cardinalis* foliage (see Fig. 2B for spectral reflectance measures). A complex green background was selected as it is known to affect the behavioral response of bees to red stimuli (Forrest and Thomson 2009). A total of 80 flowers were placed in 10 rows of eight (12 cm apart within rows and 6 cm between rows) with adjacent rows offset by half the distance between flowers in each row. On mixed arrays, 40 flowers of each type were distributed in alternating rows of two so that both types were equally available to bees upon departing most flowers. Flowers were refilled upon being drained by foragers so that reward levels associated with available flowers remained constant throughout the experiment. Flowers were also replaced between bees to control for scent markings left by previous foragers.

Experimental procedure.—Marked bees making regular foraging trips to a training feeder located inside the flight cage were collected in the colony and then individually tested on a dimorphic array of *M. lewisii*-like and *M. cardinalis*-like flowers differing in color only (Experiment 2a; *n* = 10 bees), orientation only (Experiment 2b; *n* = 10 bees), color and orientation together (Experiment 2c; *n* = 18 bees), or color and reward together (Experiment 2d; *n* = 10 bees). Flowers had a *M. lewisii*-like orientation in Experiment 2a and a *M. lewisii*-like reward (1 μL of 30% sucrose solution) in Experiments 2a–c to stimulate the emergence of "bird" traits in an ancestral

population of *M. lewisii*. For Experiment 2d, color–reward combinations were balanced among test bees (i.e., five bees were given a choice between flowers with lavender *M. lewisii*-like reward and red *M. cardinalis*-like reward, and five bees were given a choice between flowers with lavender *M. cardinalis*-like reward and red-*M. lewisii*-like reward). We digitally recorded the first 80 flower visits of each individual for later detailed analysis of their flower visit sequence.

As in Experiment 1, we assessed floral selectivity of bees by testing (1) the proportion of visits to *M. cardinalis*-like flowers (flower preference) and (2) the proportion of moves between the same flower type (flower repetition bias) against random flower selection (0.5 in both cases, given the number and distribution of flower types on the mixed array). In addition, we determined foraging rate (flower visits per minute) for each bee over the 80 consecutive flower visits, excluding all time spent performing activities other than foraging such as flying to/from the colony and grooming.

Experiment 3: Do M. cardinalis traits confer foraging costs to bees?

We used a flower discrimination assay to test the ability of bumble bee foragers to utilize *M. cardinalis*-like and *M. lewisii*-like colors (red vs. lavender; Experiment 3a; $n = 10$), orientations (horizontal vs. upright; Experiment 3b; $n = 10$), and color-orientation combinations (red-horizontal (RH) vs. red-upright (RU) vs. lavender-horizontal (LH) vs. lavender-upright (LU); Experiment 3c; $n = 12$). The assay was divided into pre-training and discrimination phases. In the pre-training phase, bees were allowed to complete a foraging run on a pure array of flowers displaying each stimulus variant in succession (bees made approximately 50 visits per foraging run). Each array consisted of 40 flowers rewarded with 2 μL of 30% sucrose solution. In this way, all individuals had equal levels of experience with each stimulus variant prior to beginning the discrimination phase. The presentation sequence of pure arrays was randomly selected for each bee to control for potential order effects on foraging performance. In the discrimination phase, which began immediately after visiting the last pure array in the pre-training phase, individual bees were digitally recorded foraging on a mixed floral array in which one of the stimulus variants was rewarded with 2 μL of 30% sucrose solution (target flowers) and the other variant(s) contained the same volume of distilled water (non-rewarding distractor flowers). As in Experiment 2, mixed arrays held a total of 80 flowers with equal numbers of each stimulus variant (40 × 2 types for Experiments 3a and b, and 20 × 4 types for Experiment 3c). Target flowers were refilled after being drained by bees so that they were always associated with sucrose reward. The stimulus variant associated with target flowers was balanced among bees; thus, five bees were tested on each target variant in Experiment 3a and b and 3 bees were tested on

each target variant in Experiment 3c. Flowers in Experiment 3a were as described for Experiment 2a. For Experiment 3b, flower color was balanced among bees (i.e., five bees experienced upright and horizontal red flowers and the other five bees experienced upright and horizontal lavender flowers).

