

**PHOTOSYNTHETIC AND GROWTH RESPONSES OF RECIPROCAL  
 HYBRIDS TO VARIATION IN WATER AND NITROGEN AVAILABILITY<sup>1</sup>**

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- *Premise of the study:* Fitness of plant hybrids often depends upon the environment, but physiological mechanisms underlying the differential responses to habitat are poorly understood. We examined physiological responses of *Ipomopsis* species and hybrids, including reciprocal F<sub>1</sub>s and F<sub>2</sub>s, to variation in soil moisture and nitrogen.
- *Methods:* To examine responses to moisture, we subjected plants to a dry-down experiment. Nitrogen was manipulated in three independent experiments, one in the field and two in common environments.
- *Key results:* Plants with *I. tenuituba* cytoplasmic background had lower optimal soil moisture for photosynthesis, appearing better adapted to dry conditions, than plants with *I. aggregata* cytoplasm. This result supported a prediction from prior studies. The species and hybrids did not differ greatly in physiological responses to nitrogen. An increase in soil nitrogen increased leaf nitrogen, carbon assimilation, integrated water-use efficiency, and growth, but the increases in growth were not mediated primarily by an increase in photosynthesis. In neither the field, nor in common-garden studies, did physiological responses to soil nitrogen differ detectably across plant types, although only *I. aggregata* and hybrids increased seed production in the field.
- *Conclusions:* These results demonstrate differences in photosynthetic responses between reciprocal hybrids and suggest that water use is more important than nitrogen in explaining the relative photosynthetic performance of these hybrids compared to their parents.

**Key words:** hybrid zone; *Ipomopsis*; nitrogen; photosynthetic rate; Polemoniaceae; water-use efficiency.

In many natural communities, the persistence of plant hybrids appears to depend upon the habitat (Emms and Arnold, 1997; Fritsche and Kaltz, 2000; Sambatti et al., 2008). Such environment-dependence of hybrid fitness is critical to the mechanisms through which species are produced and maintained. For example, maladaptation of hybrids to the home environments of the parental species can result in ecological speciation (Hatfield and Schluter, 1999). Such environment-dependence could also influence the range boundaries of plant hybrids, for example by allowing hybridization to be common in some contact sites between species but not in others (Aldridge and Campbell, 2009). In cases of hybridization between native and crop species, or between native and introduced invasives, environment-dependence might determine the conditions under which a native population is impacted by introgression (Mercer et al., 2007). However, relatively little is known about the physiological mechanisms that underlie this environmental variation in fitness of natural plant hybrids (Johnston et al., 2001; Miglia et al., 2007; Kimball and Campbell, 2009).

An understanding of the underlying mechanisms will require elucidating the links between environment and physiological processes, and then between physiology and fitness. Even for a single species, however, fitness can be affected by a large variety of

vegetative and physiological traits that mediate interactions with local environmental conditions, including leaf size, leaf initiation rate, water-use efficiency, nitrogen-use efficiency, and photosynthetic rate (reviewed by Ackerly et al., 2000), and, not surprisingly, the form of the relationship often depends sharply on the environment (Ehleringer, 1993). For example, in the annual grass *Avena barbata*, higher photosynthetic rate was associated with higher seed production in mesic environments but not in dry environments (Sherrard and Maherali, 2006). In several plant species, individuals with higher water-use efficiency achieve higher reproductive output under dry conditions but not under wet conditions (Dudley, 1996; Heschel et al., 2004), although plastic responses to drought are not always adaptive (Caruso et al., 2006). Outside of studies of *Helianthus* (Lexer et al., 2003; Ludwig et al., 2004) and of crop cultivars, little is known about links between physiological traits and fitness in interspecific hybrids and how they might differ from such links in the parental species.

Understanding relationships between environment, physiology, and fitness for a set of hybridizing species requires studies that include not just the two species, but also hybrids of diverse genetic background, as is more typical in the field. Hybrid fitness may often differ between the F<sub>1</sub> and later generations, because recombination offers the opportunity for expression of epistatic effects (Fishman and Willis, 2001). Fitness may also differ between reciprocal F<sub>1</sub> hybrids, i.e., individuals with the same nuclear makeup but with different species serving as the ovule parent. Such asymmetries can result from effects of cytoplasmic genes that are inherited through only one parent (e.g., mitochondrial genes and chloroplast genes) or from interactions between cytoplasmic and nuclear genes (Tiffin et al., 2001; Levin, 2003). Such interactions could alter the efficiency of photosynthesis, since the chloroplast genome codes for the

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large subunit of ribulose-1,5-bisphosphate (RuBP) used in photosynthesis. In addition, interactions between the mitochondria and chloroplast can influence photosynthetic and respiratory responses to drought (Atkin and Macherel, 2009).

We have been using *Ipomopsis aggregata* (Pursh) V. Grant and *I. tenuituba* (Rydb.) V. Grant (Polemoniaceae) as a model system for studying the relationships between environmental factors, physiological processes, and fitness in plant hybrids. Reciprocal transplant studies have shown that fitness of hybrids depends on the environment and on which species contributes cytoplasmic genes (Campbell and Waser, 2001, 2007; Campbell et al., 2008; Aldridge and Campbell, 2009). At our main study site in Poverty Gulch in Colorado, each of the two species achieved its highest fitness in its home environment, and  $F_1$  hybrids also had their highest fitness at the site where natural hybrids are found, largely because those with *tenuituba* cytoplasmic genes survived poorly at the other sites. To understand how these fitness relationships are produced, it is important to characterize relationships between environment and physiological processes and then between physiology and fitness. Here we pursue the first of these two steps.

Two candidates for critical environmental factors are water availability and soil N. For this *Ipomopsis* system, relative humidity and water availability vary across the contact site during the growing season. *Ipomopsis aggregata* populations experience the wettest conditions. Natural hybrid populations, consisting primarily of individuals with *tenuituba* cytoplasmic background (Wu and Campbell, 2005), experience the driest conditions. *Ipomopsis tenuituba* populations grow in areas with intermediate moisture levels (Campbell et al., 2005; Wu and Campbell, 2006). Consistent with those environmental differences, hybrids had the highest instantaneous water-use efficiency in a common garden (Campbell et al., 2005). Here we used a dry-down experiment to test the performance of both species and hybrids under varying soil moisture conditions. Assuming that there has been adaptive differentiation in response to local conditions, we predicted that hybrids, and particularly those with *tenuituba* cytoplasmic genes (because they are typically found in the driest sites) would also have the lowest optimal soil moisture for photosynthesis.

