Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*

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Abstract

We tested whether selection by pollinators could explain the parapatric distribution of coastal red- and inland yellow-flowered races of *Mimulus aurantiacus* (Phrymaceae) by examining visitation to natural and experimental populations. As a first step in evaluating whether indirect selection might explain floral divergence, we also tested for local adaptation in early life stages using a reciprocal transplant experiment. Hummingbirds visited flowers of each race at similar rates in natural populations but showed strong (>95%) preference for red flowers in all habitats in experimental arrays. Hawkmoths demonstrated nearly exclusive (>99% of visits) preference for yellow flowers and only visited in inland regions. Strong preferences for alternative floral forms support a direct role for pollinators in floral divergence. Despite these preferences, measures of plant performance across environments showed that red-flowered plants consistently survived better, grew larger and received more overall pollinator visits than yellow-flowered plants. Unmeasured components of fitness may favour the yellow race in inland habitats. Alternatively, we document a marked recent increase in inland hummingbird density that may have caused a change in the selective environment, favouring the eastward advance of red-flowered plants.

Introduction

Population differentiation across heterogeneous environments provides an excellent opportunity to study natural selection. In many plant species, differences in abiotic factors such as soil characteristics, temperature and moisture can result in adaptation to particular habitats (Clausen *et al.*, 1940, 1948; Antonovics & Bradshaw, 1971; Hiesey *et al.*, 1971; Bennington & McGraw, 1995). Adaptive differences can occur over either broad geographic regions (e.g. Clausen *et al.*, 1940, 1948) or extremely small spatial scales (Waser & Price, 1985) and may be a major factor explaining phenotypic variation between closely related species or subspecies (reviewed in Bradshaw, 1984). Gene flow will act to homogenize populations and constrain divergence

(Slatkin, 1985, 1987), but sufficiently strong selection can maintain divergence despite gene flow (Endler, 1977). Therefore, the ability for natural variation to persist among ecotypes depends both on the strength of selection and the extent of gene flow between habitats.

Examples where floral traits vary across an ecological gradient may provide additional evidence for the effects of selection. In this case, variation in pollinator behaviour, rather than adaptation to local abiotic conditions, may cause population differentiation. Adaptations that increase visitation frequency and efficiency for the most abundant or effective pollinator in different geographic locations can lead to floral divergence and reproductive isolation between closely related plant populations (Grant, 1949, 1981; Stebbins, 1970; Schemske & Bradshaw, 1999). Determining the importance that animal pollinators play in shaping variation in floral characters has a long history among plant biologists. The idea that certain pollinator types tend to specialize on particular combinations of floral traits (i.e. pollination

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syndromes; Faegri & Van der Pijl, 1971) has been widely held. However, the generality of this concept has been questioned, as plants often appear to be visited by a diverse assemblage of pollinator species, which should constrain floral divergence (Ollerton, 1996; Waser *et al.*, 1996; Waser, 1998; Chitka *et al.*, 2001). Although these criticisms have received substantial attention, the prevailing view still remains that the concept of pollination syndromes provides a useful framework for our understanding of floral evolution and diversification (Johnson & Steiner, 2000; Fenster *et al.*, 2004; Wilson *et al.*, 2004).

Mimulus aurantiacus (Phrymaceae) is a perennial, drought deciduous sub-shrub that varies widely in ecology and floral morphology throughout its range (McMinn, 1951; Tulig, 2000). In particular, there is an abrupt transition between parapatrically distributed western and eastern geographic races in extreme southwestern California. In San Diego County, western populations have red flowers with exserted stigmas, whereas eastern populations have longer and wider yellow corollas with inserted stigmas and produce little nectar (Waayers, 1996; Tulig, 2000; Streisfeld & Kohn, 2005). Roughly coincident with this transition in floral morphology is a gradient in climatic and edaphic factors. Western, coastal populations are located predominantly in deep, established soils along hillsides, whereas eastern, inland populations often grow at slightly higher elevations in shallow, sandy crevices of decomposed granite (Beeks, 1962). The coastal climate is temperate and is regulated by its proximity to the cool Pacific Ocean, whereas inland regions are slightly drier with more extreme summer and winter temperatures.

Previous work has shown that flower colour in *M. aurantiacus* is highly heritable and markedly structured between coastal and inland populations, such that yellow flowers are never seen near the coast and red flowers are absent from inland regions (Streisfeld & Kohn, 2005). The transition zone between these races is approximately 20 km wide, within which both pure and intermediate floral types segregate in a presumed hybrid zone. In contrast to this extensive divergence in flower colour, neutral loci exhibit little differentiation between the regions, indicating that habitat-specific natural selection likely maintains the differences in flower colour, despite gene flow at other loci (Streisfeld & Kohn, 2005). Grant (1993) hypothesized that the extensive divergence in floral morphology was due to adaptations to increase hummingbird pollination to red flowers near the coast and hawkmoth pollination to yellow flowers in inland regions. However, range limits of neither type of pollinator coincide with the observed transition in floral races; the same species of hummingbirds and hawkmoths occur in both coastal and inland regions.

Although flower colour is highly diverged between geographic regions, traits conferring increased viability or performance in different habitats could instead be the direct objects of natural selection. If locally adapted physiological traits are genetically correlated with flower colour, then flower colour might evolve through an indirect response to selection (Lande & Arnold, 1983; Chitka *et al.*, 2001; Armbruster, 2002; Conner, 2002). As an example, the anthocyanin floral pigments, which are responsible for red floral colours, are produced through the flavonoid biosynthetic pathway, which also produces compounds that protect plants against herbivory, ultra-violet (UV) radiation damage and heat stress (Harborne, 1976; Koes *et al.*, 1994; Shirley, 1996). Pleiotropic relationships between floral pigments and protective compounds appear common in several other groups (Fineblum & Rausher, 1997; Adler *et al.*, 2001; Armbruster, 2002; Coberly & Rausher, 2003; Irwin *et al.*, 2003; Frey, 2004; Strauss *et al.*, 2004), suggesting the possibility of indirect selection on flower colour in *M. aurantiacus*.

Strong divergence in flower colour between geographic races of *M. aurantiacus* may be due to the direct effects of adaptations to increase visitation by different pollinators or it may be an indirect result of selection to increase performance in different environments. In this paper, we experimentally test these alternative hypotheses. In order for pollinator-mediated selection to explain the current distribution of floral traits, each floral type should achieve higher pollinator-mediated reproductive success in its native habitat. To assess this hypothesis, we observe pollinator visitation to natural and experimental populations and evaluate the effect of geography and flower morphology on pollinator visitation rates. On the other hand, in order for adaptation to the local environment to maintain the variation in flower colour, two conditions must be met. First, each race should perform better in its native habitat; and second, at least some of the loci controlling these differences must be the same as, or tightly linked to, those controlling flower colour. To test the first of these two conditions, we assay for the initial signatures of local adaptation using a reciprocal transplant experiment. Finally, we examine current and historical hummingbird densities to determine how regional differences in pollinator abundance may exert selection on flower colour and how selective regimes may be changing with urbanization.

