VARIATION IN POLLINATOR PREFERENCE BETWEEN TWO *IPOMOPSIS* CONTACT SITES THAT DIFFER IN HYBRIDIZATION RATE

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Pollinator-mediated reproductive isolation is often a principal factor in determining the rate of hybridization between plant species. Pollinator preference and constancy can reduce interspecific pollen transfer between otherwise interfertile, coflowering species. The importance of this ethological isolation can be assessed by comparing the strength of preference and constancy of pollinators in contact sites that differ in the frequency of hybrid individuals. We observed visitation by hummingbirds and hawkmoths in natural single-species patches and artificial mixed-species arrays in two *Ipomopsis aggregata/I. tenuituba* contact sites—one with few hybrids, and one in which hybrids are abundant. Pollinator preference and constancy were stronger at the low-frequency hybrid site, especially for hawkmoths (*Hyles lineata*). Hawkmoths at the low-frequency hybrid site showed significant preference and constancy for *I. tenuituba*, while at the high-frequency site hawkmoths visited both species equally. One hypothesis that might explain these differences in hawkmoth foraging is that warmer nights at the low-frequency hybrid site allow for nocturnal foraging where the light-colored corollas of *I. tenuituba* have a visibility advantage. These differences in hawkmoth behavior might in turn affect hummingbirds differently at the two sites, through changes in nectar resources, leading to greater pollinator-mediated isolation at the low-frequency hybrid site. Our results suggest that differences in pollinator behaviors between sites can have both direct and indirect effects on hybridization rates between plant species.

KEY WORDS: **Ethological isolation, geographic variation, hummingbirds, hybrid zones, *Hyles lineata,* indirect effects on pollinator behavior, *Ipomopsis,* pollinator preference.**

Hybrid zones provide an opportunity to study the evolution of reproductive mechanisms that isolate species, and the range of possible outcomes following the breakdown of those mechanisms (Hewitt 1988). The strength of reproductive isolating mechanisms varies widely among pairs of taxa (Barton and Hewitt 1989; Jiggins and Mallett 2000), and even among areas of sympatry (contact sites) within single hybridizing species pairs (Williams et al. 2001; Watano et al. 2004; Aldridge 2005a). Such variation

within a species pair is useful for determining the relative importance of different isolating mechanisms. To date, however, little is known about variation in isolating mechanisms across sites and its influence on variation in hybrid formation.

Reproductive isolation in flowering plants is often associated with pollinator behaviors that reduce the interspecific transfer of pollen, termed ethological isolation (Grant 1949). Although the role of ethological isolation in speciation remains controversial (Elam and Linhardt 1988; Waser 1998; Grant 1993a; Waser and Campbell 2004), many pairs of closely related plant species exhibit contrasting suites of floral characteristics that sometimes

correlate with preferential visitation by certain functional groups of pollinators and not others (Fenster et al. 2004). One well-studied contrast is that between avian and insect pollination (Meléndez-Ackerman et al. 1998; Fulton and Hodges 1999; Emms and Arnold 2000; Bradshaw and Schemske 2003; Castellanos et al. 2004; Ippolito et al. 2004). Hummingbird pollination is typically correlated with tubular red corollas, anther and stigma exsertion, and production of large volumes of dilute nectar (Grant and Grant 1965; Wilson et al. 2004). The principal characteristics assumed to associate with hawkmoth pollination are pale color and long, narrow corolla tubes (Grant and Grant 1983). It is important to note that these suites of floral traits do not necessarily preclude effective pollination by "nonsyndrome" pollinators, nor do they always predict preferential visitation. The closely related herbs *Ipomopsis aggregata* and *I. tenuituba* have been cited as an example of ethological isolation by specialization on hummingbird (*I. aggregata*) and hawkmoth (*I. tenuituba*) pollinators (Grant and Grant 1965), although such ethological isolation is not strong in the only natural hybrid zone that has been extensively studied to date (Campbell et al. 2002b). However, that hybrid zone contains high frequencies of hybrids (Wu and Campbell 2005), whereas other natural hybrid zones between *I. aggregata* and *I. tenuituba* have a relatively low frequency of hybrids and also differ in spatial arrangement of the parent and hybrid populations (Aldridge 2005a). This variation provides an opportunity to study the importance of ethological isolation in determining rates of natural hybridization.

Ethological isolation comprises two distinct pollinator behaviors, preference and constancy (Campbell and Motten 1985; Waser 1986) that have not generally been separated in studies of hybridization (Chittka et al. 1999). Preference refers to the net overexploitation of one resource type in the presence of alternative types (Cock 1978). Preference would result in a pollinator type consistently visiting a type of flower in disproportion to the frequency of that type in the environment. Constancy refers to the tendency of an individual animal to make consecutive visits to the same type of flower, skipping intervening alternative types (reviewed in Waser 1986). It is possible for a pollinator type to show no preference even though individual pollinators tend to visit the same flower species consecutively. Both pollinator behaviors can result in assortative mating among flower types through reduced pollen transfer between types.

In addition to determining how pollinator preference and constancy change geographically, it would be useful to understand the potential mechanisms behind these changes. Pollinator behavior responds to both biotic and abiotic conditions. The plant community will affect the relative reward value of a flower (Meléndez-Ackerman et al. 1997), and the abundance and makeup of the pollinator assemblage (Moeller 2005). Climate variables such as temperature can also affect the pollinator assemblage (Fenster and

Dudash 2001; Silva-Montellano and Eguiarte 2003). Pollinator behavior varies among populations within hybrid zones (Emms and Arnold 2000), suggesting that local conditions such as spatial distribution and abundance of focal plant species can affect ethological isolation as well. Since the mechanism underlying variation in pollinator behavior often relates to the net rate of energy intake from a flower (Heinrich and Raven 1972; Pyke 1981), quantifying nectar resources offered by flowers in different sites would provide a first step toward understanding any differences in behavioral responses of pollinators to the same plant species.

In this study, we compared preference, constancy, and the resulting level of ethological isolation for the principal pollinators of *I. aggregata* and *I. tenuituba* (Polemoniaceae) in two contact sites. These sites differ in both the frequency of hybrids, and the spatial structure of the parental populations (clinal vs. mosaic; Aldridge 2005a). We observed visitation patterns of hummingbirds (*Selasphorus* spp.) and hawkmoths (*Hyles lineata*) on natural and artificial patches of flowers at both sites to address the following questions: (1) Is ethological isolation weaker at a contact site where hybrid individuals are abundant than at one where they are scarce, potentially explaining the difference in hybrid frequency? (2) Do pollinators show similar patterns of preference and constancy on plants in artificial arrays moved between sites, indicating that differences in ethological isolation between sites reflect pollinator behavior rather than differences in floral morphology? (3) Does intersite variation in temporal patterns of nectar availability influence ethological isolation?