Previous results have also suggested that soil N might be critical to hybrid performance. In our common garden study, *I. tenuituba* had higher percentage leaf N in leaves than did *I. aggregata*. In addition, progeny of crosses between natural hybrids had similarly high percentage leaf N, with the value exceeding that for  $F_2$  hybrids as well as for *I. aggregata* (Campbell et al., 2005). These results suggested that the plants of different genetic backgrounds either uptake or use nitrogen in different ways. This difference could be important for plant performance, as water-use efficiency also correlated with percentage leaf nitrogen (Campbell et al., 2005). We predicted that *I. aggregata* would show the strongest responses of leaf N, photosynthetic rate, and growth to experimental addition of soil N because its low leaf N suggests it might be N-limited. Manipulations of N were performed both in the field and for potted plants grown from seed (common garden study), the latter approach allowing genetic effects to be separated from environmental effects.

## MATERIALS AND METHODS

**Study system**—We studied *Ipomopsis* from the region of Poverty Gulch, Colorado, USA described in (Campbell and Waser, 2001). Field studies took

place in three populations: one *I. aggregata* subsp. *aggregata*, one *I. tenuituba*, and one natural hybrid population (sites L, C, and I respectively in (Campbell et al., 1997). These three sites differ in relative humidity (*aggregata* > *tenuituba* > hybrid) and available P in the soil (*aggregata* > hybrid > *tenuituba*) but not detectably so in soil N (Appendix S1, see Supplemental Data with the online version of this article). Plants were also potted from those populations for use in common garden studies. Experimental  $F_1$  and  $F_2$  hybrids were made in a screenhouse at the Rocky Mountain Biological Laboratory (RMBL) by crossing those plants with controlled hand-pollinations as described in Campbell et al. (2008).

Seeds of both species germinate the spring following production, and by the end of the summer of seedling emergence, they have produced small basal rosettes of leaves. Plants at our sites stay in that rosette stage for 2–12 yr before flowering, setting seed, and dying (Campbell and Waser, 2007). Individuals maintained in a screenhouse during the summer and sunk as pots into the ground to overwinter often flower after 2–3 yr.

Because previous comparisons of moisture conditions had relied primarily on relative humidity, we took additional environmental measurements to characterize soil moisture at the hybrid site I and *I. aggregata* site L in 2005. These two sites were chosen because they represented the two extremes in terms of moisture conditions and, because of greater accessibility, were being used for other concurrent studies of gas exchange. On six dates between 27 July and 26 August, volumetric water content was measured at a depth of 12 cm in each of five locations (the five strips in Campbell et al. [2008]) at each of the two sites. These five values were averaged, and the mean value analyzed with a randomized block ANOVA with site as a fixed factor and date as the blocking factor.

**Dry-down experiment**—To characterize photosynthetic responses to soil moisture, we employed a dry-down experiment in which potted plants were allowed to dry out progressively after withholding irrigation (Brock and Galen, 2005; Galmes et al., 2007; Easlon and Richards, 2009). Six types of plants were compared: *I. aggregata*, *I. tenuituba*, both reciprocal  $F_1$  hybrids, and both reciprocal  $F_2$  hybrids, grown from seed. On 7 July 2006, 4–8 vegetative plants of each type were carefully separated from their existing soil, transplanted into 6-in plastic pots filled with native soil from meadows at the Rocky Mountain Biological Laboratory, and watered with a dilute solution of vitamin B1 root stimulator. The soil had been sieved to remove rocks larger than 2 mm in diameter. Only single vegetative rosettes with undamaged apical meristems were used in the study, and all plants started at similar size. Potted plants were maintained inside the screenhouse and watered every other day until the start of the dry-down experiment. They were fertilized on 10 July with 1.30 mL/L Peter's 15-20-15 NPK fertilizer dissolved in water.

On 23 July, the plants were watered to saturate the soil and then allowed to dry. Photosynthetic rate (assimilation  $A$ ) and stomatal conductance ( $g$ ) were measured for each plant after 1, 4, 7, 11, 13, 15, and 18 d (the last date only for those plants that had not yet shown reductions in  $A$  by day 15). Plants were measured in a randomized order, with the same order used for each date. Gas exchange measurements were made with a LI-COR 6400 photosynthesis system (LI-COR, Lincoln, Nebraska, USA). Leaf chamber conditions were standardized with a temperature of 27°C, photosynthetically active radiation (PAR) at 1800  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  to give light-saturated photosynthesis (after Campbell et al., 2005; Wu and Campbell, 2006, 2007), and  $\text{CO}_2$  concentration of 375 ppm. Following gas exchange measurements, the measured leaves were collected and scanned to obtain leaf area using ImageJ software (National Institutes of Health freeware). All measurements are reported on a per leaf area basis. Volumetric water content (VWC) in the soil was measured in each pot immediately after each measurement of gas exchange using a soil moisture sensor (Campbell Scientific, Edmonton, Canada).

For most individual plants,  $A$  increased during the first few days as the soil dried slightly to conditions more typical of the field and then declined as the plants began to dry out, producing a nonlinear relationship between  $A$  and VWC. To analyze how  $A$  responded to VWC, we found the optimal soil moisture for photosynthetic rate for each plant individually by first fitting a quadratic regression. Because the function was usually convex, solving for the point at which the first derivative equaled zero usually gave us a maximum. For a few plants, the maximum instead equaled  $A$  at either the lowest or highest value for VWC. Once we obtained the optimal soil VWC for a particular plant, we analyzed that variable and also the value for  $A$  at the optimal soil VWC with an ANOVA, using the factor of plant type. We also performed several a priori contrasts in Proc GLM of the program SAS version 9.1.3 (Cary, North Carolina, USA) corresponding to particular genetic hypotheses (Campbell et al., 2008): (1) comparison of the two species, (2) test for heterosis (comparison of the  $F_1$  average to the species average), (3) hybrid breakdown in the  $F_2$  (comparison

of the  $F_1$  average and the  $F_2$  average), and (4) the effect of cytotype for the hybrids (comparison of hybrids with *aggregata* cytoplasm with hybrids with *tenuituba* cytoplasm).

To examine how stomatal conductance changed with soil VWC for the different types of plants, we performed a repeated measures ANOVA, where the main factor was plant type and the repeated measures were conductance 1, 4, 7, 11, 13, and 15 d after watering. We also performed the a priori contrasts described above.

