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The Birds, the Bees, and the Virtual Flowers: Can Pollinator Behavior Drive Ecological Speciation in Flowering Plants?*

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abstract: Biologists have long assumed that pollinator behavior is an important force in angiosperm speciation, yet there is surprisingly little direct evidence that floral preferences in pollinators can drive floral divergence and the evolution of reproductive (ethological) isolation between incipient plant species. In this study, we expose computer-generated plant populations with a wide variation in flower color to selection by live and virtual hummingbirds and bumblebees and track evolutionary changes in flower color over multiple generations. Flower color, which was derived from the known genetic architecture and phenotypic variance of naturally occurring plant species pollinated by both groups, evolved in simulations through a genetic algorithm in which pollinator preference determined changes in flower color between generations. The observed preferences of live hummingbirds and bumblebees were strong enough to cause adaptive divergence in flower color between plant populations but did not lead to ethological isolation. However, stronger preferences assigned to virtual pollinators in sympatric and allopatric scenarios rapidly produced ethological isolation. Pollinators can thus drive ecological speciation in flowering plants, but more rigorous and comprehensive behavioral studies are required to specify conditions that produce sufficient preference levels in pollinators.

Keywords: ecological speciation, pollinator preference, bumblebee, hummingbird, ethological isolation, floral evolution.

Despite decades of research, the major processes involved in the formation of new species remain poorly understood. Recent models of speciation have stressed the importance of ecologically based divergent selection as a factor in the evolution of reproductive isolation (Schluter 2001; Rundle and Nosil 2005). In particular, there has been much interest in the role of sexual selection as an ecological source of divergent selection and reproductive isolation (Boughman 2001, 2002; Panhuis et al. 2001; Boake 2005). Theoretical studies have concluded that speciation by sexual selection can occur when mate preferences for secondary sexual traits are sufficient to drive divergence between allopatric populations and maintain prezygotic isolation under subsequent sympatry (Turelli et al. 2001). To date, empirical evidence for speciation by sexual selection has been gained from observational and comparative studies that demonstrate correlations among mate preferences, mating traits, and prezygotic isolation (Panhuis et al. 2001; Kirkpatrick and Ravigne 2002). For example, divergence in male coloration and female preference in three-spine sticklebacks is correlated with the extent of reproductive isolation between populations (Boughman 2001). More direct empirical evidence would be obtained by conducting selection experiments to determine whether preferences for mating traits can rebuild prezygotic isolation between two species whose genomes have been blended by hybridization (Schluter 2000). Here, we use a virtual version of this approach to examine the role of animal pollinators in the speciation of flowering plants; our use of the term "virtual" throughout this article means a natural object or process simulated by a computer.

Pollinators have long been thought to play a prominent role in the speciation of flowering plants (Darwin 1876; Grant 1949, 1994; Levin 1978). One influential view attributes the speciation process to differences among pollinator groups (e.g., birds, bees, butterflies) in their preference for floral characteristics (e.g., color, odor, shape), which act to drive floral divergence between plant populations and ultimately sustain prezygotic isolation when populations come together (a process hereafter referred to as "ethological isolation" by pollinator preference; sensu Grant 1949). Because floral characters primarily influence reproduction, the process of ethological isolation by pol-

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linator preference in plants is akin to the process of speciation by sensory drive in animals. In general terms, the sensory drive hypothesis proposes that mating signals evolve in one sex as a consequence of a preexisting preference for the signals in the other sex (Endler 1992; Endler and Basolo 1998). Sensory drive can promote speciation when the biases that underlie mate preference between populations differ, and thus, it can contribute to the evolution of reproductive isolation (Boughman 2002). In ethological isolation by pollinator preference, pollinator groups selectively transfer male gametes (pollen) between plants with their preferred floral characteristics. Plant populations that are exposed to different pollinator groups will thus diverge in floral characteristics and, as a pleiotropic by-product, become reproductively isolated from one another.

Although this scenario has been invoked to explain the origin of many angiosperm taxa (see Waser 2001; Fenster et al. 2004; Waser and Campbell 2004 for reviews), it remains controversial among plant evolutionary biologists partly because of the lack of direct evidence that pollinator preferences for alternative floral characteristics are consistent and strong enough to drive floral divergence and maintain reproductive isolation between incipient plant species (Chase and Raven 1975; Waser 1998, 2001). Adequate pollinator preferences have been inferred from general associations between floral characteristics and pollinator group (Grant 1994; Fenster et al. 2004) and from observational field studies of pollinator behavior (Fulton and Hodges 1999; Schemske and Bradshaw 1999; Bradshaw and Schemske 2003; Ippolito et al. 2004). However, no study to date has empirically examined how pollinator preferences for floral characteristics affect plant populations over evolutionarily relevant time periods, presumably because of the logistical difficulties associated with conducting controlled selection experiments using plants and pollinators under natural conditions.

In this study, we circumvent these issues by using a virtual approach to evaluate key elements of pollinator-driven ecological speciation in plants. A similar approach was used by Bond and Kamil (1998, 2002) to study the evolution of crypticity and polymorphism in noctuid moths; in those studies, live blue jays acted as selective agents on populations of computer-screen moths varying in crypticity. Specifically, our aim was to empirically assess the proposition that hummingbird and bumblebee preferences for flower color can cause the evolution of ethological isolation. Although other types of pollinator behavior, such as flower constancy, have been hypothesized to influence plant speciation (see Chittka et al. 1999; Gegear and Laverty 2001; Jones 2001; Waser 2001 for discussion), here we assume that flower preference is the only behavioral factor influencing adaptive divergence and ethological isolation between red- and blue-flowered plant populations (as in the classic scenario of pollinator-driven plant speciation outlined above). We first conducted a series of selection experiments ("Part I: Simulation of Selection Exerted by Live Bumblebees and Hummingbirds") in which we created artificial ancestral plant populations with wide variation in flower color and tracked evolutionary changes in flower color resulting from preference levels expressed by live hummingbirds and bumblebees. Flower color evolved through a genetic algorithm in which pollinator color preferences in one generation determined the frequency of flower colors in the subsequent generation. Realistic flower colors were created by matching the expression of virtual alleles controlling the concentration of two pigments to colors obtained from digital images of interfertile hummingbird-pollinated (red) and bumblebee-pollinated (blue) plant species as well as F1 and F2 hybrids between them. Our genetic architecture was derived from the well-studied genetics of flower color in two closely related plant species purported to be adapted to hummingbirds and bumblebees (*Mimulus cardinalis*–*Mimulus lewisii* complex; Bradshaw et al. 1995, 1998); thus, the genetics and flower colors of our artificial plants paralleled those of natural pollination systems. We then conducted a series of selection simulations ("Part II: Simulations Involving Virtual Hummingbirds and Bumblebees"), in which we varied preferences for red and blue flowers in virtual hummingbirds and bumblebees, respectively, to determine the functional dependence of ethological isolation on the strength of divergent color preferences. For both parts I and II, we examined the effects of pollinator preferences on the trajectory of flower color evolution in each of three initial plant-pollinator scenarios: pollinator groups foraging on separate plant populations with a wide variation in flower color (scenario A: divergent selection), pollinator groups foraging on a single plant population with only red and blue flowers (scenario B: secondary contact), and pollinator groups foraging on the same plant population with a wide variation in flower color (scenario C: sympatric speciation).

