Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*)

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A paradigm of evolutionary biology is that adaptation and reproductive isolation are caused by a nearly infinite number of mutations of individually small effect. Here, we test this hypothesis by investigating the genetic basis of pollinator discrimination in two closely related species of monkeyflowers that differ in their major pollinators. This system provides a unique opportunity to investigate the genetic architecture of adaptation and speciation because floral traits that confer pollinator specificity also contribute to premating reproductive isolation. We asked: (*i*) What floral traits cause pollinator discrimination among plant species? and (*ii*) What is the genetic basis of these traits? We examined these questions by using data obtained from a large-scale field experiment where genetic markers were employed to determine the genetic basis of pollinator visitation. Observations of F_2 hybrids produced by crossing bee-pollinated *Mimulus lewisii* with hummingbird-pollinated *Mimulus cardinalis* revealed that bees preferred large flowers low in anthocyanin and carotenoid pigments, whereas hummingbirds favored nectar-rich flowers high in anthocyanins. An allele that increases petal carotenoid concentration reduced bee visitation by 80%, whereas an allele that increases nectar production doubled hummingbird visitation. These results suggest that genes of large effect on pollinator preference have contributed to floral evolution and premating reproductive isolation in these monkeyflowers. This work contributes to growing evidence that adaptation and reproductive isolation may often involve major genes.

reproductive isolation | adaptation | speciation | natural selection | pollination

One of the principal goals of evolutionary biology is to discover the genetic architecture of adaptation. Fisher's "infinitesimal" model of evolution proposes that adaptation is due to the fixation of many genes with small individual effects, and is based on the assumption that large-effect mutations move a population farther from, rather than closer to, its phenotypic optimum (1). This micromutationist view of "adaptive geometry" (2) has had widespread support, but was challenged recently by a theory suggesting that mutations of large effect can often be beneficial during the early stages of adaptation as populations move toward their optimum phenotype (3). There have been too few empirical studies to resolve the debate, and it is therefore important to identify systems in which both the genetic basis and ecological significance of adaptive traits can be identified (4, 5).

Adaptations that reduce the frequency of mating among neighboring populations are of special interest, as these may contribute to the origin of new species. Although evidence from *Drosophila* suggests that premating isolation may evolve quickly (6), and can have a simple genetic basis (7, 8), there are few comparable data from other organisms and no studies investigating the genetics of premating reproductive isolation in natural populations (9, 10).

Pollinator-mediated selection on floral traits is widely regarded as a common mechanism of adaptation and speciation in plants (11–19). The traditional view is that adaptation to the most abundant or efficient pollinators in geographically isolated populations results in floral divergence, and that pollinator preference prevents intercrossing if populations come into secondary contact. Two species that show this pattern of secondary contact are the predominantly bee-pollinated *Mimulus lewisii* and its hummingbird-pollinated congener *Mimulus cardinalis*. *M. lewisii* has pink flowers, a wide corolla with inserted anthers and stigma, a small volume of nectar, petals thrust forward to provide a landing platform for bees, and two yellow ridges of brushy hairs presumed to be nectar guides (Fig. 1*A*). *M. cardinalis* has red flowers, a narrow tubular corolla, reflexed petals, a large nectar reward, and exserted anthers and stigma to contact the forehead of hummingbirds (Fig. 1*C*). Neither species has an odor detectable by humans, and our observations suggest that pollinator visitation is influenced primarily by flower color, size, shape, and nectar reward.

Despite striking morphological differences, these two monkeyflowers are very closely related. A phylogeny based on DNA sequence from the internal transcribed spacer of nuclear ribosomal RNA places *M. cardinalis* and the Sierra Nevada form of *M. lewisii* together and distinct from Rocky Mountain and Cascade Range populations of *M. lewisii* and other members of the section *Erythranthe* (A. Yen, R. G. Olmstead, H.D.B. and D.W.S., unpublished work). Crosses between these two species produce fertile hybrids (20). Their geographic distributions are largely nonoverlapping, with *M. lewisii* found principally from mid-to-high elevation, and *M. cardinalis* found from low-to-mid elevation. The two species co-occur in a narrow altitudinal zone at 1400 m in the Sierra Nevada.