**Nitrogen addition in the field**—In 2006, we conducted a field experiment testing the effect of nutrient addition at the three field sites. At each site, we chose 16 vegetative plants with similarly sized single rosettes. Half of the plants received supplemental nitrogen (1 g potassium nitrate and 1 g ammonium sulfate) in 400 mL of water, once a week for 5 wk. The total application of 10 g N was chosen to equal amounts typically added per square meter per season (= 100 kg/ha; Treseder, 2004). This application raised leaf N above the value observed for the plant type with highest N in our previous field experiment (2.7%, Campbell et al., 2005), eliminating or reducing any variation with plant type in the degree of N limitation. The other half of the plants served as controls, receiving an equivalent amount of water only. After 36–37 d, we measured gas exchange ( $A$ ,  $g$ , and internal  $CO_2$  concentration  $c_i$ ) for each plant. All plants in the *I. aggregata* and hybrid populations were measured on August 23, and all plants in the *I. tenuituba* population on 24 August because it was not possible to measure all individuals in a single day with one LI-COR system. From each plant, we also collected leaves that were air-dried in coin envelopes, ground using a Wig-L-Bug, and sent to the UC Berkeley Center for Stable Isotope Biogeochemistry for elemental analysis of %N,  $\delta^{13}C$ , and  $\delta^{15}N$  isotope with a mass spectrometer. The carbon isotope measure is an estimate of relative water-use efficiency integrated over the leaf's lifetime, with less negative values indicating higher water-use efficiency (Farquhar et al., 1989). Differences in the ratio of  $^{15}N$  to  $^{14}N$  can indicate differences in fractionation in the soil or during N uptake or use (Nadelhoffer et al., 1996).

The size of the vegetative rosette (estimated as number of leaves  $\times$  length of the longest leaf) was measured at the start and end of the experiment and used to calculate the relative growth rate. Finally, during the following summer, we estimated reproductive success of each plant. Plants that did not survive to the next summer were assigned a value of zero because they did not live to reproduce. Plants that were still in the vegetative stage during the following summer were also assigned a reproductive success of zero. For plants that flowered, the total number of seeds produced was estimated as fixed in (Campbell and Waser, 2007). All variables were analyzed with two-way fixed effects ANOVA with the factors of fertilization and site. The univariate analyses were supplemented by MANOVA on  $A$ , growth rate, %N, and isotope measurements. Reproductive success was natural log-transformed (after adding 1) to improve normality of residuals.

To further explore how the nutrient addition influenced photosynthetic rate and growth, we employed structural equation modeling. We used Proc Calis in SAS to estimate path coefficients and compare how nitrogen affected photosynthesis and growth through increasing leaf nitrogen vs. through other mechanisms, as shown in Appendix S2 (see online Supplemental Data). We dropped the nonsignificant paths identified by stepwise multivariate Wald tests one by one, until all remaining paths were statistically significant ( $P < 0.05$ ). Once the final model was obtained, we explored the addition of all paths between these variables and reproductive success, again dropping paths with nonsignificant coefficients.

**Nitrogen manipulations in common environments**—In the field experiment, differential responses to N could reflect genetic effects or effects of the environment. To isolate genetic effects on response to nitrogen, we also performed nitrogen manipulations on plants of known genetic background that had been raised in pots. The 2007 study included plants of the six experimental types used for the dry-down study: both species, both reciprocal  $F_1$  hybrids, and both reciprocal  $F_2$  hybrids. Because the plants had been raised in pots from seeds generated from hand-pollinations in the screenhouse, environmental influences could be largely eliminated. Because natural hybrids differed in percentage leaf N from  $F_2$  hybrids in a previous study (Campbell et al., 2005), in 2008 we performed a second experiment utilizing potted plants of the two species and natural hybrids collected from the three field sites. In this case, the effects of current environmental conditions could be eliminated but not persistent effects of the environment that plants might have experienced prior to being potted. Both potted plant experiments differed from the field study in that we used ammonium nitrate as the source of N so that responses would not be influenced by addition of other nutrients. In both experiments, half of the plants were assigned at random to receive supplemental nitrogen, and half to receive

water only as a control. Fertilized plants received 0.5 g ammonium nitrate in 200 mL once per week (except as described below), and control plants always received the equivalent water. All plants were also watered every 2 d.

In the 2007 experiment with experimental hybrids, single vegetative rosettes were transplanted into native soil from the RMBL area that had been sieved as described above. During the first week only, fertilized plants received 400 mL rather than 200 mL of the fertilizer solution. After 3 wk and after 6 wk, gas exchange variables ( $A$ ,  $g$ , and  $c_i$ ) were measured for all plants. Plants were measured in randomized order spread over 2 d for each interval. In addition to gas exchange, we measured size of the vegetative rosette at the end of the experiment, as described above. Additional leaves were collected, air-dried, and ground for analysis of percentage N and  $\delta^{13}C$  isotope.

In the 2008 study with natural hybrids, plants were transplanted as before except using half soil from Poverty Gulch and half soil from the RMBL town-site, all mixed thoroughly before filling pots. Nitrogen manipulations were begun 8 d after transplanting. Gas exchange variables were measured with the LI-COR 6400 after 6 wk, as described above.

Both of the N manipulation studies in pots were analyzed with two-way fixed effects ANOVA, with plant type and N treatment as the factors. In addition, we performed contrasts comparing *I. aggregata* plants with the other types. We expected these groups to respond differently because *I. aggregata* had much lower percentage leaf N in our previous study (Campbell et al., 2005). Overall relationships between physiological variables were analyzed with path analysis as for the field manipulation of nutrients.

## RESULTS

**Environmental measurements**—On each date of measurement, soils were drier at hybrid site I than at *I. aggregata* site L (randomized block ANOVA  $F_{1,5} = 13.08$ ,  $P < 0.0153$ ). Volumetric water content (averaged over five subsamples) ranged across days from 4.6–8.6% at the hybrid site compared to a range of 5.8–10.4% at the *I. aggregata* site (Appendix S1, see online Supplemental Data).

**Dry-down experiment**—In the dry-down experiment, most plants increased their photosynthetic rate between 1 and 4 d after irrigation ceased and then declined thereafter (Fig. 1A). The optimal soil moisture for photosynthetic rate varied significantly among the six types of plants ( $F_{5,29} = 2.56$ ,  $P < 0.0493$ ; Fig. 2). Photosynthetic rate peaked at a lower soil moisture for hybrids with *I. tenuituba* cytoplasmic background (TA and F2T, optimal VWC = 14–15%) than it did for the reciprocal hybrids with *I. aggregata* cytoplasmic background (AT and F2A, optimal VWC = 17–19%; contrast  $F_{1,29} = 6.14$ ,  $P < 0.0193$ ; Fig. 2). The hybrids with *tenuituba* cytotype also had higher photosynthetic rate at their optimal soil moisture ( $F_{1,29} = 4.26$ ,  $P < 0.0481$ ), reaching 28–30 vs. 21–22  $\mu\text{mol CO}_2\text{-m}^{-2}\text{-s}^{-1}$  for those with *aggregata* cytotype (Fig. 1A). At soil moistures  $< 10\%$  VWC that are typical of those in the field,  $A$  was higher for those with *tenuituba* cytotype. None of the other a priori contrasts between types of plants were significant (all  $P > 0.05$ ).