For each bee, we assessed the ability to utilize *M. cardinalis*-like and *M. lewisii*-like floral traits and trait combinations in two ways. First, we assessed flower learnability by determining the number of visits required to reach a learning criterion of 80% visits to rewarding (target) flowers over three consecutive blocks of 10 visits. Second, we assessed flower discriminability by using data from the first 20 target flower visits after reaching the 80% learning criterion to determine (1) the amount to time required to fly between target flower types (in seconds; search time) and (2) the proportion of visits to distractor flowers (choice accuracy).

Results

Experiment 1

All bees readily collected nectar from flowers on monospecific arrays of *M. lewisii* and *M. cardinalis*; however, most individuals showed a significant species preference on the mixed array (Table 1), with 7 out of 10 bees preferring *M. lewisii* and 1 out of 10 bees preferring *M. cardinalis*. Although two bees were found to have no species preference, they did show a significant species repetition bias, only switching between species in 8 out of 50 total visits. When considered as a group, bees made only 56 out of 250 (22%) plant visits to *M. cardinalis* and switched between species at total of 12 out of 250 times (4.8%),

TABLE 1. Choice behavior of individual bumble bee foragers on mixed experimental arrays of *Mimulus cardinalis* and *M. lewisii* plants.

Bee	Species preference		Species repetition bias	
	Visits to *M. cardinalis*	Visits to *M. lewisii*	Conspecific transitions	Heterospecific transitions
1	0	25*	25	0*
2	0	25*	25	0*
3	1	24*	23	2*
4	1	24*	23	2*
5	0	25*	25	0*
6	0	25*	25	0*
7	25	0*	25	0*
8	15	10	21	4*
9	14	11	21	4*
10	0	25*	25	0*

Note: Data represent the first 25 plant choices (species preference) and transitions within and between species (species repetition bias) made by each test bee. Individuals were considered to have adopted a specialist foraging strategy if values significantly deviated from those expected given a random plant selection strategy. *$P < 0.05$.

indicating a foraging bias against *M. cardinalis* and a high degree of individual specialization.

Experiment 2

Fig. 3 shows the foraging response of bees to *M. lewisii*-like and *M. cardinalis*-like flowers differing in either color only (Experiment 2a), orientation only (Experiment 2b), or color and orientation (Experiment 2c). Overall, bees in the color-orientation combination group showed stronger avoidance of *M. cardinalis*-like flowers (Fig. 3A; one-way ANOVA, $F_{2,35} = 24.04$, $P < 0.0001$) and a higher degree of flower repetition bias (Fig. 3B; ANOVA, $F_{2,35} = 24.04$, $P < 0.0001$) than bees in the color only and orientation only groups. At the individual level, a total of 8 out of 20 foragers showed a foraging preference (more visits to one type than expected by chance) when flowers differed in color or orientation alone (*M. lewisii*-like trait preference: three bees color, three bees orientation; *M. cardinalis*-like trait preference: one bee color, one bee orientation). In contrast, 16 out of 18 bees showed a foraging preference when floral options differed in color and orientation together, with all 16 bees avoiding *M. cardinals*-like flowers. A similar pattern was observed for flower repetitions (frequency of sequential moves between similar flower types), with 2 out of 20 bees showing a repetition bias when flowers differed in color (1 bee) or orientation (1 bee) alone and 15 out of 18 bees showing a bias when flowers differed in color and orientation together.

Foraging rates were similar among color only, orientation only, and color-orientation combination groups (Experiments 2a–c; one-way ANOVA, $F_{2,35} = 2.45$, $P = 0.1$). However, there was a negative relationship between foraging rate and proportion of visits to *M. cardinalis*-like flowers in the color-orientation combination group (Fig. 3C; linear regression, $F_{1,16} = 5.94$, $P = 0.027$), indicating that visiting multi-trait *M. cardinalis*-like flowers increases bee foraging costs. No such relationship was observed when *M. lewisii*-like and *M. cardinalis*-like flowers differed in color only ($F_{1,8} = 0.01$, $P = 0.92$) and orientation only ($F_{1,8} = 2.16$, $P = 0.18$).