Materials and methods

Pollinator visitation

As flower colour is the most strongly differentiated feature between these races (Streisfeld & Kohn, 2005) and there is no consistently agreed upon taxonomy for this species group (McMinn, 1951; Munz, 1973; Thompson, 1993), we will refer to the different races of *M. aurantiacus* as the 'red' floral race and the 'yellow' floral race. In the spring of 2002, we quantified hummingbird visitation rate to five natural *M. aurantiacus* populations (Fig. 1). Two of these populations were of the red floral race, two of the yellow floral race, and the
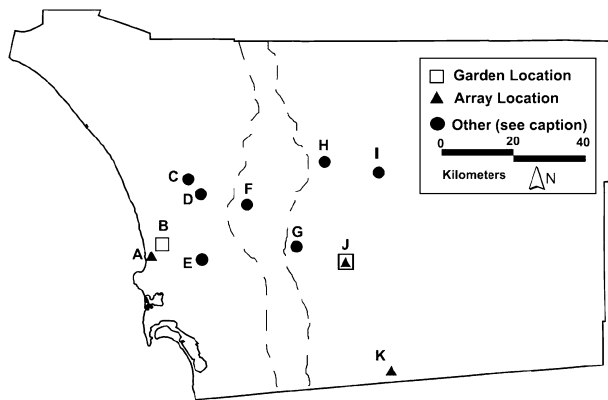
Fig. 1 Map of San Diego County, California showing the locations of *Mimulus aurantiacus* populations used in this study. Open squares denote the locations of the two transplant gardens, whereas triangles represent the locations of experimental pollinator arrays. All other populations are denoted by closed circles. Seeds for the reciprocal transplant experiments were collected from red-flowered populations A, C and E; and yellow-flowered populations G, H and I. Hummingbird observations in natural populations occurred in populations A, D, F, G and H. Evening hawkmoth observations were conducted in populations A and D on the coast and in populations G through K inland. The dashed line shows the approximate position of the hybrid zone, as determined in Streisfeld & Kohn (2005).

and floral race on the presence or absence of hawkmoths using logistic regression.

Observations in natural populations provided a means for determining which pollinators visited the different types of populations, but because all but one of these populations contained either pure red or yellow floral races, these data could not yield information on whether pollinators showed behavioural preference for different floral syndromes. In order to test this, we created experimental arrays consisting of adult plants from both red and yellow floral races placed in the alternative habitats. Plants were grown from seed generated from full-sib crosses, as part of an inheritance study to determine the genetic basis of floral traits (M. A. Streisfeld & J. R. Kohn, unpublished). Plants were grown outdoors at the UC San Diego greenhouse in 3-gallon pots until they began to produce flowers. Only plants that contained large numbers of flowers (range 20–124) were selected for these studies. Before the plants were transported to field sites, we removed all developing fruits and open flowers that had previously been visited by pollinators.

Experimental arrays consisted of sets of 30 plants (15 of each floral race) that were transported to one coastal and two inland sites. The coastal site (Fig. 1) was located alongside a pure red-flowered population and the first inland site was located within a pure yellow-flowered population approximately 5 km east of the transition zone. The second inland site was located approximately 10 km farther east than the first inland site to ensure that highly motile pollinators had not recently been feeding at red *Mimulus* flowers (Fig. 1). Plants were positioned in hexagonal arrays spaced at 1-m intervals. We observed pollinator visitation to each experimental array for 3-h periods over each of 8 days during the late afternoon and evening hours when both hummingbirds and hawkmoths were most active (between approximately 17.00 and 20.00 hours). Hawkmoth visitation could be quantified in these arrays because the spacing of potted plants allowed us to more easily follow hawkmoth movement under low light conditions. Prior to each day of observation, we randomly rearranged the location of plants within the array to prevent pollinators from learning locations of particular plants and we counted the number of open flowers per plant. We recorded the number of open flowers on each plant that was visited by each pollinator type per hour observed. To summarize pollinator preferences, we calculated the frequency of visitation to flowers of each race weighted by the frequency of each type of flower in each array.

final population was located within the transition zone and contained red, yellow and intermediate flowers. Because of the large number and high density of flowering *M. aurantiacus* plants within these populations, it was difficult to distinguish individual plants. In addition, preliminary observations indicated that visitation rates were extremely low. Therefore, we simultaneously observed hummingbird visitation to between two and four quadrats of approximately 64 m^2 in each population during several 3-h periods. Quadrat number and size varied across populations depending on the topography of the site and the density of the surrounding vegetation. Before each observation period, we counted the total number of open flowers within each quadrat. We recorded each hummingbird that entered the quadrat and visited at least one flower, counting only those visits where the bird's bill fully entered and probed the floral tube. We calculated visitation rate as the proportion of open flowers that were visited per observation hour and tested for a difference in visitation rate among populations using a nonparametric Kruskal–Wallis test. Because of the difficulty in observing evening-flying hawkmoths (*Hyles lineata*) over long distances, we were unable to quantify their visitation rates to natural populations. Instead, we recorded the proportion of evening observation periods in each population during which at least some visitation by hawkmoths occurred. We performed evening observations over a period of 3 years to five inland and two coastal sites, as indicated in Fig. 1. We tested the effects of population (nested within floral race)

Reciprocal transplants

In order to test whether adaptation to the local abiotic environment might be responsible for creating the current distributions of these geographic races, we transplanted seedlings from each race into coastal and

inland environments (Fig. 1). Each transplant site was located within approximately 200 m of a natural red- or yellow-flowered *M. aurantiacus* population. In the summer of 2002, we collected naturally pollinated fruits from five plants in each of three populations of each race (Fig. 1). We sowed the seeds at the UC San Diego greenhouse in early February 2003, which roughly coincided with the onset of seedling emergence in natural populations. Seed was germinated in small plug trays containing a mix of sphagnum peat moss, perlite, gypsum and lime, and were grown until seedlings were approximately 3–5 cm tall (7 weeks after planting). We then transplanted seedlings using a completely randomized block design into each of two common gardens (Fig. 1). In total, we transplanted 1200 seedlings per garden (10 seedlings per family × 5 maternal families × 6 seed source populations × 4 blocks). Transplants were spaced every 25 cm and planted in 15 rows of 20 per block. Rows were staggered by 12.5 cm to minimize competition between plants. We watered plants twice daily for the first week after planting, once daily for the second week and every other day for the third week to reduce mortality due to transplanting. Afterwards, no additional water was provided. During the period of watering, there was no effect of floral race ($P > 0.7$) or source population ($P > 0.5$) on the proportion of surviving seedlings per family in either garden. Therefore, we considered any mortality during these first 3 weeks to have occurred because of the effects of transplanting and not natural selection. These plants were eliminated from subsequent analysis.