Methods: Virtual Plants and Flowers

The flower color of plants was generated from information contained on a homologous pair of virtual chromosomes. The algorithm (programmed in Fortran using Lahey ED, ver. 3.80) included the important features of the genetics of the *Mimulus cardinalis* (red flower, hummingbird pollinated) and *Mimulus lewisii* (pinkish purple flower, bumblebee pollinated) system described by Bradshaw et al. (1995, 1998). In their quantitative trait locus (QTL) analysis of floral characteristics, Bradshaw et al. (1995, 1998) observed multiple QTLs for production of the anthocyanin and carotenoid pigments that contribute to flower color. We used these QTLs as a guideline for our genetic algorithm (table 1). In our simulations, flower color was determined by the relative concentrations of two virtual pigments (hereafter referred to as pigment 1 and pigment 2) decoded from 10 loci on eight linkage groups. Low concentrations of both pigments 1 and 2 led to blue flowers, and high concentrations of both pigments led to red flowers. Each locus had 64 alleles (this number of alleles was chosen because $64 = 4^3$, the number of possible nucleotide combinations per codon) that were expressed in an additive, complete dominance, partial dominance, or epistatic fashion. Table 1 shows the linkage group and allelic expression associated with each locus, and the appendix provides an example of how pigment concentrations were calculated. Additive expression results simply in the production of pigment based on the average of the two alleles. In complete dominance expression, the allele for lower pigment production is dominant to the allele for higher pigment production (which is the case for flower pigments in the *M. lewisii–M. cardinalis* system). In partial dominance expression, the pigment concentration is calculated using the formula $(\text{allele}_1 + \text{allele}_2)/[(\text{allele}_1/\text{allele}_2) + (\text{allele}_2/\text{allele}_1)]$, which favors the allele with the lower value. Epistatic interactions occur when alleles at locus 5 are homozygous dominant or heterozygous and act to limit the production of pigment 2. Thus, variation in pigment 2 concentration is expressed only in flowers that are homozygous recessive at locus 5.

Sixteen flower colors were created based on the concentrations of pigments 1 and 2 decoded from plant genotypes (fig. 1), ranging from low concentrations of pigment 1 and 2 (flower 1, typical of bumblebee flowers) to high concentrations of pigments 1 and 2 (flower 16, typical of hummingbird flowers). In part I, we matched artificial paper flowers to 16 virtual colors, while in part II, we used virtual colors only. We chose 16 flower colors as a compromise between using all the continuous variation in flower color and the logistics of creating artificial flowers for part I. The genotype of virtual flowers 1 and 16 (parental types) were created such that the progeny of F1 and F2 hybrid crosses had the same mean and variance in pigment concentrations as the hybrid generations in the *M. lewisii–M. cardinalis* system (Bradshaw et al. 1998).

Part I: Simulation of Selection Exerted by Live Bumblebees and Hummingbirds

Subjects

Bumblebees. From August to October 2004, we examined bumblebee-mediated selection on flower color in virtual plant populations in our laboratory at the University of Toronto. Subjects were *Bombus impatiens* (Cresson) workers obtained from colonies either provided by Biobest Biological Systems Canada (Leamington, ON) or raised in the laboratory from spring queens. Colonies were connected to a screened flight cage (2.2-m cube) with a gated tunnel so that we could control entry of bees into the flight cage. Bees were trained to collect 30% sucrose (w/w) solution from clear feeders located within the cage, and individuals that made regular foraging trips between the colony and feeders were marked on the dorsal surface of the thorax and/or abdomen for identification. Colonies were supplied with pollen ad lib. throughout testing. Preliminary experiments using bees with experience foraging outdoors showed that their color preferences on experi-

Table 1: Details of the loci determining pigment concentration

Locus	Pigment	Interaction	Linkage group	Percentage of variance explained	Source
Locus 1	Pigment 2	Dominance	A	43	Bradshaw et al. 1998
Locus 2	Pigment 1	Dominance	B	8	Bradshaw et al. 1995
Locus 3	Pigment 1	Additive	B	8	Bradshaw et al. 1995
Locus 4	Pigment 2	Dominance	C	8	Bradshaw et al. 1998
Locus 5	Pigment 2	Epistatic	D		Bradshaw et al. 1995, 1998
Locus 6	Pigment 1	Dominance	D	20	Bradshaw et al. 1998
Locus 7	Pigment 2	Partial dominance	Not defined	49	…
Locus 8	Pigment 1	Partial dominance	Not defined	22	…
Locus 9	Pigment 1	Partial dominance	Not defined	21	…
Locus 10	Pigment 1	Partial dominance	Not defined	21	…

Note: Loci 7–10 were created for this study to account for the phenotypic variance left unexplained by Bradshaw et al. (1995, 1998). Note that loci 2 and 3 were identified by Bradshaw et al. (1995) but not later by Bradshaw et al. (1998). They were included in our study to account for the possibility that they are truly present but that their percentages of variance explained (PVEs) were below the detection threshold (8%) in the second study. The PVE for locus 4 was set at 8% because it was detected only during some analyses by Bradshaw et al. (1998).

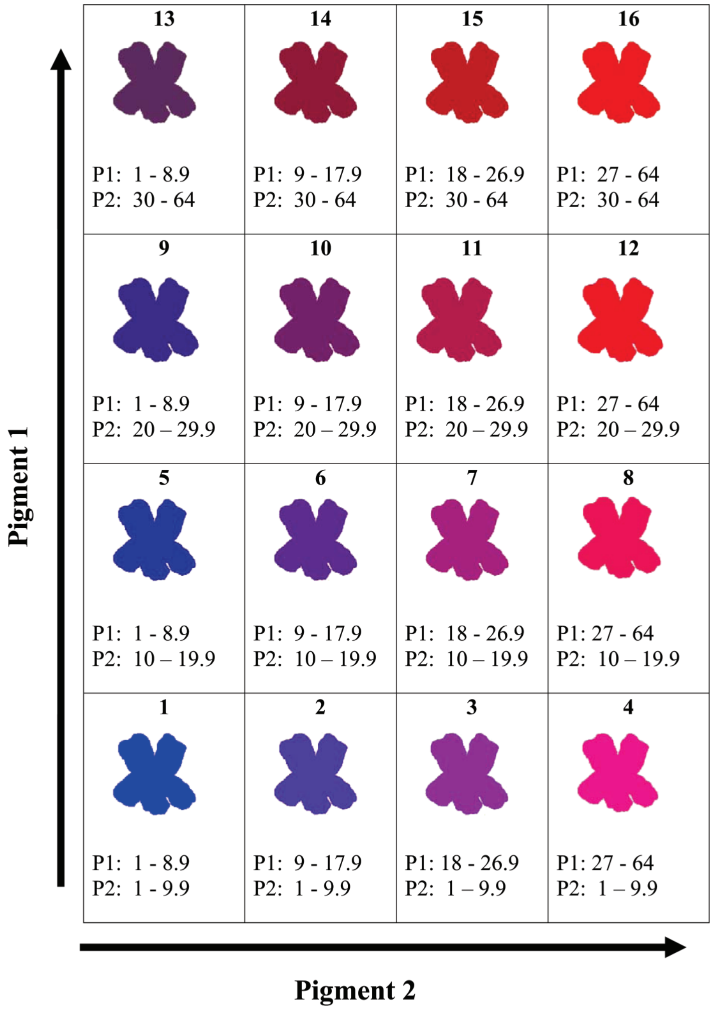


Figure 1: The 16 flower colors used in artificial flower selection experiments, with their corresponding pigment concentrations (P1 and P2). Flower colors 1, 2, and 5 made up category 1 flowers; colors 12, 15, and 16 made up category 3 flowers. Pigment concentration is in arbitrary units.

mental arrays of artificial flowers were similar to those of naive, lab-reared bees. Because colonies of bees with outdoor experience were more difficult to obtain, we used flower-naive individuals for all experiments. Bees were tested only once; thus, different bumblebee populations determined the mating success of virtual plants for each generation.