When flowers differed in both color and reward traits (Experiment 2d), bees as a group showed a strong avoidance of *M. cardinalis*-like reward (one-sample *t* test, $t = 14.41$, $df = 9$, $P < 0.001$), making only 18% of total visits to flowers containing such rewards. Bees also moved between flowers containing the same reward type more often than expected given random reward selection (mean proportion of repetitions ± SE = 0.71 ± 0.02; one-sample *t* test, $t = 14.41$, $df = 9$, $P < 0.001$), indicating a flower repetition bias. However, the strength of flower avoidance and repetition bias shown by bees depended on the specific reward-color pairing, with red coloration significantly increasing avoidance of *M. cardinalis*-like rewards (Fig. 4A left; $t = 3.38$, $df = 8$, $P = 0.01$) and reducing repetition frequency (Fig. 4A right; $t = 3.04$, $df = 8$, $P = 0.02$). Red coloration also reduced bee
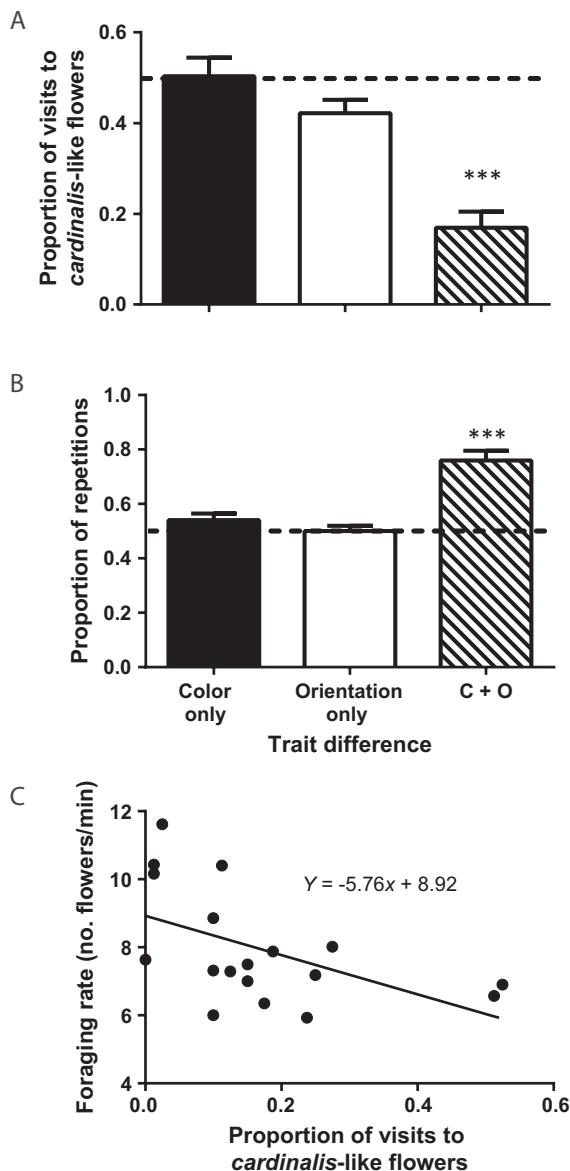

FIG. 3. Choice behavior of bumble bees on mixed arrays of *Mimulus cardinalis*-like and *M. lewisii*-like floral models differing in color only (Experiment 2a; $n = 10$), orientation only (Experiment 2b; $n = 10$), and color and orientation together (C + O; Experiment 2c; $n = 18$). (A) Proportion of bee visits to floral models containing *M. cardinalis*-like traits. (B) Proportion of sequential flower choices to the same flower type (repetitions). Dashed line represents proportion expected given random flower selection on the experimental array. (C) Functional relationship between foraging rate (number of flowers visited per minute) and proportion of visits to the *M. cardinalis*-like color-orientation trait combination in Experiment 2c. Values correspond to mean ± SE. ***$P < 0.001$.

