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Ecophysiology of first and second generation hybrids in a natural plant hybrid zone

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Abstract Hybrids between related species vary widely in relative fitness, and that fitness can depend upon the environment. We investigated aspects of physiology that might influence fitness patterns in a plant hybrid zone. Seeds of *Ipomopsis aggregata*, *I. tenuituba*, F1 hybrids, F2 hybrids, and offspring of crosses between natural hybrids were planted into the relatively mesic site of origin for *I. aggregata* and the drier site for natural hybrids. We measured rates of photosynthesis (A_{max}), transpiration (E), instantaneous (A/E) and long-term ($\delta^{13}C$) indices of water use efficiency (WUE), and leaf nitrogen and carbon. We also examined correlations of these traits with plant size. Photosynthetic rate and A/E were higher in vegetative than flowering plants. WUE varied between sites and years, but differences among genotypic classes were spatially and temporally consistent. Instantaneous WUE was higher for F1 hybrids than for the average of the parental species, thereby showing heterosis. There was no evidence of hybrid breakdown, as WUE was no different in the F2 than the average across the F1 and parental species. Nor did WUE depend on cross direction in producing F1 progeny. Carbon isotope discrimination revealed higher long-term water use efficiency in *I. tenuituba* than *I. aggregata*. Leaf nitrogen was higher in *I. tenuituba* than *I. aggregata*, and higher in offspring of natural hybrids than in the F2. Results indicate heterosis for water use efficiency, with no hybrid breakdown. Heterosis in WUE may help to explain the relatively high survival of both reciprocal F1 hybrids in dry sites within the natural hybrid zone.

Keywords Carbon isotope discrimination · Hybridization · *Ipomopsis* · Photosynthetic rate · Water use efficiency

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Introduction

Hybrids between some pairs of species exhibit high mortality or sterility (Stebbins 1958; Dobzhansky 1970), while crosses between other pairs result in hybrid vigor, with reproductive isolation primarily prezygotic (Jiggins and Mallet 2000) or weakly developed. Since relative fitness of hybrids can vary with the environment, an accurate assessment of hybrid fitness often requires measurement under a range of experimental conditions or ideally under natural conditions in the field. Recognition of this point (Rieseberg and Carney 1998) has led to a number of reciprocal transplant studies of hybrids, particularly with plants (review in Arnold 1997; also Emms and Arnold 1997; Wang et al. 1997; Fritsche and Kaltz 2000; Campbell and Waser 2001). These studies have a wide range of outcomes, from low to high hybrid fitness, which in turn support different evolutionary models for hybrid zones (Barton and Hewitt 1985) and make different predictions about further evolutionary change due to hybridization. However, remarkably little is known about physiological aspects of plant performance that might explain this variation.

These characteristics of plant performance, can in principle, involve developmental or physiological capacity to live in a particular habitat, interactions with other species, or a combination of the two that influences survival or reproduction, such as a reduction in carbon gain through photosynthesis due to competition for light, water or nutrients (e.g., Norman and Martin 1999). In plants, the physiological match to a particular habitat may relate to efficient photosynthesis, or to the

ability to maximize photosynthesis relative to water, nitrogen, or carbon costs. As a result, the study of photosynthetic rate and water use efficiency (carbon assimilated through photosynthesis per water lost to transpiration) provide a reasonable starting point for investigating aspects of performance that may influence fitness (Donovan and Ehleringer 1994; Arntz et al. 2000). Because interactions between physiology and competitors or natural enemies are possible, it is important to examine such physiological traits under natural conditions. For natural hybrids, studies of physiological performance have been largely restricted to the greenhouse where the relationship to field environmental conditions is usually unclear (Johnston et al. 2001; Silim et al. 2001; Schwarzbach et al. 2001). The rare exceptions have not employed hybrids of known genetic background (McArthur et al. 1998). Knowing genetic background is important because low fitness may not appear until the F2 generation when new recombinant genotypes can appear that show some negative effects of interactions between loci (negative epistasis; Fenster et al. 1997). The resulting hybrid breakdown (lower fitness in the F2 compared to the average of the parental species and F1 hybrids) is thought to be critical to the postzygotic isolation seen in speciation (Dobzhansky 1970).

In this study we compared photosynthetic rate, water use efficiency, and leaf nitrogen and carbon in two parental species of the montane herb *Ipomopsis* (Polemoniaceae) and several types of hybrids. We used hybrids of known genetic background (F1 and F2), as well as offspring of crosses between natural hybrids. The plants were grown from seed at two field sites so we could test hybrid physiology under natural conditions: the source site for the *I. aggregata* parents and a site at the middle of a hybrid zone between *I. aggregata* and *I. tenuituba*, where natural hybrids are the most abundant. In this system, F1 hybrid survival is on average as high as that of the parental species, but also depends on environment and on which species is used as the maternal parent (Campbell and Waser 2001), so we included F1 of both cytoplasmic types. Our study had several goals. First, we characterized the two physical environments used in our study. The hybrid site is relatively dry, making water use efficiency a candidate for a trait that influences growth or survival. Second, we compared the hybrids to the parental species using leaf water potentials as an index of water status, gas exchange methods to measure photosynthetic rate and instantaneous water use efficiency, carbon isotope discrimination to measure integrated water use efficiency over the lifetime of a leaf (Farquhar et al. 1989), and elemental analysis of leaf nitrogen and carbon. We tested specifically for heterosis in the F1 generation (defined as an F1 mean exceeding that of the mid-parent) and for hybrid breakdown in the F2 generation. Finally, we examined correlations between the physiological measures, plant size and reproductive traits.

Materials and methods

Study system

Our field sites were located in two areas in Poverty Gulch, Gunnison County, CO (USA). In this region, pure populations of *I. aggregata* occur up to 2,900 m (site L and below in Campbell et al. 1997), *I. tenuituba* occurs at and above 3,200 m (sites A-C), and a natural hybrid zone occurs in the intermediate elevations (Campbell et al. 1997). The first site (latitude 38°57′67″, longitude 107°05′20″) consists of a steep talus slope with relatively low vegetative cover in the center of the hybrid zone (hereafter "hybrid site" or site I, Campbell et al. 1997) and contains an experimental planting of *Ipomopsis* plants of known genetic background. The second site (latitude 38°57′33″, longitude 107°04′57″), containing another experimental planting, consists of a gentle slope with higher vegetative cover, where non-introgressed *I. aggregata* grows (hereafter "*aggregata* site" or site L, Campbell et al. 1997). Plantings (see below) were designed to compare the fitness of different kinds of hybrids and the parental species, and data on survival, reproduction, and lifetime fitness are still being collected. Here we examined physiological performance of the hybrids.