Materials and Methods
STUDY SYSTEM

This study was conducted in two contact sites of *I. aggregata* subsp. *aggregata* (Pursh) V. Grant and *I. tenuituba* (Rydb.) V. Grant in western Colorado. These monocarpic perennials occur widely throughout the western United States and form variable hybrid zones in contact (Grant and Wilken 1988; Aldridge 2005a). Flowers of both species have tubular corollas, and exhibit the characteristic colors and morphologies of hummingbird and hawkmoth pollination syndromes, respectively (Grant and Grant 1965). The study sites were chosen because they represent a contrast between a clinal site with high hybridization and a mosaic site with low hybridization (Aldridge 2005a) and thus provide an opportunity for a comparison of the importance of individual reproductive isolating mechanisms in determining rates of natural hybridization (Williams et al. 2001).

Poverty Gulch (PG) is a tributary of the Slate River in Gunnison County, Colorado. Populations of *I. aggregata* and *I. tenuituba* lie at opposite ends of an altitudinal cline, with *I. aggregata* growing in meadows up to 2900 m, *I. tenuituba* growing on rocky slopes at 3100–3250 m, and an extensive hybrid swarm in between

(Grant and Wilken 1988; Wu and Campbell 2005). Grizzly Ridge (GR) lies on the north rim of the Black Canyon of the Gunnison, in Montrose County, Colorado. Elevations at GR range from 2375–2450 m, and both *Ipomopsis* species occur there in scattered, discrete patches within sagebrush and oak scrub vegetation at similar elevations. Although patches of different species grow within meters of each other, morphologically and genetically intermediate individuals are rare at GR (Aldridge 2005a). Analysis of molecular data by the admixture program STRUCTURE (Pritchard et al. 2000) suggests two groups (corresponding to two species) at GR rather than three groups as at PG (Aldridge 2005b). Similarities in elevation, as well as in soils and vegetation, between patches of the two species at GR suggest that species sorting due to environmental heterogeneity is unlikely to explain the low frequency of hybrids at that site.

Plants at both sites exhibit the patterns of floral morphology that distinguish the species (see Aldridge 2005a for description of morphological data), with *I. aggregata* (Fig. 1–filled symbols) having shorter, wider corollas than *I. tenuituba* (see Fig. 1–open symbols). However, there is a potentially important difference between the sites in that the two species are more divergent for corolla width at GR (see Fig. 1–circles) and for corolla length at PG (see Fig. 1–squares). Hummingbirds at PG preferentially visit flowers with wider corollas, and selection for that trait is typically directional in both species due to scarcity of hawkmoths at that site (Campbell et al. 1997; Meléndez-Ackerman et al. 1997). In contrast, hawkmoths are consistently abundant at GR, perhaps

due to the warmer climate. During the period 10 July 2004-18 Aug. 2004, daytime temperatures at GR averaged 8.9°C higher ($t_{78} = 7.30$, $P < 0.0001$), and nighttime lows 2.8°C higher ($t_{78} = 31.11$, $P < 0.0001$) than at PG. There is considerable overlap in flowering time between species at both sites, but flowering starts and finishes earlier at GR (late May–early July) than at PG (late June–early August). This is likely due to the hotter, dryer climate at GR, as potted plants from there will continue flowering into September when kept watered in a greenhouse.

POLLINATOR OBSERVATIONS IN NATURAL POPULATIONS

In order to quantify natural premating isolation at each site, observations of pollinator visits to natural populations were conducted in 2001. To factor out effects of time of observation, we used Latin square designs to assign patches to observation times. At GR, four patches of each species were observed at four times of day, across four days in a 4×4 Latin square design. Observations were in 2-h time blocks beginning at 0600, 1000, 1400, and 1900 h. One Latin square was conducted for each species separately, and one conducted on pairs of patches, one of each species, observed simultaneously. Observations were conducted on *I. tenuituba* from 28 May to 31 May, both species from 6 June to 9 June, and *I. aggregata* from 16 June to 19 June, for a total of 64 h of observation of each species. Observations at PG were conducted in a similar manner, except that the midday time blocks were eliminated, resulting in only two patches of each species being observed at two
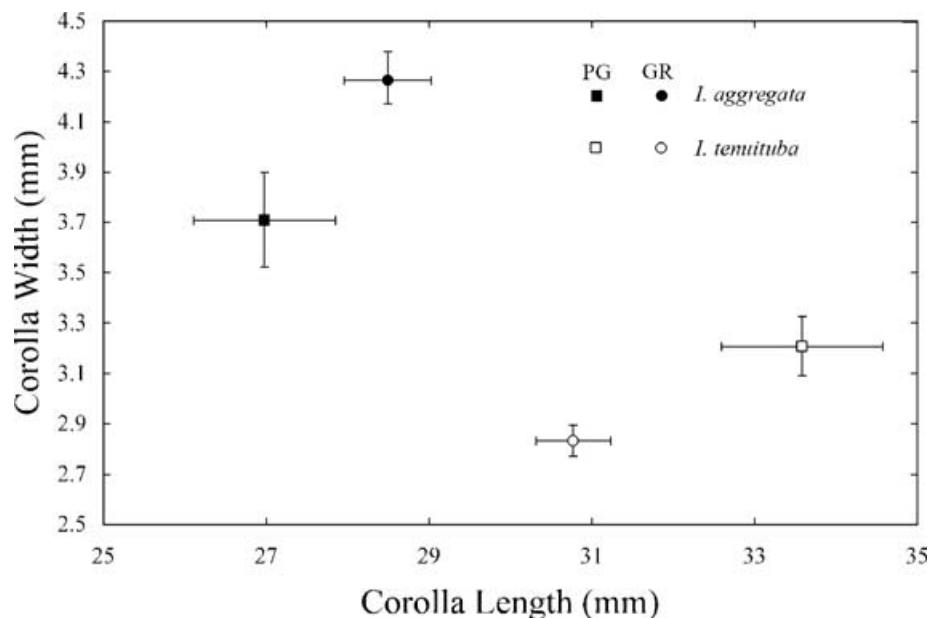