In 1998, we conducted observations (>80 hr) in a sympatric area along the South Fork of the Tuolumne River, California, and found that bees were the only visitors to *M. lewisii* (100% of 233 visits), and that hummingbirds were the primary visitors to *M. cardinalis* (97% of 146 visits). Only once did we observe a pollinator visit both *Mimulus* species in succession. These results show that pollinator discrimination results in strong premating reproductive isolation in the zone of sympatry.

Two experiments are required to elucidate the genetic architecture of reproductive isolation by pollinator-mediated selection. First, the genetic basis of traits such as flower color, size, shape, and nectar reward must be determined for plant species with different pollinators. Second, the response of wild pollinators to each floral trait must be evaluated in a geographic region where the plant species co-occur. We have completed the first experiment, using linkage mapping with molecular markers to identify quantitative trait loci (QTL) that control complex floral traits in *M. lewisii* and *M. cardinalis*. We found that most floral traits had at least one QTL of large effect (explaining >25% of the F_2 phenotypic variance), suggesting that pollinator-mediated selection in this system could involve "major" genes (21, 22). Here, we report results from the second experiment, identifying the ecological significance of floral traits and the effect of simple genetic changes on pollinator visitation in nature.

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Abbreviation: QTL, quantitative trait loci.

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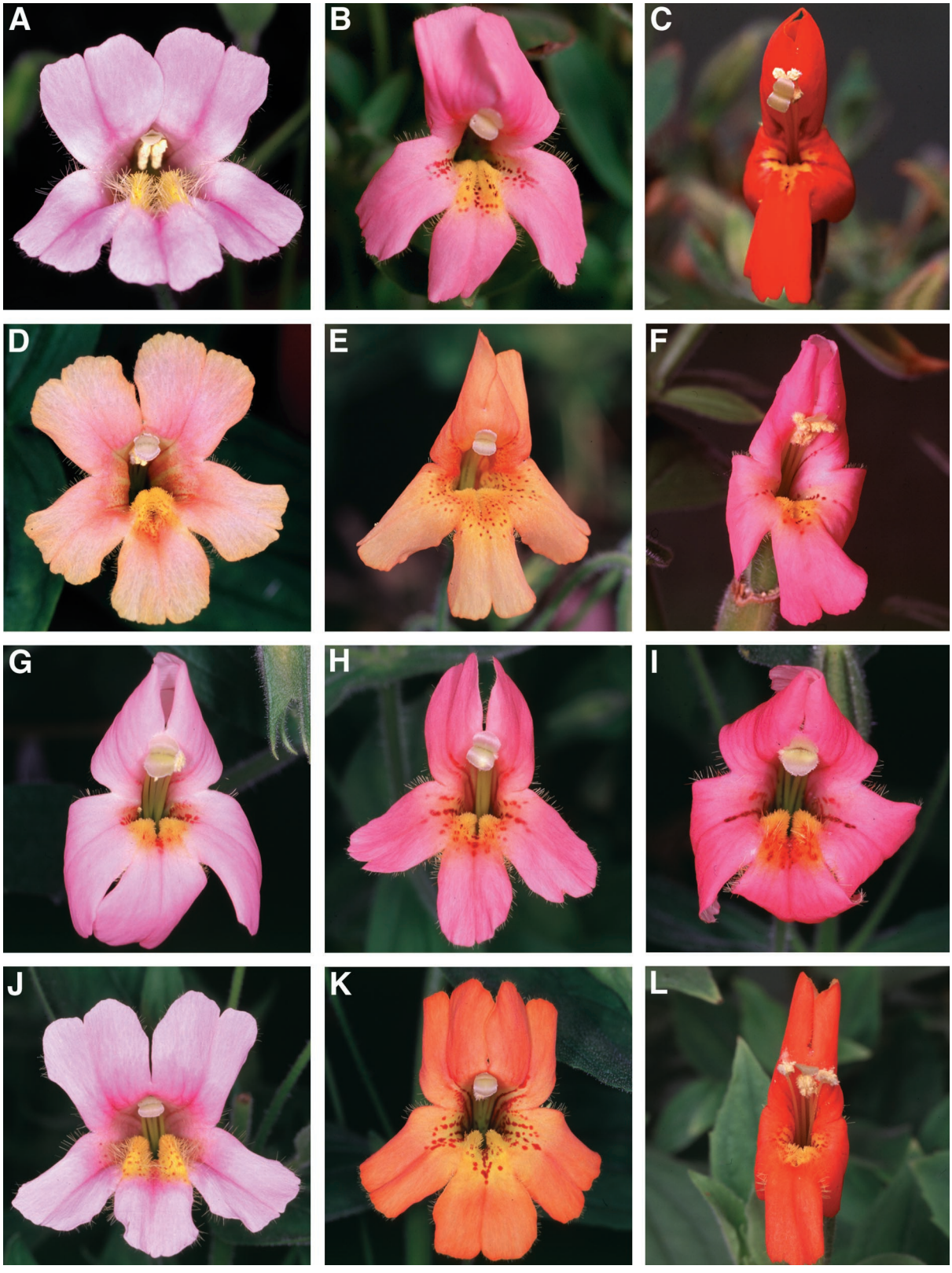