In Poverty Gulch, individuals of both plant species are monocarpic with rare exception. Seeds produced in late summer overwinter in the soil and germinate in late June or early July to form a small rosette of leaves. The plants spend 2–10 years or more in this vegetative stage before blooming during a single season, setting seed, and dying (Campbell 1997; Campbell and Waser 2001). Plants are self-incompatible, facilitating the use of hand-pollinations in a greenhouse setting to control matings. There is no seed bank (Campbell 1997).

Experimental plantings

The plantings consisted of progeny of known genetic background produced by hand-pollinating potted plants in a screenhouse at the Rocky Mountain Biological Laboratory (RMBL) and planted as seed into the field. Both plantings relied on a 1995 collection of *I. aggregata* and *I. tenuituba* plants potted from pure populations in Poverty Gulch (L and C). Potted plants were crossed by hand to produce F1 plants that served as parents for second generation hybrids. We crossed each of the *I. aggregata* plants to a different *I. tenuituba* plant to yield independent full-sib seed families and raised the F1 progeny in pots at the RMBL.

Seeds for the first experimental planting were generated in 1997. We used flowering plants from five of the F1 families to produce sets of F2 seeds and backcross seeds. An additional 15 plants of each of the two species were potted from Poverty Gulch to serve as parents in

conspecific crosses (*I. aggregata* and *I. tenuituba*), interspecific crosses to make F1 (*I. aggregata* × *I. tenuituba*), and backcrosses (not considered further in this paper). We employed a diallel mating design with the five F1 lines to produce the F2 plants. Omitting selfs, this design included 20 crosses. We used reciprocal factorial designs to produce the other five types of progeny, with each type represented by 25 crosses (five plants × five different plants). The use of these designs maximized the diversity of the crosses possible with our five F1 lines and allowed us to investigate whether the direction of hybridization affects physiological performance.

In September 1997 we planted seeds from these crosses into ten replicate randomized blocks at the hybrid site. Where possible, each block included one seed from each of the crosses generating F2 progeny and two seeds from each of the other crosses, one seed representing each direction of cross. Seeds were planted at 10 cm intervals using a planting frame of one meter square, following Campbell and Waser (2001). Corners of each square meter were marked to allow relocation of the planted individuals in subsequent summers. Because there is no seed bank, we needed only to remove fruits from nearby *Ipomopsis* plants to ensure that planted areas contained only our planted seeds.

Seeds placed in the *aggregata* (site L) habitat were generated from crosses made in 1998 using the same diallel and reciprocal factorial mating designs, but involving plants potted from the field in 1998 along with the screenhouse-grown F1. We also added a set of reciprocal factorial crosses between natural hybrids potted from site I at Poverty Gulch.

Measurement of environmental variables

We examined physiological performance of surviving individuals from the two experimental plantings during the summers of 2001 and 2002. Based on a nearby EPA weather station (latitude 38°57′29″, longitude 106°59′06″), July 2002 was unusually dry compared to July 2001, with 79.8 vs. 136.9 mm total precipitation and 26 vs. 16 days with <1 mm precipitation (http://www.epa.gov/castnet/metdata.html). To further characterize environmental differences between the two sites, in 2002 we recorded temperature, relative humidity, and light intensity. Temperature and relative humidity were recorded every 10 min between July 1 and August 2 using Hobo data loggers placed in partial shade at the hybrid and *aggregata* sites. We downloaded the data using BoxCar Pro software and determined daily maxima and minima for temperature, relative humidity, and vapor pressure deficit (following Murray 1967). Measurements were made 4 cm above the ground similar to the height grown by the small vegetative rosettes of *Ipomopsis*. Photosynthetically active radiation (PAR) was measured with an Apogee sensor and averaged over points within five randomly chosen blocks of each planting between 1000 hours and 1500 hours during July 29–August 8.

Water potentials

Pre-dawn (0400–0600 hours) and mid-day (1100–1300 hours) leaf water potentials (ψ) were measured in 2001 using a pressure chamber with a 0–80 bar operating range (Soilmoisture Equipment Model 3005-xx2x, Plant Water Status Console). Water potentials were measured on July 27 for 28 plants at the *aggregata* site, and on July 28 for 18 plants at the hybrid site. Measurements were timed to coincide with days on which we measured gas exchange parameters. Both days were dry and sunny, with the last precipitation on July 26. Only conspecific *aggregata* (AA), conspecific *tenuituba* (TT), and both types of F1 (AT and TA, where the first letter designates the maternal parent) were included. Pre-dawn and mid-day leaf water potentials were measured for the same individuals by sampling the longest basal leaf on each plant at the time of measurement.

Variation in leaf water potentials was analyzed using a repeated measures two-way ANOVA with the factors of site and type of cross. Since no interaction was detected ($P=0.366$) and because of small sample sizes and a large site effect, we also conducted one-way repeated measures ANOVAs for each site separately to examine patterns of variation among cross types.

Gas exchange measurements

During July 23–29, 2001 and July 9–15, 2002 we measured instantaneous rates of photosynthesis (A_{max}), transpiration (E), stomatal conductance (g), and internal CO_2 concentration (c_i) for vegetative and flowering plants at the two field sites using an infrared gas-analysis system (IRGA) model LI-COR 6400 (Li-COR, Lincoln, NE, USA). In 2001, we measured nearly all individuals with a leaf large enough to use the standard leaf chamber of the LI-COR. Plants representing the following types of crosses were sampled at the *aggregata* site: conspecific *aggregata* (AA), conspecific *tenuituba* (TT), both reciprocal F1 hybrids (AT and TA), F2 hybrids (F2), and crosses between natural hybrids from site I (II; $n=117$ plants total). At the hybrid site we sampled plants of AA, AT, TA, and TT genotypes. Only 29 were of sufficient size for the leaf chamber. In 2002, we sampled an additional 29 vegetative individuals at the *aggregata* site that had reached sufficient size to accommodate the chamber, and re-measured 20 of the plants sampled in 2001.