Hummingbirds. From June to August 2004, we examined hummingbird-mediated selection on flower color at the Rocky Mountain Biological Laboratory (Crested Butte, CO). Subjects were wild adult male and female and juvenile broad-tailed hummingbirds (*Selasphorus platycercus*) and male and female rufous hummingbirds (*Selasphorus rufus*). Birds were trained to collect 30% sucrose (w/w) solution from a hummingbird feeder located in a small meadow near our field laboratory. To control for the possibility that the choice behavior of birds during testing was influenced by the color of the training feeder, the feeder was covered with white tape. The feeder was available to birds at all times except during testing. Individual birds that regularly visited the feeder were identified based on species, sex, age (adult, juvenile), and distinguishing marks. Only birds that were consistently identified by three experimental observers were used, in order to maximize the chance that color preferences of different birds determined the mating success of virtual plants for each generation. In addition to visiting the feeder, all of the birds frequently visited the flowers of several plant species in the surrounding area during and outside of testing periods, including *Aconitum columbianum* (bluish purple), *Delphinium barbeyi* (bluish purple), and *Ipomopsis aggregata* (red).

Flowers and Arrays

Artificial flowers were constructed by removing the caps from clear 1.5-mL polypropylene microcentrifuge tubes (Fisher Scientific) and fixing a paper collar (corolla) around the entrance of the tube (fig. 2). Adobe Photoshop 6.0 software was used to create 16 different-colored corolla templates (numbered 1–16) that were then printed with a Xerox Docucolor 12-color laser printer (fig. 1). Color stimuli were duplicated from digital images of flowers from plant species supposed to be adapted to bumblebees (color 1) and hummingbirds (color 16) as well as digital images of actual F1 (color 2) and F2 (remaining flower colors) hybrids between them, including *Penstemon spectabilis* and *Penstemon centranthifolius*, *Lobelia siphilitica* and *Lobelia cardinalis*, and *Mimulus lewisii* and *Mimulus cardinalis*. Thus, flower colors represent a composite of typical bird- and bee-pollinated plants and not one genus in particular. In all cases, flower color number corresponded to pigment concentrations derived from the genetic algorithm described above. We generated spectral reflectance curves for flowers 1, 4, 13, and 16 (fig. 3) with an Ocean Optics spectrometer (USB 2000; Dunedin, FL).

Flowers were presented to test subjects by placing them in 96 numbered holes drilled into a vertical sheet of plastic poster board (175 cm × 123 cm) that was covered in green construction paper, thereby creating a population of virtual plants in which the interflower movement of pollinators could be easily tracked. Holes were distributed in 16 rows of six (spaced 15 cm apart within rows and with 7.5 cm between rows), with alternating rows offset to the left by 7.5 cm. Thus, the distance from any flower to its nearest neighbor was equal for all 96 positions. Flowers were made rewarding by dispensing a 4-μL drop of 30% sucrose solution (hereafter, "nectar") halfway down the inner bottom surface of the tube (fig. 2) and refilled either immediately after the test subject entered the next flower (bumblebees) or after it visited a maximum of 30 flowers (approximately one-third of the flowers on the array; hummingbirds). A different refilling procedure was used for hummingbirds because, unlike bumblebees, they collected the nectar while hovering in front of flowers and were thus more likely to be disturbed by the experimenter during refilling.

For bumblebee experiments, the floral array was fastened to the inner wall of the flight cage and illuminated by one General Electric 40-W black-light fluorescent tube, one Sylvania 40-W Growlux fluorescent tube, and two Sylvania 32-W Octron fluorescent tubes positioned above the array. This illumination did not exactly match sunlight, but it was closer than ordinary fluorescent lighting would have been. Hummingbird experiments were conducted outside, illuminated by daylight, and were not run if it was raining. Birds were not caged.

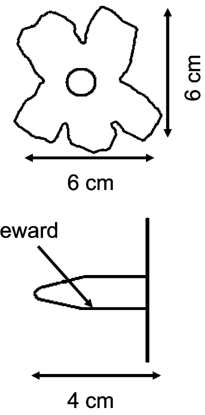


Figure 2: Design of the virtual flowers used in bumblebee and hummingbird selection experiments (*top*, front view; *bottom*, side view).

Experimental Procedure

The initial plant population presented to bumblebees and hummingbirds consisted of an array containing 96 flowers randomly distributed and with the following abundances: six flowers of each of the 16 colors (scenarios A and C) or 48 flowers each of colors 1 and 16 (scenario B). The genotype of each flower was generated independently so that flowers of the same color could have different combinations of alleles. Only one identified pollinator was allowed to visit flowers on the array at a time, and all flowers were replaced between individuals. That individual was allowed to forage on the array until nectar had been collected from 100 flowers. We recorded which flowers were visited and used this information in our genetic algorithm. The algorithm created virtual seeds based on the color of flowers selected by individuals from each pollinator group and randomly selected 96 seeds for the next generation of plants. This new generation of plants was then randomly arranged on the array for the new set of experimentally naive pollinators to forage on. We included in the genetic algorithm a step calculating pollen carryover (male gametes) between flowers. This step was used to determine seed production based on an exponential decay curve with 30% fewer pollen grains delivered at each subsequent flower (a stochastic term drawn from a normal distribution with a standard deviation of 25% of the expected deposition value was also included at each visit to mimic natural variation in pollen deposition). For example, we assumed a pollinator would pick up 100 pollen grains upon visiting a flower for the first time and transfer 30 grains (±7.5 grains, rounded) to the next flower visited, 21 grains

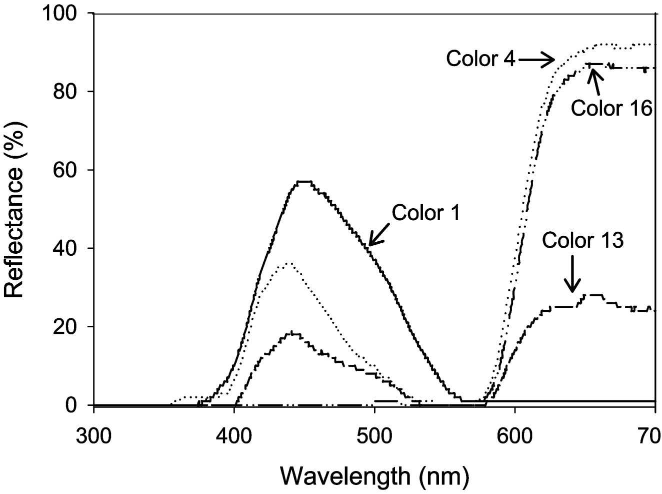


Figure 3: Reflectance spectra of some of the artificial flowers used in live bumblebee and hummingbird selection experiments.

(±5.3) to the flower after that, and so on, up to a maximum carryover of 10 flowers. We recorded the flower choices of at least five bees and five birds for each of the six generations of the virtual plants. A new colony of bumblebees was used for each generation. Unfortunately, the abundance of hummingbirds at the time of our experiment allowed for only three experimentally naive birds to be used in later generations.

For simplicity, the strength of color preference was based on three color categories within the original 16 colors: category 1 corresponded to blue flowers (low concentrations of pigments 1 and 2; colors 1, 2, and 5), category 2 corresponded to intermediate types (intermediate concentrations of pigments 1 and 2; colors 3, 4, 6–11, 13, and 14), and category 3 corresponded to red flower types (high concentrations of pigments 1 and 2; colors 12, 15, and 16; fig. 1). Flower preferences for each pollinator type were calculated by taking the ratio of observed to expected number of visits to color categories, which changed with each generation based on selection in the previous generation, and then standardizing the values with the most preferred color category having a preference value of 1 (0% rejection). The preference value for nonpreferred color categories was the proportion of times each category was accepted by pollinators in relation to the preferred color category.