Figure 1. Corolla length and width of *Ipomopsis aggregata* and *I. tenuituba* from two contact sites in western Colorado. Points are means for each species at each site; bars are 95% confidence intervals. Sample sizes: Grizzly Ridge (GR) *tenuituba*, 107; GR aggregata, 70; Poverty Gulch (PG) tenuituba, 30; PG aggregata, 20. Symbols are: open, *I. tenuituba*; filled, *I. aggregata*; circles, Grizzly Ridge, Montrose, Co.; squares, Poverty Gulch, Gunnison, Co. Note that the species are more divergent for width at GR, and for length at PG.

time blocks each day, over two days (2 × 2 Latin square). Results from GR indicated that visitation was very low during midday, so observations at PG took place at 0600 and 1900 h only. Patches at PG were always observed in pairs, one of each species, one observer per patch. Observations at PG occurred on 07/12–07/13 and 07/16–07/17, for a total of 8 h per species. In all cases, the patches observed contained 15–20 flowering plants. Data for each observation period consisted of total flowers open in the patch, the type of visitor, sequence of plants visited, and number of flowers probed on each plant for every visitor. For each patch, we calculated a visit rate (visits per flower per hour) by each pollinator type. These rates were calculated from pooled visit data from all observations (paired and unpaired at GR). We conducted a two-way analysis of variance (ANOVA) for each site, testing the effects of plant species, visitor type, and their interaction, on visit rate, with patches as replicates. A significant interaction would indicate that the two plant species tend to be visited by different visitor types. Only hummingbirds and hawkmoths were observed visiting. Other potential pollinators are extremely rare; for example, over 95% of all visits to *I. aggregata* in areas near PG are by hummingbirds or hawkmoths (Price et al. 2005). Observations were repeated in 2002 in two patches of each species over four days to confirm that visitation patterns were similar to those observed in 2001.

POLLINATOR PREFERENCE AND CONSTANCY AT ARRAYS

Natural visitation patterns by pollinators ultimately determine premating reproductive isolation, but observations of pollinator visitation to single-species natural patches within a hybrid zone cannot distinguish preference from constancy, nor can they tell us if differences in behavior between two sites reflect site differences or plant differences. In order to measure preference and constancy, we presented pollinators with immediate choices of >1 flower type in random spatial arrangement in a single foraging bout (Waser 1986). Exposing pollinators at both sites to plants from both source sites also made it possible to determine if differences in pollinator preferences between natural patches are due to differences in plant characteristics or differences in pollinator responses to the same plants. To do this, artificial mixed-species arrays were constructed using potted plants. Arrays consisted of 12 plants of each species, with approximately equal total numbers of flowers, evenly spaced in random order in a 4 m × 6 m rectangular area. To avoid genetic contamination of natural populations, all flowers in the array were emasculated prior to placing out for observation. Arrays of plants from GR were placed at GR and at PG in summer 2003, and arrays of PG plants placed at both sites in summer 2004. In 2003, arrays could be placed at each site during peak flowering (GR: 05/31–06/13; PG: 07/11–07/21); however, because the later flowering season at PG delayed availability of

those plants, arrays could not be set out at GR until well past peak flowering (07/08–07/15). Despite the lateness of the experiment at GR, plants of both species were still in flower at that site during the array observations, and both hummingbirds and hawkmoths were still present at the site and actively foraging. Arrays were rotated among three different locations at each site to avoid repeated sampling of the same visitors.

Unlike natural populations, array observations were not conducted at regular times, but rather continued until approximately 20 foraging bouts by each pollinator type were recorded at each site. Potted plants tended to wilt in midday heat, so hours of observation concentrated in the morning and evening. Typically, observations began around 0530, ceased around 1000, resumed around 1800, and ended after dark around 2130 h. In rare cases at GR, visits were recorded (by flashlight) between 0200 and 0400 h. Array observations totaled 151 h in 2003 and 45 h in 2004. Arrays were re-randomized each time they were set out, and visitation data were collected from a total of 45 different array configurations. For each visitor to the array, the pollinator type, sequence of plants visited, and number of flowers probed on each plant were recorded. These data were used to calculate an overall measure of ethological reproductive isolation (ERI) as: 1–(heterospecific plant transitions/conspecific plant transitions) (Ramsey et al. 2003). We calculated ERI for each of the four combinations of site of origin and site of observation in the array experiment, pooling data for all pollinators in each case.

We employed three statistical approaches to test for pollinator preference, pollinator constancy, and the resulting level of assortative mating, for hummingbirds and hawkmoths separately. To test pollinator preference, for each type of visitor and array, the proportion of visits to *I. aggregata* was compared to the null expectation of 0.5 using one-sample t-tests with a foraging bout as a replicate. Significant departure from the null expectation was interpreted as pollinator preference for the species visited more frequently. Variation in strength of preference across the two sites and two sources of plants was tested using two-way ANOVA on the proportion of visits to *I. aggregata.* All proportions were arcsine-square-root transformed prior to analysis. Our second approach tested constancy, defined as rejection of the null hypothesis that the next species a pollinator visits is independent of the species just visited (Waser 1986). Thus defined, constancy results in a long series of conspecific plant-–plant movements within a foraging bout that might still include an equal number of visits to all available species. In such a case a pollinator type could lack preference and yet enforce ethological isolation by constancy. So, in cases where both plant species were visited more than occasionally (>10%), we tested constancy using G-tests of independence to see whether the type of plant visited depended on the type last visited (Sokal and Rohlf 1995). Third, the combined influence of preference and constancy on assortative mating was analyzed by

comparing the proportion of conspecific movements in a foraging bout to the null expectation of 0.5. Variation in the strength of assortative mating was assessed by two-way ANOVA, on the proportion of conspecific movements, using the factors of site and source of plants.

NECTAR RESOURCES AND FRUIT SET

Nectar resources both affect and reflect pollinator visitation patterns. The standing crop of nectar available in a population of flowers will reflect recent visitation and affect it in the immediate future. Nectar production rate was measured over 48 h ($n = 30$ plants/species at GR, 06/01/04–06/03/04; $n = 21$ plants/species at PG, 07/26/04–07/28/04). Three unopened buds per plant were enclosed in 2-inch lengths of plastic drinking straw crimped at one end to exclude pollinators, and collected 48 h later for measurement of nectar volume and sugar concentration. Nectar standing crop was measured in natural populations in the evening (∼1900 h), before the start of heavy foraging activity by hawkmoths, and the following morning (∼0600 h), just prior to the appearance of hummingbirds. Measurements were averaged across three flowers per plant for 30 plants per species at GR and 40 plants per species at PG. Different flowers on the same plants were used for each time period, and all plants of both species were measured within approximately 2 h. Standing crop was compared between evening and morning using repeated measures ANOVA (SPSS Inc., Chicago, IL) to account for individual plant effects. All nectar measurements were performed during peak flowering at each site.