Eight weeks after planting, but before plants began to produce flowers, we measured the size of each surviving seedling. On each plant, we counted the total number of branches with two or more fully expanded leaf nodes and we measured the length of the three longest branches, to the nearest 0.1 cm. As an index of plant size, we calculated the average length of the three longest branches multiplied by the number of branches. We used the length of the longest branch as an alternative measure of plant size and obtained similar results. As plants became dormant at the end of the season (15–18 weeks after planting), we determined the proportion of surviving seedlings from each family within each block. Finally, we counted the total number of fruits set by a random subset of 20 plants per population ($n = 120$) in the coastal garden. We were unable to perform this analysis in the inland garden. Plants there did not produce any flowers during this season and the garden was later destroyed by wildfire precluding further fitness measurements.

The data were analysed using linear mixed models (PROC-MIXED; SAS Institute, Cary, NC, USA), which allow for both fixed and random effects. We tested for an effect of floral race on the proportion of surviving seedlings per family per block using arcsine square root transformed viability data. We also tested for an effect of floral race on plant size using logarithm transformed growth data. Higher success of each floral race in its native geographical region would suggest local adaptation. This can be detected by assessing the interaction between floral race and garden location. We assessed statistical significance using the model that best fit the data according to likelihood ratio tests (LRT; Littell *et al.*, 1996). We ran the model iteratively, beginning with the full model and each time removing the next highest order random effect. Two times the difference in log-likelihood scores between full and reduced models were tested against a chi-squared distribution with degrees of freedom equal to the difference in the number of estimated parameters. Each time a reduced model did not significantly better fit the data, we removed the next highest-order random effect until we could no longer improve model fit, retaining significant random effects and all fixed effects. Then, significance of the fixed effects was tested using this model. Fixed effects were tested using Type III F-tests based on the Satterthwaite estimation to approximate the degrees of freedom in the denominator term. Random effects were tested using LRT (Littell *et al.*, 1996). For both analyses, we tested the fixed effects of garden location (coast/inland), floral race (red/yellow) and the interaction between them. Random effects included population (nested within floral race) and block (nested within location). Family (nested within population within floral race) was included only in the analysis of plant size. All higher order interactions involving at least one random effect were considered random effects. For the fruit set data, we tested for an effect of floral race and population (nested within floral race) on square root transformed fruit number per plant.

Relative hummingbird densities

Variation in pollinator abundance in each region could affect selection on the floral races. Using data on current hummingbird densities from Unitt (2004; raw data kindly provided by the author), we determined the relative abundance of each of the common hummingbird species that visits *M. aurantiacus* flowers in coastal and inland regions. These species included *Calypte anna* (Anna's Hummingbird), *Archilochus axexandri* (Black-chinned Hummingbird) and *Calypte costae* (Costa's Hummingbird). The data consisted of breeding season bird counts from each 25 km^2 quadrat from a grid covering the entire county. We selected forty quadrats (20 each from coastal and inland regions) where red- or yellow-flowered *M. aurantiacus* populations occur. In each quadrat we tabulated the number of observations per observation hour over the census years 1997 to 2002 for each species and then tested for a significant difference in overall hummingbird density between coastal and inland regions using Mann–Whitney *U*-tests.

Because the selective regime imposed by pollinators might vary over time if densities of either type of pollinator change, we also compared historical estimates of hummingbird densities from coastal and inland sites using North American Christmas Bird Counts (CBC; National Audubon Society, 2002 http://www.audubon.org/bird/cbc/hr). Data consisted of annual observations where volunteers recorded the numbers of all bird species encountered within circles of 12 km radius at various sites across North America on a single winter day. We compared the hummingbird count data from a coastal (San Diego) and an inland (Escondido) recording site within the ranges of the red and yellow floral races from 1953 to 2004. No data were available from the inland site prior to 1953 and no data were recorded at this site from 1960 to 1985. To account for differences in observation effort across sites and years, we standardized each count by the number of observation hours at each site. We then analysed the rate of increase in hummingbird densities in coastal and inland locations over this period using ANCOVA. The most common hummingbird species currently seen in our study area, *C. anna*, is about as widespread and abundant in the winter (December to February) as in the spring and summer when *M. aurantiacus* blooms (Unitt, 2004). Changes over time in CBC data for this species should reflect changes in summertime densities. Because no census data of any kind were available for hawkmoths, we could not perform similar population density analyses for that pollinator.

Results

Pollinator visitation

In total, we observed natural *M. aurantiacus* populations for 159 h, recording 8674 flower visits by various hummingbird species. There were also infrequent visits by nonnative honey bees. We did not include these in our analyses, as honey bees rarely contacted stigmas. The primary hummingbird visitor was *C. anna* (Anna's Hummingbird), but *C. costae* (Costa's Hummingbird) and *A. axexandri* (Black-chinned Hummingbird) were also frequent visitors. Hummingbird visitation rate was similar in all populations, regardless of floral race (Fig. 2; Kruskal–Wallis $\chi_4^2 = 4.64$; $P = 0.326$) and ranged across observation periods between 0 and 0.07 visits per flower per hour. Over a 3-year period, we frequently observed visitation by the hawkmoth *H. lineata*, during evening observations in yellow-flowered populations, but never in red-flowered populations. Visitation occurred in populations of the yellow race during 16 of 22 (73%) evening observation periods. Although *H. lineata* were seen flying in the evenings in coastal regions, they were never seen visiting *M. aurantiacus* flowers (0 of 10 evening observation periods; LRT $\chi_1^2 = 18.58$; $P < 0.0001$). No hawkmoth species other than *H. lineata* were observed in either habitat.
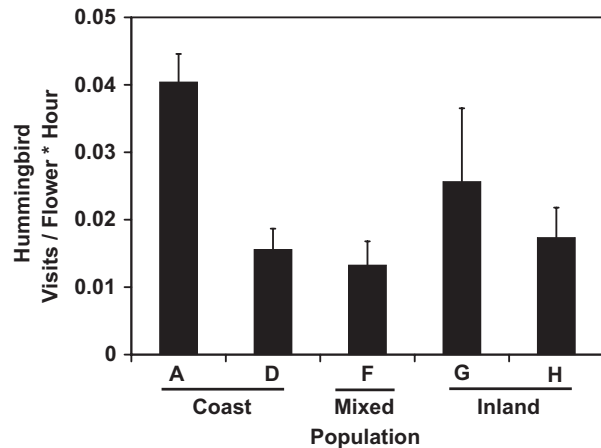

