

# Phenotypic selection on leaf ecophysiological traits in *Helianthus*

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## Summary

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**Key words:** desert dunes, *Helianthus* (sunflower), leaf size, nitrogen, selection differentials, selection gradients, succulence, water-use efficiency.

- Habitats that differ in soil resource availability are expected to differ for selection on resource-related plant traits.
- Here, we examined spatial and temporal variation in phenotypic selection on leaf ecophysiological traits for 10 *Helianthus* populations, including two species of hybrid origin, *Helianthus anomalus* and *Helianthus deserticola*, and artificial hybrids of their ancestral parents. Leaf traits assessed were leaf size, succulence, nitrogen (N) concentration and water-use efficiency (WUE).
- Biomass and leaf traits of artificial hybrids indicate that the actively moving dune habitat of *H. anomalus* was more growth limiting, with lower N availability but higher relative water availability than the stabilized dune habitat of *H. deserticola*. Habitats differed for direct selection on leaf N and WUE, but not size or succulence, for the artificial hybrids. However, within the *H. anomalus* habitat, direct selection on WUE also differed among populations. Across years, direct selection on leaf traits did not differ.
- Leaf N was the only trait for which direct selection differed between habitats but not within the *H. anomalus* habitat, suggesting that nutrient limitation is an important selective force driving adaptation of *H. anomalus* to the active dune habitat.

## Introduction

Species and population differences in plant ecophysiological traits related to carbon gain and resource use have long been observed and are often interpreted as potentially adaptive (Ackerly *et al.*, 2000; Aerts & Chapin, 2000; Arntz & Delph, 2001). Phenotypic selection analysis provides a means of estimating the strength of direct and indirect selection on individual traits, and thus testing adaptive hypotheses for the evolution of these traits (Lande & Arnold, 1983; Brodie *et al.*, 1995; Kingsolver *et al.*, 2001). Detection of contrasting selection between habitats provides the strongest inference that a trait is adaptive in a particular habitat and that selection may drive population differentiation (Wade & Kalisz, 1990; Dudley, 1996a). Selection has been shown to vary spatially and temporally for morphological and life-history traits (Kalisz, 1986; Kelly, 1992; Bennington & McGraw, 1995; Maad & Alexandersson, 2004; Young, 2008). However, there have been relatively few tests of whether selection on plant ecophysiological traits differs by resource environment (Dudley, 1996a; Heschel & Riginos, 2005; Sherrard & Maherali, 2006; Saldana *et al.*,

2007). Here we used a compilation of unpublished and published data for *Helianthus* to investigate the spatial and temporal variation in direct selection on plant ecophysiological traits.

*Helianthus anomalus* and *Helianthus deserticola* are annual sunflowers of recent homoploid hybrid origin (170 000–63 000 yr BP) from the same ancestral parent species: *Helianthus annuus* and *Helianthus petiolaris* (Schwarzbach & Rieseberg, 2002; Gross *et al.*, 2003). The two hybrid species are endemic to desert southwest USA in habitats that differ from those of their ancestral parent species (Gross *et al.*, 2003; Rosenthal *et al.*, 2005a). Studies of naturally occurring plants in the hybrid species habitats indicate that the actively moving dune habitat of *H. anomalus* has lower N and higher water availability than the adjacent stabilized dune habitat of *H. deserticola* (Rosenthal *et al.*, 2005a; Donovan *et al.*, 2007). In addition, the growth of naturally occurring *H. anomalus* seedlings is enhanced by the addition of nitrogen (N) and water, but not by water alone (Ludwig *et al.*, 2006). This raises the question of whether selection on ecophysiological traits differs between these two habitats and thus may have contributed to local adaptation and hybrid speciation. We address this question

**Table 1** Summary of *Helianthus* populations analysed for phenotypic selection of leaf ecophysiological traits

Population codes	Plant material	Habitat	Natural population or experimental plot	Year measured	Population sample size
BC2ann-ANO	BC2ann	<i>H. anomalus</i>	ANO plot <sup>1</sup>	2002	114
BC2pet-ANO	BC2pet	<i>H. anomalus</i>	ANO plot <sup>1</sup>	2002	104
BC2ann-DES	BC2ann	<i>H. deserticola</i>	DES plot <sup>2</sup>	2002	198
BC2pet-DES	BC2pet	<i>H. deserticola</i>	DES plot <sup>2</sup>	2002	192
BC2annF1-ANO-03	BC2ann-F1	<i>H. anomalus</i>	ANO plot	2003	175
<i>H. anomalus</i> -ANO	<i>H. anomalus</i>	<i>H. anomalus</i>	ANO plot <sup>1</sup>	2002	130
<i>H. anomalus</i> -ANO-03	<i>H. anomalus</i>	<i>H. anomalus</i>	ANO plot	2003	141
<i>H. anomalus</i> -wet	<i>H. anomalus</i>	<i>H. anomalus</i>	Natural population-wet <sup>3</sup>	2002	114
<i>H. anomalus</i> -dry	<i>H. anomalus</i>	<i>H. anomalus</i>	Natural population-dry <sup>3</sup>	2002	114
<i>H. deserticola</i>	<i>H. deserticola</i>	<i>H. deserticola</i>	Natural population <sup>3</sup>	2002	135

ANO plot and DES plot refer to the experimental plots in the *H. anomalus* and *H. deserticola* habitats, respectively. BC2ann and BC2pet were interspecific artificial hybrids, backcrosses toward *H. annuus* and *H. petiolaris*, respectively (see the Materials and Methods section).

<sup>1</sup>Data originally published in Ludwig *et al.* (2004).

<sup>2</sup>Part of data originally published in Gross *et al.* (2004).

<sup>3</sup>Data originally published in Donovan *et al.* (2007).

with a compilation of data for 10 *Helianthus* populations (Table 1), including artificial hybrids (BC2ann, BC2pet) planted into both *H. anomalus* and *H. deserticola* habitats, natural populations in both habitats, and *H. anomalus* planted into its own habitat. BC2ann and BC2pet are artificial hybrids of *H. annuus* and *H. petiolaris* (see the Materials and Methods section for details) that represent the ancestral hybrid populations from which the hybrid species arose and contain a range of values for individual traits and trait combinations, including ‘*H. anomalus*-like’ and ‘*H. deserticola*-like’ multi-trait phenotypes (Rieseberg *et al.*, 2003; Rosenthal *et al.*, 2005b). Hybrid speciation may have been facilitated by ‘escape’ of early generation hybrid phenotypes into the new habitats currently occupied by the *H. anomalus* and *H. deserticola*, and subsequent environmental selection (Rieseberg *et al.*, 2003; Karrenberg *et al.*, 2007).