After the first week, stomatal conductance in all plants declined as soil moisture dropped. The repeated measures ANOVA revealed different temporal responses for conductance of the two plant species (contrast of time  $\times$  species interaction in univariate method with Greenhouse–Geisser correction,  $F_{5,125} = 3.08$ ,  $P < 0.0282$ ). To explore this relationship further, we regressed conductance as a function of soil moisture. The plant types differed significantly in linear regression coefficient (VWC  $\times$  plant type interaction in analysis of covariance,  $F_{5,193} = 3.00$ ,  $P < 0.0124$ ). Fitting separate regressions for each type revealed that stomatal conductance was more sensitive to VWC in *I. aggregata* than in *I. tenuituba* (slopes = 0.021 vs. 0.000, contrast  $P < 0.0207$ ), but we found no evidence for differential

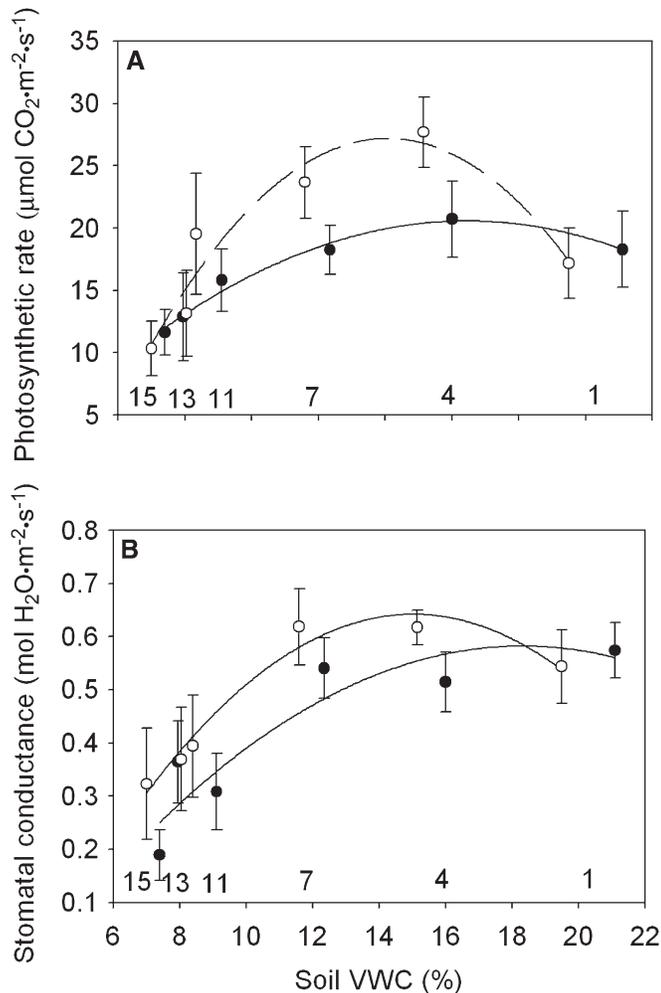


Fig. 1. Changes in gas exchange with soil moisture in the dry-down experiment. (A) Photosynthetic rate. (B) Stomatal conductance. Means and standard errors are shown as a function of mean soil volumetric water content (VWC) at 1, 4, 7, 11, 13, or 15 d after last watering for hybrids with *Ipomopsis aggregata* cytoplasmic background (closed symbols) and hybrids with *I. tenuituba* cytoplasmic background (open symbols). Curves represent quadratic fits to the data. Numbers indicate days since last watering.

responses of hybrids with different cytoplasmic backgrounds (Fig. 1B).

**Nutrient manipulation in the field**—Plants growing in the field responded significantly to the nutrient addition (MANOVA, Wilks lambda  $F_{5,36} = 12.99$ ,  $P < 0.0001$ ). Univariate analyses showed increases in leaf N (Table 1,  $F_{1,40} = 59.90$ ,  $P < 0.0001$ ), photosynthetic rate ( $F_{1,40} = 7.59$ ,  $P < 0.0088$ ), relative growth rate ( $F_{1,41} = 6.93$ ,  $P < 0.0119$ ; Fig. 3), and  $\delta^{15}\text{N}$  ( $F_{1,40} = 19.33$ ,  $P < 0.0001$ ), but not in integrated water-use efficiency as estimated by  $\delta^{13}\text{C}$  ( $F_{1,40} = 2.42$ ,  $P < 0.128$ ). The path model shown in Fig. 4A explained the data well (departure  $P > 0.8134$ ) and yielded significant positive path coefficients for the effect of the treatment on percentage leaf N and growth as well as indirect effects on photosynthetic rate and integrated water-use efficiency mediated by the effect on leaf N (Fig. 4A). Whereas relative growth rate and photosynthetic rate showed similar patterns of response to the nutrient addition (Fig. 3), the path anal-

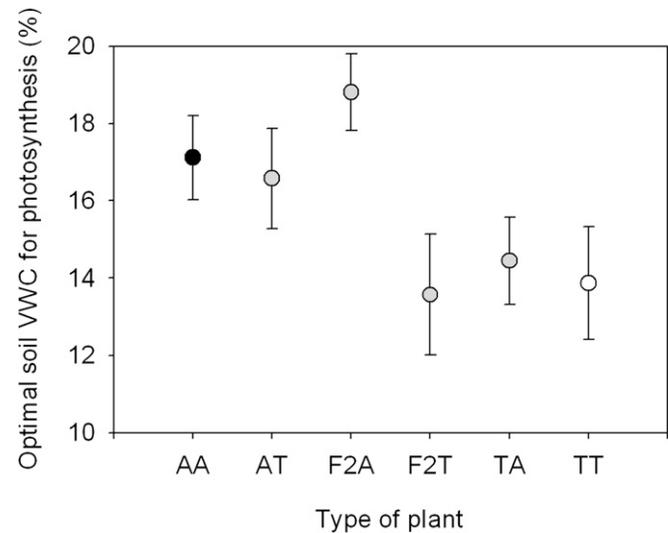


Fig. 2. Optimal soil moisture for photosynthesis in the dry-down experiment. Means and standard errors of the optima are shown for six types of plants. AA: *Ipomopsis aggregata*. AT: F<sub>1</sub> hybrids with *I. aggregata* as the maternal parent. F<sub>2</sub>A: F<sub>2</sub> hybrids with *I. aggregata* cytoplasm. F<sub>2</sub>T: F<sub>2</sub> hybrids with *I. tenuituba* cytoplasm. TA: F<sub>1</sub> hybrids with *I. tenuituba* as the maternal parent. TT: *I. tenuituba*.

ysis suggested that most of the growth response was not due directly to an increase in photosynthesis, because the direct path was not significant and not retained in the final model.

The nutrient treatment  $\times$  site interaction was not significant for any of the physiological variables or growth rate (all  $P > 0.05$ ), nor did the plants at the *I. aggregata* site respond to nutrients significantly differently in these variables from plants at the other two sites. Seed production did, however, show a treatment  $\times$  site interaction ( $F_{2,41} = 3.78$ ,  $P < 0.0311$ ), with fertilization increasing reproductive success only at the *I. aggregata* and hybrid sites (Fig. 3). The path analysis suggested that the primary effect on seed production occurred via the increase in % leaf nitrogen in a way that was not mediated directly by aboveground growth rate in the same season (Fig. 4B).