foraging rates when it was paired with *M. lewisii*-like vs. *M. cardinalis*-like reward (Fig. 4B; $t = 4.08$, $df = 8$, $P = 0.003$), indicating a color-reward interaction effect on foraging proficiency.
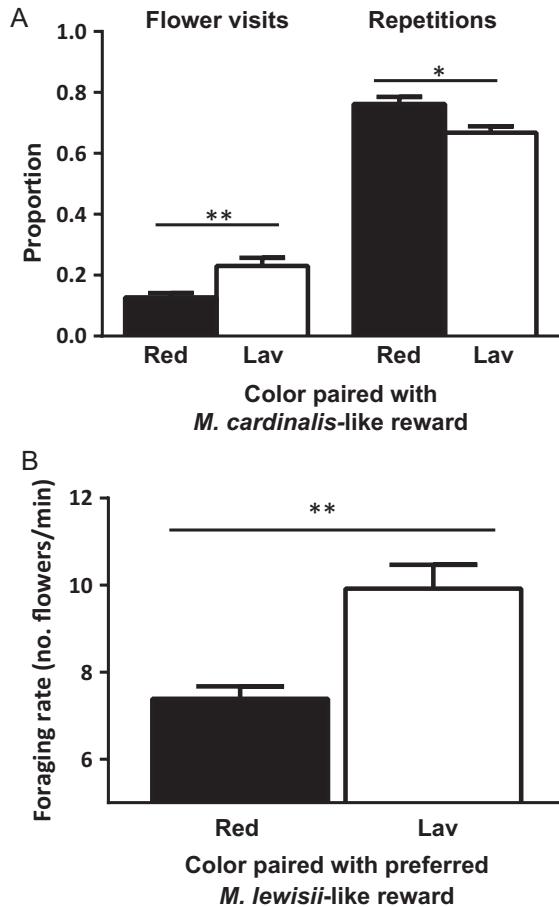
FIG. 4. Choice behavior of bumble bees on mixed arrays of *Mimulus cardinalis*-like and *M. lewisii*-like floral models differing color and reward traits (Experiment 2d). (A) Proportion of flower visits (left) and moves between the same flower type (repetitions, right) when *M. cardinalis*-like rewards were paired with red vs. lavender (LAV) flower color (alternative flowers had *M. lewisii*-like reward with lavender vs. red flowers, respectively). (B) Bee foraging rates when preferred *M. lewisii*-like reward was paired with red vs. lavender flower color. Rate is expressed as the number of flowers visited per minute. Values correspond to mean ± SE. **$P < 0.01$, *$P < 0.05$.

Experiment 3

All bees in Experiments 3a–c (n = 10, 10, and 12, respectively) reached the learning criterion of 80% visits to rewarding flower for three consecutive blocks of 10 flower visits. Two bees in Experiment 3a were excluded from further data analysis because they did not sample both color variants during the discrimination phase. Fig. 5A shows the learning rates of bees when discriminating *M. lewisii*-like and *M. cardinalis*-like flowers based on color cues only (Experiment 3a), orientation cues only (Experiment 3b), and color and orientation cues together (Experiment 3c). Learning rates of bees on the color only discrimination task did not depend on whether flowers were red or lavender (Fig. 5A, left bars); however, bees took longer to learn the orientation task when rewarding

flowers were red vs. lavender in coloration (Fig. 5A, middle bars; t = 2.65, df = 8, P = 0.03). When target flowers were defined by a specific *M. lewisii*-like and *M. cardinalis*-like color and orientation combination (Experiment 3c), bees took much longer to reach the learning criterion when color-orientation compound contained red vs. lavender stimuli (RH-RU pooled) vs. lavender (LH-LU pooled; Fig. 5A, right bars; t = 3.41, df = 10, P = 0.007), representing an average increase of 23 flower visits. In contrast, learning rates did not differ between *M. lewisii*-like and *M. cardinalis*-like orientation cues when ignoring differences in color cues (mean [±SE] number of visits to reach learning criterion for RU-LU pooled = 36.67 ± 4.216 and RH-LH pooled = 41.67 ± 9.804; t = 0.4685, df = 10, P = 0.65). Together, these results indicate that color and orientation traits function as an integrated unit to reduce the learnability of *M. cardinalis*-like flowers, with the orientation component of the compound stimulus affecting the ability of bees to learn the associated red color component.

After reaching the learning criterion, floral differences in *M. lewisii*-like and *M. cardinalis*-like color alone (Fig. 5B, left bars) and orientation alone (Fig. 5B, middle bars) had no effect on bee search times. However, bees spent significantly more time searching for red-orientation combinations (RH-RU pooled) than lavender-orientation combination (LH-LU pooled; Fig. 5B, right bars; t = 3.65, df = 10, P = 0.005), increasing from 1.49 to 2.65 s per visit. In contrast, flower orientation had no effect on search times while controlling for differences in flower color (RH-LH pooled vs. RU-LU pooled; t = 1.376 df = 10, P = 0.199). Target color had no effect on choice accuracy across experimental treatments (mean errors for lavender and red target flowers, color only = 0.01 ± 0.01 vs. 0.05 ± 0.03; t = 1.265, df = 6, P = 0.25; orientation only = 0.03 ± 0.02 vs. 0.04 ± 0.019, t = 0.3625, df = 8, P = 0.73; color-orientation combination = 0.06 ± 0.03 for LH-LU vs. 0.12 ± 0.03 for RH-RU, t = 1.414, df = 10, P = 0.19).