Using this procedure, we were able to test the proposition that bumblebee and hummingbird preferences for blue and red flowers, respectively, independent of reward level, are a sufficient selective force to drive floral divergence and the evolution of ethological isolation (e.g., Bradshaw and Schemske 2003; Fenster et al. 2004; Waser and Campbell 2004). In contrast to the supposed bumblebee preference for blue flowers, we found that both flower-naive and field-experienced bumblebees had a weak preference for red flowers under these reward conditions (see "Results"). In an attempt to get a stronger bumblebee preference for blue flowers and thus determine whether divergent pollinator preferences can lead to the evolution of ethological isolation, we subsequently ran a sucrose supplementation experiment. Individual bees were presented with an array containing 48 blue (category 1) and 48 red (category 3) flowers that contained rewards of 35% and 10% sucrose solution, respectively (nectar concentrations that are commonly found in bumblebee- and hummingbird-pollinated plant species; Fenster et al. 2004), and the first 120 flower visits were recorded. We assessed changes in the frequency of visits to blue flowers over the 120 visits and found that a consistent preference for blue flowers developed after 90 visits. We therefore calculated bumblebee preference for blue flowers based on the frequency of visits to blue flowers over last 30 visits to the array. Preference for intermediate flower colors was estimated as the preference value midway between the more preferred and less preferred flower values. As expected, higher nectar concentrations in blue flowers substantially increased bumblebee preference for blue flowers. Hereafter, we refer to bumblebee preference for blue flowers based on the outcome of sucrose supplementation as a sucrose-based preference.

Using the average preference for all hummingbirds and bumblebees over the six generations and average sucrose-based preferences in bumblebees, we ran simulations for 100 generations under the conditions outlined for scenarios A–C. This procedure allowed us to extrapolate the effects of live pollinators on floral divergence and the evolution of ethological isolation.

Part II: Simulations Involving Virtual Hummingbirds and Bumblebees

For all three scenarios, the initial plant populations consisted of 3,200 individuals: 200 flowers of each of the 16 colors for scenarios A and C and 1,600 flowers of colors 1 and 16 for scenario B. Virtual bumblebees and hummingbirds were programmed to visit a randomly selected sequence of flowers (a "foraging bout") that included all flowers in the population. Depending on the strength of the pollinator's color preference, each flower was either accepted or rejected. Mating between plants occurred only by pollen transfer from a visited flower to the next visited flower. We did not retain pollen carryover as a factor in the model because pilot simulation runs revealed that there was no qualitative effect of including pollen carryover on the results of simulations. Also, running simulations of the 96-flower live pollinator experimental setup (using the same initial flower genotypes) with and without pollen carryover produced very similar results. The decrease in processing time for simulations due to the removal of pollen carryover allowed us to increase the total number of simulation runs and thus greatly increase our confidence in the mean final flower proportions. Mating continued until all flowers in the population had been presented to the virtual pollinator at least once and until 3,200 plant matings had occurred. If the 3,200 matings did not occur in the first foraging bout because of flower rejections, the pollinator began a new foraging bout on the same population of flowers with a new random visit sequence until satisfying the criterion of 3,200 plant matings.

Virtual bumblebees were programmed to always accept flowers in color category 1 when encountered, and virtual hummingbirds were programmed to always accept flowers in category 3 when encountered. The strength of preference for the two less preferred color categories was generated for each pollinator group by a variable P, with P_B for virtual bumblebees and P_H for virtual hummingbirds. Values of P_B and P_H ranged from 0 to 1, with larger values representing a greater fidelity to the most preferred color category. Values of P_B and P_H were varied independent of one another, allowing bumblebees and hummingbirds to have different preference levels. Category 2 flowers (which were always preferred at an intermediate level by both pollinator groups) were assigned a preference of $1 - P_B$ or $1 - P_H$ (where P_B and P_H vary between 0 and 1 in increments of 0.1), and categories 1 (for birds) and 3 (for bees) flowers were assigned preferences of $1 - 2P_H$ and $1 - 2P_B$ (with 0 being the lowest possible preference level, representing 100% rejection of flowers in the category when encountered), respectively. Thus, when P_B was 0.5, the preferences of bumblebees for flowers in color categories 1, 2, and 3 were 1, 0.5, and 0, respectively, whereas when P_H was 0.5, the preferences of hummingbirds were 0, 0.5, and 1, respectively. Using this method, we independently assigned 11 strengths of preference for the less preferred color category to each pollinator group, for a total of 121 preference combinations (values in each group ranged from 0 [0% rejection of flowers when encountered] to 1 [100% rejection of flowers when encountered]). These preference values directly correspond to calculations used for live bumblebee and hummingbird preferences: the preferred color category was assigned a preference of 1 in both cases, and nonpreferred color categories were assigned values that represented the proportion of times they were accepted by pollinators in relation to the preferred color category. Thus, the live pollinator color preferences can be fit into the scheme used for the simulations of virtual pollinators and plants.

For each generation, virtual plants were mated by first simulating gametogenesis in the diploid parent, using a process that included independent assortment of linkage groups and crossing over within a linkage group. At syngamy, at each locus there was a 1×10^{-6} probability of mutation that would result in an inversion of the allele (e.g., from 1 to 64 or from 40 to 25). We chose 1×10^{-6} because it falls within the range of estimated allelic mutation rates and nucleotide substitution rates in plants (Muse 2000; Thuillet et al. 2002) The mutation rate was also not too high to prevent true breeding within type 1 flowers or type 16 flowers (*Mimulus lewisii* and *Mimulus cardinalis* breed truly in the field and lab), a prerequisite for our model. Based on the visitation patterns of virtual pollinators within the plant population, the algorithm then mated plants to produce seeds. The algorithm assumes that pollinators pick up a single pollen grain on each flower visit and deposit it on the next flower visited, resulting in the production of a seed, and that there is no limit to the number of times that a flower can mate (i.e., produce and receive pollen).

Once all 3,200 seeds had been created by the algorithm, they became the next generation of plants. Thus, each new generation of plants represents biases (if any) in the choice behavior of pollinators while foraging on flowers in the preceding generation. Simulations ran for 500 generations, and all simulations had stabilized within 500 generations (i.e., the mean proportion of each flower type over all the simulations did not change by more than 0.001 per generation over the final 50 generations). Our criterion for defining a population as containing only one flower type was that the percentage of that flower type was greater than 99% of the whole population (minus two standard deviations, to account for variation in the simulations) averaged over 100 simulations. For floral divergence to