**Nitrogen manipulations in common environments**—Plants growing in pots also responded to the addition of nitrogen

TABLE 1. Percentage leaf N as a response of *Ipomopsis* to soil nutrients in the field and in common garden studies in pots. Values are means  $\pm$  SE.

Experiment	Type of plant	Control	Nutrients added
Field	<i>I. aggregata</i> in situ	2.31 $\pm$ 0.12	3.23 $\pm$ 0.13
	Natural hybrid in situ	1.88 $\pm$ 0.12	3.09 $\pm$ 0.19
Pots 2007	<i>I. tenuituba</i> in situ	2.10 $\pm$ 0.14	2.79 $\pm$ 0.16
	<i>I. aggregata</i> (AA)	2.40 $\pm$ 0.32	4.66 $\pm$ 0.19
	<i>I. aggregata</i> $\times$ <i>I. tenuituba</i> (AT)	2.82 $\pm$ 0.45	4.60 $\pm$ 0.39
	F <sub>2</sub> A	2.25 $\pm$ 0.19	4.79 $\pm$ 0.16
	F <sub>2</sub> T	2.05 $\pm$ 0.11	4.20 $\pm$ 0.20
	<i>I. tenuituba</i> $\times$ <i>I. aggregata</i> (TA)	1.95 $\pm$ 0.48	4.63 $\pm$ 0.24
Pots 2008	<i>I. tenuituba</i> (TT)	2.43 $\pm$ 0.26	4.94 $\pm$ 0.36
	<i>I. aggregata</i>	3.04 $\pm$ 0.24	5.22 $\pm$ 0.18
	Natural hybrid	2.77 $\pm$ 0.25	5.71 $\pm$ 0.15
	<i>I. tenuituba</i>	3.24 $\pm$ 0.32	5.08 $\pm$ 0.20

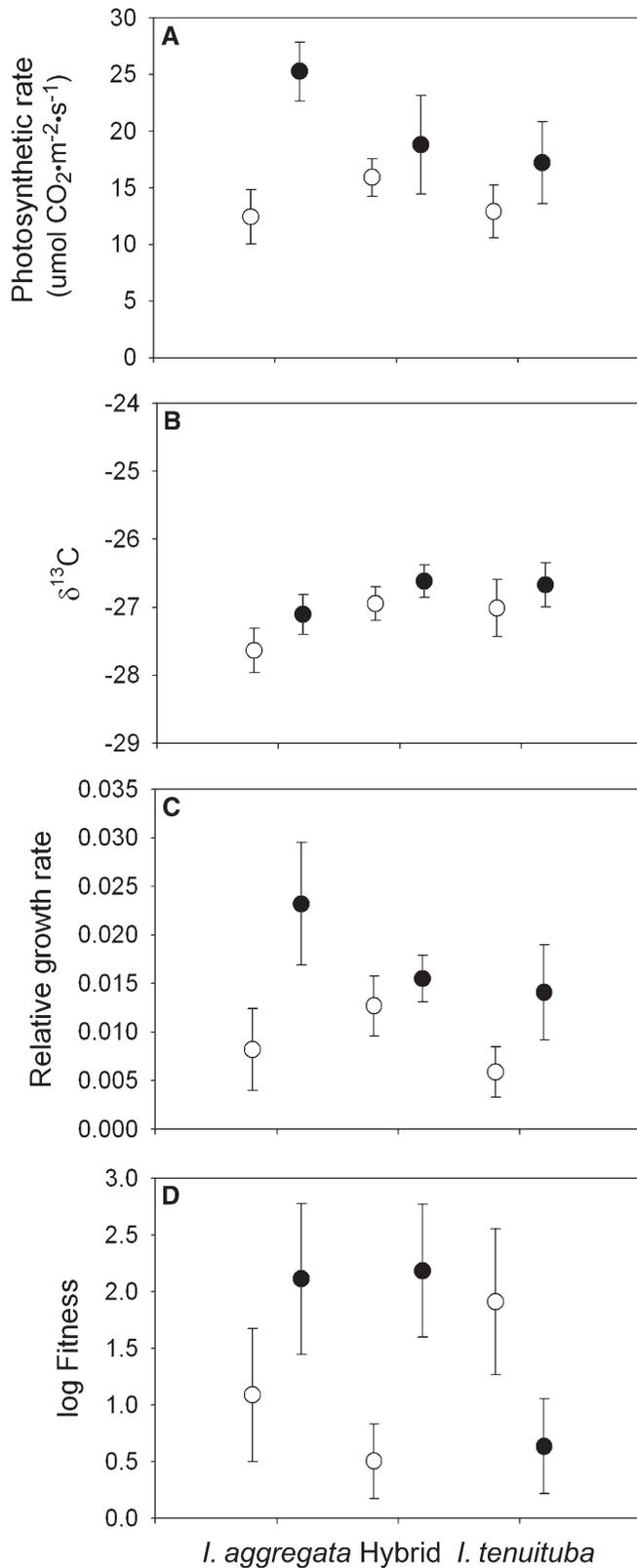


Fig. 3. Effects of nutrient addition on plants growing in situ in the *Ipomopsis aggregata*, *I. tenuituba*, and hybrid field sites. (A) Photosynthetic rate. (B)  $\delta^{13}\text{C}$ . (C) Relative aboveground growth rate. (D) Fitness based on number of seeds produced the following year. Means and standard errors are shown for control plants (open symbols) and fertilized plants (closed symbols).