Instantaneous water-use efficiency (WUE) was calculated as A_{max}/E (hereafter A/E), since ambient CO_2 concentration, leaf temperature, and vapor pressure deficit (vpd) were similar among all individuals (Farquhar et al. 1989; Donovan and Ehleringer 1994). This measure (A/E) correlated tightly with A/g ($r=0.96$ in

2001, $n = 146$, $P < 0.0001$), and with c_i ($r = -0.98$), so these two alternative indices are not considered further. Measurements were taken using the following settings for the LI-COR 6400: ambient CO_2 concentration 350 ppm, leaf temperature 27°C, and photosynthetically active radiation (PAR) of 1,800 μm m^{-2} s^{-1} . Differences in photosynthetic rates between the hybrids and parental species are similar at CO_2 levels of 350 and 375 ppm (unpublished data). A PAR of 1,800 was representative of sunny conditions at these sites (see Table 1), and in a pilot study, potted plants of AA, TT, AT, and TT showed similar differences in A_{max} at 1800 PAR as they did at 1200 and 500 PAR. Measurements were made between 0930 hours and 1500 hours under full sun. After measurement in the IRGA, leaves were collected in glassine envelopes, and their areas determined using a CID-leaf area meter (2001, model 201) and a scanner with ROOTEDGE Software (2002, National Soil Tilth Laboratory, Ames, IA, USA). Reported gas exchange parameters have been corrected for leaf area. Our analyses focused on the parameter WUE although we also considered A_{max} and E separately to aid in interpretation of the patterns in water use efficiency.

It was not possible to run a single factorial analysis with the factors of cross type, site, year, and stage of plant (vegetative vs. flowering), as not all combinations were represented. Instead we ran several simpler analyses. First, we tested for differences in gas exchange parameters between sites using separate two-way ANOVAs for each parameter (A_{max}, E, and WUE) with the factors of site and cross type, and keeping stage and year constant by using measurements from vegetative plants of AA, AT, TA, TT genotypes in 2001. These were the only types of plants sampled in both sites. These analyses and all other ANOVAs employed type III SS from Proc GLM, which is appropriate for unbalanced designs with fixed effects (SAS 8.2, SAS Institute, Cary, NC, USA). We found no significant site × type interactions ($P > 0.50$ for all), and for simplicity restrict the remainder of the analyses to plants from the *aggregata* site where the sample size is much higher.

Second, we examined differences in gas exchange parameters between vegetative and flowering plants at the *aggregata* site in 2001 using two-way ANOVAs with the factors of cross type and stage. There were no significant type × stage interactions ($P > 0.20$ for all), suggesting that our decision to restrict sampling to vegetative plants in 2002 would not bias the comparison of different types of crosses. Third, we used one-way ANOVAs to examine differences in gas exchange parameters among all cross types at the *aggregata* site in 2001, pooling all vegetative and flowering individuals. A priori contrasts in Proc GLM were used to test for: differences between species, heterosis (difference between the F1 and the mid-parent value), effect of the direction of the F1 cross, F2 hybrid breakdown (difference between the F2 and the mean of the F1 and mid-parent), difference between F2 and natural hybrids, and an overall difference between hybrids and the parental types (Table 2). The tests for species differences, heterosis, and hybrid breakdown are statistically independent of each other and form the standard approach to examining genetic architecture of quantitative traits (Lynch and Walsh 1998). We added a test for the direction of the F1 cross because previous work in this hybrid zone found that it influenced survival (Campbell and Waser 2001). We added the remaining two contrasts to test whether natural hybrids have diverged from the array of recombinant types present in the F2 and to examine a more general form of hybrid vigor.

Fourth, we explored consistency of these results over 2 years by analyzing WUE with two-way ANOVA using the factors of cross type and year. Our analysis included vegetative plants only, as flowering plants were not measured in 2002 and in the previous year they showed markedly lower water use efficiency. We recognize that the 2 years of data are not completely independent as 20 of the plants were measured in both years. However, our main purpose was to examine whether our results held across 2 years that were different in water availability.

Integrated water use efficiency and elemental analyses

The instantaneous measure of water use efficiency was supplemented by a measure of integrated water use efficiency over the lifetime of the leaf. Leaves used for

Table 1 Environmental measurements at the hybrid and *aggregata* sites

Variable	Hybrid site			*I. aggregata* site		
	Mean	SE	*n*	Mean	SE	*n*
Maximum temperature (°C)	35.9	1.0	33	25.5	0.5	33
Minimum temperature (°C)	6.9	0.4	33	6.2	0.3	33
Max RH (%)	86.4	3.1	33	94.2	1.9	33
Min RH (%)	15.0	1.8	33	31.6	2.2	33
Max vpd (kPa)	5.32	0.39	33	2.26	0.14	33
Min vpd (kPa)	0.16	0.05	33	0.06	0.02	33
Light (PAR)	1,700	121	4	1,641	114	4

Temperatures 4 cm above ground, relative humidity (RH), and vapor pressure deficit (vpd) are daily maximum and minimum values from July 1 to August 2, 2002. Light values are photosynthetically active radiation measured between 1000 hours and 1500 hours during July 29 to August 8, 2002

Table 2 Mean values for physiological traits across all plants measured at the two sites in 2001 and the *aggregata* site in 2002

	2001		2002
	Hybrid site	*aggregata* site	*aggregata* site
A_{max} (μmol CO_2 m^{-2} s^{-1})	20.0	17.5	24.4
E (mmol H_2O m^{-2} s^{-1})	21.3	15.9	14.0
A/E	0.94	1.15	1.85
$\delta^{13}C$ (‰)	−27.51	−27.00	−25.91
C (%)	43.00	43.24	44.00
N (%)	2.44	2.46	2.46

$\delta^{13}C$ is reported as deviation from the international standard

the instantaneous measures were combined with additional leaves collected from the same plants, air dried, ground using a Wig-L-Bug (Bratt Technologies Inc., East Orange, NJ, USA), and sent to the UC Berkeley Center for Stable Isotope Biogeochemistry for elemental analyses. For each plant, the leaf carbon isotope ratio ($\delta^{13}C$) was determined as an integrated measure of leaf-lifetime WUE (Farquhar et al. 1989). Plants discriminate against $^{13}CO_2$ over the more abundant $^{12}CO_2$ during photosynthesis, and there is an inverse relationship between this isotope discrimination and A_{max}/E (Fahrquar et al. 1989) such that a lower (more negative) $\delta^{13}C$ indicates a lower integrated WUE. Additionally, % leaf nitrogen (N) content and % carbon (C) content were measured on the same leaf samples. Pearson correlation coefficients were calculated between the two measures of water use efficiency (A/E and $\delta^{13}C$), and also between these and N and C, to see whether water use efficiency increased with percent nitrogen as might be expected from the relationship between photosynthetic rate and nitrogen (Field and Mooney 1986; McDowell 2002). Correlations were calculated separately for vegetative and flowering plants in 2001 and for all vegetative plants in both years. We used two-way ANOVA to examine the influence of cross type and year on the elemental measures. These analyses employed plants of all cross types at the *aggregata* site, and were complemented by a priori contrasts as for the analysis of gas exchange parameters. The univariate analyses were supplemented with one-way MANOVA (ignoring year) and canonical discriminant analysis (Proc CANDISC of SAS) to determine which combination of A_{max}, E, $\delta^{13}C$, N, and C maximized relative variance among the cross types.