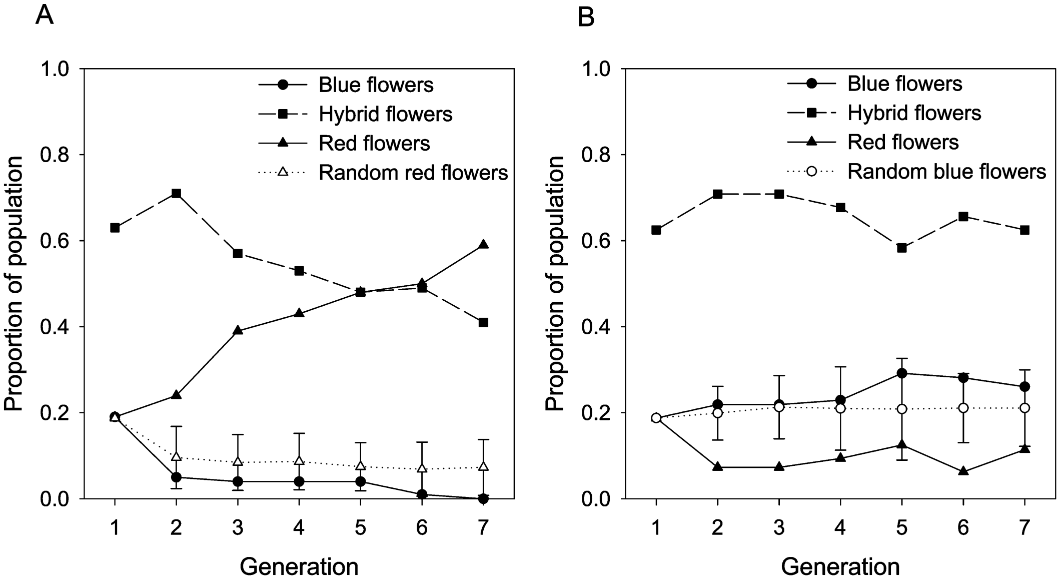


Figure 4: Live hummingbird- and bumblebee-mediated selection on flower color in isolated virtual plant populations over six generations, from an initial population of 96 flowers consisting of 18 blue flowers, 60 intermediates, and 18 red flowers (generation 1). *A*, Proportion of red flowers in the hummingbird-only population increased over generations because of a strong red preference. Dashed line represents the proportion of red flowers produced in multiple simulations of random foraging (i.e., no preferences), with error bars representing 3 SD from the mean (99% confidence intervals). *B*, There was little change in the proportion of red or blue flowers in the bumblebee-only population over generations because of weak color preferences. Dashed line represents the proportion of blue flowers produced in multiple simulations of random foraging (i.e., no preferences), with error bars representing 3 SD from the mean (99% confidence intervals).

have occurred in the population, less than 1% of plants (mean of simulations plus two standard deviations) must be of intermediate phenotypes, and 1% or more of plants (mean of simulations minus two standard deviations) must be of each extreme phenotype (category 1 and category 3). Simulations were run with varying ratios of virtual bumblebees (blue preference) to virtual hummingbirds (red preference; 10%, 25%, 50%, 75%, and 90% of the mixed pollinator populations were bumblebees with a preference for blue flowers) to show the how changes in the composition of mixed pollinator assemblages affect final flower composition.

Results

Hummingbirds strongly preferred red flowers when foraging on the array of 96 flowers (ratios of observed to expected visits: blue flowers = 0.21, intermediate flowers = 0.93, red flowers = 1.33; standardized values: blue flowers = 0.16, intermediate flowers = 0.70, red flowers = 1.00), while, as reported in "Methods," bumblebees tested in the laboratory had a weak preference for red flowers (observed to expected visits: blue flowers = 0.91, intermediate flowers = 1.00, red flowers = 1.18; standardized values: blue flowers = 0.77, intermediate flowers = 0.84, red flowers = 1.00). These preferences led to an increase in the proportion of red flowers in the seventh flower generation in the hummingbird-only population (fig. 4*A*) but little change in the bumblebee-only population (fig. 4*B*). Sucrose supplementation of blue flowers increased the strength of preference for blue flowers in bumblebees (observed to expected visits: blue flowers = 1.63, red flowers = 0.37; standardized values: blue flowers = 1.00, red flowers = 0.24). Unless otherwise stated, we used sucrose-based preferences for bumblebees in simulations.

Scenario A (Divergent Selection)

When only one pollinator group is present, weak color preference can drive a population toward just one flower color. The color preferences of live hummingbirds and bumblebees resulted in floral divergence between plant populations after 100 generations in a simulation: the hummingbird-only plant population had only red flowers after 60 generations, and the bumblebee-only plant population had only blue flowers after 84 generations. We simulated color preferences as low as 0.025, wherein pollinators would reject intermediates 2.5% of the time and the least preferred flower 5% of the time (i.e., the proportions of flowers visited by a bee would be 0.342 blue flowers, 0.333 intermediates, and 0.325 red flowers). We found that even this low level of preference led to the favored flower color dominating the population (>99%) after 500 generations (results not shown).

Scenario B (Secondary Contact)

In simulations that represented well-differentiated blue- and red-flowered populations returning to sympatry after previous geographical isolation (allopatry) with different pollinators, strong color preferences are required to maintain reproductive isolation (fig. 5, *scenario B*). Thus, although very weak color preferences can drive floral divergence between allopatric populations with different pollinator groups, only strong color preferences can maintain reproductive isolation of bird- and bee-flowered types within mixed populations upon secondary contact. Observed color preferences of live bumblebees and hummingbirds were not strong enough to maintain reproductive isolation within such populations upon secondary contact for even a single generation (fig. 5, *scenario B*; fig. 6). However, the combination of a weak color preference by one pollinator group (e.g., 0%–60% rejection of the red flowers) and a relatively strong color preference by the other pollinator group (e.g., 20%–100% rejection of blue flowers) can lead to a population of plants with only one flower color (e.g., upper left of middle plot in fig. 5, *scenario B*). Note that the proportion of red flowers (category 3) observed during random foraging in figure 6 decreased so rapidly because blue (category 1) flowers are homozygous dominant at the epistatic locus on linkage group 5. Therefore, 50% of these alleles in the population are dominant for low concentrations of pigment 2, and the mixture of these alleles into the population after the first generation results in limited expression of pigment 2 in most of the flowers.

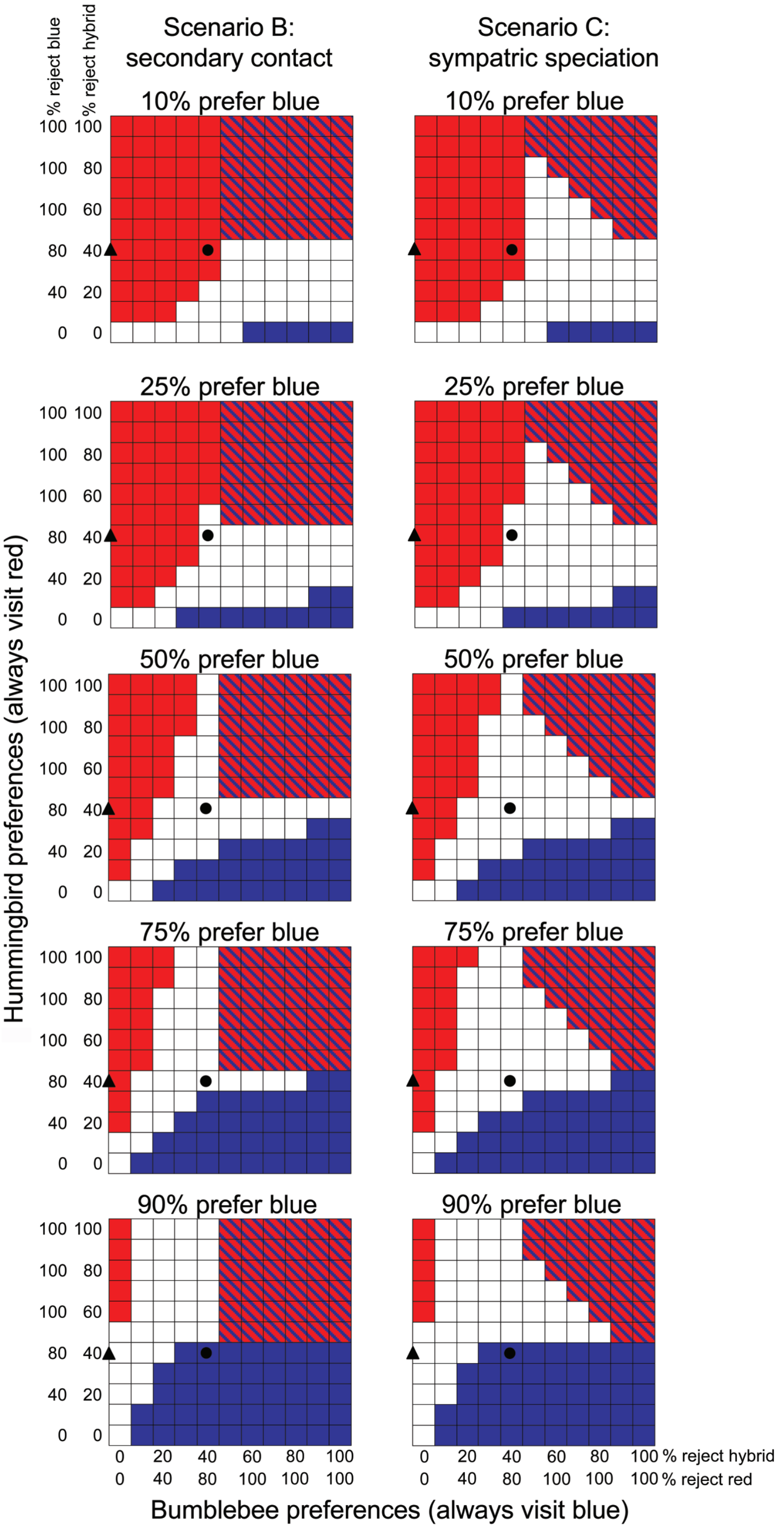
Scenario C (Sympatric Speciation)