Fig. 1. *M. lewisii* (*A*), an F_1 hybrid (*B*), *M. cardinalis* (*C*), and examples of variation in floral traits found in F_2 hybrids (*D–L*).

Materials and Methods

Seed of both parental species was collected in Yosemite National Park. We crossed *M. lewisii* (Fig. 1*A*) with *M. cardinalis* (Fig. 1*C*) to produce F_1 hybrids, then mated unrelated F_1s to produce an outcrossed F_2 population. The F_1 hybrids have pink flowers and moderately reflexed petals, with nectar guides similar to those of *M. lewisii*, but lacking hairs (Fig. 1*B*), whereas the F_2 generation displays a wide range of flower colors and morphologies (Fig. 1 *D–L*).

We examined the visitation by bees and hummingbirds to the parental species and hybrids in an experimental population. We grew parental, F_1, and F_2 individuals to flowering in the University of Washington greenhouses as part of our QTL studies (22), and transported a subset of these plants to the study site (Wawona Ranger Station, Yosemite National Park, elevation 1300 m) where the two species co-occur. We arranged plants randomly in a 5 x 15 m plot, with 0.5-m spacing ($n = 24$ for each of the parents and the F_1, and $n = 228$ for the F_2 generation). We used fewer parentals and F_1s than F_2s to reduce the likelihood that pollinators would develop a preference for F_2s that resembled the parental species. Our observation period (June 1996) preceded the flowering time of natural populations of *M. lewisii* and *M. cardinalis*. This schedule prevented gene flow from our study population and ensured that pollinators had not yet encountered the study species in natural populations in 1996.

We conducted observations of bee and hummingbird visitation from dawn to dusk in separate 30-min periods, three to four times a day (mean = 3.7 periods per day for each pollinator type) on 7 days from June 18 to June 27, for a total of 26 hr. Three to five observers watched the plot during each observation period, using tape recorders to record flower visits by bees and hummingbirds. We recorded the number of open flowers for each plant on each day of observation. To obtain a daily "rate" of pollinator visitation (visits per flower per day), we divided the daily total number of visits for each pollinator by flower number. There were more bees than could be recorded during some observation periods, but this is likely to result in only a slight underestimate of the relative frequency of bee visitation, so we did not attempt to correct for the unobserved bee visits. Voucher specimens of the most common bees were identified by E. Sugden (Department of Zoology, University of Washington).

Four floral traits were chosen for analysis: (*i*) petal anthocyanin concentration (purple pigments), (*ii*) petal carotenoid concentration (yellow pigments), (*iii*) nectar volume, and (*iv*) projected area (a composite measure of the petal surface exposed to pollinators). These traits are highly diverged in the two parental species (21–23), and were expected to affect pollinator visitation rates because of their contribution to pollinator attraction and reward. We cannot exclude the possibility that other, unmeasured traits may contribute to pollinator visitation, and that these may be linked to the traits included in our study, or have pleiotropic effects on those traits.