Observations of pollinators provide a snapshot of pollinator behavior, but plants might receive visits during hours when observations are not being performed. Measurements of female reproductive success integrate visitation over a season to the extent that seed production is pollen limited. Seed production is pollen limited, at least in *I. tenuituba* populations at PG (Campbell et al. 1997, 2002b). Variation in seed production should reflect variation in estimated rates of pollinator visitation if observations are capturing an accurate sample of visitation. In order to estimate indirectly the pollination services to the species at each site, inflorescence stalks were collected in 2002 late in the season after seed dispersal. Aborted buds, calyces, and dehisced capsules remain on the inflorescence stalk after the plant dies, allowing measurement of the numbers of flowers and successful fruits made by a plant during its one reproductive season. Stalks were collected at random from one patch of *I. aggregata* ($n = 15$) and three patches of *I. tenuituba* ($n = 29$) at each site. We compared total flowers, fruits initiated per flower, and dehisced capsules per initiated fruit for the two species using separate *t*-tests for each site. All proportions were arcsine square-root transformed before analysis.

Results

POLLINATOR OBSERVATIONS IN NATURAL POPULATIONS

Pollinator assemblages were similar at both GR and PG; we observed one species of hawkmoth (*Hyles lineata*) and three species of hummingbird (*Selasphorus platycercus*–"Broad tailed" and *S. rufus*–"Rufous" at GR and PG, and *Archilocus alexandri*–"Black chinned" at GR). Visitation rates to natural populations by hawkmoths at GR were much higher than those by hummingbirds, and pollinators showed complete fidelity to one species or the other at that site (see Fig. 2). Hummingbirds visited only *I. aggregata* patches, and hawkmoths only *I. tenuituba*, although both types of visitor were observed flying through patches of
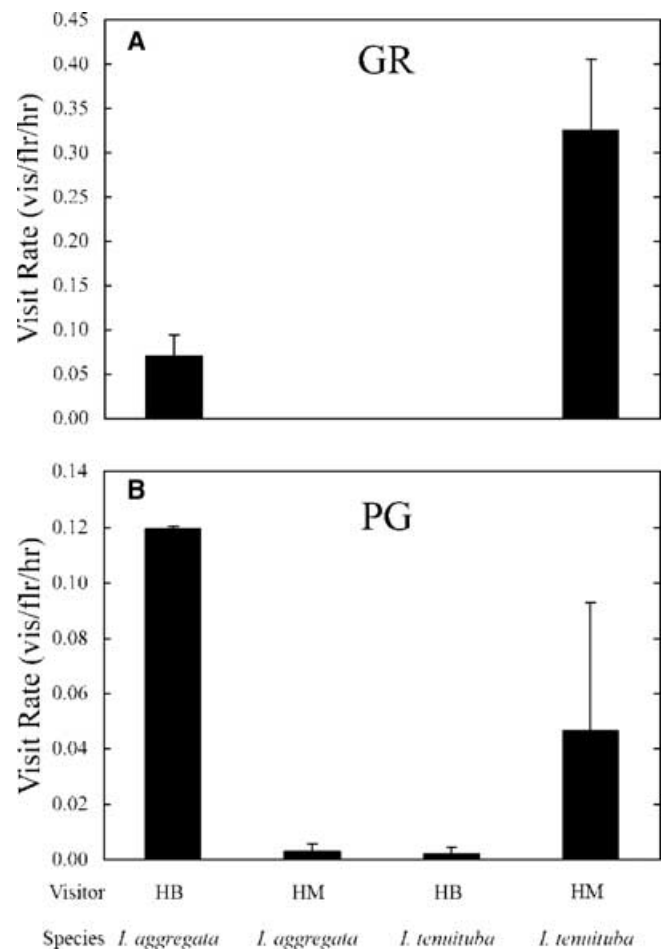

Figure 2. Visitation rates of principal pollinators to natural populations of *Ipomopsis aggregata* and *I. tenuituba* at two contact sites in western Colorado, Grizzly Ridge (GR), Montrose Co., and Poverty Gulch (PG), Gunnison Co. Visitors: HB = hummingbirds (*Selasphorus platycercus*, *S. rufus*); HM = hawkmoths (*Hyles lineata*). Bars are ± 1 standard error of the mean (SEM) of repeated observations of multiple populations (GR: $n = 4$/sp.; PG: $n = 2$/sp.). Note change of scale on y-axis.

both plant species without stopping. This pattern suggested strong ethological isolation at GR due to contrasting pollinator preferences, as indicated by a plant $\times$ visitor type interaction on visit rate to a patch ($F_{1,12} = 9.160$, $P = 0.001$). Preferences were also strong at PG (ANOVA plant $\times$ visitor interaction: $F_{1,4} = 11.895$, $P = 0.026$), but with some overlap of both visitors onto both plant species (Fig. 2b). We observed many more hawkmoth visits at GR than at PG (118 foraging bouts, 2054 flowers at GR vs. 11 foraging bouts, 224 flowers at PG), while hummingbird visitation was similar (26 foraging bouts, 349 flowers at GR vs. 28 foraging bouts, 446 flowers at PG). However, correcting for the much greater amount of time spent observing at GR indicated that hawkmoth visitors to *Ipomopsis* were approximately 50% more numerous at GR than at PG (0.922 foraging bouts/h vs. 0.688 foraging bouts/h, respectively), and hummingbird visitors to *Ipomopsis* were much more common at PG than at GR (1.625 bouts/h vs. 0.219 bouts/h, respectively).

POLLINATOR PREFERENCE AND CONSTANCY AT ARRAYS

As in the natural populations, hawkmoths were less common than hummingbirds at arrays set up at PG in both 2003 (12 vs. 29 foraging bouts, respectively) and 2004 (5 vs. 17 foraging bouts,

respectively). At GR the pattern changed between 2003 (39 vs. 15 foraging bouts, respectively) and 2004 (10 vs. 18 foraging bouts, respectively) perhaps because the experiments were conducted there after the peak of flowering in the latter year. Regardless of their abundance, hawkmoths tended to forage in late evening (both sites) and early morning (GR), especially in hotter weather at GR (2004), when all hawkmoth visits were observed between 2030 and 0530 h. Ethological reproductive isolation was highest for GR plants at GR (0.962), followed by PG plants at PG (0.689) and GR plants at PG (0.582), and lowest for PG plants at GR (0.297).