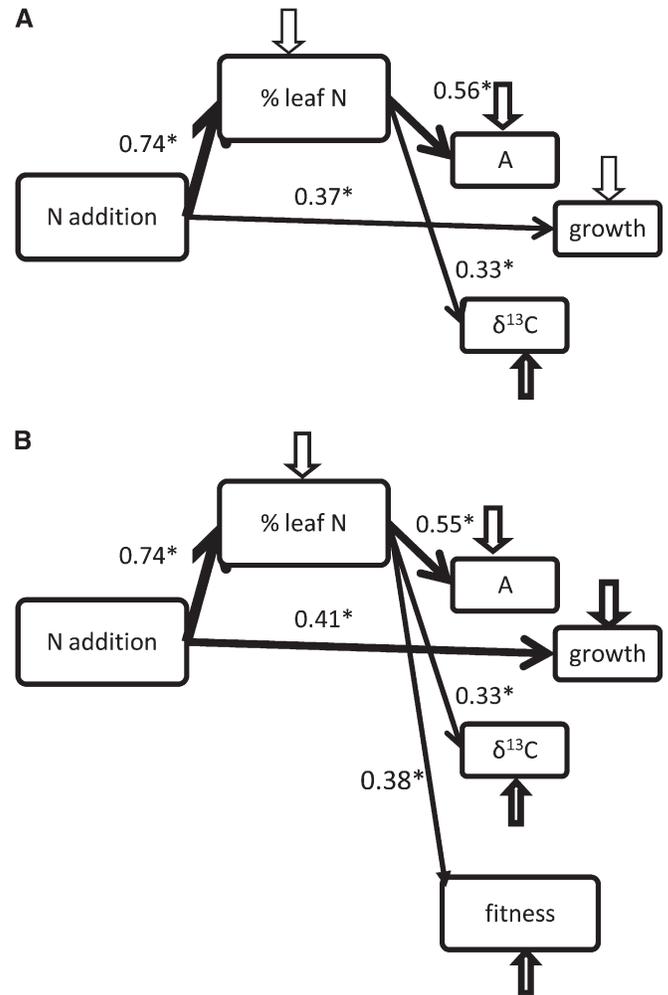


Fig. 4. Solved path analyses for the effects of nutrient additions in the field. The width of the arrow indicates the size of the standardized path coefficient (also given as a number). Unlabelled arrows that do not have a starting box show the relative size of variation unexplained by the model. \*  $P < 0.05$ . (A) Without the fitness estimate. (B) With fitness (seeds produced) included.

(MANOVA Wilks lambda,  $F_{4,32} = 53.76$ ,  $P < 0.0001$  in 2007,  $F_{4,39} = 58.11$ ,  $P < 0.0001$  in 2008). For the 2007 study with experimental hybrids raised from seed, univariate analyses showed a doubling of leaf N from 2.3 to 4.6% on average (Table 1,  $F_{1,35} = 178.61$ ,  $P < 0.0001$ ) and increases in photosynthetic rate ( $F_{1,46} = 5.07$ ,  $P < 0.0291$ ) and size after 6 wk ( $F_{1,35} = 21.96$ ,  $P < 0.0001$ , Fig. 5). Although the integrated measure of water-use efficiency was not significantly altered by nitrogen ( $P = 0.1407$ , Fig. 5C), intrinsic water-use efficiency, regardless of whether measured as  $A/g$  or  $c_i$ , did increase significantly ( $F_{1,46} = 5.41$  and  $5.55$ , both  $P < 0.05$ ) as stomatal conductance was largely unchanged ( $P = 0.1957$ , Fig. 5B). We could not detect differences between types of plants in their responses of any of these variables to nitrogen (all type  $\times$  treatment interactions,  $P > 0.05$ ). Neither did plants of *I. aggregata* show a significantly stronger response than the average for the other types of plants combined (all  $P > 0.05$ ). However, there was a main effect of plant type on  $\delta^{13}\text{C}$  ( $F_{5,35} = 2.63$ ,  $P < 0.0406$ ), with plants of *I. tenuituba* showing the highest integrated water-use efficiency (mean of  $-25.85$ , Fig. 5C).

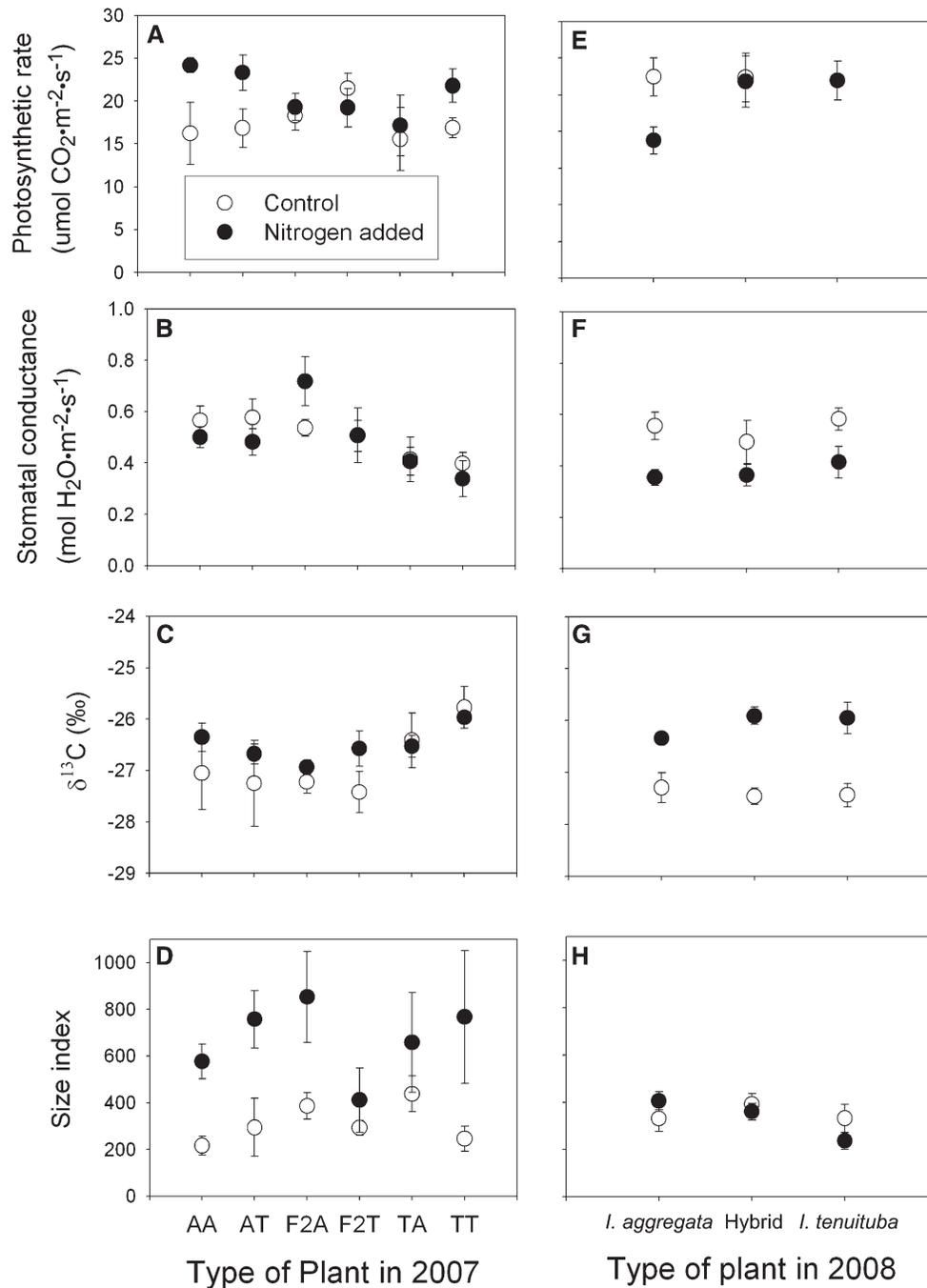


Fig. 5. Effects of nitrogen additions measured after 6 wk in the common garden experiments in 2007 and 2008. (A and E) Photosynthetic rate. (B and F) Stomatal conductance. (C and G)  $\delta^{13}\text{C}$ . (D and H) Size index. Conventions follow those in Fig. 3.