For these *Helianthus* populations, we chose focal traits based on their potential relevance to the differing habitats and potential importance based on previous comparisons of these species in common environment experiments: individual leaf size (individual leaf area), leaf succulence, leaf N concentration, and time-integrated photosynthetic water-use efficiency (WUE, ratio of photosynthetic carbon gain to transpirational water loss) estimated from leaf carbon isotope ratio ( $\delta^{13}\text{C}$ ). We expected water and N limitations in the *H. anomalus* and *H. deserticola* habitats to influence selection on these resource related traits (Ehleringer & Clark, 1988; Dudley, 1996a; Geber & Dawson, 1997; Heschel *et al.*, 2002; Wright *et al.*, 2003; Heschel & Riginos, 2005; Maron *et al.*, 2007). Greater leaf size indicates more area for photosynthesis. In some species, leaf area is strongly correlated with plant size, although the causality is unclear (Dudley & Schmitt, 1996). Higher leaf succulence, as generally found in *H. anomalus*, may contribute to drought tolerance (Jennings, 1976; Brouillette *et al.*, 2006).

Leaf size, leaf N and leaf WUE are physiologically linked to each other and to carbon gain, water use and WUE. A smaller leaf size may increase WUE by reducing the leaf boundary layer and thus the leaf temperature and transpirational water loss for a given stomatal conductance (Jones, 1992; Nobel, 1999). Leaf N is a major determinant of the biochemical capacity of photosynthesis (Field & Mooney, 1986). Plants can increase leaf WUE by increasing leaf N invested in photosynthetic capacity and by decreasing stomatal conductance (Wright *et al.*, 2001, 2003). However, only decreasing stomatal conductance will reduce water loss (Cohen, 1970; Ludlow, 1989). Smaller leaf size and greater WUE are generally expected to be favored in water-limited habitats, although there is debate about whether water limitation might also select for drought escape with lower WUE and short lifespan, depending on the timing of water availability (Geber & Dawson, 1990, 1997; Dudley, 1996a; Stanton *et al.*, 2000; McKay *et al.*, 2003; Heschel & Riginos, 2005; Sherrard & Maherali, 2006; Angert *et al.*, 2007; Huxman *et al.*, 2008).

Results from several of the study populations were previously published to address other objectives, as summarized here. Ludwig *et al.* (2004) compared the populations planted into the *H. anomalus* habitat (hereafter designated ‘ANO’ experimental plot) in 2002, including *H. anomalus* and artificial hybrids (BC2ann and BC2pet). There was selection on all of the leaf traits in at least one of the populations, and selection on some of the traits in the artificial hybrids was similar to, or could move the phenotype towards, the current *H. anomalus* phenotype. Gross *et al.* (2004) compared the artificial hybrids planted in to the *H. deserticola* habitat (hereafter designated ‘DES’ experimental plot) in 2002 for a different set of leaf traits and came to a similar conclusion that selection on some of the traits in the artificial hybrids could move the phenotype towards the current *H. deserticola* phenotype. Donovan *et al.*

(2007) compared the wild populations in 2002 and found no evidence that the longer-lived *H. anomalous* is under selection for lower WUE characteristics consistent with drought tolerance, or that the shorter-lived *H. deserticola* is under direct selection on WUE consistent with drought escape.

By adding the previously unpublished 2003 ANO plot data and reanalysing the 2002 DES plot data for a comparable set of traits, we now have a compilation of data from 10 populations to ask several new questions. (1) Does selection differ spatially among habitats for artificial hybrids planted into both habitats? A spatial comparison of selection across habitats for the same traits and the same plant material provides the strongest inference of selective forces driving trait differentiation and local adaptation (Wade & Kalisz, 1990; see discussion in Dudley, 1996a; Geber & Griffen, 2003). To provide a context for habitat comparison, we ask two further questions: (2) Does direct selection differ spatially among planted and natural populations of *H. anomalous* within the *H. anomalous* habitat; and (3) Does direct selection differ temporally across years for planted populations within the *H. anomalous* habitat? Selection that varies between habitats, but not within a habitat or across years, is a likely candidate for driving population differentiation and speciation. Finally, we report the relationship of direct to total selection for each trait in order to address potential constraints on evolutionary response to selection.

## Materials and Methods

The study populations (Table 1) were located in central Utah, USA, at the Little Sahara Recreation Area (LSRA, 39°41'N, 112°22'W) managed by the Bureau of Land Management. The Little Sahara study area has a cold desert climate with an average rainfall of 299 mm (24-yr average, 1979–2006). The studies were conducted in 2002, which was a relatively dry year (120 mm), and 2003, which was closer to average (289 mm). In 2002, the *H. anomalous* habitat had lower soil N and higher late summer soil moisture compared with the *H. deserticola* habitat (Rosenthal *et al.*, 2005a). The natural populations at the LSRA were described in Donovan *et al.* (2007) and the experimental plots were located near the natural populations (Gross *et al.*, 2004; Ludwig *et al.*, 2004).

### Sources for ANO and DES experimental plot plants

In 2002, *H. anomalous* Blake seedlings were planted into the ANO plots, and BC2ann and BC2pet seedlings were planted into the ANO and DES plots (Gross *et al.*, 2004; Ludwig *et al.*, 2004). The seed source for *H. anomalous* was the site of the ANO plot. BC2ann and BC2pet are interspecific artificial hybrids (backcrosses toward *H. annuus* and *H. petiolaris*, respectively) used in glasshouse studies of hybrid trait expression and quantitative trait loci studies (Rieseberg *et al.*, 2003; Rosenthal *et al.*, 2005b; Brouillette *et al.*, 2007).

In order to compare selection across years, we also planted *H. anomalous* and artificial hybrids in the ANO plot in 2003. For *H. anomalous*, the same seed source was used in 2002 and 2003. For BC2ann, the seed source used in 2002 had been depleted, so for comparison in 2003 we used BC2ann-F<sub>1</sub> that had been generated during earlier studies at University of Georgia (glasshouse-grown BC2ann plants described in Rosenthal *et al.* (2005b) were crossed randomly to generate BC2ann-F<sub>1</sub>). Because the BC2ann-F<sub>1</sub> was generated in the glasshouse with non-limiting water and nutrients, and no environmental selection, we expect it to represent a similar genetic mix as BC2ann. For both years, the seedlings were germinated in April (to coincide with germination in natural populations) in glasshouses (Indiana University in 2002, University of Georgia in 2003), transported to UT by truck, and transplanted into the experimental plots (13–14 May in 2002, and 6–17 May in 2003).