These differences in the overall measure of ethological isolation reflected behavioral responses by both hummingbirds and hawkmoths (see Fig. 3). Hummingbirds exhibited strong preference for *I. aggregata* in artificial arrays at both sites, while hawkmoths weakly preferred *I. tenuituba* (proportion of total visits by all pollinators to *I. aggregata* ANOVA: $F_{1,143} = 314.7$, $P < 0.001$). Hummingbird preference was weakest on PG plants at GR, but significant in all cases (see Fig. 3). Hawkmoth preference was significant only on GR plants at GR; nowhere else did the proportion of hawkmoth visits to *I. aggregata* differ significantly from 0.5 (see Fig. 3). There was a significant interaction of site and source on proportion of visits to *I. aggregata* for both hawkmoths ($F_{1,62} = 9.850$, $P = 0.003$) and hummingbirds ($F_{1,75} = 6.655$,
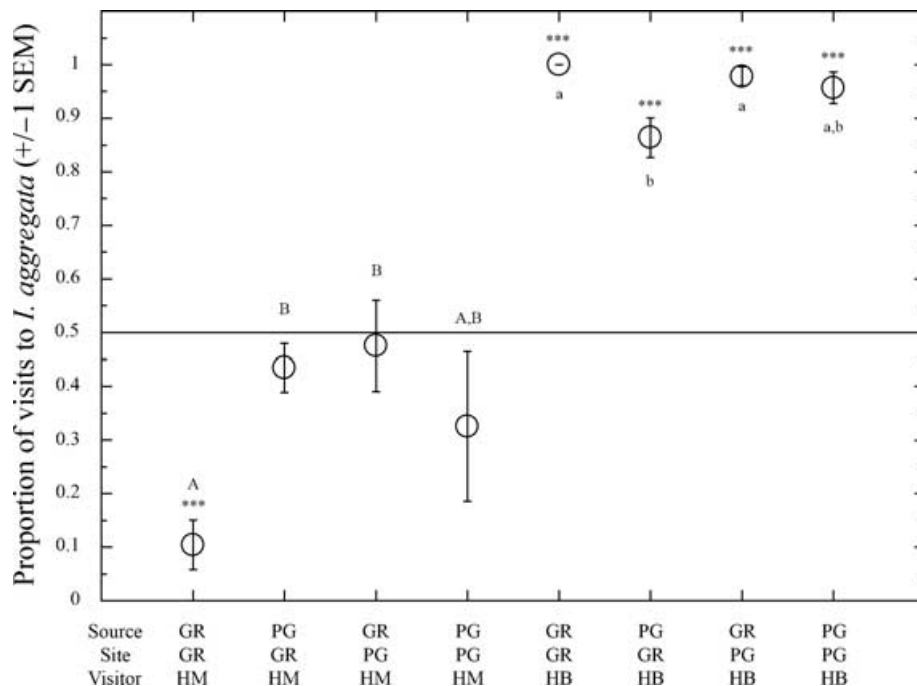

Figure 3. Pollinator preferences measured as proportion of visits to *Ipomopsis aggregata* by hummingbirds (HB) and hawkmoths (HM) visiting artificial populations of *I. aggregata* and *I. tenuituba*. Observations were conducted at two sites in Colorado, Grizzly Ridge, Montrose County (GR), and Poverty Gulch, Gunnison County (PG). Populations consisted of equal numbers of each species in random arrangement, drawn from either of two sources: PG or GR. *** indicates significance of 1-sample *t*-tests comparing mean proportion to 0.5 (arcsine square-root transformed): $P < 0.001$. Letters indicate Tamhane's T2 post hoc groupings from 1-way ANOVA comparing proportion of visits to *I. aggregata* (arcsine square-root transformed) across the four site and source combinations for each pollinator type. Upper case letters show groupings for hawkmoths; lower case letters show groupings for hummingbirds.

Table 1. Plant–plant movements by hummingbird (HB) and hawkmoth (HM) visitors to experimental arrays of *Ipomopsis aggregata* (a) and *I. tenuituba* (t) plants in western Colorado. Arrays consisted of equal numbers of each plant species, drawn from one of two sources: Grizzly Ridge, Montrose County (GR), and Poverty Gulch, Gunnison County (PG). Observations were conducted at those same sites. G-values are from tests of independence for 2 × 2 tables of moves (from – to). Dashes indicate where some expected values were too small to perform G-tests. Bateman's 1951 index quantifies constancy on a scale of -1 (complete disassortative mating) to 1 (complete assortative mating) (Waser 1986).

Site	Source	Visitor	Move				G	P	Bateman's index
			a-a	a-t	t-a	t-t			
GR	GR	HB	79	0	0	0	–	–	–
		HM	11	5	5	175	45.3	< 0.001	0.795
	PG	HB	46	12	12	2	0.313	0.576	− 0.112
		HM	24	25	29	39	0.461	0.497	0.064
PG	GR	HB	71	1	2	1	4.09	< 0.05	0.713
		HM	16	20	20	15	1.14	0.284	0.020
	PG	HB	37	2	4	0	–	–	–
		HM	11	5	7	10	2.56	0.109	0.279

$P = 0.012$), as both types of pollinators showed stronger preference on GR plants than on PG plants only when tested at the GR site.

Patterns of constancy were more complex. Hummingbirds visited >1 species in excess of 10% only when foraging on PG plants at GR (see Fig. 3). In that case, they exhibited no significant constancy (likelihood ratio G = 0.313, $P < 0.576$; Table 1). In all other cases, hummingbirds either showed significant constancy (GR plants at PG: G = 4.1, $P < 0.05$) or the contingency tables were too unbalanced to perform G tests because of strong preference (see Table 1). Hawkmoths showed constancy only when foraging on GR plants at GR (G = 45.3, $P < 0.001$; for the other three combinations, $P > 0.10$; see Table 1).

The combined effects of preference and constancy determine the proportion of conspecific moves. Hawkmoth visitation produced no assortative mating at PG, with the proportion of conspecific moves not significantly different from 0.5 (see Fig. 4). At GR, hawkmoth behavior depended on the source of the plants, producing a site × source interaction in two-way ANOVA ($F_{1,58} = 21.032$, $P < 0.001$). The proportion of conspecific moves by hawkmoths at GR was significantly more than 0.5 when foraging on plants from GR, but not when foraging on plants from PG (see Fig. 4). Conversely, hummingbird behavior produced strong assortative mating in all cases except at GR when foraging on PG plants, again leading to a site × source interaction ($F_{1,65} = 6.497$, $P = 0.013$). In the case of hummingbirds, there was also a main effect of source ($F_{1,65} = 20.371$, $P < 0.001$) in which the proportion of conspecific moves was higher on GR plants (see Fig. 4).