Our 2008 experiment with natural hybrids contrasted with our other experiments in that nitrogen addition had no significant overall effect on photosynthetic rate ( $F_{1,42} = 2.57$ ,  $P = 0.1165$ ), even though percentage N in the leaves was increased from 3.02 to 5.34% (main effect in ANOVA,  $F_{1,42} = 151.98$ ,  $P < 0.0001$ ). Photosynthetic rate was remarkably high even in the control plants, with a mean of  $27 \mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for each of the three plant types, *I. aggregata*, natural hybrids, and *I. tenuituba*, a value similar to that obtained only with fertilization in

our other studies. Because control plants had very high levels of %N compared to in our other studies (Table 1), this result likely reflected unusually high levels of N already in the soil used for this study. Although N addition did not increase A in this particular experiment, it did dramatically increase integrated water-use efficiency ( $F_{1,42} = 55.46$ ,  $P < 0.0001$ , Fig. 5G), consistent with the decrease in stomatal conductance ( $F_{1,42} = 13.64$ ,  $P < 0.0006$ , Fig. 5F). Again, no interactions were detected between plant type and N addition for %N, A,  $\delta^{13}\text{C}$ , or size (all  $P > 0.05$ ).

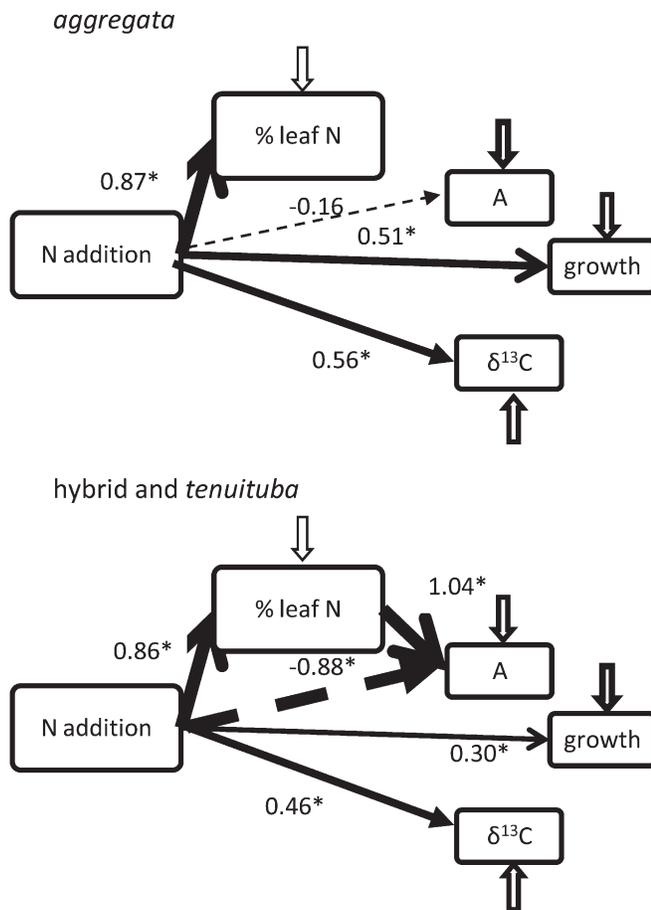


Fig. 6. Solved path analyses of the common garden experiments, separately for (A) *Ipomopsis aggregata* plants and for (B) all other types. Conventions follow those in Fig. 4.

We saw no main effect of plant type on %N ( $F_{2,42} = 0.11$ ,  $P = 0.8980$ ), nor did natural hybrids have higher %N than *I. aggregata* in control plants (Table 1), as had been observed in a previous common garden study (Campbell et al., 2005).

Structural equation modeling led to similar path diagrams for the 2007 and 2008 experiments with potted plants. In both cases, photosynthetic rate responded strongly to leaf N (standardized path coefficients = 0.78 and 0.61 for 2007 and 2008 respectively, both  $P < 0.05$ ), despite the absence of a detectable effect in the ANOVA for 2008.

Because path diagrams were similar for the 2 years, we combined data from both experiments to increase sample sizes for comparing *I. aggregata* with all other plants. The hybrids (including experimental hybrids and natural hybrids) and *I. tenuituba* were lumped into a single category because preliminary separate analyses produced similar path coefficients for the model in Fig. 6B, with the exception of no significant paths to growth for *I. tenuituba* (standardized coefficients = 0.01 and 0.16 for paths from A and N addition, respectively). The best-fitting path diagrams were different for the two sets of plants retained in the final analyses (Fig. 6). For *I. aggregata*, leaf N, water-use-efficiency, and plant size all responded independently to N addition, and photosynthetic rate did not show a significant path. In contrast, for the other plants, photosynthetic rate (A) responded strongly to leaf N, and there was also a

negative direct effect of nitrogen addition on A, suggesting a mechanism not present in the field data (Fig. 4). Each path fit its own data set well (departure  $\chi^2$ ,  $P = 0.8702$  and  $0.5685$  for *aggregata* and other plants). In contrast, the data for hybrid and *tenuituba* plants would be poorly fit by the path diagram shown for *I. aggregata* (departure  $\chi^2$ ,  $P < 0.0001$ ).

## DISCUSSION

**Responses to water**—In the hybrid zone at the Poverty Gulch contact site, plants of *Ipomopsis tenuituba* and hybrids occupy drier habitats than do plants of *I. aggregata*. Previous studies had shown that these plants have higher water-use efficiencies than do plants of *I. aggregata* both in the field (Wu and Campbell, 2007) and when grown under common conditions (Campbell et al., 2005). Furthermore, most natural hybrids, which grow in the driest sites, have cytoplasmic genes inherited from *I. tenuituba* (Wu and Campbell, 2005), an asymmetry that suggested the hypothesis that such hybrids would perform better in response to drought than do plants with *I. aggregata* cytoplasmic genes. Here we found that photosynthetic responses to simulated drought varied markedly among different types of hybrids. As predicted, those hybrid plants with *I. tenuituba* cytoplasmic background and thus with chloroplast and mitochondrial genomes inherited from that species had a lower optimal soil moisture for photosynthesis. In addition, stomatal conductance was more sensitive to soil moisture for *I. aggregata* than it was for *I. tenuituba*. These results are consistent with the higher integrated water-use-efficiency for *I. tenuituba* in the 2007 potted plant study, as well as an earlier study (Campbell et al., 2005). They also offer a mechanistic explanation for why hybrids with *I. tenuituba* cytoplasm have relatively high fitness when planted into the dry hybrid site, even though they have very low fitness when planted into the more mesic *I. aggregata* site. Similar context-dependence for the performance of hybrids with different cytotypes has been documented in *Brassica rapa* (Allainguillaume et al., 2009). In that case, the crop-specific chloroplast appears adaptive in riverside populations of crop-wild hybrids but not in populations found in weedy agricultural fields, potentially explaining the high level of chloroplast acquisition in the riverside populations.