Although the combination of a strong color preference by one pollinator group and weak color preference by the other pollinator group can drive the evolution of flower color in the population (upper left and lower right corners of each plot in fig. 5, *scenario C*), both groups must reject flowers with a nonpreferred extreme color (i.e., red flowers for bees and blue flowers for birds) and reject the intermediate flowers at a high rate to mediate the evolution of ethological isolation (see upper right corner of fig. 5, *scenario C*). That is, only when there is little or no "indirect" gene flow from a blue flower, via an intermediate, to a red flower (or vice versa) does the population diverge into typical bee- and bird-flower colors. Consider a situation where bees reject red flowers 100% of the time and intermediates 80% of the time, while birds reject blue flowers 100% of the time and intermediates 60% of the time. In this case, only 16.7% of bee visits are to intermediates and only 28.5% of bird visits are to intermediates, visitation rates low enough to restrict the gene flow that would prevent disruptive selection. The color preferences of live hummingbirds and bumblebees were not strong enough to initiate disruptive selection on flower color and, thus, the evolution of ethological isolation (fig. 5, *scenario C*; fig. 7). In fact, the ratio of extreme to intermediate flower colors after 100 generations was the same as that expected based on random foraging, indicating that the moderate preferences for divergent colors in both birds and bees had little effect on flower color in the plant population.

Of particular interest is that pollinators with preferences for red flowers have a greater effect on the flower population than pollinators with blue preferences. This is evident from two related trends. First, there is an asymmetry in the range of preferences that will drive a population to all blue or all red flowers. For example, when pollinator proportions are equal, a red preference of 10% and a blue preference of 0% drove the population to all red flowers, but the reverse was not true at a blue preference of 10% and a red preference of 0% (fig. 5). Also, a flower population will go to all red flowers more rapidly (in fewer generations) with a set of strong red preference and weaker blue preference than it will go to blue flowers with an equivalent set of strong blue preference and weaker red preference (fig. 8). We confirmed this asymmetry by running simulations on hypervariable plant populations with only blue-preferring or only red-preferring pollinators with moderate preferences of 40%. Again, the plant population went to all red flowers more rapidly (67 generations) than it went to all blue flowers (81 generations). The asymmetry arises because the genetics of the system hides genetic color variation for red color in blue flowers but not vice versa. The epistatic effect of locus 5 hides variation in pigment 2 unless this locus is homozygous recessive. Selection for blue flowers is slowed because the dominance of alleles for low concentrations of pigment 2 at locus 5 results in the maintenance of recessive alleles that are expressed when they come together. Dominance allele interactions also always favor blue flowers in this system, so the same effect holds for loci 1, 2, 4, and 6. Selection for red flowers is not slowed by such an effect because red flowers are already homozygous recessive (as are colors 5–16 in fig. 1), so no genetic variation is hidden.

Discussion

Our virtual pollination system, which draws together genetic, phenotypic, and behavioral elements of natural


% reject blue
% reject hybrid
Scenario B:
secondary contact
Scenario C:
sympatric speciation
10% prefer blue
25% prefer blue
50% prefer blue
75% prefer blue
90% prefer blue
100 100
100 80
100 60
80 40
40 20
0 0
Hummingbird preferences (always visit red)
0 20 40 60 80 100 % reject hybrid
0 40 80 100 100 100 % reject red
Bumblebee preferences (always visit blue)

pollinator-plant systems, was designed to empirically evaluate the long-standing view that pollinator preferences for floral characteristics can drive two key processes necessary for plant speciation: floral divergence and the evolution of ethological isolation. Our selection experiments with live pollinators revealed that, all else being equal, hummingbird preferences were strong enough to drive the evolution of red flowers in hummingbird-only populations, but bumblebee preferences for blue flowers were too weak to drive the evolution of blue flowers, even in populations visited only by bumblebees. Observed preference levels would also not drive divergence within plant populations visited by both pollinators. Indeed, bumblebees showed a weak preference for red over blue flowers independent of nectar rewards, which is surprising considering that bees have been assumed to be predisposed to prefer colors in the blue-violet range of the visual spectrum (Schemske and Bradshaw 1999; Fenster et al. 2004) and have a reduced ability to see red flowers (e.g., Bradshaw et al. 1995; Proctor et al. 1996; but see Chittka and Waser 1997). When bumblebee preference for blue flowers was strengthened by increasing sucrose levels in blue flowers (category 1) compared to red flowers (category 3), pollinator preferences resulted in rapid floral divergence between allopatric hummingbird-only and bumblebee-only plant populations. Despite the fact that pollinator preferences were sufficient to generate floral divergence, they were not strong enough to either maintain reproductive isolation between incipient red- and blue-flowered plant species upon secondary contact or drive the evolution of ethological isolation in sympatry. Together, these results indicate that hummingbirds and bumblebees can develop color preferences strong enough to initiate adaptive divergence in flower color; however, these preferences do not lead to plant speciation.

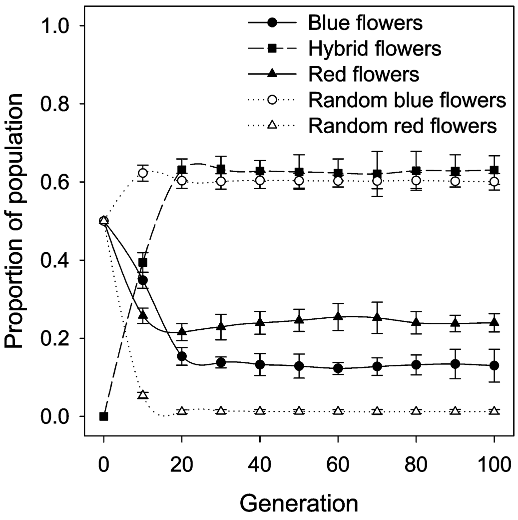


Figure 6: Effect of bumblebee (sucrose-based) and hummingbird preferences on sympatric populations of 1,600 red-flowered and 1,600 blue-flowered plants (proportions of the initial population: red = 0.5, intermediate = 0, blue = 0.5; scenario B, secondary contact). Color preferences were not strong enough to maintain ethological isolation between the two color morphs. Dashed lines represent the proportions of red and blue flowers produced by random foraging (no preferences).