Fig. 2 Hummingbird visitation rates (measured as the proportion of open flowers visited per hour observed) to five natural *Mimulus aurantiacus* populations. Observations were performed simultaneously within two to four quadrats in each population during 3 h intervals. The coastal populations (A and D) contain plants exclusively from the red floral race, whereas the inland populations (G and H) represent pure yellow floral races. The one mixed population (F) occurs within the transition zone between red and yellow floral races and contains red, yellow and intermediate flower colours. Vertical bars represent standard errors.

In experimental arrays of red and yellow floral races, pollinators demonstrated striking differences. Hummingbirds were the only visitors to plants at the coastal array and they visited the red race 94% of the time (Table 1). In the inland arrays, 90% (Inland 1) and 97% (Inland 2) of hummingbird visits were to red flowers. Hummingbird preference, measured as the frequency of visitation to red flowers weighted by the frequency of available red flowers, was even stronger (95–98%; Table 1). On the other hand, *H. lineata* visited flowers only in the inland arrays and all but one of 749 visits were to yellow flowers (Table 1). Overall, the red race was visited far more frequently than the yellow race in all experimental arrays (Table 1), including those in inland locations.

Reciprocal transplants

We detected a small but significant effect of floral race on survival, with plants from the red race surviving better overall (Table 2a; Fig. 3a). In the coastal garden, average survival for each of the three red-flowered populations exceeded that of the yellow race. In the inland garden, two of the three red-flowered populations showed higher survival than the yellow race (Fig. 3a). All of the populations from the red race showed increased size relative to the yellow race in both gardens (Table 2b; Fig. 3b). The red race also set significantly more fruits than the yellow race in the coastal garden ($F_{1,118} = 31.91$; $P < 0.0001$) and there was no significant effect of population of origin (nested within floral race; LRT $\chi_1^2 = 0.1$; $P = 0.376$). Relative success of each floral

Table 1 Pollinator preferences for the red and yellow floral races in three experimental arrays of *Mimulus aurantiacus*.

	Coast (pop. A)		Inland 1 (pop. J)		Inland 2 (pop. K)	
	Red	Yellow	Red	Yellow	Red	Yellow
Hummingbird	1384 (0.95)	96 (0.05)	363 (0.95)	41 (0.05)	1424 (0.98)	37 (0.02)
Hawkmoth	0 (N/A)	0 (N/A)	1 (0.02)	109 (0.98)	0 (0.00)	639 (1.00)
Total visits	1384 (0.95)	96 (0.05)	364 (0.85)	150 (0.15)	1424 (0.74)	676 (0.26)

The number of visits by hummingbirds and hawkmoths to each floral race in each of the three arrays is indicated. The proportion of total open flowers from the red race in each array was 0.456, 0.301 and 0.422 for the Coast, Inland 1 and Inland 2 arrays, respectively. Pollinator preference values (in parentheses) are the relative frequency of visitation weighted by the frequency of each type of flower in each array. Total visitation refers to the relative frequency of visits to each floral type regardless of pollinator identity.

Table 2 Linear mixed model for (a) the proportion of surviving seedlings from each maternal family and (b) plant size 8 weeks after transplanting in two common gardens in *Mimulus aurantiacus*.

Source	d.f.	Statistic	*P*-value
(a) Viability			
Fixed effects			
Location	1, 6	32.1	0.0013
race	1, 230	5.68	0.018
Location × race	1, 230	0.56	0.4556
Random effects			
Population (race)	1	0	>0.5
Block (location)	1	6.3	0.006
Location × population (race)	1	0	>0.5
Block (location) × race	1	0	>0.5
Block (location) × population (race)	1	0	>0.5
(b) Plant size			
Fixed effects			
Location	1, 6.85	51.07	0.0002
Race	1, 4.05	12.54	0.0235
Location × race	1, 3.39	2.43	0.2067
Random effects			
Population (race)	1	58.7	<0.0001
Family (population, race)	1	22.5	<0.0001
Block (location)	1	234.4	<0.0001
Location × population (race)	1	4.2	0.0202
Location × family (population, race)	1	31.4	<0.0001
Block (Location) × race	1	0	>0.5
Block (location) × population (race)	1	0	>0.5
Block (location) × family (population, race)	1	0.4	0.2635

Viability data were arcsine square-root transformed and plant size data were log-transformed before analysis to meet the assumptions of the models.

Fig. 3 (a) Proportion of transplanted seedlings that survived to the end of the growing season and (b) size of transplanted seedlings 8 weeks after transplanting to coastal and inland common gardens of *Mimulus aurantiacus*. Each point represents the mean value from the six source populations in both coastal and inland gardens. Closed diamonds represent populations of the red floral race and open triangles denote populations of the yellow floral race. The lines connect the average value for each floral race in each garden to show the similarity in slopes. The red floral race is represented by the solid line and the yellow floral race is represented by the dashed line.

race did not depend on environment, as there was no interaction between garden location and floral race for either measurement (Table 2).

Survival in both gardens was high, but it was higher in the coastal garden. Ninety-one percent of plants from the red race survived in the coastal garden to 88% from the yellow race. These results were significantly greater than for the inland garden, where survival of the red race averaged 69% and the yellow race equalled 62% (Table 2a; Fig. 3a). Similar results were found for meas-
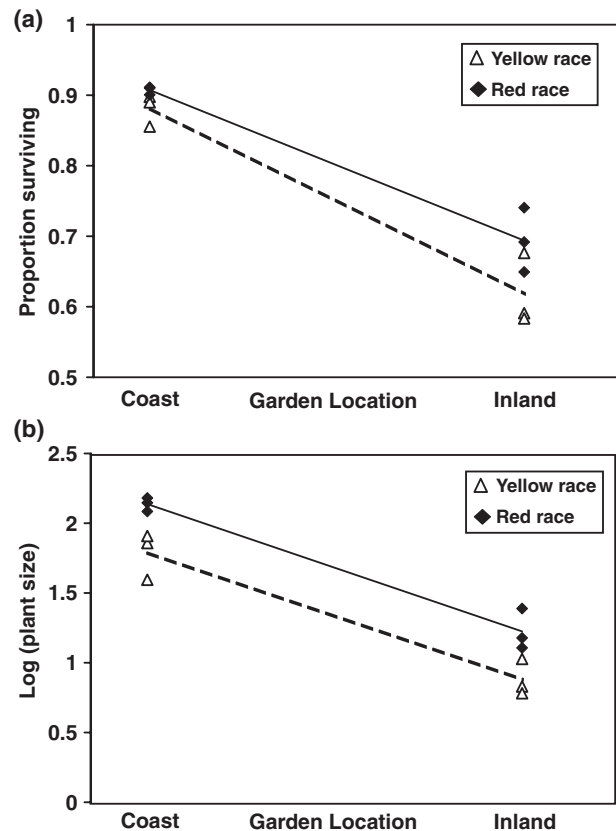
ures of plant size, as plants in the coastal garden again outperformed plants in the inland garden (Table 2b; Fig. 3b). Significant block effects were detected in both