While the major goal of our study was to compare the ecophysiology of hybrids to the parental species, a secondary goal was to examine correlations between performance and other characters of the plant related to size and reproduction. For vegetative rosettes in 2001, we recorded the length of the longest leaf and total number of leaves and multiplied these to calculate an index of rosette size (Campbell 1997). For plants in bloom that year, we determined the number of flowers produced over the season, mean length of the corolla, mean width of the corolla, and number of seeds/flower (methods in Campbell 1989, 1991). Pearson correlations were calculated between these measures and both instantaneous and integrated WUE.

Results

Environmental variables at the two sites

Minimum daily relative humidity was lower at the hybrid site than at the *aggregata* site on all measured days but one and averaged only 15% (Table 1). Minimum nighttime temperatures were similar at the two sites, but maximum temperatures at leaf height 4 cm above the ground were higher every day at the hybrid site, averaging over 35°C compared to 25°C at the *aggregata* site (Table 1). Light intensity was only slightly higher at the hybrid site than at the *aggregata* site (Table 1). In general, conditions experienced by *Ipomopsis* rosettes were hotter and drier at the hybrid site, placing more demand for water from leaves. This site difference is due likely to steeper slope aspect, lower shading from vegetative cover, and a greater amount of bare rock substrate compared to the *aggregata* site.

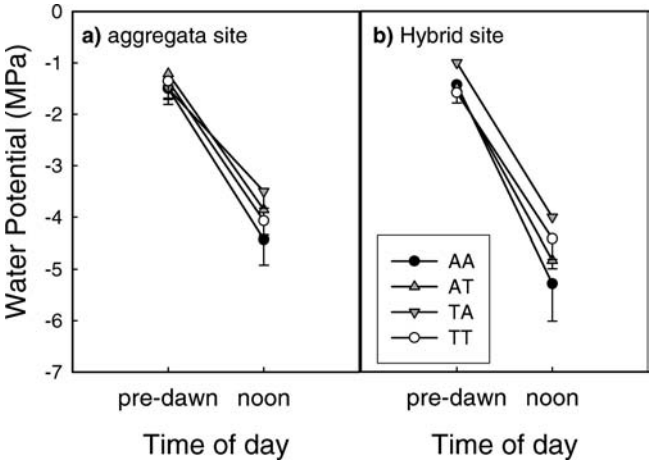

Fig. 1 Pre-dawn and noon water potentials for AA, AT, TA, and TT plants at the **a** *aggregata* and **b** hybrid sites. Means−SE are shown

Water potentials

In 2001, differences in water potentials among genotypes were consistent across the two sites; there was no detectable interaction in a two-way repeated measures ANOVA ($P = 0.75$). For simplicity, one-way repeated measures ANOVAs were used to look separately at site and cross type effects. Pre-dawn water potentials were similar for all four types of plants and for both sites (means ranged from −1.0 MPa to −1.6 MPa). Water potentials dropped sharply by mid-day, and largely because of the difference in mid-day measurements (Fig. 1), water potentials were significantly more negative at the hybrid site than at the *aggregata* site (between subjects effect in repeated measures ANOVA, $P = 0.0148$). F1 hybrids with *tenuituba* as the mother (TA) tended to have the highest water status at mid-day compared to other genotypes (repeated measures ANOVA, $P = 0.0566$; Fig. 1). *I. aggregata* plants at the hybrid site appeared under the greatest mid-day water stress, judging by their mean water potential of −5.3 Mpa at this dry, rocky site.

Gas exchange parameters

In 2001, water use efficiency (A/E) was significantly higher at the *aggregata* than at the hybrid site ($F_{1,54} = 10.67$, $P = 0.0019$ in a two-way ANOVA with site and type). The difference was due to lower rates of transpiration (E; $P < 0.0001$) at the *aggregata* site rather than higher rates of photosynthesis (A_{max}; $P = 0.3383$; Table 2). There was no significant site × type interaction for any of the three gas exchange parameters (all $P > 0.50$), suggesting that AA, AT, TA, and TT plants all responded similarly to environmental differences between the sites. The remainder of the analyses are restricted to the *aggregata* site, where the sample size was much larger.

Plants in the vegetative stage had much higher WUE than flowering individuals ($F_{1,105} = 10.47$, $P = 0.0016$; Fig. 2). This stage effect resulted largely from higher photosynthetic rate for vegetative rosettes (A_{max}; P value for stage effect = 0.0019) rather than reduced transpiration rate (E; $P = 0.3445$). A_{max} averaged 19.1 μmol CO_2 m^{-2} s^{-1} for vegetative plants compared to 14.3 μmol CO_2 m^{-2} s^{-1} for flowering plants. The differences between stages were similar for all types of plants (Fig. 2), with no significant stage × type interaction for any of the gas exchange parameters (all $P > 0.20$).

With vegetative and flowering plants sampled in 2001 included in the analysis, water use efficiency showed significant heterosis, with mean WUE for the F1 hybrids higher than the mean for the two parental species (Fig. 3, Table 3, $P = 0.0412$). In addition, hybrids in general (F1, F2, and offspring of crosses between natural hybrids) tended to have higher WUE than the two pure parental species as a group ($P = 0.0666$). There was no indication of F2 hybrid breakdown ($P = 0.7236$, Fig. 3). A posteriori comparisons revealed higher WUE for the F1 than for *I. aggregata* (Tukey test; $P < 0.05$), but not *I. tenuituba*. While A_{max} and E correlated equally well with WUE ($r = 0.38$ and $r = -0.36$, both $P < 0.0001$), neither of those parameters alone exhibited significant heterosis (both $P > 0.10$). An ANCOVA of photosynthetic rate (A_{max}), using transpiration (E) as the covariate, provided