### Plant trait methods

Leaf measurements were made on 7–19 June 2002, and 27 June 2003. For the experimental plot populations, all live plants that produced  $\geq 4$  leaves after transplants were sampled. The youngest fully expanded leaf was collected at dawn, when maximally hydrated, and measured for leaf wet mass and leaf area (CID, Inc., Pullman, WA, USA). Leaves were dried at 60°C for determination of dry mass. Leaf succulence was calculated as (wet biomass – dry biomass)/leaf area (Jennings, 1976). Dried leaves were individually ground and analysed for N concentration (mg N g<sup>-1</sup> dry biomass) (NA 1500: Carlo Erba Instruments, Milan, Italy) and leaf carbon isotopic composition (leaf  $\delta^{13}\text{C}$ ) (continuous flow mass spectrometer; Finnegan, Bremen, Germany). Leaf  $\delta^{13}\text{C}$  provides an integrated measure of leaf intercellular CO<sub>2</sub> concentration ( $C_i$ ) over the lifetime of the leaf. Integrated  $C_i$  is, in turn, a relative measure of integrated instantaneous WUE, provided that leaf temperatures are similar (Farquhar *et al.*, 1989; Ehleringer, 1993). A higher (less negative) leaf  $\delta^{13}\text{C}$  reflects greater WUE.

For each population, plants were harvested for aboveground biomass either during the study if they died, or at the end of the growing season harvest (4–6 September 2002, 9 September 2003). For each plant, the biomass was sorted into vegetative biomass (stem and leaf) and reproductive biomass (bud, flower, and seeds), dried at 60°C, and weighed. We used aboveground vegetative biomass as our proxy for fitness because that was the most integrative measure of performance available for comparison across all 10 naturally occurring and planted populations (Ludwig *et al.*, 2004). Vegetative biomass is a reasonable proxy for fitness based on the expectation that leaf traits affect fitness primarily through vegetative biomass (Samson & Werk, 1986; Ehleringer & Clark, 1988; Ferris & Lechowicz, 1990; Dudley, 1996a). As a measure of fitness, biomass incorporates both survivorship and fecundity, because plants that died at a younger age should be smaller and plants that are larger should produce more seeds. For the ANO plot in 2003, the

correlation between vegetative biomass and reproductive biomass was significant for *H. anomalous*-ANO-03 ( $r^2 = 0.80$ ,  $P < 0.001$ ,  $n = 141$ ) and BC2annF1-ANO-03 ( $r^2 = 0.23$ ,  $P < 0.001$ ,  $n = 171$ ). In addition, *H. anomalous*-ANO-03 seed head biomass was correlated with seed number ( $r^2 = 0.72$ ,  $P < 0.001$ ,  $n = 70$  seed heads) for a subset of seed heads collected as seeds matured and before they dispersed. For the other populations, the vegetative and reproductive biomass correlation coefficients range from  $r^2 = 0.39$  to  $r^2 = 0.93$  ( $P < 0.001$  for all) (Ludwig *et al.*, 2004; Donovan *et al.*, 2007).

### Phenotypic selection analysis

Selection differentials ( $S$ ) indicate total selection for a trait that includes direct selection on that trait and indirect selection through correlated traits. Selection gradients ( $\beta$ ) indicate direct selection, provided that relevant traits have been incorporated into the analyses (Lande & Arnold, 1983; Mitchell-Olds & Shaw, 1987). Standardized selection differentials and selection gradients were calculated for each of the 10 populations. For each selection analysis, the trait data were standardized (mean = 0, variance = 1) with no further transformations applied. Aboveground vegetative biomass, our proxy for fitness, was relativized to population mean (mean = 1). Standardized linear selection differentials ( $S$ ) were estimated for each leaf trait as the covariance between standardized traits and relative fitness (Lande & Arnold, 1983). Standardized linear selection gradients ( $\beta$ ) were estimated for the leaf traits with multiple regressions, with relative fitness as the dependent variable and standardized traits as independent variables (Lande & Arnold, 1983). For  $S$  and  $\beta$  the residuals of the regression analyses were not normally distributed, so significance levels were determined by a bootstrap analysis with 10 000 estimates (Dixon, 2000). The 95% confidence intervals were then estimated from the bootstrap distribution.

Pairwise contrasts of populations for the linear selection gradients were done with an additional term in the general linear model (e.g. plot or year) that allows for comparison of slopes (Kalisz, 1986; Dudley, 1996a), with significance again assessed with bootstrap analysis and 95% confidence intervals. Pairwise contrasts of populations for ecophysiological traits and biomass were done with one-way analysis of variance (SAS, 2001).

For each population, nonlinear selection gradients were estimated using a general linear model with relative fitness as the dependent variable and all trait values as linear terms, but with quadratic terms (nonlinear selection) and all two-way interactions between traits (correlational selection) included. Significance levels were determined by a bootstrap analysis with 10 000 estimates (Dixon, 2000). These results are not addressed in the results and discussion because there were no new examples of nonlinear selection and only one new example of correlational selection (leaf size and N, BC2ann-

F1) beyond those already reported (Ludwig *et al.*, 2004; Donovan *et al.*, 2007).

For each population, phenotypic correlations among traits were assessed with Pearson correlations. For each trait, the relationship between direct and indirect selection across the 10 populations was assessed with a Pearson correlation.

## Results

### Spatial comparison of artificial hybrids in contrasting habitats: ANO and DES plots

The artificial hybrid populations (BC2ann and BC2pet) were compared for trait expression and selection in the ANO and DES experimental plots in 2002. The BC2ann and BC2pet plants accumulated 60–90% less biomass in the ANO plot than in the DES plot (for population means and statistical comparison see Table 2; for individual plant data see Ludwig *et al.* (2004) for the ANO plot plants and the Supporting Information, Fig. S1, for the DES plot plants). The lower biomass in the ANO plot was accompanied by smaller leaf size, lower leaf succulence, lower leaf N and lower WUE (more negative  $\delta^{13}\text{C}$ ).