NECTAR RESOURCES AND FRUIT SET

Nectar sugar concentrations were higher in *I. tenuituba* (36.04% ± 0.76 SEM) than in *I. aggregata* (29.94% ± 0.72 SEM) at the two sites combined, and higher at GR (34.21% ± 0.69 SEM) than at PG (31.17% ± 1.04 SEM; see Table 2). Sugar concentrations were similar in *I. tenuituba* at both sites, but significantly lower in *I. aggregata* at PG, leading to a site × species interaction (see Table 2). Forty-eight-hour nectar production was lowest in *I. tenuituba* from GR and PG (mean for both sites: 1.22 μL ± 0.10 SEM), intermediate in *I. aggregata* at PG (3.59 μL ± 0.42 SEM) and highest in *I. aggregata* at GR (8.16 μL ± 0.59 SEM; Table 2). The very high nectar reward of GR *I. aggregata* might help explain the strong preference of GR hummingbirds.

Nectar standing crop was higher in *I. aggregata* than in *I. tenuituba* at both sites (see Fig. 5). There was a significant effect of time (evening–morning) on nectar standing crop at both sites, and a significant interaction of time and type of plant (see Table 3; Fig. 5). Nectar standing crop increased between evening and morning in all combinations of species and site except *I. tenuituba* at GR, in which it decreased to nearly 0 μL by morning (see Fig. 5), suggesting activity of nocturnal visitors only on *I. tenuituba* at GR. All rates of overnight increase were similar in the other three site–species combinations, though standing crop was highest overall in *I. aggregata* at GR (2.5–3.5 μL vs. 1.0–2.0 μL for the others), which might reflect the low abundance of hummingbirds at GR.

Fruit set reflected the patterns of pollinator abundance and behavior at the two sites. Although *I. aggregata* and *I. tenuituba* made similar numbers of flowers per plant at GR in 2002 (48.7 vs. 50.6, respectively; $t_{42} = -0.221$, $P = 0.826$), a much smaller proportion of those flowers formed fruits in *I. aggregata* (0.08 vs. 0.332, respectively; $t_{42} = -5.20$, $P < 0.001$), consistent with the lower visitation rates of hummingbirds. At PG, fruit set was higher in *I. aggregata* (0.41 vs. 0.26 for *I. tenuituba*; $t_{42} = 3.647$, $P = 0.001$) despite higher flower production in *I. tenuituba* (108.9 flr/plant
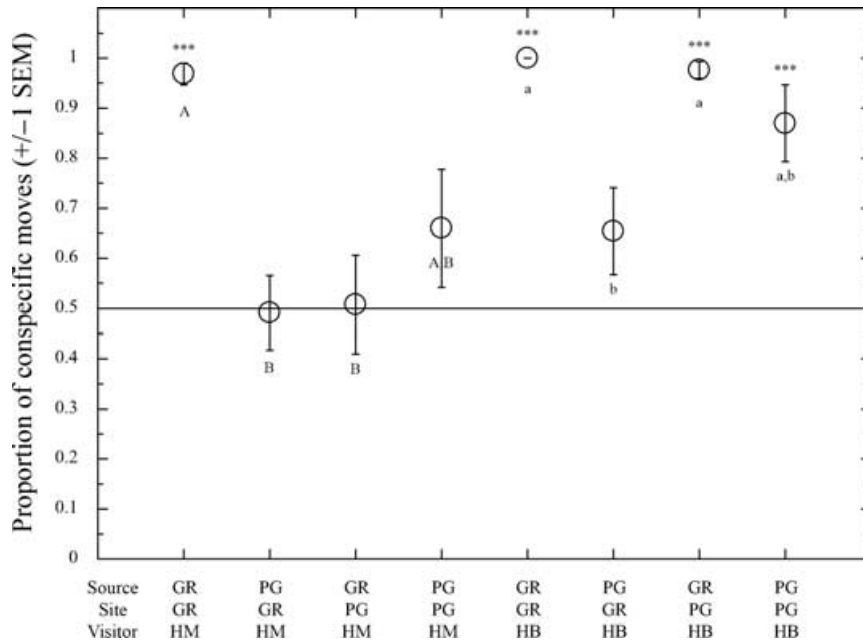
Figure 4. Assortative mating due to pollinator movements measured as proportion of plant–plant moves that were conspecific in artificial populations of *Ipomopsis aggregata* and *I. tenuituba*. Artificial populations consisted of equal numbers of each species in random arrangement, taken from either of two sources in Colorado, USA: Grizzly Ridge, Montrose County (GR), or Poverty Gulch, Gunnison County (PG). Observations of hummingbird (HB) and hawkmoth (HM) visitors were conducted at those same two sites. *** indicates significance of 1-sample *t*-tests comparing mean proportion to 0.5 (arcsine-square root transformed): *P* < 0.001. Letters indicate Tamhane's T2 post hoc groupings from one-way ANOVA comparing proportion of conspecific moves (arcsine square-root transformed) across the four site and source combinations for each pollinator type. Upper case letters show groupings for hawkmoths; lower case letters show groupings for hummingbirds.

vs. 63.5 flr/plant; $t_{41} = -3.187, P < 0.001$), reflecting the paucity of hawkmoth pollinators and the tendency of hummingbirds to avoid *I. tenuituba*. Once initiated, fruits on both species at both sites matured to dehiscence at very high rates (0.89–0.98; *t*-tests for species effects were nonsignificant at both sites).

Discussion

Patterns of pollinator visitation to natural populations suggest that ethological isolation is stronger at GR than at PG, which probably contributes to the low frequency of hybrid individuals at that contact site. Stronger preference by hummingbirds for *I. aggregata*

Table 2. Results of ANOVA comparing (a) sugar concentration (%) and (b) volume (μL) of nectar produced over 48 h by *Ipomopsis aggregata* and *I. tenuituba* at two sites in western Colorado.