gardens for both traits, demonstrating substantial spatial heterogeneity within each garden. The interactions between garden location and source population and family were both significant for the plant size data set (Table 2b), indicating that the relative performance of populations and families differed among gardens.

Relative hummingbird densities

Data from the *San Diego County Bird Atlas* (Unitt, 2004) show that total hummingbird density is significantly greater in coastal, red-flowered regions, compared with inland, yellow-flowered regions (Table 3). Anna's Hummingbird, the most abundant species, is found in significantly greater numbers in the coastal region, whereas there is no clear regional difference in density for either Black-chinned or Costa's Hummingbird. CBC data are consistent with the data from Unitt (2004), in that Anna's Hummingbirds are more frequent at the coastal than the inland site (Fig. 4). However, during the period from 1953 to 2004, Anna's hummingbird densities increased markedly in both regions. The average encounter rate of Anna's Hummingbird recorded at the inland site during the past decade (1995–2004) was 2.3 (SD = 0.59) birds h^{-1}, which is more than 3.5 times higher than the average encounter rate for this species at the coastal site in the past (1954–1963; mean = 0.64; SD = 0.27; t_{18} = 8.23; $P < 0.001$; Fig. 4). Analysis of total birds encountered, uncorrected for the number of observer hours, provided similar population trends (data not shown).

Discussion

Pollinator preference

We have detected strong evidence for the possibility of direct selection on floral traits by alternative pollinators.

Table 3 Densities of the three most common hummingbird species, data from Unitt (2004).

Hummingbird species	Geographic region	Mean	SD	P-value
Calypte anna	Coast	2.267	2.225	<0.0001
	Inland	0.585	0.460	
Calypte costae	Coast	0.301	0.378	0.068
	Inland	0.577	0.586	
Archilochus axexandri	Coast	0.152	0.253	0.404
	Inland	0.089	0.122	
Total	Coast	2.719	2.397	0.009
	Inland	1.251	0.875	

Mean counts and standard deviations per observation hour are presented from 40 quadrats from coastal and inland regions in San Diego County where either pure red-flowered or yellow-flowered *Mimulus aurantiacus* occur. Mann–Whitney *U*-tests are used to test for a significant difference in density between coastal and inland regions.
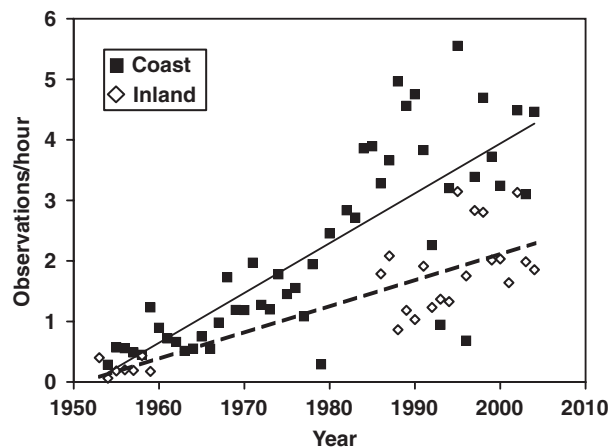

Fig. 4 Encounter rate for *Calypte anna* (Anna's hummingbird) at coastal and inland recording sites in San Diego County, California, during Christmas Bird Counts from 1953 to 2004. Density increased significantly in both sites, but more rapidly in the coastal site (site by year interaction $F_{1,1}$ = 10.04; $P < 0.003$). The lines denote the best-fit linear regression of bird observations per hour against observation year in each site ($P < 0.001$ for each regression). No data were available from the inland site prior to 1953 and no data were recorded at this site from 1960 to 1985. One outlying point (1981) was removed from the coastal data set, as observations per hour were more than 2.5 times greater than the maximum seen in any other year.

Hummingbirds were the predominant visitors to natural *Mimulus aurantiacus* populations, but we also observed hawkmoths visiting in yellow-flowered, inland populations. The presence of two different pollinator types with strong and opposing preferences for alternative floral types is consistent with Grant's (1993) interpretation of the pollination system in these taxa. Although hummingbirds visited the yellow race in natural inland populations (Fig. 2), they displayed a dramatic preference for the red race in experimental arrays in the same habitat. This shift in visitation occurred immediately, such that the first flowers that hummingbirds visited in the two inland arrays were to red flowers. Strikingly, hummingbirds that had recently been foraging 10–20 km beyond the range of red-flowered *Mimulus* and that frequently visited yellow flowers in nearby natural populations, exhibited a strong and immediate bias in favour of red flowers in the arrays. In contrast, hawkmoths nearly exclusively visited yellow flowers in the east (Table 1) and were never seen visiting *Mimulus* flowers near the coast, even when yellow flowers were present in the experimental array.

In these floral races, flower colour is correlated with several other floral traits that may be important in attracting pollinators, including nectar volume and flower size and shape (Waayers, 1996; Tulig, 2000; Streisfeld & Kohn, 2005). All flowers produce limited quantities of nectar, but on average, flowers from the red race produce 1.5 µL of nectar, whereas flowers of the

yellow race produce 0.2 µL (Streisfeld & Kohn, 2005). Hummingbirds are generally believed to choose flowers based on the availability of the nectar reward (Schemske & Bradshaw, 1999; Healy & Hurley, 2001), so it is possible that the dramatic preference that hummingbirds exhibit even in the inland sites is because of an increase in nectar and not differences in flower colour (Meléndez-Ackerman *et al.*, 1997). However, recent studies have demonstrated that differences in flower colour alone may be sufficient to alter hummingbird preferences (Meléndez-Ackerman *et al.*, 1997; Bradshaw & Schemske, 2003). Additionally, hawkmoths visit yellow flowers possessing little nectar reward despite the presence of more rewarding, red flowers in the arrays, suggesting that they may choose flowers based on other stimuli. There is no noticeable odour emitted by either floral type (M. A. Streisfeld, personal observation), but spectral reflectance patterns of red and yellow flowers differ dramatically. Yellow flowers show peak reflectance at two wavelengths (approximately 380 and 580 nm), whereas red flowers reflect maximally at approximately 700 nm (Streisfeld & Kohn, 2005). Hawkmoths, including *H. lineata*, have been shown to possess colour vision under extremely low light conditions (Kelber *et al.*, 2002), suggesting that they may distinguish between the floral types based on colour differences.