DISCUSSION

Research on the adaptive significance of floral syndromes has mainly focused on how single traits affect the attraction and utilization of a plant's "most effective pollinator" group (Stebbins 1970, Faegri and Van Der Pijl 1979), with a only handful of studies considering the effect of multi-trait interactions on their choice behavior (Raguso and Willis 2002, 2005, Campbell et al. 2014, Fenster et al. 2015). The goal of our study was to gain insight into the potential functional importance of secondary pollinators in the evolution of syndrome trait complexes, which we investigated through the quantitative assessment of bumble bee foraging responses to individual traits and trait combinations of a classic "hummingbird" flower, *Mimulus cardinalis*. We found that bees readily visited *M. cardinalis* flowers in monospecific greenhouse populations but mostly avoided them
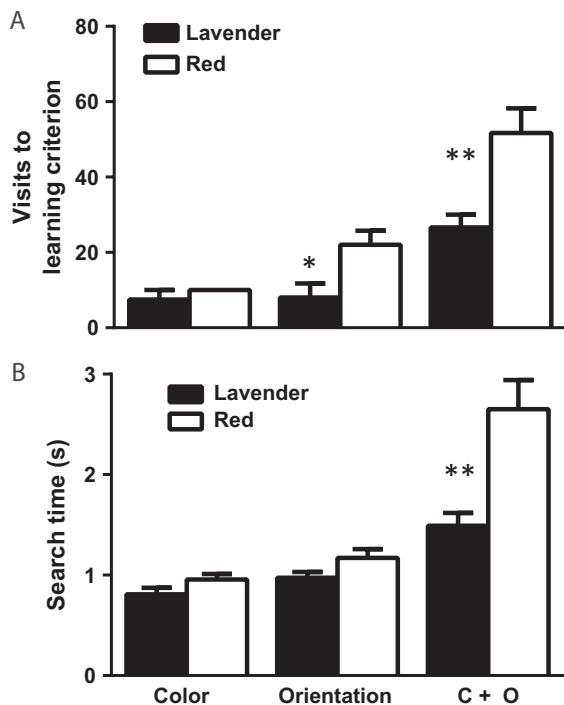
Fig. 5. Performance of bees on *Mimulus cardinalis*-like and *M. lewisii*-like color (Experiment 3a; *n* = 8), orientation (Experiment 3b; *n* = 10), and color-orientation compound (C + O; Experiment 3c; *n* = 12) discrimination tasks. (A) Number of visits required to reach the learning criterion of 80% visits to the rewarding (target) flower type. (B) Time (in seconds) spent searching for rewarding (target) flower types subsequent to reaching the 80% learning criterion. Search time reflects the average amount of time taken to make 20 consecutive visits to rewarding flowers. Solid and open bars correspond to lavender (*M. lewisii*-like) and red (*M. cardinalis*-like) target (rewarding) flowers respectively. Values correspond to mean ± SE. **P < 0.01.

when melittophilous flowers of *M. lewisii* were also available (only 22% of overall bee visits to plants in mixed populations were to *M. cardinalis*). Despite suggestions to the contrary (Bradshaw and Schemske 2003, Lunau et al. 2011), bumble bees clearly have the capacity to detect bird syndrome flowers and learn to associate them with a nectar reward, a finding well supported by previous work (Chittka and Waser 1997, Mayfield et al. 2001, Martinez-Harms et al. 2010), but choose to avoid them in mixed floral environments when it makes economic sense to do so.