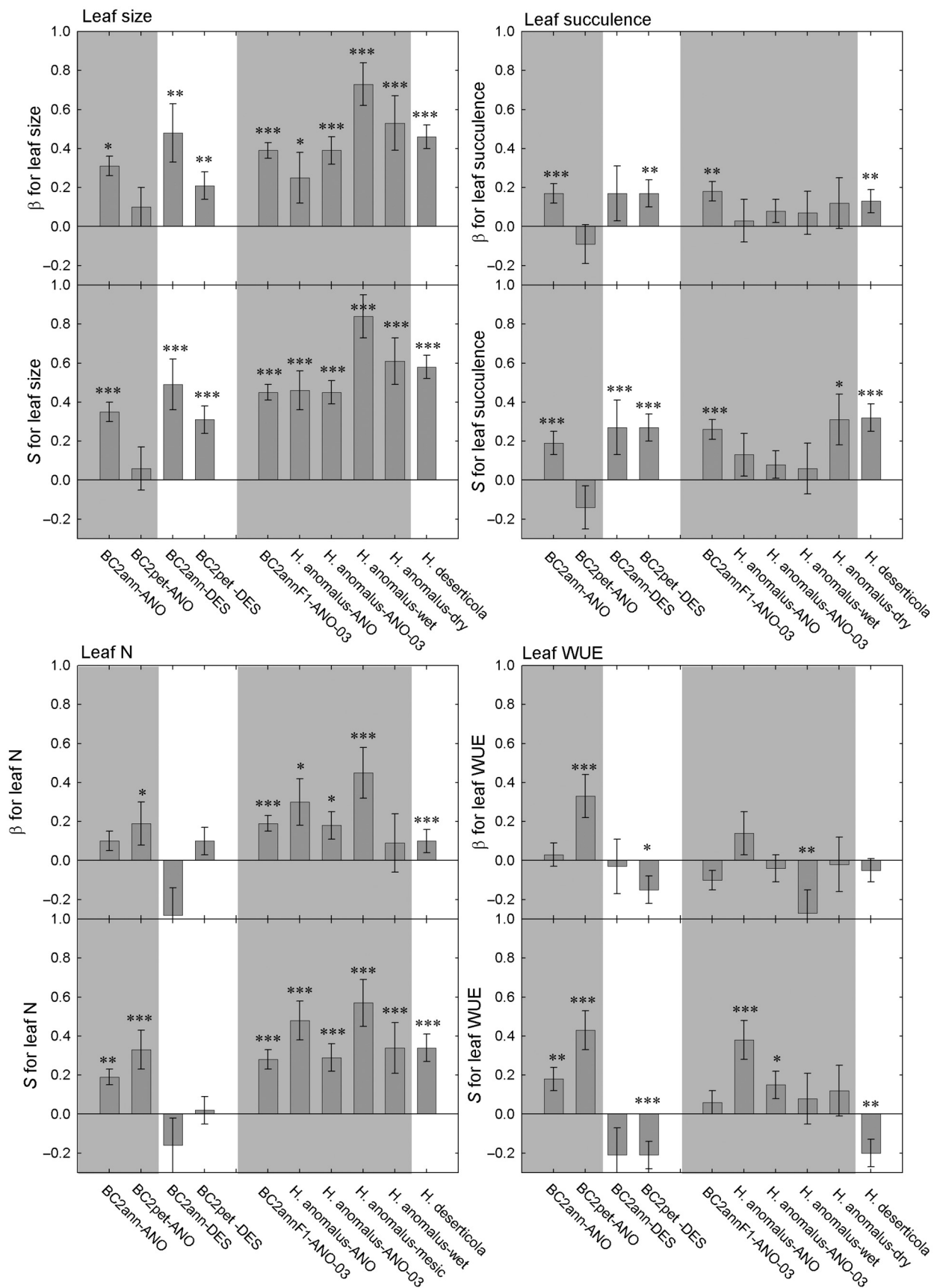
Direct selection ( $\beta$ ) on leaf N differed between the ANO and DES plots for BC2ann (Table 2), even though the  $\beta$ -values in each plot were not significantly different from zero (Fig. 1). Lower soil N in ANO plot was associated with selection favoring higher leaf N, whereas the higher soil N in the DES plot was associated with selection favoring lower leaf N.

The  $\beta$  for leaf WUE also differed between the ANO and DES plots (Table 2). For BC2pet, higher WUE was favored in the ANO plot whereas lower WUE was favored in the DES plot. The  $\beta$  for leaf size and succulence did not differ by habitat (Fig. 1, Table 2).

### Spatial comparison of planted and natural populations within *H. anomalous* habitat

*Helianthus anomalous* planted in the ANO plot (*H. anomalous*-ANO) were compared with two naturally occurring *H. anomalous* populations (*H. anomalous*-wet, *H. anomalous*-dry) for trait expression and selection in 2002. The planted population had smaller leaves, higher succulence, lower leaf N and lower WUE than both natural populations (for population means and statistical comparison see Table 3; for individual plant data see Ludwig *et al.* (2004) for planted *H. anomalous* and Donovan *et al.* (2007) for natural populations of *H. anomalous*). By the end of the season, the planted population had accumulated less biomass than the adjacent wet natural population, but more biomass than the more distant dry natural population (Table 3).

The  $\beta$  for the planted population differed from the wet natural population for leaf size (positive in both, but stronger in the natural population) and WUE (not significantly different from zero in the planted population and negative in natural population; Fig. 1, Table 3). The  $\beta$  for the planted population



**Table 2** Comparison of ANO and DES Experimental Plots for leaf traits, aboveground vegetative biomass and reproductive biomass (mean  $\pm$  1 SE) of the *Helianthus* artificial hybrids (BC2ann and BC2pet)

	ANO plot	DES plot	F for comparison of means	Difference in $\beta$ for traits
<b>BC2ann</b>				
Population sample size	BC2ann-ANO 114	BC2ann-DES 198		
Leaf size (cm <sup>2</sup> )	6.32 $\pm$ 0.25	12.81 $\pm$ 0.50	<b>125.2***</b>	0.17 <sup>ns</sup>
Leaf succulence (g cm <sup>-2</sup> )	0.032 $\pm$ 0.005	0.036 $\pm$ 0.004	<b>58.1***</b>	0.00 <sup>ns</sup>
Leaf N concentration (mg g <sup>-1</sup> )	22.83 $\pm$ 0.55	34.5 $\pm$ 0.38	<b>321.2***</b>	<b>0.38*</b>
Leaf $\delta^{13}\text{C}$ (‰)	-27.27 $\pm$ 0.07	-26.24 $\pm$ 0.05	<b>132.0***</b>	0.06 <sup>ns</sup>
Aboveground vegetative biomass (g)	0.93 $\pm$ 0.06	6.07 $\pm$ 0.83	<b>186.9***</b>	
Reproductive biomass (g)	0.43 $\pm$ 0.04	2.65 $\pm$ 0.30	<b>145.2***</b>	
<b>BC2pet</b>				
Population sample size	BC2pet-ANO 104	BC2pet-DES 192		
Leaf size (cm <sup>2</sup> )	3.57 $\pm$ 0.14	7.94 $\pm$ 0.21	<b>280.5***</b>	0.10 <sup>ns</sup>
Leaf succulence (g cm <sup>-2</sup> )	0.034 $\pm$ 0.003	0.039 $\pm$ 0.004	<b>62.2***</b>	0.26 <sup>ns</sup>
Leaf N concentration (mg g <sup>-1</sup> )	25.45 $\pm$ 0.74	35.78 $\pm$ 0.47	<b>149.6***</b>	0.10 <sup>ns</sup>
Leaf $\delta^{13}\text{C}$ (‰)	-27.85 $\pm$ 0.08	-26.75 $\pm$ 0.05	<b>130.2***</b>	<b>0.48***</b>
Aboveground vegetative biomass (g)	1.28 $\pm$ 0.14	15.22 $\pm$ 1.05	<b>542.4***</b>	
Reproductive biomass (g)	0.32 $\pm$ 0.03	4.63 $\pm$ 0.25	<b>593.1***</b>	