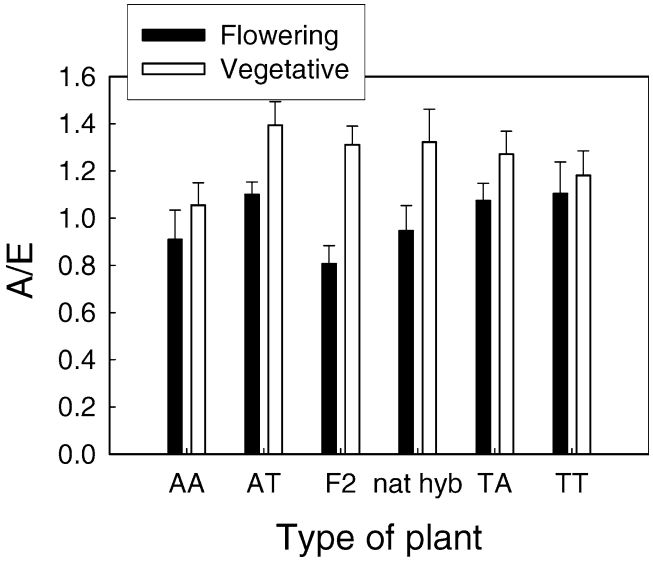

Fig. 2 Comparison of flowering and vegetative individuals of all cross types for instantaneous water use efficiency (A/E). Data shown are means + SE for a total of 117 plants at the *aggregata* site

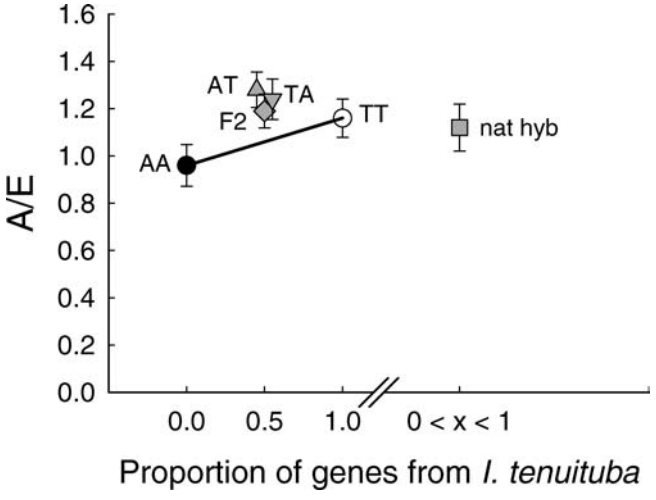

Fig. 3 Instantaneous water use efficiency for all plants at the *aggregata* site in 2001. A/E is plotted against the proportion of genes from *I. tenuituba*. Offspring of crosses between natural hybrids are shown for comparison. Data shown are means and SE ($n = 117$ plants in total). The *mid-parent* line connecting AA and TT plants predicts the value for the F1 and F2 if variation is due to additive effects of genes. Types AT, TA, and F2 all have 0.5 of genes from *I. tenuituba*. The symbols are offset slightly for visualization. F1 values above the line indicate heterosis

Table 3 ANOVAs comparing A/E and c_i among cross types using all plants measured at the *aggregata* site in 2001

Source	df	SS	F	P
Instantaneous WUE (A/E)				
Model	5	1.1378	1.52	0.1882
AA vs. TT (1 0 0 0 0 −1)	1	0.3448	2.31	0.1315
AT vs. TA (0 1 0 0 −1 0)	1	0.0100	0.07	0.7962
Heterosis (−0.5 0.5 0 0 0.5 −0.5)	1	0.6371	4.27	0.0412
Breakdown (0.25 0.25 −1 0 0.25 0.25)	1	0.0188	0.13	0.7236
F2 vs. II (0 0 −1 1 0 0)	1	0.0456	0.30	0.5819
Hybrids high (−0.5 0.25 0.25 0.25 0.25 −0.5)	1	0.5125	3.43	0.0666
Error	111	16.5800		
Intercellular CO_2 concentration (c_i)				
Model	5	5729.5	1.94	0.0936
AA vs. TT (1 0 0 0 0 −1)	1	1,095.2	1.85	0.1762
AT vs. TA (0 1 0 0 −1 0)	1	184.4	0.31	0.5776
Heterosis (−0.5 0.5 0 0 0.5 −0.5)	1	3,527.7	5.97	0.0162
Breakdown (0.25 0.25 −1 0 0.25 0.25)	1	223.5	0.38	0.5398
F2 vs. II (0 0 −1 1 0 0)	1	398.8	0.67	0.4132
Hybrids high (−0.5 0.25 0.25 0.25 0.25 −0.5)	1	2,916.4	4.93	0.0284
Error	111	65,614.8		

Six a priori contrasts were specified, using the coefficients in *parentheses*. Cross types were ordered alphabetically: AA, AT, F2, II, TA, TT, where II refers to offspring of crosses between natural hybrids from population I

further support for heterosis in water use efficiency by demonstrating higher adjusted photosynthetic rates for F1 hybrids (P value for contrast = 0.0149) and for hybrids in general ($P = 0.0449$), as well as a trend towards higher water use efficiency in *I. tenuituba* than in *I. aggregata* ($P = 0.0729$, Fig. 3).

Reciprocal F1 hybrids had similar water use efficiency (Fig. 3) but appeared to achieve it in different ways. Hybrids with *tenuituba* as the mother (TA) had an unusually high photosynthetic rate (A_{max} mean = 21.7 - μmol CO_2 m^{-2} s^{-1} compared to 15.2–18.3 for the other cross types including 16.6 for the reciprocal AT hybrid; $P = 0.07$). In contrast, hybrids with *aggregata* as the mother (AT) had unusually low transpiration (mean = 13.7 mmol H_2O m^{-2} s^{-1} compared to 14.3–17.3 for the other cross types, including 17.3 for TA; $P = 0.11$).

For the 20 plants measured in both years at the *aggregata* site, individual plants had higher instantaneous WUE in the drought year of 2002 than in 2001 (mean = 1.95 vs. 1.33 μmol CO_2 mm H_2O^{-1}; paired t test; $P = 0.0018$). The increases in WUE reflected primarily changes in maximal photosynthetic rate, which increased on average from 20.7 to 25.1 μmol CO_2 m^{-2} s^{-1} for the vegetative plants ($P = 0.05$), with no significant change in transpiration rate (E mean = 16.8 and 13.9 mmol H_2O m^{-2} s^{-1} in 2001 and 2002, respectively, $P = 0.09$). A similar year effect was

Table 4 Two-way ANOVAs for A/E, $\delta^{13}C$ and N at the *aggregata* site. Conventions as in Table 3