Despite strong pollinator preferences for alternate floral types, total visitation is consistently greater to plants of the red race in experimental arrays in both environments (Table 1). After we scale the total number of visits to each floral race by the frequency of available flowers in each array, red flowers are visited 5.7 and 2.8 times more frequently than yellow flowers in each of the two inland arrays and 19 times more frequently in the coastal array (Table 1). We have focused here only on one component of pollination, visitation frequency. Pollen transfer efficiency can also differ between the races and pollinators (e.g. Campbell *et al.*, 1998; Thomson *et al.*, 2000; Thomson, 2003). For example, if hawkmoths are more efficient at removing pollen and subsequently transferring it to stigmas of other flowers, then under-visitation of yellow flowers in inland sites may be balanced by an increase in the efficiency of pollen transfer. Given current data, hawkmoth pollination efficiency would have to be three to six times greater than for hummingbirds in order to favour yellow flowers in the east. Additionally, because hummingbirds visit *Mimulus* flowers throughout the day and hawkmoths are active for <1 h during dusk and early evening, our late-afternoon 3-h observation periods have only captured a portion of the overall daily visitation by hummingbirds, but include all of the daily hawkmoth visitation. Therefore, these are likely to be conservative estimates of the ratio of hawkmoth to hummingbird visitation, requiring an even greater difference in pollinator efficiency to favour yellow flowers in inland regions. Large differences in pollinator efficiency among floral visitors have been reported in various systems (Schemske & Horvitz, 1984; Campbell *et al.*, 1998; Mayfield *et al.*, 2001; Castellanos *et al.*, 2003), suggesting that such differences may be possible in our system. However, to our knowledge, there are no examples where the relative pollen transfer efficiencies of hummingbirds and hawkmoths have been compared.

Variation in pollinator abundances across regions could affect selection on the floral races and current selection could differ from historical selection if these abundances have changed. Despite the occurrence of hummingbirds throughout the county, they reach higher densities in the west where the red floral race predominates (Table 3). This pattern is driven mainly by the prevalence of Anna's hummingbird in coastal regions, as Costa's and Black-chinned are found at far lower densities and do not show strong differences in current abundances across the regions we consider here. Greater abundance of hummingbirds along the coast is also seen in historical data (Fig. 4). Higher relative abundance of hummingbirds on the coast, combined with the strong hummingbird preference for red flowers (Table 1), indicates that the red race is likely to be currently, as well as historically, favoured in coastal regions. We do not have similar estimates of hawkmoth densities, although they are seen in coastal as well as inland regions. The presence of hummingbirds and hawkmoths in both geographic locations suggests that the extensive floral differentiation may have occurred despite overlapping pollinator ranges.

Reciprocal transplants

We currently have little evidence supporting the hypothesis that indirect selection has favoured divergence in floral traits. The red floral race exhibited higher survival and greater growth than the yellow race in both regions and we detected no interaction between floral race and transplant site for these measures of performance (Fig. 3). Furthermore, the red race showed significantly higher fruit set than the yellow race in the coastal garden. These results demonstrate a consistent advantage for the red race in both regions, suggesting no local adaptation to environmental conditions for early performance measures in inland regions. It remains possible that measurements of performance in later life stages might show an advantage for the yellow race at inland sites. Unfortunately, because of the wildfire at the end of the first season, we were unable to measure the long-term fitness of each floral race in the inland environment.

Although further experiments are clearly needed before we can rule out the possibility of local adaptation in unmeasured growth stages, there is little *a priori* evidence to suggest a genetic correlation between germination, survival, or growth differences and floral pigmentation. First, the anthocyanin pigments, which are end-products of flavonoid synthesis (Koes *et al.*,

1994; Shirley, 1996), are found in red flowers near the coast but are absent in inland yellow flowers (Streisfeld & Kohn, 2005). These pigments occur opposite from their apparent need as protective agents if abiotic stressors are the selective forces that prevent the advance of the red floral race eastward. Abiotic conditions become more severe as distance from the coast increases, with drier, warmer, less cloudy summers, more UV radiation exposure and colder winter low temperatures.

Second, although anthocyanin pigmentation is present in red flowers but absent in yellow flowers, both races express anthocyanin pigments and flavonoids in vegetative tissues (Lincoln, 1980; Lincoln & Walla, 1986; Hare, 2002; M. A. Streisfeld, personal observation). Therefore, hypotheses concerning indirect selection acting on reproductive rather than vegetative structures are the most tenable. For example, Irwin et al. (2003) found that the presence of anthocyanin floral pigments in Raphanus sativus significantly reduced the preference and performance of various leaf and floral herbivores on plants with pigmented flowers and increased the concentration of inducible defensive secondary compounds in leaf tissue. These data were taken to suggest that herbivores could act to oppose selection by pollinators for flowers lacking anthocyanin floral pigments. Although this indicates that changes in floral pigments can have a substantial effect on nonpollinator mediated selection and herbivore damage can help to maintain a floral colour polymorphism, the colour morph that contains the anthocyanin pigments in M. aurantiacus also is visited more frequently by pollinators. In addition, we observed no obvious difference and overall low levels of insect damage to flowers and fruits in coastal and inland regions in San Diego County (M. A. Streisfeld, personal observation). This makes it unlikely that indirect selection on floral pigments mediated through differences in insect damage to reproductive structures is occurring here. Nevertheless, future work should examine if levels of defensive compounds differ among floral tissues of each morph and whether this is associated with any differences in insect damage.

Conclusions

In M. aurantiacus, strong geographic differentiation in flower colour is accompanied by little corresponding divergence at neutral loci (Streisfeld & Kohn, 2005). This suggests that strong natural selection has been responsible for differentiation of floral traits. The strong preferences by different pollinators for alternative floral forms (Table 1) support Grant's (1993) view that selection imposed by different pollinators may have brought about the observed pattern of floral divergence. However, experiments in the current study demonstrate a consistent advantage for the red floral race in survival, growth and pollinator visitation and we are unable to identify factors that currently favour yellow flowers in inland regions.