Although bumblebee and hummingbird preferences for flower color are unlikely to sustain ethological isolation, combinations of color and other floral characteristics typically associated with each pollinator group, such as flower orientation, shape, odor, and size, may produce stronger preference levels than color alone. This hypothesis seems reasonable considering that pollination systems are distinguished by suites of floral characteristics (Fenster et al. 2004) that may be genetically linked (Bradshaw et al. 1995; Hodges et al. 2002). Recent studies have shown that pollinator preference levels are stronger when flowers differ in multiple floral characteristics than when they differ in a single characteristic (Gegear and Laverty 2001, 2005; Raguso and Willis 2002, 2005; Gegear 2005). Indeed, two floral characteristics commonly used to define bumblebee and hummingbird pollination syndromes (flower color and orientation) have been shown to induce strong foraging preferences in bumblebees independent of reward level when presented to bees together but not when presented alone (R. J. Gegear, unpublished data). The strength of pollinator preference for certain combinations of floral stimuli may also be enhanced by preferences for nectar components such as volume, concentration, and com-

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Figure 5: Simulated effects of divergent color preference in virtual bumblebees and hummingbirds on interfertile red- and blue-flowered virtual plants at multiple ratios of blue-preferring bumblebees to red-preferring hummingbirds. The *X*-axis represents color preferences of virtual bumblebees and the *Y*-axis the color preferences of virtual hummingbirds. Each square represents one plant population after 500 generations of selection by birds and bees with a given strength of preference; the color (red, blue, or striped) corresponds to the flower colors found in the population (striped squares indicate coexistence of both extreme blue- and extreme red-flowered plants, while white squares indicate coexistence of all color types). Captions above each plot indicate the percentage of bumblebees in the mixed hummingbird-bumblebee population. Scenario B simulations represent a zone of secondary contact between red- and blue-flowered plant populations. Scenario C simulations represent a hypervariable population of flowers visited by both hummingbirds and bumblebees (sympatric speciation). The triangles represent the flower color of plants in the populations based on the preferences of live hummingbirds and bumblebees, while the circles represent populations based on the preference of live hummingbirds and sucrose-based preference of live bumblebees.

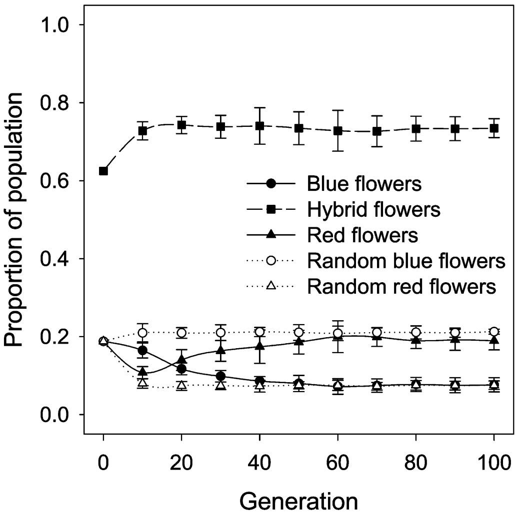
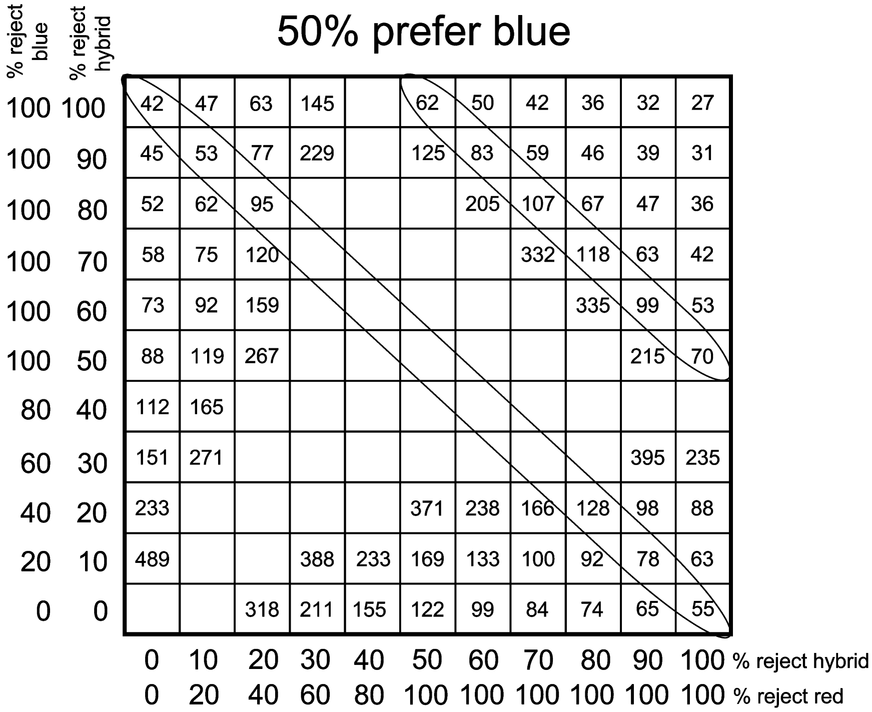


Figure 7: Effect of bumblebee (sucrose-based) and hummingbird foraging preferences on a plant population containing 600 red flowers, 2,000 intermediates, and 600 blue flowers (proportion of initial population: blue = 0.2, intermediate = 0.6, red = 0.2; scenario C, sympatric speciation). Color preferences were not strong enough to lead to floral divergence and the evolution of ethological isolation. Dashed lines represent the proportions of red and blue flowers produced by random foraging (no preferences).

position (e.g., Cresswell and Galen 1991; Fenster et al. 2006). For example, the tendency of bird-pollinated plant species to have greater hexose : sucrose ratios in floral nectar than insect-pollinated plant species may reflect differences in pollinator sugar preferences (Dupont et al. 2004). These points underscore the importance of using a behavioral approach to examine the adaptive significance of floral characteristics in animal-pollinated plants.

What strength of pollinator preference for flower characteristics will cause floral divergence and plant speciation? Given the genetics of flower color in the *Mimulus lewisii*–*Mimulus cardinalis* system, our simulations indicate that pollinator groups with divergent preferences must reject intermediate phenotypes at least 50% of the time and completely reject the extreme phenotypes (red or blue flowers in our study) while foraging to produce the minimum strength of divergent selection required for ethological isolation to evolve as a pleiotropic by-product of floral divergence. Given these conditions, we found that speciation occurs regardless of whether pollinators are foraging on separate plant populations that later come into contact with one another (allopatric; scenarios A and B) or a single plant population (sympatric; scenario C). Thus, pollinator preferences can include a wide range of floral phenotypes (e.g., 13 of 16 colors) within the same plant population and still generate a selective force strong enough to build reproductive isolation between diverging floral phenotypes. In addition, we found that plant speciation occurred very rapidly when threshold strengths of divergent preferences for floral phenotypes were achieved by each pollinator group. For example, it took only 27 generations (given our model's parameters) for ethological isolation to evolve between incipient plants species in ecological contact when virtual hummingbirds visited only red flowers (category 1) and virtual bumblebees visited only blue flowers (category 3). These results indicate not only that pollinator preferences for floral characteristics can cause ecological speciation in plants but also that they can do so over very short evolutionary time periods and regardless of whether incipient species are geographically isolated.

One ecologically important condition of our simulations was that pollinator densities were low enough (or plant densities were high enough) to prevent the depletion of nectar rewards from plants expressing the preferred flower color. Whether higher pollinator densities will influence the probability of floral divergence and the evolution of ethological isolation in our simulations depends on how pollinators respond to a decrease in relative availability of rewarding plants with the preferred flower color (see Sargent and Otto 2006 for a similar discussion from the plant's perspective). One possibility is that pollinators will visit the initially nonpreferred flower type (i.e., adopt a more generalized foraging strategy), thereby weakening preferences and preventing the evolution of ethological isolation. Alternatively, a decrease in the frequency of rewarding plants with the preferred flower color may have little or no effect on pollinator choice behavior. For example, pollinators may simply leave the patch in search of another patch of rewarding plants with the preferred flower color (Goulson 1999). If pollinators decide to continue to forage within the patch, they may maintain their color preferences despite the corresponding decrease in the frequency of rewarding flowers of the preferred color. Heinrich et al. (1977) found that bumblebees foraging on a color-dimorphic floral array maintained a strong preference for blue over novel white flowers over three consecutive days despite encountering as many as 75 empty blue flowers during a foraging bout, indicating that color preferences can persist independent of rewards. Pollinators may also learn to associate the less preferred color with higher rewards and, in doing so, switch their color preferences. Based on our simulations (fig. 5), such "assortative" movement of pollinators could still facilitate the evolution of ethological isolation as long as new color preferences were formed quickly and there were few subsequent transitions between extreme floral phenotypes (although we did not vary preference levels among individuals within each pollinator group in our simulations).