Source	df	P		
		A/E	$\delta^{13}C$	N
Vegetative plants only ($n = 124$)				
Cross type	5	0.0759	0.5033	0.0038
AA vs. TT	1	0.5197	0.0771	0.0340
AT vs. TA	1	0.2072	0.1436	0.2972
Heterosis	1	0.0068	0.5861	0.2157
Breakdown	1	0.2637	0.7234	0.0791
F2 vs. II	1	0.4175	0.5247	0.0014
Hybrids high	1	0.0075	0.3698	0.1714
Year	1	0.0001	0.0001	0.0032
Cross type × year	5	0.3590	0.5033	0.1010
Vegetative and flowering plants ($n = 159$–166)				
Cross type	5	0.0191	0.0561	0.0021
AA vs. TT	1	0.3135	0.0268	0.0170
AT vs. TA	1	0.0949	0.4010	0.2413
Heterosis	1	0.0022	0.2321	0.0733
Breakdown	1	0.3533	0.4517	0.1360
F2 vs. II	1	0.1942	0.2248	0.0065
Hybrids high	1	0.0047	0.0538	0.0752
Year	1	0.0001	0.0001	0.5605
Cross type × Year	5	0.3559	0.7203	0.2094

seen when all vegetative plants, or all vegetative and flowering plants, were analyzed (Table 4, Fig. 4). For the vegetative plants, WUE was higher in 2002 (mean = 1.85, $N = 49$ plants) than. in 2001 (mean = 1.27, $N = 75$ plants, $P < 0.0001$ in two-way ANOVA), even though percent leaf nitrogen was slightly lower in 2002. There was no interaction between the cross type and year (Table 4), indicating that all types of plants responded similarly to the drier conditions. With the larger sample of plants from both years, F1 hybrids again showed significantly higher WUE than the parental types, as did hybrids as a whole (all $P < 0.01$, Table 4), with no evidence for hybrid breakdown.

Integrated water use efficiency and elemental analyses

The integrated measure of water use efficiency ($\delta^{13}C$) was significantly correlated with the instantaneous measure (A/E) in vegetative plants ($r = 0.30$, $P < 0.01$ in 2001 and $r = 0.49$, $P < 0.0001$ in both years combined) but not in flowering individuals ($r = 0.05$, Table 5). Like the instantaneous measure, $\delta^{13}C$ indicated higher water use efficiency at the *aggregata* site than the hybrid site in 2001 (Table 2) and, for this site, higher yet water use efficiency in 2002 (mean = −25.91‰). ANCOVA with site as the categorical value suggested that instantaneous WUE scales with integrated WUE within as well as across sites ($P = 0.0523$).

Especially in 2001, instantaneous water use efficiency also correlated with percent nitrogen in the leaf (Table 5). ANCOVA revealed a within-site increase of A/E with N in 2001 ($P < 0.01$) and a similar increase within the three combinations of year and site represented in the full data set ($P < 0.05$) even though the correlation of A/E with N did not appear significant when the 2002 data were added in (Table 5). This discrepancy largely reflects the much higher water use efficiency for the same nitrogen content in 2002 compared to 2001 (effect of year-site combination in ANCOVA, $P < 0.0001$).

Comparing $\delta^{13}C$ among cross types revealed a less negative mean value for *I. tenuituba* than *I. aggregata*, suggesting higher long term water use efficiency in the former species ($P < 0.05$ for vegetative and flowering plants combined, Table 4, Fig. 5). However, we found no statistical support for heterosis in integrated water use efficiency (Table 4). Percent nitrogen in the leaves was higher in *I. tenuituba* than *I. aggregata* ($P < 0.05$, Table 4) and higher in offspring of crosses between natural hybrids than in the F2 ($P < 0.01$, Table 4, Fig. 5). Percent C showed no difference among the six cross types in a similar two-way ANOVA with year as the other factor ($P = 0.9079$). In the multivariate analysis, the first canonical variate explained 72% of the variance ($P = 0.0208$) and was most highly correlated with percent nitrogen ($r = 0.87$, $N = 149$). It also showed a significant negative correlation with transpiration rate ($r = -0.40$). Offspring of natural hybrids and *I. tenuituba* had the highest class means (0.49 and 0.42), reflecting their higher nitrogen content and water use efficiency compared to *I. aggregata* (class mean = −0.72).

The instantaneous measure of water use efficiency (A/E) showed no significant correlation with any of the other vegetative or reproductive characters (Table 6), nor did photosynthetic rate (A_{max}) or transpiration rate (E) with these same characters. However, the integrated measure, $\delta^{13}C$, did correlate weakly with rosette size in vegetative plants ($r = 0.21$, $P < 0.05$). This correlation appeared similar within the *aggregata* site ($r = 0.21$, $n = 65$) and the hybrid site ($r = 0.22$, $n = 20$). Most correlations between the elemental measures and other vegetative and reproductive characters were weak or nonexistent, but C correlated negatively with rosette size, and N correlated positively with flower number (uncorrected P values, Table 6).

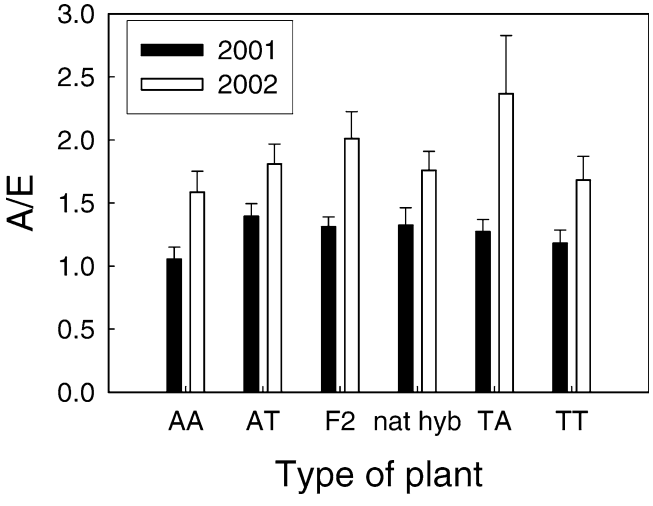

Fig. 4 Comparison of A/E in the 2 years of study at the *aggregata* site. Data shown are means + SE for a total of 124 vegetative plants

Discussion

This study of *Ipomopsis* hybrids revealed heterosis for instantaneous water use efficiency under natural conditions; that is, F1 hybrids significantly exceeded the mean A/E for the two parental species. In addition, plants of *I. tenuituba* tended toward higher water use efficiency than plants of *I. aggregata*. The data on water potentials, although limited in scope, are consistent with these findings in that the F1 hybrids appeared to be under less water stress in the mid-day heat. Because all types of offspring were grown from seed at the same field sites and thus shared a common environment, as did the parents of the pure species and the F1, it is possible to make genetic inferences from the high instantaneous water use efficiency of the F1. In principle, such an observation of heterosis may be due to dominance, overdominance, or positive epis-