F-values are presented for comparison of traits in the ANO and DES plots. The difference in selection gradients ( $\beta$ ) between ANO and DES plots (see Fig. 1 for  $\beta$  values) is presented for each leaf trait. ns, nonsignificant; bold type, statistical significance (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

**Table 3** Comparison of planted and naturally occurring *Helianthus anomalus* for leaf traits, aboveground vegetative biomass and reproductive biomass (mean  $\pm$  1 SE) in 2002

	Planted population	Natural population	F for comparison of means	Difference in $\beta$ for traits
<b>Planted vs wet natural population</b>				
Population sample size	<i>H. anomalus</i> -ANO 130	<i>H. anomalus</i> -wet 114		
Leaf size (cm <sup>2</sup> )	5.38 $\pm$ 2.30	7.24 $\pm$ 4.19	<b>12.1***</b>	<b>0.48*</b>
Leaf succulence (g cm <sup>-2</sup> )	0.050 $\pm$ 0.005	0.045 $\pm$ 0.005	<b>63.8***</b>	0.15 <sup>ns</sup>
Leaf N concentration (mg g <sup>-1</sup> )	20.07 $\pm$ 4.93	34.13 $\pm$ 5.94	<b>407.5***</b>	0.15 <sup>ns</sup>
Leaf $\delta^{13}\text{C}$ (‰)	-27.15 $\pm$ 1.06	-26.62 $\pm$ 0.98	<b>16.3***</b>	<b>0.41**</b>
Aboveground vegetative biomass (g)	5.64 $\pm$ 6.92	7.84 $\pm$ 11.45	0.3 <sup>ns</sup>	
Reproductive biomass (g)	0.73 $\pm$ 1.31	2.40 $\pm$ 3.28	<b>33.9***</b>	
<b>Planted vs dry natural population</b>				
Population sample size	<i>H. anomalus</i> -ANO 130	<i>H. anomalus</i> -dry 114		
Leaf size (cm <sup>2</sup> )	5.38 $\pm$ 2.30	8.23 $\pm$ 4.54	<b>29.6***</b>	0.27 <sup>ns</sup>
Leaf succulence (g cm <sup>-2</sup> )	0.050 $\pm$ 0.005	0.046 $\pm$ 0.006	<b>23.3***</b>	0.09 <sup>ns</sup>
Leaf N concentration (mg g <sup>-1</sup> )	20.07 $\pm$ 4.93	34.96 $\pm$ 5.62	<b>485.8***</b>	0.20 <sup>ns</sup>
Leaf $\delta^{13}\text{C}$ (‰)	-27.15 $\pm$ 1.06	-26.69 $\pm$ 0.97	<b>12.3***</b>	0.16 <sup>ns</sup>
Aboveground vegetative biomass (g)	5.64 $\pm$ 6.92	2.28 $\pm$ 3.11	<b>35.5***</b>	
Reproductive biomass (g)	0.73 $\pm$ 1.31	0.41 $\pm$ 1.31	0.0 <sup>ns</sup>	

F-values are presented for comparison of traits for planted and naturally occurring *H. anomalus*. The difference in selection gradients ( $\beta$ ) between planted and naturally occurring *H. anomalus* (see Fig. 1 for  $\beta$  values) is presented for each leaf trait. ns, nonsignificant; bold type, statistical significance (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

**Fig. 1** Selection gradients ( $\beta$ , direct selection) and selection differentials (S, total selection) (mean  $\pm$  1 SE) for 10 populations of *Helianthus* (wild species and artificial hybrids). The leaf ecophysiological traits are leaf size, leaf succulence, leaf nitrogen (N) concentration, and leaf water-use efficiency (WUE) estimated from  $\delta_{13}\text{C}$ . Asterisks above bars indicate that  $\beta$  or S is significantly different from zero (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). The tinted background indicates *Helianthus anomalus* habitat and the white background indicates *Helianthus deserticola* habitat. From left to right in each panel, the first four populations (bars) are BC2ann-ANO and BC2pet-ANO (both in the ANO experimental plot) and BC2ann-DES and BC2pet-DES (both in the DES experimental plot). The remaining bars are BC2ann-F1-ANO-03, *H. anomalus*-ANO, *H. anomalus* ANO-03, *H. anomalus*-wet and *H. anomalus*-dry (all in *H. anomalus* habitat) and *H. deserticola* (*H. deserticola* habitat). See Table 1 and the Materials and Methods section for further information on each population. For statistical comparison of  $\beta$  among populations, see Tables 2–4.

**Table 4** Comparison of 2002 and 2003 for leaf traits, aboveground vegetative biomass and reproductive biomass (mean  $\pm$  1 SE) for *Helianthus anomalus* and artificial hybrids planted in the ANO experimental plot

	2002	2003	F for comparison of means	Difference in $\beta$ for traits
<i>H. anomalus</i>				
Population sample size	<i>H. anomalus</i> -ANO 130	<i>H. anomalus</i> -ANO-03 141		
Leaf size (cm <sup>2</sup> )	5.38 $\pm$ 2.30	6.67 $\pm$ 3.28	<b>14.0***</b>	0.14 <sup>ns</sup>
Leaf succulence (g cm <sup>-2</sup> )	0.050 $\pm$ 0.005	0.051 $\pm$ 0.009	0.3 <sup>ns</sup>	0.05 <sup>ns</sup>
Leaf N concentration (mg g <sup>-1</sup> )	20.07 $\pm$ 4.93	28.98 $\pm$ 5.82	<b>183.7***</b>	0.12 <sup>ns</sup>
Leaf $\delta^{13}\text{C}$ (‰)	-27.15 $\pm$ 1.06	-27.91 $\pm$ 0.72	<b>48.5***</b>	0.28 <sup>ns</sup>
Aboveground vegetative biomass (g)	5.64 $\pm$ 6.92	13.60 $\pm$ 11.62	<b>73.0***</b>	
Reproductive biomass (g)	0.73 $\pm$ 1.31	3.55 $\pm$ 3.11	<b>91.5***</b>	
Artificial hybrids				
Population sample size	BC2ann-ANO 114	BC2annF <sub>1</sub> -ANO-03 175		
Leaf size (cm <sup>2</sup> )	6.32 $\pm$ 2.62	4.32 $\pm$ 2.28	<b>53.0***</b>	0.08 <sup>ns</sup>
Leaf succulence (g cm <sup>-2</sup> )	0.032 $\pm$ 0.003	0.034 $\pm$ 0.006	<b>21.3***</b>	0.01 <sup>ns</sup>
Leaf N concentration (mg g <sup>-1</sup> )	22.83 $\pm$ 5.88	22.63 $\pm$ 5.96	0.1 <sup>ns</sup>	0.09 <sup>ns</sup>
Leaf WUE ( $\delta^{13}\text{C}$ , ‰)	-27.27 $\pm$ 0.77	-27.59 $\pm$ 0.96	<b>9.0**</b>	0.13 <sup>ns</sup>
Aboveground vegetative biomass (g)	0.93 $\pm$ 0.62	1.05 $\pm$ 0.78	0.9 <sup>ns</sup>	
Reproductive biomass (g)	0.43 $\pm$ 0.47	0.40 $\pm$ 0.31	0.4 <sup>ns</sup>	