We used the mean of two randomly drawn flowers per plant to estimate the phenotypic value of each trait. Petal anthocyanin concentration was estimated by punching 6-mm disks from the lateral petals, extracting the anthocyanins with 0.5 ml of methanol/0.1% HCl, and determining the absorbance at 510 nm. Petal carotenoid concentration was estimated similarly, using methylene chloride for extraction and measuring absorbance at 450 nm. To estimate projected area of the corolla, we recorded video images of flowers from the perspective of approaching pollinators, i.e., in a plane perpendicular to the long axis of the corolla tube, and analyzed these with image analysis software (National Institutes of Health IMAGE; http://rsb.info.nih.gov/nih-image). Nectar volume was measured with a graduated pipette tip. For practical reasons, all measurements were conducted while the study plants were growing in the University of Washington greenhouse. We remeasured a subset of plants in the field plot, and found that the greenhouse and field values were positively correlated for all morphological traits ($P < 0.01, n = 56$) and for nectar volume ($P < 0.0001, n = 31$).

To examine the relationship between pollinator visitation and floral traits in the F_2 population, we treated the proportion of bee visits and the daily visitation rates of bees and hummingbirds as dependent variables in separate multiple regressions, with the four floral traits as independent variables. Analyzing the proportion of bee visits evaluates the effects of floral characters on the composition of the pollinator assemblage, whereas analyzing daily visitation rates by bees and hummingbirds identifies the mechanisms responsible for differences in pollinator composition, i.e., increasing bee visitation vs. decreasing hummingbird visitation. We performed an angular transformation on the proportion of visits by bees and a square-root transformation on all floral traits. The transformed variables were then standardized (mean = 0, SD = 1) to provide a direct comparison of the magnitudes of the regression coefficients for different analyses.

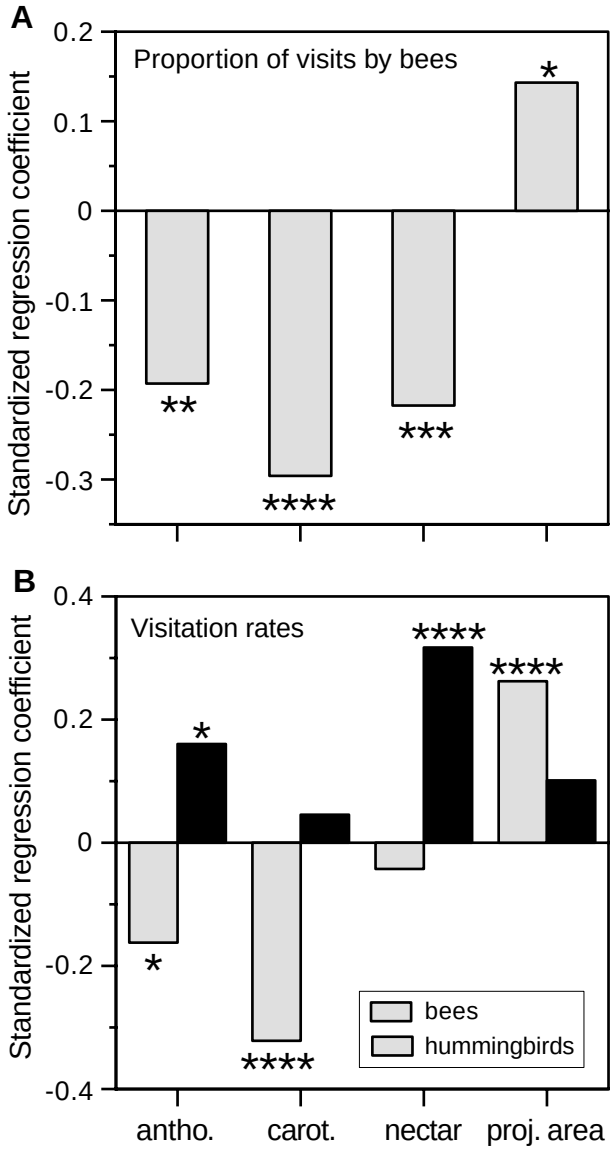

Fig. 2. Contribution of floral traits to pollinator visitation, as determined by multiple regression analysis (antho., petal anthocyanin concentration; carot., petal carotenoid concentration; nectar, nectar volume per flower; proj. area, projected area of petals). Bars give the standardized regression coefficients; ⋆, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. $n = 228$ F_2 plants for all analyses. (*A*) Multiple regression of floral traits on the proportion of visits by bees ($F = 24.2$, $P < 0.0001$, $R^2 = 0.31$). (*B*) Multiple regression of floral traits on the mean daily visitation rates by bees ($F = 22.1$, $P < 0.0001$, $R^2 = 0.28$) and hummingbirds ($F = 13.7$, $P < 0.0001$, $R^2 = 0.20$).