One limitation to our study is that the results are based primarily on leaf-level traits. Our analyses of responses to N suggested that aboveground growth and reproductive success are not necessarily tightly coupled with leaf-level gas exchange (see below), and so further experiments are required to see if plants with *I. tenuituba* cytoplasmic genes (both the pure species and hybrids) also have relatively higher fitness under drought conditions. If so, these differences in physiological responses to soil moisture could help to explain the restriction of hybrids to particular environmental sites.

Responses to soil moisture could also, in principle, help to explain differences in the frequency of hybridization across different contact sites, as these vary greatly in habitat types. Whereas the Poverty Gulch contact site studied here occurs in the subalpine, other contact sites across the western United States are dominated by sagebrush and sage oak and still others occur at a transition to the alpine (Aldridge, 2005). Because such contact sites may differ in soil moisture, the consequences of drought tolerance in hybrids may play out differently in these various localities and might be an additional factor, along with pollination, explaining the geographic variation in frequency of hybrids seen in this group.

**Responses to nitrogen**—Soil N is another environmental factor that we had predicted to influence the relative performance of these two species and their hybrids, in part because leaf N was much higher in the progeny of crosses between natural hybrids than in *I. aggregata* in a common garden study conducted in the field (Campbell et al., 2005). Addition of soil N led to overall increases not only in leaf N per area but also in photosynthetic rate, as expected and commonly observed, since photosynthetic proteins represent the majority of leaf N (Evans, 1989). In some experiments, extra soil N also led to an increase in integrated water-use efficiency, although the mechanism appeared to vary. In the field study with in situ plants, water-use efficiency was positively related to leaf N (Fig. 4), whereas in the common-environment studies, the increase in water-use efficiency was not mediated by leaf N (Fig. 6). In addition to environmental differences, the field study differed from the common-garden studies in that the source of nutrients included not only N but also K and sulfate, which conceivably could have led to differences in response. In the 2006 and 2007 experiments, aboveground growth also responded positively to nutrient addition, but not via a path of increased photosynthetic rate. The path analyses also suggested that growth did not relate directly to leaf N, even though the two traits were correlated ( $r = 0.32$ ,  $N = 46$ ,  $P = 0.0301$  in 2006 and  $r = 0.51$ ,  $N = 47$ ,  $P = 0.0002$  in 2007). Similar correlations of growth and leaf N were also observed in a study of *Helianthus* hybrids (Ludwig et al., 2004).

Although overall responses to soil N were strong, we saw little evidence to support the hypothesis that the two species and hybrids respond in fundamentally different ways, at least when grown in a common environment. Indeed, our 2008 experiment with plants in pots was unable to replicate the earlier common garden finding in which natural hybrids had higher leaf nitrogen than *I. aggregata*, instead finding no significant difference between control plants of these two types ( $P > 0.40$ ). This discrepancy could reflect the overall higher N levels even without supplementation, or other differences between growth conditions in pots and in the field. The only response variable for which we detected a plant type  $\times$  nutrient interaction was fitness in the field. Plants of *I. aggregata* and hybrids lived to produce more seeds if they had received fertilizer, whereas the nutrient addition made no difference or even decreased fitness for plants of *I. tenuituba*, suggesting that something other than N limits seed production in the latter species. One candidate is P, since levels in the soil were lower at that site (online Appendix S1), and a second candidate is pollination level because hummingbirds are less common visitors at that higher elevation (Campbell et al., 1997, 2002). The fitness measure related positively to leaf N but not to aboveground growth in the year of nutrient addition, suggesting that the main effect of N on seed production may be mediated through acquisition of C stored in belowground tissues. In our field experiment, plants at the hybrid site not only showed the biggest increase in fitness; they also had the largest increase in  $\delta^{15}\text{N}$  isotope ratio (from  $-0.76$  to  $1.20$  ‰). At all sites, the N addition increased  $\delta^{15}\text{N}$  in leaves, consistent with the commonly observed association between  $\delta^{15}\text{N}$  and N supply (Craine et al., 2009). In some habitats,  $\delta^{15}\text{N}$  is higher in nonmycorrhizal plants and also increases with soil depth (Nadelhoffer et al., 1996), suggesting the hypothesis that fertilized plants at the hybrid site may have especially enhanced root growth that allows acquisition from deeper soil layers and/or more reduced dependence on mycorrhizal associations.

The 2008 experiment differed from the others in that photosynthesis did not increase with N addition to the soil. This dis-

crepancy may be explained by a high leaf N level already in the control plants (3.0%), a level equal to that in the field only for fertilized plants. Photosynthetic rate often relates linearly to leaf nitrogen over a wide range of % N, (Evans, 1989; Marshall and Vos, 1991), but a lack of increase in response to N is sometimes observed with N at levels higher than normally encountered (Field et al., 1983). This N-saturation of photosynthesis may help to explain the negative path from N addition to photosynthetic rate (A in Fig. 6), as in combination with the positive path from leaf N, it could reflect a less than linear increase of A with soil N. Despite any saturation of photosynthetic rate, in 2008 addition of soil N did increase water-use efficiency, presumably via the observed decrease in stomatal conductance. Such effects of soil nitrogen on water-use efficiency suggest that interactions between water and N availability may influence growth in the natural hybrid zone.

The only indication that physiological variables responded in different ways to N for different types of plants came from path diagrams showing a weaker response of photosynthetic rate to leaf N for *I. aggregata* than for hybrids and *I. tenuituba*, when grown in a common environment (Fig. 6). This difference was not sufficient, however, to generate a detectable interaction between effects of type of plant and N addition on photosynthetic rate.

**Conclusions**—Our results suggest that variation in soil moisture is more important than N levels in explaining the relative photosynthetic performance of these *Ipomopsis* hybrids compared to their parental species. In particular, plants with a cytoplasmic background of *I. tenuituba*, including hybrids, had a lower optimal soil moisture for photosynthetic rate than did those with the alternate cytoplasmic background. This pattern is consistent with their distribution in the field at the Poverty Gulch contact site and helps to explain the previously demonstrated patterns in fitness in this hybrid zone, as plants with *I. tenuituba* cytoplasm not only occupy drier sites than does *I. aggregata* but also achieve higher relative fitness in the drier sites than they do when planted into *I. aggregata* territory. This study represents a first step in elucidating the mechanisms by which an environmental variable mediates the relative fitness of hybrids; the next step is to measure the consequences of the physiological responses to soil moisture for fitness. In general, studies that evaluate the linkages between environmental variables, physiological performance, and fitness are sorely needed to understand how the fitness of plant hybrids depends on the environment.

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