Table 5 Correlation coefficients between instantaneous water use efficiency (A/E) and integrated water use efficiency ($\delta^{13}C$), percent carbon (C), and percent nitrogen (N) in the leaf

	$\delta^{13}C$	C	N
Flowering plants 2001			
A/E	0.05	0.08	0.28
$\delta^{13}C$		0.09	−0.07
C			0.26
Vegetative plants at both sites 2001			
A/E	0.30**	0.13	0.33**
$\delta^{13}C$		0.22*	−0.08
C			0.19
Vegetative plants at both sites 2001 and *aggregata* site 2002			
A/E	0.49****	0.12	0.02
$\delta^{13}C$		0.27**	−0.19*
C			0.12

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$
[a] Flowering plants in 2001 at both sites ($n = 47$)
[b] Vegetative plants in 2001 at both sites ($n = 87$–100)
[c] Vegetative plants in 2001 at both sites and in 2002 at the *aggregata* site (total $n = 127$–137)

tastis, in which specific interactions between genes from the two species increase the trait value in a hybrid individual (Lynch and Walsh 1998). In this case, dominant genes from *I. tenuituba* is a more likely explanation than overdominance, as water use efficiency in the F1 significantly exceeded that of *I. aggregata* but not that of *I. tenuituba*.

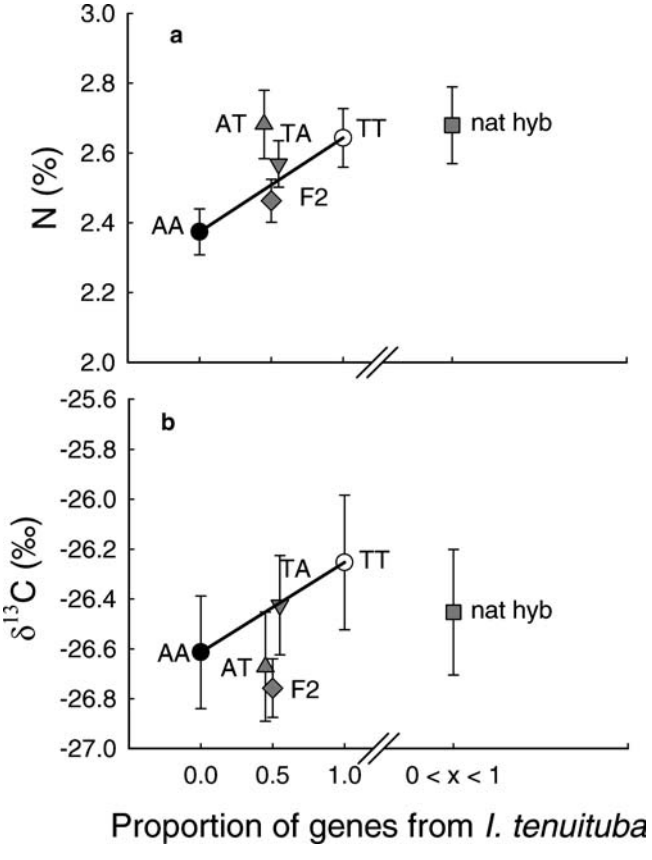

Fig. 5 Elemental measures for vegetative plants in both years at the *aggregata* site. Percent nitrogen (N) and integrated water use efficiency ($\delta^{13}C$) are plotted against the proportion of genes from *I. tenuituba*. Offspring of crosses between natural hybrids are shown for comparison. Data shown are means and SE ($n = 124$ plants in total). The *mid-parent* line connecting AA and TT predicts the value for the F1 (AT and TA) and F2 if variation is due to additive effects of genes

Previous studies had found dramatic differences in survival between the two types of reciprocal F1 hybrids (Campbell and Waser 2001), leading us to test for associated differences in physiological performance. We found similar water use efficiencies for these two genetic classes. However, they may achieve it in different ways. Although the differences were not statistically significant, the trend in means suggests the hypothesis that F1 with *aggregata* as the mother achieve high water use efficiency by lowering transpiration, while those with *tenuituba* as the mother achieve high water use efficiency by an increase in photosynthetic rate. If this is so, it in turn suggests the hypothesis that cytoplasmic genes transferred through the mother, such as in chloroplast DNA (Wolf et al. 1997), interact with nuclear genes in affecting photosynthesis.

Genetic inferences from the performance of the F2 require one additional caveat, as the F1 parents of these individuals were raised under greenhouse rather than field conditions like the *I. aggregata* and *I. tenuituba* parents. However, the lack of any detectable difference between water use efficiency of the F2 and the mean of the F1 and mid-parent suggests the absence of hybrid breakdown due to negative epistastic interactions between genes from the two species when recombined in the F2. Instead hybrids of all genetic backgrounds considered as a whole have high instantaneous water use efficiency compared to the mid-parent, with all classes exceeding the performance of *I. aggregata*. This idea is consistent with the hypothesis of dominant or partially dominant genes from *I. tenuituba*.

Integrated water use efficiency ($\delta^{13}C$) correlated significantly with instantaneous water use efficiency in vegetative plants, although not detectably so in flowering plants, perhaps because the integrated measure

Table 6 Correlation coefficients in 2001 between physiological characters (A/E, δ^{13}C, C, N) and rosette size of vegetative plants ($n = 85$–96 for both sites), and between physiological characters and reproductive characters of flowering plants (corolla length, corolla width, flower number, seeds/flower, $n = 41$ for plants at the *aggregata* site)

	Vegetative plants Rosette size	Flowering plants Corolla length	Corolla width	Flower number	Seeds per flr
A/E	0.01	0.00	−0.01	0.23	−0.22
δ^{13}C	0.21*	0.04	0.00	−0.03	0.15
C	−0.26*	0.15	0.31	0.03	0.10
N	−0.17	0.13	0.09	0.34*	−0.20