Specialized foraging by pollinators has long been thought to plant a prominent role in the diversification of flowers (Grant 1949, 1994); however, this view has been criticized due to the fact that most pollinators are floral generalists at the level of species and above (Waser et al. 1996, Waser 1998). A major assumption made by both sides of this ongoing debate is that pollinator visitation patterns observed at the taxonomic level also occur at the individual level (i.e., individuals of generalist species will indiscriminately visit any flower (generalize) that they have the behavioral capacity to exploit, assuming that it contains sufficient

reward). We found that bumble bees visited both *M. lewisii* and *M. cardinalis* flowers at the species level, yet the majority of individual bees restricted their visits to one of the two species (seven bees on *M. lewisii* and one bee on *M. cardinalis*). Interestingly, 2/10 bees visited both species but infrequently switched between them (i.e., they temporarily specialized on one species followed by the other). Thus, pollinators can be considered "generalist" at the species level based on overall frequency of flower visitation (flower preference) but actually be highly "specialist" at the individual level based on the temporal sequence of flower visitation. In fact, bees only switched between *M. cardinalis* and *M. lewisii* plants a total of 12 out of 250 times (4.8% of total moves). Such individual specialization in bees would confer reproductive benefits to plants in mixed populations through assortative mating, and would also substantially reduce hybridization between *M. cardinalis* and *M. lewisii* in areas of sympatry. Recent studies on plant–pollinator interaction networks have shown that individual specialization, although a common behavioral attribute in generalist pollinators, is often overlooked by traditional species-level indices of specialization (e.g., visitation rates to a focal plant) due to inter-individual variability in floral resource use (Tur et al. 2014, 2015). These findings, combined with the present work, suggest that our understanding of pollinator-mediated floral adaptation, microevolution, and plant speciation would be greatly enhanced by "scaling down" the definition and quantification of pollinator specialization to the individual level.

Results of our trait manipulation experiments revealed that *M. cardinalis*-like color, orientation, and reward properties operate as an integrated functional unit to generate foraging selectivity in bumble bees. When *M. cardinalis*-like and *M. lewisii*-like flowers differed in color or orientation alone, only 6 out of 20 bees showed an avoidance response to *M. cardinalis*-like flowers (three avoided red color, three avoided horizontal orientation) and 2 out of 20 bees showed a flower repetition bias. In contrast, the majority of bees avoided *M. cardinalis*-like flowers (16 out of 18 bees) and showed a repetition bias (15 out of 18 bees) when color and orientation traits were combined together, representing a more than two-fold increase and an almost eight-fold increase in each form of foraging selectivity compared to single trait effects, respectively. We also found that *M. cardinalis*-like reward (low concentration–high volume) alone was sufficient to deter bees from visiting flowers independent of flower coloration (Experiment 2d), which is consistent with previous work on floral reward preferences in bumble bees (Cnaani et al. 2006). However, the percentage of bees avoiding *M. cardinalis*-like reward was much lower when it was paired with lavender vs. red coloration (77% vs. 87% of total visits to *M. cardinalis*-like rewards, respectively). Given that bees showed no color preference when floral reward levels were equivalent (Fig. 3A), these results indicate that color and reward traits also interact non-additively to deter bumble bee visitation from *M. cardinalis*-like flowers and encourage individuals to move sequentially between similar flowers.

This functional redundancy between color-orientation and color-reward synergistic trait interactions in *M. cardinalis* may provide a "backup system" of bee deterrence in situations where nectar properties are altered by extrinsic factors such as pollinator density, evaporation, or soil conditions to make them more *M. lewisii*-like, or enable plants to also deter bees foraging for pollen instead of nectar. Interestingly, we found that *M. cardinalis* two-trait combinations were sufficient to generate levels of avoidance observed in natural flowers (Experiment 1), but failed to generate the same degree of repetition bias (0.76 for two-trait models vs. 0.92 for natural flowers). This reduction may be due to the fact that model flowers lacked another important trait distinguishing *M. cardinalis* and *M. lewisii* flowers (Gegear and Laverty 2001, Gegear and Burns 2007), such as odor (Byers et al. 2014). Regardless, our results indicate that *M. cardinalis* color, orientation, and reward traits interact synergistically to discourage bumble bee visitation and force individuals to adopt a highly specialist foraging strategy. To our knowledge, the present study is the first to show that bird syndrome flowers adaptively manipulate bumble bee decision-making processes through multi-trait synergisms.