% reject blue	% reject hybrid												
100	100	42	47	63	145		62	50	42	36	32	27	
100	90	45	53	77	229		125	83	59	46	39	31	
100	80	52	62	95				205	107	67	47	36	
100	70	58	75	120					332	118	63	42	
100	60	73	92	159						335	99	53	
100	50	88	119	267							215	70	
80	40	112	165										
60	30	151	271								395	235	
40	20	233					371	238	166	128	98	88	
20	10	489			388	233	169	133	100	92	78	63	
0	0			318	211	155	122	99	84	74	65	55	
		0	10	20	30	40	50	60	70	80	90	100	% reject hybrid
		0	20	40	60	80	100	100	100	100	100	100	% reject red

Figure 8: Number of plant generations required for virtual pollinators with different degrees of color preference to drive the evolution of flower color and ecological speciation in scenario C (sympatric speciation). Numbers in the squares are the numbers of generations are required before the flower population reaches the criteria either for one flower type dominating the population (*upper left corner*, *lower right corner*) or for ecological speciation (*upper right corner*). When red-preferring pollinators have a stronger preference value than blue-preferring pollinators, they drive the flower population to all red flowers more rapidly than when blue-preferring pollinators have a stronger preference value than red-preferring pollinators. The extended ovals delineate zones of equivalent but opposite preferences.

Further controlled behavioral experiments on how pollinator preferences are affected by variation in nectar rewards at different spatial and temporal scales are required to examine these potential scenarios and provide insight into the ecological conditions necessary for plant speciation.

Our simulation of the genetics from a naturally occurring pollination system revealed an unexpected asymmetry in the effects of red-preferring and blue-preferring pollinators on floral evolution. Because blue flowers can hide genetic variation for red coloration, it is easier for pollinators to drive selection toward red flowers than toward blue flowers from an initially hypervariable plant population. Thus, the *M. lewisii*–*M. cardinalis* genetic system we used is predisposed to shift from blue (note that flowers of *M. lewisii* are actually lavender-pink) to red flowers when exposed to selection from red-preferring pollinators, which, if these systems have a similar genetic basis for flower color variation, may help to explain the shift from bee to hummingbird pollination in *Mimulus* (Beardsley et al. 2003), as well as in other systems such as *Penstemon* (Thomson et al. 2000) and *Schizanthus* (Perez et al. 2006).

The importance of pollinators in plant speciation has recently been questioned because of reports that many pollination interactions that are supposed to be specialized (e.g., birds specialize on red flowers and bees specialize on blue flowers) are actually more generalized (e.g., each group visits many plant species and each plant species is visited by both groups; Waser et al. 1996; Johnson and Steiner 2000; Aigner 2001; Waser 2001). In this apparent paradox of plant-pollinator systems (Ollerton 1996), it is assumed that convergence of floral characteristics among plant species (evolutionary specialization) cannot reflect selection pressures exerted by one pollinator group unless that group shows strong preferences for plants with those characteristics (ecological specialization). Our simulations do not support this view. Floral divergence between allopatric bird-only and bee-only plant populations occurred even when pollinator preferences were extremely weak. Furthermore, ethological isolation resulted in mixed pollinator populations (sympatry) despite moderate preferences for intermediate colors (50% probability of accepting an intermediate color). Thus, pollinators can appear to be generalized in their foraging patterns and still drive adaptive divergence and speciation. The results of our simulations also demonstrate that floral characteristics can reflect the preference of a single pollinator group despite the fact that plant species are visited by many pollinator groups. For example, plant populations visited by both hummingbirds and bumblebees will diverge in floral characteristics depending on the strength of pollinator preference and the abundance of each pollinator group in the population (fig. 5). Evolutionary convergence in floral characteristics can therefore reflect mechanisms of foraging in pollinators even in the absence of extreme ecological specialization, either past or present.

Pollinator-driven plant speciation continues to be a contentious issue among plant evolutionary biologists because key elements of pollinator behavior such as floral preference, sensory capabilities, information processing, and decision making are assumed rather than empirically evaluated under controlled conditions. By creating hypervariable populations of artificial flowers, as per Schluter (2000), and imposing pollinator-mediated selection through both computer simulations and selection experiments using live pollinators, we have outlined the circumstances necessary for ethological isolation and have begun a more rigorous and integrative investigation of how pollinators influence key ecological and evolutionary processes in flowering plants. In addition, our work suggests that adaptive divergence and ecological speciation in plants and animals can occur via analogous mechanisms, which we hope will foster new research perspectives and a more comprehensive approach to the study of phenotypic diversity.

Acknowledgments

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APPENDIX

Sample Calculation for Determining Concentration of Pigments 1 and 2

Genotype: $\{L1_a, L1_b\}$, $\{\{L2_a, L2_b\}, \{L3_a, L3_b\}\}$, $\{L4_a, L4_b\}$, $\{\{L5_a, L5_b\},\{L6_a, L6_b\}\}$, $\{L7_a, L7_b\}$, $\{L8_a, L8_b\}$, $\{L9_a, L9_b\}$, $\{L10_a, L10_b\}$.

Example: {24, 24}, {{34, 31}, {20, 20}}, {24, 1}, {{0, 0}, {3, 14}}, {44, 48}, {50, 35}, {62, 62}, {14, 32}.

Dominance: the allele with a lower value is completely dominant over the other allele (loci 1, 2, 4, 6).

Partial dominance: the allele with a lower value is partially dominant over the other allele, so the output of that locus is lower than a mean of the two alleles (loci 7–10).

Additive: a mean of the two alleles is used (locus 3).

Epistasis: only if alleles are homozygous recessive (locus 5).

Pigment 1 = (if $L2_a < L2_b$, then $L2_a$, else $L2_b$) $\times 0.08$ (this is a complete dominance interaction) + $[(L3_a + L3_b)/2] \times 0.08$ (this is an additive interaction) + (if $L6_a < L6_b$, then $L6_a$, else $L6_b$) $\times 0.2 + (L8_{a+}L8_b)/[(L8_a/L8_b) + (L8_b/L8_a)] \times 0.22$ (this is a partial dominance interaction) + $(L9_a + L9_b)/[(L9_a/L9_b) + (L9_b/L9_a)] \times 0.21 + (L10_a + L10_b)/[(L10_a/L10_b) + (L10_b/L10_a)] \times 0.21$.

Example: pigment 1 concentration is $(31 \times 0.08) + (20 \times 0.08) + (3 \times 0.2) + (39.9 \times 0.22) + (62 \times 0.21) + (16.9 \times 0.21) = 30.03$.

Pigment 2 = if $L5_a + L5_b = 1$ or 2, then (random value between 1 and 6); if $L5_a + L5_b = 0$, then (if $L1_a < L1_b$, then $L1_a$, else $L1_b$) $\times 0.43$ + (if $L4_a < L4_b$, then $L4_a$, else $L4_b$) $\times 0.2 + (L7_a + L7_b)/[(L7_a/L7_b) + (L7_b/L7_a)] \times 0.21$.

Example: $L5_a + L5_b = 0$, so pigment 2 concentration is $(24 \times 0.43) + (1 \times 0.2) + (45.8 \times 0.21) = 20.14$. So the example has a pigment 1 concentration of 30.03 and a Pigment 2 concentration of 20.14. From figure 1, the flower is therefore color 12 (a reddish flower).

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