Results and Discussion

We observed a total of 12,567 pollinator visits in the experimental population. The non-native honeybee *Apis mellifera*

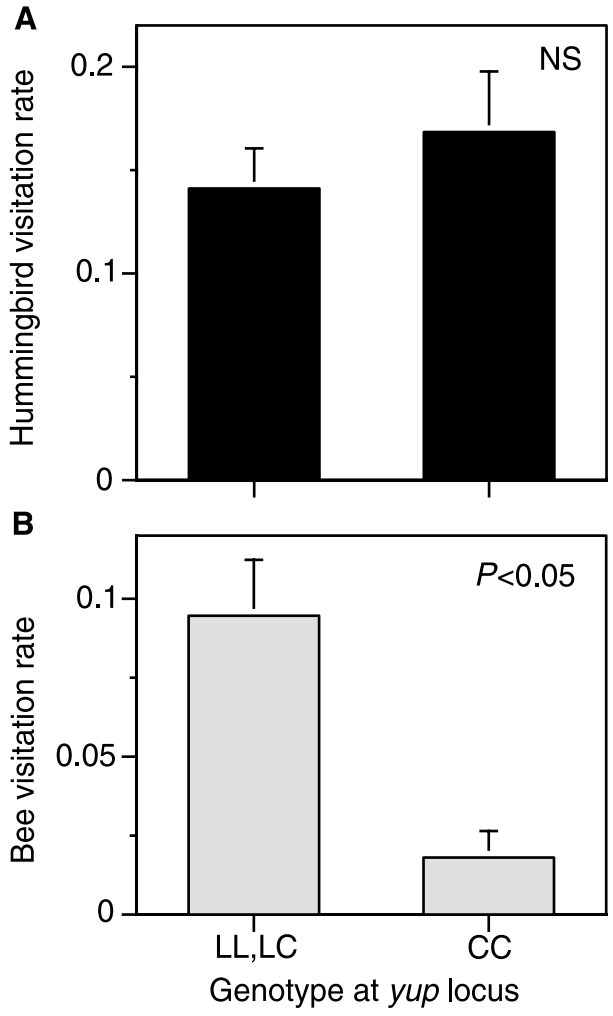

Fig. 3. Effect of allelic differences at the *yup* locus on the visitation rate (visits per flower per day) of hummingbirds (*A*) and bees (*B*). Heterozygous individuals (LC) or those homozygous for the *M. lewisii* allele (LL) lack carotenoids in their upper petals and are pink-flowered (n = 165), whereas individuals homozygous for the *M. cardinalis* allele (CC) have petal carotenoids and vary in color from light orange to red (n = 63). Bars denote the mean + 2 SE. Significance levels were determined by Mann–Whitney *U* tests.