F-values are presented for comparison of traits in 2002 and 2003. The difference in selection gradients ( $\beta$ ) between 2002 and 2003 (see Fig. 1 for  $\beta$  values) is presented for each leaf trait. ns, nonsignificant; bold type, statistical significance (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

did not differ from the dry natural population for any leaf traits (Fig. 1, Table 3).

#### Temporal comparisons between 2002 and 2003 for planted populations in the ANO plot

For *H. anomalus* in the ANO plot, the 2003 plants (*H. anomalus*-ANO-03) had larger leaves, higher leaf N, lower WUE, and more biomass than the 2002 plants (*H. anomalus*-ANO) (for population means and statistical comparison see Table 4; for individual plant data see Ludwig *et al.*, 2004 for the ANO plot in 2002 and Fig. S2 for the ANO plot in 2003). Although selection favored larger leaf size and higher leaf N concentration in both years,  $\beta$  did not differ between years for any leaf traits (Fig. 1, Table 4).

For the artificial hybrids in the ANO plot, the 2003 plants (BC2annF<sub>1</sub>-ANO-03) had smaller leaves and lower WUE, but did not differ in biomass, from the 2002 plants (BC2ann-ANO). Although selection favored larger leaf size and greater succulence in both years, and higher leaf N in 2003,  $\beta$  did not differ between years for any of the leaf traits (Fig. 1, Table 4).

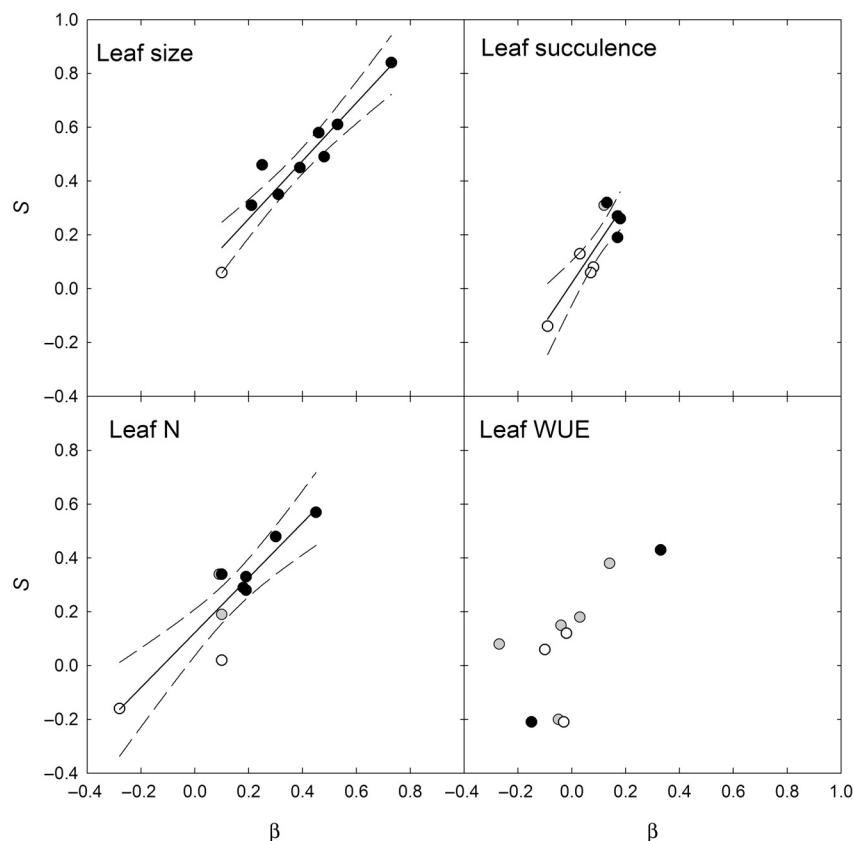
#### Comparing direct and total selection

There was a significant positive relationship between  $S$  and  $\beta$  for leaf size, leaf succulence, and leaf N concentration (Fig. 2,  $r^2 = 0.90$ ,  $r^2 = 0.77$ ,  $r^2 = 0.81$ , respectively,  $P < 0.01$ ,  $n = 10$  for each). There was no significant relationship between  $S$  and  $\beta$  for leaf  $\delta^{13}\text{C}$  ( $r^2 = 0.45$ ,  $P > 0.05$ ,  $n = 10$ ).

## Discussion

The contrast of the artificial hybrids (BC2ann, BC2pet) planted into the two habitats provides a direct test of the habitat differences. The hybrid plants grown in the ANO plot had less biomass than plants grown in the DES plot. Given that competition in both plots was minimized by the low plant cover characteristic of both habitats, and weeding of volunteers and planting design within the plots, the lower biomass in the ANO plot was most likely caused by abiotic factors such as N limitation and unstable sand surfaces (burial, excavation, abrasion from blowing sand). Although dunes look dry, they generally store more water than adjacent stabilized areas, provided that root growth is sufficient to access deeper soils (Pavlik, 1980; Rosenthal *et al.*, 2005a). Lower biomass in the ANO plot was accompanied by lower leaf succulence, much lower leaf N and lower WUE compared with the DES plot, consistent with our expectation that the *H. anomalus* habitat has lower N availability and higher water availability than the *H. deserticola* habitat (Rosenthal *et al.*, 2005a; Donovan *et al.*, 2007). The two habitats differed for direct selection for leaf N and WUE in the BC2ann and BC2pet, respectively, but did not differ for direct selection on leaf size and succulence.