Source	Type III SS	df	MS	F	P
(a) Sugar concentration					
Site	234.509	1	234.509	9.969	0.002
Species	1066.634	1	1066.634	45.343	<0.001
Site × Species	203.327	1	203.327	8.644	0.004
Error	2258.264	96	23.524		
Total	3762.734	99			
(b) Volume					
Site	155.009	1	155.009	38.118	<0.001
Species	544.377	1	544.377	133.866	<0.001
Site × Species	104.869	1	104.869	25.788	<0.001
Error	398.525	98	4.067		
Total	2746.413	102			

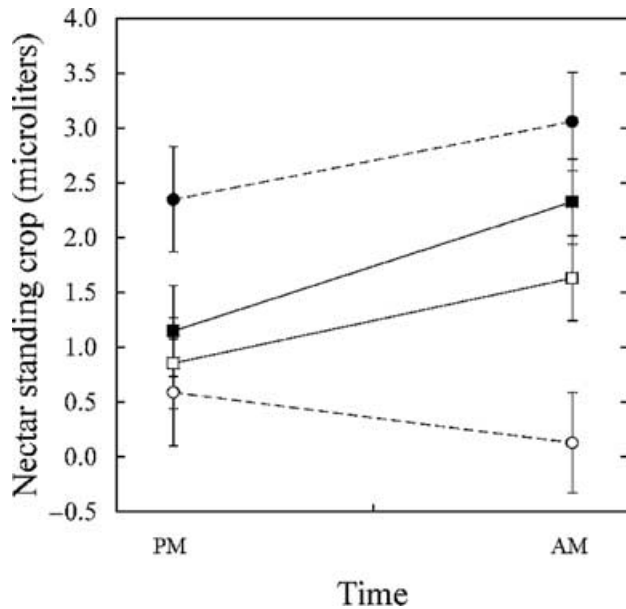Sugar concentration was measured using a refractometer.

Figure 5. Standing crop of nectar measured in two *Ipomopsis aggregata*–*I. tenuituba* contact sites in western Colorado. Nectar measurements were made in the evening (PM: ~1900 h) and the following morning (AM: ~0500 h) on the same plants but different flowers. Contact sites are indicated by circles (Grizzly Ridge, Montrose County) and squares (Poverty Gulch, Gunnison County); species by closed (*I. aggregata*) and open (*I. tenuituba*) symbols.

Table 3. Repeated measures ANOVA of nectar standing crop at two *Ipomopsis aggregata* –*I. tenuituba* contact sites in western Colorado. Nectar was measured on the same plants at ~1900 h and ~0600 h, on three different flowers per plant at each time. The sites were: Grizzly Ridge, Montrose County, and Poverty Gulch, Gunnison County. "Type" refers to each of four combinations of plant species × site. Means are shown in Figure 5.

Source	Type III SS	df	MS	F	P
Type	172.231	3	57.410	22.923	<0.001
Error	338.112	135	2.505		
Time	20.667	1	20.667	26.208	<0.001
Time × Type	23.759	3	7.920	10.043	<0.001
Error	106.459	135	0.789		
Total	150.885	139			

and hawkmoths for *I. tenuituba*, even when presented with both species in the same foraging bout, likely reduces interspecific pollen movement compared to its value at PG. In models of pollen movement at PG (Campbell et al. 2002a) and at GR (Campbell and Aldridge 2006), varying levels of ethological isolation produces model results quite similar to empirical observations of visitation at arrays, while varying the efficiencies of pollinator types as vectors (mechanical isolation; Grant 1949) has little effect on pollen movement. Ethological isolation can be the principal isolating mechanism between species (Husband and Sabara 2003; Ramsey et al. 2003), but it is rarely complete (Grant 1993b, Waser and Campbell 2004); however, GR appears to be a case of nearly total pollinator-mediated reproductive isolation, as each species is exclusively visited by a different pollinator (see Fig. 2). Less obvious is whether the very low number of visits each plant species receives from both pollinator types at PG is sufficient to explain the abundance of hybrids at that contact site. Observations of natural populations at PG indicate only slightly less pollinator fidelity there than at GR, yet hybrids are much more abundant. A possible explanation is that observations at PG were conducted only on patches of parent species and not hybrids. Even when F_1 hybrid formation is very rare, intermediate hybrid individuals can facilitate gene flow by attracting the pollinators of both parent species (Grant 1993b; Emms and Arnold 2000; Wesselingh and Arnold 2000; Broyles 2002; Campbell 2003; Ippolito et al. 2004; but see

Straw 1956). In an earlier study, hybrid patches at PG received a more even mix of visits from hummingbirds and hawkmoths than pure parental patches (Campbell et al. 1997), which could result in higher amounts of gene flow than at GR where the "hybrid bridge" is absent. Similar array experiments conducted at PG that included hybrids showed that pollen transfer from hybrids to parents was as great as intraspecific transfer within parent species, and that hummingbirds were not constant on *I. aggregata* in the presence of hybrids (Campbell et al. 2002a). Hybrids were excluded from the arrays in the present study, as they do not occur at GR, and thus were unavailable. Future studies using artificially produced F1 and F2 hybrids from GR could assess the effect of morphologically intermediate individuals on pollinator movement patterns at that site. Were a "hybrid bridge" effect to be seen at GR, it would suggest either that postzygotic selection prevents the establishment of hybrid patches, or that prezygotic isolation is sufficiently strong to as yet prevent the formation of sufficient numbers of F1 hybrids to serve as a bridge.

Pollinator behavior in artificial arrays differed between sites much more than pollinator behavior in natural patches. Hawkmoths at the two sites especially demonstrated very different responses to mixed-species patches of plants from GR. Hawkmoths at PG tended to visit without regard to plant species, a pattern observed in other studies of hawkmoth foraging on *Ipomopsis* (Elam and Linhart 1988; Campbell et al. 1997; but see Paige and Whitham 1985), while they strongly preferred *I. tenuituba* in similar arrays at GR. This result indicates that floral differences alone cannot explain the differences in level of hawkmoth preference. Although hummingbirds at both sites favored *I. aggregata*, preference was more pronounced on plants from GR than from PG (see Fig. 3), suggesting that perhaps the narrower corollas of GR *I. tenuituba* act as a stronger deterrent (Thomson and Thomson 1992) to hummingbird visitation (Grant 1992; Grant and Temeles 1992). Corolla width in both species at PG is under positive

selection by hummingbirds except in rare years of abundant hawk-moths, when it is under disruptive selection (Campbell et al. 1997). The higher abundance and interannual reliability of hawkmoths at GR, which we noted each summer between 2001 and 2004 (this study, and unpublished data for 2002), might exert consistent diversifying selection, leading to more divergence in corolla width and greater pollinator fidelity. This suggests an intriguing potential case of fairly fine-scale adaptation between populations of the same species to local pollinator assemblages, although the strength of selection on corolla width at GR remains to be measured. However, floral morphology alone cannot explain the lack of preference or constancy by hawkmoths foraging on GR plants at PG. The presence of hybrids at PG could reduce ethological isolation by weakening the link between floral morphology and nectar reward.