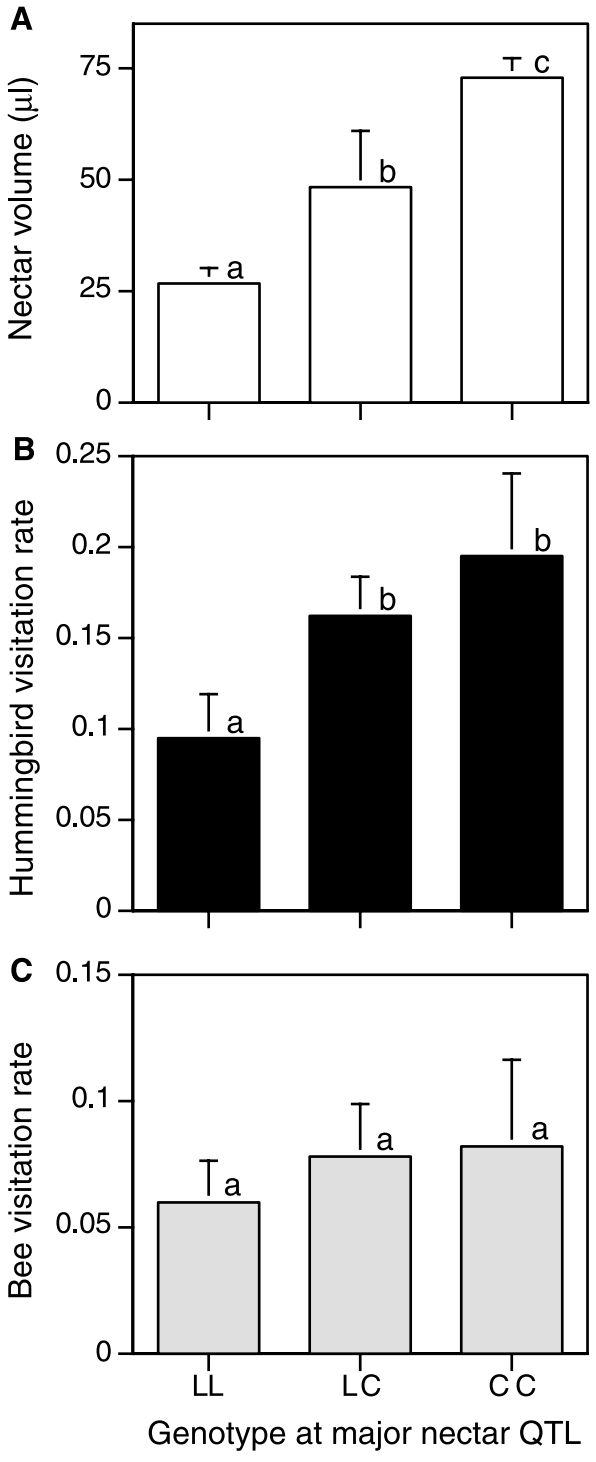

Fig. 4. Effect of marker genotype for the major nectar QTL (RAPD marker L04co; ref. 22) on nectar volume per flower (*A*), and the visitation rate (visits per flower per day) of hummingbirds (*B*) and bees (*C*). Genotypes are: LL, individuals homozygous for the *M. lewisii* allele (n = 61); LC, heterozygotes (n = 130); CC, individuals homozygous for the *M. cardinalis* allele (n = 36). Bars denote the mean + 2 SE, and bars with different letters identify means that are significantly different ($P < 0.01$) based on Mann–Whitney *U* tests corrected for multiple comparisons (31).

comprised <5% of the total visits to F_2s and was excluded from our analyses. We combined all other bee species to form a single category. The bumblebee *Bombus vosnesenski* was responsible for >95% of all bee visits, with the remaining visitation by *Osmia* (*Monilosmia*) sp. and an unknown bumblebee. Bumblebees generally visited flowers for nectar and made only passive contact with the anthers, whereas *Osmia* (*Monilosmia*) sp. actively collected pollen during its foraging bouts. Pollen-collecting bumblebees were observed most often on plants with red or orange flowers. Anna's hummingbird (*Calypte anna*) was the only species of hummingbird observed. Although we did not mark hummingbirds, chases between individuals with different plumage were common, suggesting that several different hummingbirds were visiting the experimental plants.

M. lewisii was visited primarily by bees (82% of 78 visits), and *M. cardinalis* was visited by hummingbirds (99.6% of 2,097 visits), establishing that pollinator behavior in our experimental plots is similar to that observed in natural populations. The composition of the visitors to F_1 hybrids (59% bees; 1,744 visits) was exactly intermediate to that of the parental species, indicating a strong genetic component to visitation. The composition of pollinators visiting the F_2s (8648 visits) varied widely, from plants visited only by bees to those visited only by hummingbirds, with a mean of 38% bee visitation per plant.

Increased petal anthocyanins, petal carotenoids, and nectar volume significantly reduced the proportion of bee visitation, whereas greater projected area increased the proportion of bee visitation (Fig. 2*A*). These results provide clear evidence that flower color contributes to reproductive isolation in this system, despite recent statements to the contrary (24, 25). Petal anthocyanin concentration significantly affected both bee and hummingbird visitation rates, but with opposite effects, whereas each of the other floral traits had a significant effect on one pollinator, but not on the other (Fig. 2*B*). Bee visitation rate was negatively associated with petal anthocyanin and carotenoid concentration and positively associated with projected area, whereas hummingbird visitation rate was positively associated with both petal anthocyanin concentration and nectar volume (Fig. 2*B*).