Several scenarios could explain these results. First, indirect selection could still favour yellow flowers in inland regions if selective differences expressed at seed germination or after the first growing season favour the yellow race in inland regions and are genetically correlated with flower colour differences. Because the apparent need for abiotic stress protection by flavonoids occurs in reverse of regional differences in floral anthocyanin expression and because both floral morphs express these compounds in vegetative tissues, indirect selection on prereproductive components of the life-cycle appears unlikely. Indirect selection acting on reproductive structures could explain these results if flavonoids in flowers protect reproductive structures from insect damage. If unmeasured fitness components are found to favour the yellow race inland, experiments using recombinant individuals will be needed to show that fitness differences are due to genes underlying or linked to flower colour.

Another possibility is that environmental factors that differ between regions could act directly on flower colour to cause differences in fitness among floral races. For example, hotter inland temperatures during flowering could cause a reduction in nectar production or pollen viability in red flowers, as they are likely to attain higher temperatures than yellow flowers. This could have an effect on subsequent pollinator visitation and/or flower fertility in red flowers in the inland habitat (Jewell et al., 1994). Although we have not directly tested these hypotheses, warmer inland temperatures did not cause reduced visitation to red flowers during the array experiments, as might be expected if nectar production was impacted. Finally, very high pollinator efficiencies of hawkmoths relative to hummingbirds could favour the yellow morph in the east, despite lower rates of visitation.

Alternatively, current selection may not reflect the pattern of historical selection that created the observed differentiation in floral forms. Estimates indicate that the density of hummingbirds, particularly Anna's, has increased markedly in recent decades in San Diego County (Unitt, 2004; Fig. 4), such that current densities in inland regions greatly exceed those recorded in the coastal region 50 years ago. Increased densities of Anna's hummingbirds occur both in urban and natural settings (Bolger et al., 1997; Unitt, 2004). Therefore, selection may currently favour red flowers in both regions and the red-flowered morph will advance eastward.

Future studies of patterns of variation surrounding the genes controlling floral traits hold the potential to tell us much about the extent of historical selection that created floral divergence. Tests of the effect of flower colour in different environments on insect damage, pollen fertility and nectar production would help further evaluate the possibility of nonpollinator mediated selection. Further trials with pollinators that disassociate flower colour, size and nectar differences can help us dissect which traits are most important to visitation by each pollinator. Finally,

studies of the effects of urbanization on the abundances of different pollinators can help us predict changes in the selective regime and future evolution, of this and other species.

Acknowledgments

References

Adler, L.S., Karban, R. & Strauss, S.Y. 2001. Direct and indirect effects of alkaloids on plant fitness via herbivory and pollination. *Ecology* **82**: 2032–2044.

Antonovics, J. & Bradshaw, A.D. 1971. Evolution in closely adjacent plant populations. VIII. Clinal patterns at a mine boundary. *Heredity* **25**: 349–362.

Armbruster, W.S. 2002. Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. *J. Evol. Biol.* **15**: 468–486.

Beeks, R.M. 1962. Variation and hybridization in southern California populations of *Diplacus scrophulariaceae*. *Aliso* **5**: 83–122.

Bennington, C.C. & McGraw, J.B. 1995. Natural selection and ecotypic differentiation in *Impatiens pallida*. *Ecol. Monogr.* **65**: 303–323.

Bolger, D.T., Scott, T.A. & Rotenberry, J.T. 1997. Breeding bird abundance in an urbanizing landscape in coastal southern California. *Conserv. Biol.* **11**: 406–421.

Bradshaw, A.D. 1984. Ecological significance of genetic variation between populations. In: *Perspectives in Plant Population Biology* (R. Dirzo & J. Sarukhan, eds), pp. 213–228. Sinauer, Sunderland, MA, USA.

Bradshaw, H.D., Jr & Schemske, D.W. 2003. Allele substitution at a flower color locus produces a pollinator shift in monkeyflowers. *Nature* **426**: 126–128.

Campbell, D.R., Waser, N.M. & Wolf, P.G. 1998. Pollen transfer by natural hybrids and parental species in an *Ipomopsis* hybrid zone. *Evolution* **52**: 1602–1611.

Castellanos, M.C., Wilson, P. & Thomson, J.D. 2003. Pollen transfer by hummingbirds and bumblebees, and the divergence of pollination modes in *Penstemon*. *Evolution* **57**: 2743–2752.

Chitka, L., Spaethe, J., Schmidt, A. & Hickelsberger, A. 2001. Adaptation, constraint, and chance in the evolution of flower color and pollinator color vision. In: *Cognitive Ecology of Pollination: Animal Behavior and Floral Evolution* (L. Chitka, &

J. D. Thomson, eds), pp. 106–126. Cambridge Univ. Press, Cambridge.

Clausen, J., Keck, D.D. & Hiesey, W.M. 1940. *Experimental Studies on the Nature of Species I. The Effects of Varied Environments on Western American Plants*. Carnegie Inst. Washington Publ. No. 520.

Clausen, J., Keck, D.D. & Hiesey, W.M. 1948. *Experimental Studies on the Nature of Species III. Environmental Responses of Climatic Races of Achillea*. Carnegie Inst. Washington Publ. No. 581.

Coberly, L.C. & Rausher, M.D. 2003. Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. *Mol. Ecol.* **12**: 1113–1124.

Conner, J.K. 2002. Genetic mechanisms of floral trait correlations in a natural plant population. *Nature* **420**: 407–410.

Endler, J.A. 1977. *Geographic Variation, Speciation, and Clines*. Princeton University Press, Princeton, New Jersey, USA.

Faegri, K. & Van der Pijl, L. 1971. *The Principles of Pollination Ecology*. 2nd edn. Pergamon, Oxford.

Fenster, C.B., Armbruster, W.S., Wilson, P., Dudash, M.R. & Thomson, J.D. 2004. Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Syst.* **35**: 375–403.

Fineblum, W.L. & Rausher, M.D. 1997. Do floral pigmentation genes also influence resistance to enemies? The W locus in *Ipomoea purpurea*. *Ecology* **78**: 1646–1654.

Frey, F.M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia viginica* (Portulacaceae). *Evolution* **58**: 2426–2437.

Grant, V. 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* **3**: 82–97.

Grant, V. 1981. *Plant Speciation*, 2nd edn. Columbia University Press, New York, USA.

Grant, V. 1993. Origin of floral isolation between ornithophilous and sphingophilous plant species. *Proc. Natl Acad. Sci. U. S. A.* **90**: 7729–7733.