One possibility for the difference in hawkmoth behavior between sites relates to climatic conditions. Hawkmoths might be limited to largely diurnal foraging at PG by colder nighttime temperatures (Campbell et al. 1997), while at GR they can forage throughout the night, when white flowers have a visibility advantage over dark red ones (Paige and Whitham 1985). Although *Hyles lineata* can distinguish color even under dim starlight, they prefer light-colored flowers in low-light conditions (Kelber et al. 2003). In contrast, *H. lineata* shows no preference for color while foraging diurnally at PG (Campbell et al. 1997); however, it has not yet been shown directly that these differences in foraging behavior are the result of differences in light availability rather than other differences between experiments. Although 2003 and 2004 were years of low hawkmoth abundance at PG, they were observed only in the evenings around 2100 h. Out of all array observations at GR, we observed seven hawkmoth foraging bouts after 2100 h, and five hawkmoth bouts before 0600 h, whereas at PG, we observed two hawkmoth bouts after 2100 h and none before 0600 h, when temperatures are low. In years when hawkmoths are abundant at PG (e.g., 2001; personal observation), they are seen mostly during the day (Campbell et al. 1997). While these data are consistent with our temperature hypothesis, it remains speculative without quantitative data on hawkmoth abundance at PG and GR at all times of day (e.g., light trap studies).

Hawkmoth foraging might indirectly affect hummingbird behavior by changing the nectar resources in the two plant species. Patterns of changes in nectar standing crop indicate that GR *I. tenuituba* receives nocturnal visitors, while all other groups are visited diurnally. In the morning at GR, when hummingbirds start foraging, *I. tenuituba* flowers are nearly empty, while *I. aggregata* flowers contain around 3 μL of nectar. In contrast, both species at PG follow the same pattern of diurnal nectar depletion and overnight replenishment, making *I. tenuituba* rewarding in the morning. At that site *I. aggregata* does have more dilute nectar, which is thought to associate with hummingbird pollination,

though mostly as a deterrent to insect visitation, so concentration will not necessarily drive hummingbird preference. Moreover, hummingbirds will visit hawkmoth flowers opportunistically even if other more rewarding flowers are available in the vicinity as well (Campbell et al. 1997; Aigner and Scott 2002). Thus, hummingbirds might encounter very different rewards in *I. tenuituba* when they begin their daily foraging at PG compared to at GR, and carry over that tendency to either visit opportunistically or avoid that species for the rest of the day. Experimental manipulation of nectar rewards and flower color has shown that hummingbirds will shift preferences to pale flowers based on reward (Meléndez-Ackerman et al. 1997), and thus their preferences depend in part on recent experience. The spatial structure of the sites might affect this process as well, as in a mosaic (Harrison 1986) site such as GR, where populations of either species are separated by only a few meters throughout the site, pollinators can easily move to patches of their preferred flower at low cost. In contrast, in a lengthy clinal site such as PG, such discriminate movement would require long-distance travel.

For both hummingbirds and hawkmoths, patterns of preference were very similar to patterns of conspecific movements (see Figs. 3 and 4). Overall ethological isolation by hawkmoths was nearly identical to preference, likely because in most cases hawkmoths showed so little preference or constancy. Ethological isolation by hummingbirds was substantially weaker than preference only when foraging on PG plants at GR, the one case in which hummingbirds showed no constancy. Unfortunately, the effect of the lateness of the season at GR when we were able to observe these arrays might confound these results. In this case, hummingbirds might have been more willing to forage on *I. tenuituba* in our arrays, as overall abundance of flowers at GR was lower than at peak season and foragers could not afford to be as discriminating despite their morphological preferences. It should be noted, however, that *I. aggregata* peaks later than *I. tenuituba* at GR and therefore was still flowering in greater abundance than *I. tenuituba* when we conducted our array experiment in 2004. Consequently, hummingbirds at that time would have been encountering *I. tenuituba* far less frequently than at peak season, and so might be expected to show even lesser tendency to forage on them. Visitation patterns suggest that preference alone accounted for most of the ethological isolation at these sites, though a lack of constancy could reduce ethological isolation despite moderately strong preference.

In the absence of ethological isolation, other isolating mechanisms might also limit hybridization despite interspecific pollen transfer. Postmating isolation such as conspecific pollen advantage can reduce the seed siring success of heterospecific pollen in the presence of conspecific pollen on the same stigma. There is evidence for an asymmetrical competitive advantage for *I. aggregata* pollen from GR in mixed pollen loads; however, the

asymmetry does not appear to reduce hybrid formation enough to account for the difference in hybrid frequency between GR and PG (Aldridge and Campbell 2006). Asymmetrical pollen success does appear to limit hybridization between *I. aggregata* and another close relative, *I. arizonica*. In this case, style length differences between the species contribute both to reduced pollen transfer and asymmetrical pollen success (Wolf et al. 2001). Postzygotic isolation that reduces the survival or fecundity of hybrid sporophytes seems unlikely at GR due to similarities in soils and vegetation among patches of both species at that site. In contrast, environmental species sorting does appear to help maintain the integrity of the species boundaries at PG, and to favor hybrids in certain parts of that contact site (Campbell and Waser 2001).

Variation in hybridization among contact sites of a pair of animal pollinated species has been reported in *Aquilegia* (Chase and Raven 1975), *Epimedium* (Suzuki 1984), and *Platanthera* (Nilsson 1983). In the former case, the variation was found to result from variation in postdispersal selection against hybrids, and in the latter two cases from variation in pollinator assemblage. In the case of this study, the pollinator assemblages were essentially the same at the two sites, but pollinator behavior was markedly different. Similar variation in hybridization has also been reported in nonanimal pollinated systems. In *Pinus* (Watano et al. 2004), presence of other tree species acted as a barrier to pollen dispersal at one site, while in *Quercus* (Williams et al. 2001), differences in pollen fitness between dry and mesic sites reduced hybridization in the mesic site. In this study of *Ipomopsis*, inter-site variation in hybridization results from variation in ethological isolation.

In summary, we found evidence for ethological isolation as an explanation for variation in hybridization rate among *Ipomopsis* contact sites. Complex patterns of hawkmoth preference and constancy among the sites could affect foraging behavior of hummingbirds to produce stronger ethological isolation at a site where hybrids are rare. These behavioral patterns might reflect greater morphological divergence between populations of the plant species driven by more consistent selection exerted by hawkmoths at the latter site. Differences in spatial structure between the sites might also reinforce these behavioral patterns, further strengthening the isolation at the mosaic site. This study demonstrates the utility of using natural variation in hybridization rate among contact sites within a species pair for assessing the importance of individual reproductive isolating mechanisms, and reinforces the importance of considering spatial variation in species interactions before drawing general conclusions.

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