We tested the hypothesis that adaptation to different pollinators may involve genes with large phenotypic effects by comparing visitation rates as a function of QTL marker genotype for petal carotenoid concentration and nectar volume, the two traits with the greatest impact on bee and hummingbird visitation, respectively (Fig. 2*B*). A single Mendelian locus controls the distribution of carotenoid pigments in the petals (20). F_2 plants homozygous for the recessive *M. cardinalis* allele at the *yup* locus (yellow upper; ref. 20) have carotenoids distributed throughout the petals, and are orange- or red-flowered (Fig. 1 *D*, *E*, *K*, and *L*), whereas F_2s carrying the dominant *M. lewisii* allele are pink-flowered (Fig. 1 *F–J*). There was no effect of *yup* genotype on hummingbird visitation rate (Fig. 3*A*), but bee visitation was 80% lower in plants homozygous for the *M. cardinalis* allele (Fig. 3*B*). This clearly shows that genetic variation for petal carotenoid concentration affects bee visitation and supports earlier findings that bees visiting *Mimulus* species in the section *Erythranthe* strongly prefer pink over red flowers (26).

Although hummingbirds have been shown to exert strong selection for red coloration (27), we found only a weak relationship between hummingbird visitation and flower color. Hummingbirds had a slight, but significant preference for flowers with high petal anthocyanin concentration (Fig. 2*B*), but exhibited no preference for flowers high in petal carotenoids. That petal carotenoids significantly decrease bee visitation but have no effect on hummingbirds suggests that the high concentration of these pigments in the flowers of *M. cardinalis* (22) may function primarily to discourage bee visitation. The hypothesis that the red coloration of many hummingbird flowers functions primarily to reduce visitation by insects (28) is consistent with the finding that hummingbirds do not have an innate preference for red (29, 30).

To examine the effect of nectar reward on pollinator visitation, we compared hummingbird and bee visitation rates for the three F_2 genotypic classes at the major nectar QTL (22). Our previous genetic mapping study found that this QTL explains 41% of the difference in nectar volume between the two parental species and has an additive mode of action, with the *M. cardinalis* allele causing an increase in nectar (22). Segregation of the parental alleles at this locus produced a nearly 3-fold range in mean nectar volume per flower in our F_2 field population (Fig. 4*A*). The average nectar volume of the heterozygous genotypic class was intermediate to that of the two homozygous classes (Fig. 4*A*), and the visitation rate of hummingbirds closely matched this distribution of nectar volume (Fig. 4*B*). Plants homozygous for the *M. cardinalis* allele had twice the rate of hummingbird visitation as *M. lewisii* homozygotes, whereas heterozygotes had an intermediate value (Fig. 4*B*). These results demonstrate that despite the bewildering array of floral variation in the F_2 population (Fig. 1 *D–L*), hummingbirds have the remarkable ability to distinguish the phenotypic effects of allele substitutions at the major nectar QTL. In contrast, there was no relationship between bee visitation rate and marker genotype at the nectar QTL (Fig. 4*C*). The ability of hummingbirds to quickly find rich nectar sources, and to return to them often, has also been documented in experiments on spatial learning (29, 32, 33) and suggests that hummingbirds are capable of exerting strong selection on the nectar rewards of flowers.

Taken together, our results provide evidence of striking differences in the floral preferences of bees and hummingbirds, and considerable opportunity for the adaptive divergence of floral traits through pollinator-mediated selection. This stands in contrast to recent suggestions that pollinators typically have broad preferences, and are therefore unlikely to contribute to floral evolution or the reproductive isolation of sympatric taxa (25, 34, 35). Floral traits associated with bumblebee and hummingbird pollination, such as petal carotenoid pigments and nectar volume, appear to be under relatively simple genetic control, with major QTLs responsible for pollinator discrimination and reproductive isolation in nature. This work contributes to the growing body of evidence that adaptation may often involve genes of large effect (3, 5, 36–39). Further studies are needed to determine whether our results can be generalized to other plant taxa where closely related species differ in their major pollinators.

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