Why do *M. cardinalis* trait combinations function as an effective deterrent to bumble bee visitation? For opportunistic pollinators such as bees, optimal foraging theory predicts that individuals should adopt floral specialization as an optimal strategy when it yields a greater rate of nectar intake than generalization (Stephens and Krebs 1986). In support of this prediction, we found that there was a negative relationship between visit frequency to two-trait *M. cardinalis*-like flowers and foraging rate (Fig. 4C), indicating that bees incur a significant time cost when utilizing such flowers. Our subsequent series of flower discrimination experiments revealed that these time costs are incurred when bees learn and subsequently process information on multiple *M. cardinalis*-like floral traits at the same time (Fig. 5). Interestingly, these multi-trait interaction effects on the learnability and discriminability of flowers were driven primarily by limitations on the ability to combine a red color stimulus with either an upright or a horizontal orientation trait, forcing bees to make 25 additional visits to reach our 80% learning criterion and to increase their search times from 1.49 to 2.65 s per flower compared to a combination of lavender coloration and either orientation trait. These findings, combined with the effects of red flower coloration on reward preference and foraging rate found in Experiment 2 (Fig. 4A, B), support the long-standing hypothesis that the red coloration typical of classic bird syndrome flowers functions in bee deterrence rather than bird attraction (Schemske and Bradshaw 1999, Lunau et al. 2011, Rodriguez-Girones et al. 2015). However, our results do not support the pervasive mechanistic view that red flower color by itself generates aversive responses in bees. Instead, our results show that red coloration works in synergy with other bird syndrome traits to exploit a specific limitation on the multi-sensory processing abilities

of bees, thereby increasing foraging costs to levels that favor flower selectivity as an adaptive decision strategy.

Evolutionary transitions from bee to bird flowers are thought to occur through a "cascade" of changes to individual traits, with changes to flower color or reward providing the critical first step in the process (Fenster et al. 2004, Thomson and Wilson 2008). However, our results, and those of Gegear and Burns (2007), indicate that variation in color alone would not produce the specialization responses in bumble bees needed to drive floral divergence. Rather, our results indicate that simultaneous changes in color and at least one other trait would be required in order to produce any evolutionarily meaningful level of specialization in bumble bees. Multi-trait changes would also be required if flowers initially varied in reward traits as bees cannot detect nectar rewards remotely and would therefore need an associated change in a display trait (e.g., color, odor, shape, or size) to generate sufficient specialization through learned preferences. The well-characterized genetics underlying floral trait differences in the *Mimulus lewisii–M. cardinalis* system provide additional support for this view. Bradshaw et al. (1998) found that 9 of 12 floral traits differing between *M. lewisii* and *M. cardinalis*, including those affecting color and orientation, were controlled by at least one major quantitative trait locus (QTL). In fact, Bradshaw and Schemske (2003) attempted to experimentally decouple color and reward traits in the *Mimulus* system and failed to do so (see Wilson et al. [2006] for discussion), which is consistent with the view that a simultaneous change in color and reward (or orientation) traits initiated floral divergence between *M. cardinalis* and *M. lewisii*. Bird floral syndromes are therefore adaptive trait complexes (Stebbins 1970, Fenster et al. 2004) maintained by synergistic trait interaction effects on bumble bee behavior.

Recent field studies have shown that hummingbirds also prefer classic "bird" floral traits to a greater extent when present together vs. alone (Fenster et al. 2006, 2015), suggesting that floral trait changes associated with bee-to-bird evolutionary transitions may serve the dual function of attracting primary bird pollinators and deterring secondary bumble bee pollinators. Such reciprocal trait interaction effects on bee and bird behavior would "speed up" floral divergence by strengthening correlational selection on sensory trait complexes while at the same time restricting pollinator-mediated interspecific gene flow between diverging floral forms (Campbell 2009). The notion that floral syndromes are adaptive trait complexes is not a new one (Stebbins 1970), but only a handful of studies have experimentally tested for trait interaction effects on primary pollinators (Raguso and Willis 2002, 2005, Sletvold and Agren 2011, Campbell et al. 2014, Bischoff et al. 2015, Junker and Parachnowitsch 2015). We contribute to this growing body of work by showing that floral syndrome traits can interact in a non-additive way to adaptively manipulate the behavior of secondary pollinators, and therefore represent complex adaptations to multiple pollinator groups. Our study also highlights the importance of

adopting a rigorous behavioral approach for establishing the functional role of trait interaction effects in pollinator-mediated floral diversification.

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