* Uncorrected $P < 0.05$. None of the correlations are significant using a sequential Bonferroni correction for the four entries for vegetative plants or the 16 entries for flowering plants

also reflected conditions for some time prior to formation of the flowers. Flowering individuals also differed from the rosettes in being much less water use efficient, due primarily to a 25% lower photosynthetic rate. This lower photosynthetic rate suggests the absence of sink driven photosynthesis from reproductive organs and instead senescence of vegetative organs as these monocarpic plants divert resources into flower and seed production. At least one resource, leaf nitrogen, was lower in flowering than vegetative individuals (means = 2.2 vs. 2.6%; $P < 0.0001$ in two-way ANOVA with stage and cross type as factors). While the two measures of water use efficiency correlated in vegetative plants, there were discrepancies in comparisons of various genotypic classes. The integrated measure revealed higher water use efficiency in *I. tenuituba* than in *I. aggregata* (when data from both years at the *aggregata* site were combined), but it did not provide support for heterosis in water use efficiency. There are at least two possible reasons for these discrepancies. In theory, the instantaneous measure could have been taken under conditions that are not typical of the entire growth period of the leaf, but this seems unlikely as the observation of heterosis in the instantaneous measure was consistent across 2 years of study made during peak growth times. The second possibility is that the integrated measure has low statistical power. This explanation is more tenable, as the integrated measure appeared to have little resolution for detecting fine scale differences between genetic classes, as judged by the higher standard error relative to the difference between means in Fig. 5 (despite its higher sample size) compared to Fig. 3. In contrast, the carbon isotope method easily revealed differences at a coarser level, such as the higher water use efficiency in 2002 rather than 2001.

Instantaneous water use efficiency increased with an increase in percent leaf nitrogen. This is a common finding, presumably because photosynthetic enzymes and pigments represent a major investment in leaf nitrogen (Field and Mooney 1986). However, the genotypic class differences in n did not match completely the differences in water use efficiency. Percent nitrogen was higher in *I. tenuituba* than *I. aggregata*, but heterosis was weaker for this trait. Unlike the results for water use efficiency, offspring of natural hybrids were very rich in nitrogen compared to the range of genotypes present in the F2 (Fig. 5a), suggesting that the hybrid habitat may have selected for altered nitrogen use or uptake. The role of nitrogen is underscored by the finding that n correlated most highly of all the physiological traits with the canonical discriminant variate maximizing relative variance among the six genotypic classes.

While the physiology of crop hybrids is relatively well-studied, few other investigators have examined these traits in hybrids between native species. The studies that exist provide mixed results; water use efficiency like other physiological and morphological traits is not necessarily intermediate and can be transgressive (Rosenthal et al. 2002). For example, natural hybrids between *Pinus sitchensis* and *P. glaucus* had higher δ^{13}C than either of the parental species, suggesting higher water use efficiency under growth chamber conditions (Silim et al. 2001). In contrast, the putative hybrid sunflower *Helianthus anomalus* had lower instantaneous water use efficiency than either of the parental species, as measured by c_i under greenhouse conditions (Schwarzbach et al. 2001). A greenhouse study of *Iris brevicaulis* and *I. fulva* was exceptional in its inclusion of hybrids of known genetic background, in this case backcrosses to the parental species (Johnston et al. 2001). Backcrosses to *I. fulva* had the lowest photosynthetic rate, and other ecophysiological characters did not differ detectably across the genotypic classes.

These studies were mostly conducted under greenhouse or growth chamber conditions. To our knowledge, our study represents the first test of photosynthetic rate and water use efficiency of F1 and F2 hybrids between native species under natural field conditions. Because the study was performed in the field, we can examine the extent to which these physiological results are associated with patterns in survival and other fitness components at these same sites. In a reciprocal transplant study, 5-year survival from seed was at least as high for F1 hybrids with *aggregata* as the mother as for pure *I. aggregata* in the *aggregata* site, while F1 hybrids with *tenuituba* as the mother had very poor survival at that site (Campbell and Waser 2001). At the hybrid site, in contrast, both types of F1 hybrids had relatively high

survival similar to the mean of the parental species. These results indicated that fitness of hybrids was strongly dependent on both genetic background and environment. The high hybrid survival at the dry hybrid site could be explained in part by the high water use efficiency of both reciprocal F1 hybrids seen in the current study, at least as judged by gas exchange. The hybrid site experiences higher ground temperatures and vpd than either the *I. aggregata* site or the *I. tenuituba* site (unpublished data), which may place a premium on high water use efficiency. It is possible that the high water use efficiency of F1 hybrids allows high survival at that dry site, while conferring no advantage at the more mesic *aggregata* site. The dependency of F1 hybrid survival on maternal parent at the *aggregata* site is not explained by water use efficiency and instead suggests selection for other kinds of traits specific to hybrids with *aggregata* cytoplasmic background. It is unclear at this point what those critical traits are.

If the hybrid and *I. aggregata* sites differ in their selective milieu in accordance with the environmental measurements, we predict a correlation between water use efficiency in vegetative individuals and subsequent survival to reproduction at the dry hybrid site, but no such correlation or a weaker relationship at the more mesic *aggregata* site. Here we found that integrated water use efficiency correlated weakly with size in vegetative plants. Furthermore, previous work shows that vegetative rosette size at an early age correlates with survival to flowering in *I. aggregata* (Campbell 1997). However, we do not yet know if water use efficiency affects future growth and survival in the hybrid zone. Studies that have examined selection on this trait have found that high water use efficiency sometimes increases fitness components and other times is selected against, depending on the environment (reviewed in Arntz and Delph 2001). Few of those studies employed specific genotypes, however Arntz et al. (2000) found that a mutation reducing photosynthetic carbon assimilation and water use efficiency in *Amaranthus* also lowered survivorship during early establishment.

While this study has focused on differences in physiological traits between hybrids and the parental species, it is important to note that other differences also influence hybrid fitness in this system. For example, hybrids have pollination success as high as that of *I. aggregata* and exceeding that of *I. tenuituba*, due primarily to preferences of hummingbird pollinators (Campbell et al. 2002a). On the other hand, hybrid pollen suffers slightly in pollen competition (Campbell et al. 2003). Rates of seed predation on natural hybrids are typically intermediate between the two species (Campbell et al. 2002b). Other physiological traits are likely also involved in hybrid fitness; as we argued above water relations do not explain the higher survival of hybrids with *aggregata* rather than *tenuituba* cytoplasm in the *I. aggregata* site.

A longstanding question about plant hybrid zones concerns the extent to which they are maintained by habitat selection involving physiological responses, such as those measured here (Chase and Raven 1975), versus selection on floral traits mediated by pollinators (Grant 1949). Comparing aspects of physiology, as we have done here, and pollination of hybrids to the parental species (Campbell et al. 1997, 2002a) is a first step. In addition, future investigations should evaluate the relationships of both physiological characters and floral characters to overall fitness in both parental environments. Such analyses would illuminate the extent to which divergent selection on these two kinds of traits maintains species differences.

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