Harborne, J.B. 1976. Functions of flavonoids in plants. In: *Chemistry and Biochemistry of Plant Pigments* (T. W. Goodwin, ed), pp. 736–778. Academic Press, London.

Hare, J.D. 2002. Geographic and genetic variation in the leaf surface resin components of *Mimulus aurantiacus* from southern California. *Biochem. Syst. Ecol.* **30**: 281–296.

Healy, S.D. & Hurley, A.T. 2001. Foraging and spatial learning in hummingbirds. In: *Cognitive Ecology of Pollination: Animal Behavior and Floral Evolution* (L. Chitka & J. D. Thomson, eds), pp. 127–147. Cambridge University Press, Cambridge.

Hiesey, W.M., Nobs, M.A. & Bjorkman, O. 1971. *Experimental Studies on the Nature of Species V. Biosystematics, Genetics, and Physiological Ecology of the Erythranthe Section of Mimulus*. Carnegie Inst. Washington Publ. No. 628.

Irwin, R.E., Strauss, S.Y., Storz, S., Emerson, A. & Guibert, G. 2003. The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* **84**: 1733–1743.

Jewell, J., McKee, J. & Richards, A.J. 1994. The keel colour polymorphism in *Lotus corniculatus* L.: differences in internal flower temperatures. *New Phytol.* **128**: 363–368.

Johnson, S.D. & Steiner, K.E. 2000. Generalization versus specialization in plant pollination systems. *Trends Ecol. Evol.* **15**: 140–143.

Kelber, A., Balkenius, A. & Warrant, E.J. 2002. Scotopic colour vision in nocturnal hawkmoths. *Nature* **419**: 922–925.

Koes, R.E., Quattrocchio, R. & Mol, J.N.M. 1994. The flavonoid biosynthetic pathway in plants: function and evolution. *Bioessays* **16**: 123–132.

Lande, R. & Arnold, S.J. 1983. The measurement of selection correlated characters. *Evolution* **37**: 1210–1226.

Lincoln, D.E. 1980. Leaf resin flavonoids of *Diplacus aurantiacus*. *Biochem. Syst. Ecol.* **8**: 397–400.

Lincoln, D.E. & Walla, M.D. 1986. Flavonoids from *Diplacus aurantiacus* leaf resin. *Biochem. Syst. Ecol.* **14**: 195–198.

Littell, R.C., Milliken, G.A., Stroup, W.W. & Wolfinger, R.D. 1996. *SAS System for Mixed Models*. SAS Institute, Cary, North Carolina, USA.

Mayfield, M.M., Waser, N.M. & Price, M.V. 2001. Exploring the 'most effective pollinator principle' with complex flowers: bumblebees and *Ipomopsis aggregata*. *Ann. Bot.* **88**: 591–596.

McMinn, H.E. 1951. Studies in the genus *Diplacus*, Scrophulariaceae. *Madroño* **11**: 1–32.

Meléndez-Ackerman, E., Campbell, D.R. & Waser, N.M. 1997. Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecology* **78**: 2532–2541.

Munz, P.A. 1973. *A California Flora and Supplement*. University of. California Press, Los Angeles, California, USA.

National Audubon Society. 2002. *The Christmas Bird Count Historical Results [Online]*. Available at http://www.audubon.org/bird/cbc/hr.

Ollerton, J. 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-pollinator systems. *J. Ecol.* **84**: 767–769.

Schemske, D.W. & Bradshaw, H.D., Jr 1999. Pollinator preference and the evolution of floral traits in monkey flower *Mimulus*. *Proc. Natl Acad. Sci. U. S. A.* **96**: 11910–11915.

Schemske, D.W. & Horvitz, C.C. 1984. Variation among floral visitors in pollination ability: a precondition for mutualism specialization. *Science* **225**: 519–521.

Shirley, B.W. 1996. Flavonoid biosynthesis: new functions for an old pathway. *Trends Plant Sci.* **1**: 377–382.

Slatkin, M. 1985. Gene flow in natural populations. *Annu. Rev. Ecol. Syst.* **16**: 393–430.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. *Science.* **236**: 787–792.

Stebbins, G.L., Jr 1970. Adaptive radiation of reproductive characteristics in Angiosperms I: pollination mechanisms. *Annu. Rev. Ecol. Syst.* **1**: 327–354.

Strauss, S.Y., Irwin, R.E. & Lambrix, V.M. 2004. Optimal defense theory and flower petal colour predict variation in the secondary chemistry of wild radish. *J. Ecol.* **92**: 132–141.

Streisfeld, M.A. & Kohn, J.R. 2005. Contrasting patterns of floral and molecular variation across a cline in *Mimulus aurantiacus*. *Evolution* **59**: 2548–2559.

Thompson, D.M. 1993. *Mimulus*. In: *The Jepson Manual; Higher Plants of California* (J. C. Hickman, ed.), pp. 1037–1047. University of California Press, Los Angeles, California, USA.

Thomson, J.D. 2003. When is it mutualism? 2001 presidential address, American Society of Naturalists. *Am. Nat.* **162**: S1–S9.

Thomson, J.D., Wilson, P., Valenzuela, M. & Malzone, M. 2000. Pollen presentation and pollination syndromes, with special reference to *Penstemon*. *Plant Species Biol.* **15**: 11–29.

Tulig, M. 2000. Morphological variation in *Mimulus* section *Diplacus* (Scrophulariaceae). MSc Thesis, California State Polytechnic University, Pomona.

Unitt, P. 2004. *San Diego County Bird Atlas*. Ibis Publishing Co., San Diego, California, USA.

Waayers, G.M. 1996. Hybridization, introgression, and selection in *Mimulus aurantiacus* ssp. *australis* and *Mimulus puniceus*. MSc Thesis, San Diego State University, San Diego.

Waser, N.M. 1998. Pollination, angiosperm speciation, and the nature of species boundaries. *Oikos* **81**: 198–201.

Waser, N.M. & Price, M.V. 1985. Reciprocal transplant experiments with *Delphinium nelsonii* (Ranunculaceae): evidence for local adaptation. *Am. J. Bot.* **72**: 1726–1732.

Waser, N.M., Chitka, L., Price, M.V., Williams, N.M. & Ollerton, J. 1996. Generalization in pollination systems, and why it matters. *Ecology* **77**: 1043–1060.

Wilson, P., Castellanos, M.C., Hogue, J.N., Thomson, J.D. & Armbruster, W.S. 2004. A multivariate search for pollination syndromes among penstemons. *Oikos* **104**: 345–361.