Selection on leaf N differed between habitats as expected on the basis of habitat resource availability. For BC2ann, direct selection tended to favor higher leaf N in the ANO plot with lower N availability, but tended to favor lower N in the DES plot with higher N availability. This habitat difference is consistent with previously reported habitat differences in selection



**Fig. 2** Relationship of selection gradients ( $\beta$ ) to selection differentials ( $S$ ) across 10 populations of *Helianthus* (wild species and artificial hybrids) for each leaf trait. Closed circles, both  $\beta$  and  $S$  are significantly different from 0; tinted circles, either  $\beta$  or  $S$  significantly different from 0; open circles, neither  $\beta$  or  $S$  significantly different from 0 (see Fig. 1). Significant correlations are represented by solid line ( $P < 0.01$ ), with dashed lines indicating 95% confidence intervals. The correlation is not significant ( $P > 0.05$ ) for leaf water-use efficiency (WUE).

on leaf N for naturally occurring populations: stronger selection for high leaf N in naturally occurring *H. anomalus*-wet than for *H. deserticola* (Donovan *et al.*, 2007). The habitat differences in selection on leaf N are set against a background of relatively few differences within each habitat. Within each of the experimental plots, selection on N did not differ between BC2pet and BC2ann (Ludwig *et al.*, 2004 for ANO plot;  $P > 0.05$  for DES Plot). Within the ANO plot, the selection on leaf N did not differ between years (Table 4). The difference in selection on leaf N between habitats, but not within the *H. anomalus* habitat or across years, suggests that N limitation and selection on leaf N, or unmeasured traits related to achieving a higher leaf N, may be contributing to population differentiation and speciation.

Does direct selection on leaf N indicate that leaf N itself is the important trait, or could it be, instead, that underlying unmeasured traits allow the plant to gather and use N more efficiently? It is difficult to say because there are currently only a few studies that have investigated selection on leaf N and related traits. Maron *et al.* (2007) found total selection for higher leaf N, but not direct selection, for European *Hypericum perforatum* planted outside of its native range. Agrawal *et al.* (2008) found no direct selection for leaf C : N ratio for *Asclepias incarnata* planted into a common environment. However, for plants in the *H. anomalus* habitat, with such low fertility, it seems reasonable to hypothesize that the direct selection on

leaf N reflects selection on an underlying suite of traits that enhance access and efficiency of uptake and subsequent use. For example, *H. anomalus* is capable of hydraulic lift (Howard *et al.*, 2009), where water is transferred at night from wetter to drier soils via roots. This may allow plants to maintain root growth in dry soils and continue nutrient uptake by affecting root uptake capacity, nutrient mobility in the soil, and nutrient availability via microbial communities and decomposition (Caldwell *et al.*, 1998; Bauerle *et al.*, 2008). Also, plants in low nutrient habitats may use N more efficiently by prolonging leaf lifetime and reabsorbing more N before senescence (Aerts & Chapin, 2000). We are currently investigating whether *H. anomalus* differs from its ancestral parents for leaf lifetime and nutrient-use efficiency, and following nutrient-related traits in an artificial selection experiment under low soil N conditions.

Direct selection on WUE differed between habitats and within habitat. For BC2pet, higher WUE was favored in the ANO plot and lower WUE was favored in the DES plot. This was opposite to the expectation, based on water availability in the two habitats, that that greater water limitation generally favors more conservative water use (Dudley, 1996a; Heschel *et al.*, 2002; Casper *et al.*, 2005). One interpretation might be that selection on WUE and closely related parameters is dependent on the strength and timing of water deficits; for example, drought earlier in the growing season may favor a live fast, die young strategy (Geber & Dawson, 1990, 1997; Stanton *et al.*,

2000; McKay *et al.*, 2003; Heschel & Riginos, 2005; but see Sherrard & Maherali, 2006). This would be consistent with the earlier flowering of *H. deserticola* and selection for only lower WUE or no selection in the *H. deserticola* habitat. However, there was also substantial variation in selection on WUE within the sand dune habitat of *H. anomalus*. First, the *H. anomalus* planted in the ANO plot (*H. anomalus*-ANO) was only c. 20 m from the mesic natural population (*H. anomalus*-wet), and yet there was strong selection for higher and lower WUE, respectively, in these adjacent populations. Second, BC2ann and BC2pet in the ANO plot differed in selection on WUE (Ludwig *et al.*, 2004). These differences may be caused by localized variation in water availability and plant rooting depth. The within-habitat variation in selection on WUE makes this less likely to be a candidate for driving population differentiation and speciation. In addition, among the focal traits in this study, the relationship between direct and total selection was weakest for WUE, indicating a larger contribution of indirect selection. This result fits into a larger context of a relative lack of indirect selection for most traits in wild populations, but the potential for greater indirect selection on plant physiological traits (Kingsolver *et al.*, 2001; Geber & Griffen, 2003).

Larger leaf size was favored by direct and total selection in most of the planted and natural *Helianthus* populations, but direct selection did not differ by habitat for the BC2ann and BC2pet planted into both habitats. Other studies have varied as to whether drought results in direct selection for smaller leaf size (Bennington & McGraw, 1995; Dudley, 1996a; Heschel & Riginos, 2005; Caruso *et al.*, 2006). For *Helianthus* species in these desert habitats, and within the context of these traits, differences in water availability did not result in differential selection on leaf size.

Direct selection on leaf succulence also did not differ between habitats. This was surprising because *H. anomalus* has a substantially higher leaf succulence than its ancestral parents and *H. deserticola*, suggesting that some factor in the *H. anomalus* habitat directly or indirectly favors greater leaf succulence (Schwarzbach *et al.*, 2001; Rosenthal *et al.*, 2002; Brouillette *et al.*, 2006; Donovan *et al.*, 2007). We found no evidence of strong indirect selection on succulence through other traits included in this study. In our current studies we have shifted the focus to related traits that have also been suggested as potentially adaptive because of their correlation with plant functional types, such as leaf toughness, leaf thickness, leaf dry mass per area and leaf dry matter content (Garnier & Laurent, 1994; Cornelissen *et al.*, 2003; Diaz *et al.*, 2004; Ishida *et al.*, 2008; but see Volaire, 2008). Leaf dry matter content is of particular interest because it can be correlated with other traits important for nutrient stress tolerance, such as relative growth rate and leaf life span (Cornelissen *et al.*, 2003), but varies in the strength of correlation with succulence across the *Helianthus* study populations (Table S2).

Would we expect the habitat differences in phenotypic selection on leaf N to result in trait differentiation if we followed

the artificial hybrids for several generations in these habitats? This requires consideration of the amount of standing genetic variation for selection to act on and the genetic correlations among traits. For these traits, genetic variation for the selection to act on is likely, as demonstrated by quantitative trait loci identified for the BC2pet *Helianthus* population in this study (Rieseberg *et al.*, 2003; Brouillette *et al.*, 2007) and heritability estimates reported for other species (Geber & Griffen, 2003; Caruso *et al.*, 2005; Culley *et al.*, 2006; Agrawal *et al.*, 2008). However, heritability estimates vary by species, populations and environment, and are expected to be lower in natural populations where the environment is usually more variable than for common environment experiments. Unfortunately, we do not know whether the genetic correlations among traits would facilitate or constrain selection for these populations. We did not detect strong indirect selection for these traits (except for WUE). Although we did report phenotypic correlations among traits (for phenotypic trait correlations, see Ludwig *et al.* (2004) for ANO plot 2002, Donovan *et al.* (2007) for natural populations in 2002, and Tables S1 and S2 for DES plot 2002 and ANO plot 2003, respectively), this does not tell us the underlying genetic correlations for each population and whether these correlations are likely to constrain or facilitate trait evolution in *Helianthus* in these environments. Reports to date conflict on whether correlations among ecophysiological traits will likely constrain evolution (Dudley, 1996b; Geber & Griffen, 2003; Caruso *et al.*, 2005; Agrawal *et al.*, 2008).

Are the habitat differences in phenotypic selection reflected in differences in traits for the native *Helianthus* species endemic to these habitats? In the natural populations compared at the same time selection was estimated (June), *H. anomalus* did have higher leaf N than *H. deserticola*, despite the lower soil N in the *H. anomalus* habitat, consistent with the divergent phenotypic selection (Rosenthal *et al.*, 2002; Donovan *et al.*, 2007). Although species comparisons in glasshouse experiments under high- and low-nutrient treatments have not found a consistent species difference in leaf N relations (Schwarzbach *et al.*, 2001; Rosenthal *et al.*, 2002; Brouillette *et al.*, 2006), the expression of the species differences may depend on environmental factors not replicated in glasshouse conditions.

There are several caveats that should be kept in mind when interpreting these results. One caveat is that in this study we were limited to using vegetative biomass as our proxy for fitness. We feel that it is a reasonable proxy for fitness when exploring traits expected to affect fitness through vegetative growth (Samson & Werk, 1986; Ehleringer & Clark, 1988; Ferris & Lechowicz, 1990; Dudley, 1996a). However, measures of reproductive biomass or seed production would have been preferred and may have changed the results if ecophysiological traits affect seed production more directly.

A second caveat is that, when estimating direct selection on traits, the choice of traits to be included can influence the results. As an example, our results for selection on leaf size differ from a previous analysis of leaf size for the artificial hybrids in the

DES plot (Gross *et al.*, 2004), where the total selection was still positive, but direct selection was negative. We attribute difference in results among studies to the choice of traits included in the selection analysis. Gross *et al.* (2004) included stem diameter, which appears to be a surrogate for plant size, and thus mixes both 'morphology' and 'performance' traits (*sensu* Arnold, 1983). Another example is that including leaf N in the analysis may affect the detection and interpretation of direct selection on WUE (Donovan *et al.*, 2007). Plants can increase WUE either by increasing photosynthetic capacity (e.g. via increased leaf N) or by decreasing water loss. When both WUE and leaf N are incorporated in a selection analysis, selection for increased leaf N may include selection for increased photosynthetic capacity, and selection on WUE may only measure selection on reducing water loss. However, for studies that assess selection on WUE but not leaf N (Dudley, 1996a), selection on WUE should include selection for increasing photosynthetic capacity through increased leaf N as well as for reducing water loss, increasing the apparent adaptive value of the WUE. For a third example, leaf N concentration (mass basis) and leaf N per unit area are correlated for these populations ( $r^2 = 0.47\text{--}0.86$ ,  $P < 0.001$  for all; Table S2) and both have received attention with regard to WUE, plant nutrient relations and leaf economics (Field & Mooney, 1986; Wright *et al.*, 2001, 2003, 2004; Cornelissen *et al.*, 2003; Huxman *et al.*, 2008). We used leaf N concentration in all the analyses presented, but could have replaced it with leaf N on an area basis, and could also have included specific area in the analyses. We chose to use leaf N concentration and ignore specific leaf area because initial exploration of the data indicated that the combination of leaf area, N concentration, succulence and WUE gave us the greatest explanatory power for fitness for this study. However, this makes it less comparable to any studies that focus on leaf N on an area basis. While the choice of traits to be included in any given phenotypic selection study is usually influenced by the biology of the focal species and the question being asked, the growing number of studies of selection on ecophysiological traits will, it is hoped, lead to a consensus on traits worth exploring.

A third caveat is that our point measure of traits misses the complexity of how both traits and the environment change. While we did not find a difference in direct selection on traits when they were measured in June of 2002 and 2003, we did find within-season variation in selection on traits measured in June vs. July for the *H. anomalous* native populations (Donovan *et al.*, 2007). In addition, selection on WUE may differ between juveniles and adults of long-lived perennials (Donovan & Ehleringer, 1994; Casper *et al.*, 2005), differ across years for adults (Casper *et al.*, 2005) and depend on when drought occurs during the growing season (Heschel & Riginos, 2005). Thus, shifting trait values as plants respond to their environment and shifting selection as the environment changes needs to be further explored to understand selection on, and evolutionary responses of, ecophysiological traits.

In summary, we detected substantial direct and total selection on the four-leaf ecophysiological traits for desert annual sunflowers. This adds to the growing evidence for selection on ecophysiological traits (Dudley, 1996a; Heschel *et al.*, 2002; Casper *et al.*, 2005; Heschel & Riginos, 2005; Caruso *et al.*, 2006; Sherrard & Maherali, 2006; Maron *et al.*, 2007; Saldana *et al.*, 2007; Agrawal *et al.*, 2008), although strengths and directions of natural selection vary. For the traits studied, except for WUE, direct and total selection were in good agreement, indicating little effect of indirect selection. The differences in selection on traits in the *H. anomalous* and *H. deserticola* habitat, combined with differences in plant biomass and trait means, suggest that nutrient limitation has been more important than water limitation as a driving force for adaptation of *H. anomalous* to the desert dune habitat. More studies assessing spatial and temporal variation in selection are needed to understand the evolution of ecophysiological traits.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Relationship of leaf traits to aboveground vegetative biomass for artificial hybrid populations in the DES experimental plot in 2002.

**Fig. S2** Relationship of leaf traits to aboveground vegetative biomass for the ANO experimental plot in 2003: *Helianthus anomalous* (*H. anomalous*-ANO-03), and artificial hybrids (BC2annF<sub>1</sub>-ANO-03).

**Table S1** (a) Phenotypic correlations of traits for artificial hybrids in the DES experimental plot in 2002; (b) phenotypic correlations of traits for plants in the ANO experimental plot in 2003: *Helianthus anomalous* (*H. anomalous*-ANO-03) and BC2annF<sub>1</sub> (BC2annF<sub>1</sub>-ANO-03)

**Table S2** Phenotypic correlations of traits used in the selection analysis, and related traits of interest.

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