Strong ecological but weak evolutionary effects of elevated CO_2 on a recombinant inbred population of *Arabidopsis thaliana*

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Summary

• Increases in atmospheric CO_2 concentration have an impact on plant communities by influencing plant growth and morphology, species interactions, and ecosystem processes. These ecological effects may be accompanied by evolutionary change if elevated CO_2 (eCO_2) alters patterns of natural selection or expression of genetic variation.

• Here, a statistically powerful quantitative genetic experiment and manipulations of CO_2 concentrations in a field setting were used to investigate how eCO_2 impacts patterns of selection on ecologically important traits in *Arabidopsis thaliana*; heritabilities, which influence the rate of response to selection; and genetic covariances between traits, which may constrain responses to selection.

• CO_2 had strong phenotypic effects; plants grown in eCO_2 were taller and produced more biomass and fruits. Also, significant directional selection was observed on many traits and significant genetic variation was observed for all traits. However, no evolutionary effect of eCO_2 was detected; patterns of selection, heritabilities and genetic correlations corresponded closely in ambient and elevated CO_2 environments.

• The data suggest that patterns of natural selection and the quantitative genetic parameters of this *A. thaliana* population are robust to increases in CO_2 concentration and that responses to eCO_2 will be primarily ecological.

Key words: *Arabidopsis thaliana*, carbon dioxide (CO_2), climate change, contemporary evolution, G-matrix, genetic variation, global change, natural selection.

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Introduction

Atmospheric concentrations of carbon dioxide (CO_2) are rising rapidly and are expected to be *c.* 40% higher in 2050 than they are today (Houghton *et al.*, 2001). Given that CO_2 is the raw material of photosynthesis, this historically unprecedented rate of increase, along with accompanying changes in global climate, is expected to have profound effects on plant physiology and growth, community dynamics, species distributions, and probabilities of extinction (Bazzaz, 1990; Davis & Shaw, 2001; Poorter & Navas, 2003; Niklaus & Körner, 2004; Reich *et al.*, 2006). In particular, elevated CO_2 (eCO_2) stimulates photosynthesis and can alter light compensation points, often resulting in increased plant growth (Körner, 2006). The effects of CO_2 concentration on plant physiology and growth can impact ecological interactions in several ways, including allowing plants to grow in deeper shade (Körner, 2006), altering competitive interactions (Brooker,

2006; Körner, 2006), and influencing interactions with herbivores, pathogens, and mutualists (Bazzaz, 1990; Bezemer & Jones, 1998; Coviella & Trumble, 1999; Mitchell *et al.*, 2003; Johnson *et al.*, 2005). As experimental evidence documenting these ecological consequences has accumulated, it has stimulated interest in the potential for elevated CO_2 (eCO_2) concentrations to alter the evolution of plant populations.

Rapid evolutionary responses may be important because genetic changes within species could alter predicted ecological responses to eCO_2 and other types of environmental change (Geber & Dawson, 1993; Bazzaz *et al.*, 1995; Curtis *et al.*, 1996; Thomas & Jasienski, 1996; Yoshida *et al.*, 2003). While evolution is often assumed to proceed slowly relative to ecological change, evolutionary responses over a few decades have been documented in response to heavy metal contamination of soils (McNeilly & Bradshaw, 1968; Wu & Bradshaw, 1972) and even over a few years in response to drought (Grant & Grant, 2002) and predation (Reznick *et al.*, 1990; Arendt & Reznick, 2005). Evidence of rapid evolutionary change in still other contexts is accumulating steadily (e.g. global warming (Reale *et al.*, 2003) and biological invasions (Strauss *et al.*, 2006)). Understanding how the CO_2 environment affects evolutionary dynamics is necessary for a full understanding of the biological impacts of increasing CO_2 concentrations, as well as for evaluating the robustness of ecological predictions.

Several lines of evidence suggest that atmospheric CO_2 concentrations influence the evolution of vascular plant populations, although the importance of elevated CO_2 as a selective agent remains an open question. First, several studies have documented that the effects of CO_2 concentrations on plant growth or fitness are genetically variable within species (Table 1), indicating either that genotypes with highest fitness in an eCO_2 environment will be different from those today or that patterns of selection will differ with CO_2 environment. Several studies, however, have failed to detect genetic variation in responses to eCO_2 (Table 1). Second, surveys of herbaria specimens reveal correlated changes in CO_2 concentrations and traits putatively involved in CO_2 uptake (e.g. stomatal densities) over the past 150–300 yr (Woodward, 1987; Penuelas & Matamala, 1990; Radoglou & Jarvis, 1990, but see Körner, 1988). The magnitude of change in herbaria specimens is similar, however, to plastic responses to eCO_2; therefore, genetic changes need not be invoked to explain the observed changes (Woodward, 1987, 1993). Third, plants from populations growing near geothermal vents where concentrations of CO_2 are naturally elevated have, in some instances, expressed higher fitness when grown in eCO_2 than those from populations that grow in more typical conditions (Woodward *et al.*, 1991; Woodward, 1993). These experiments, however, have been conducted with limited replication, making it difficult to disentangle the effects of CO_2 from other environmental variables, such as temperature and soil type, that also differ among locations. Moreover, other studies fail to detect adaptation to elevated CO_2 (Collins & Bell, 2006) or only demonstrate differences in growth between populations at subambient CO_2 concentrations (Ward & Strain, 1997).

Despite suggestive evidence that evolutionary responses could occur, experiments that have artificially selected for increased fitness in eCO_2 environments have found no evidence that plant populations will adapt to eCO_2 (Maxon Smith, 1977; Potvin & Tousignant, 1996; Ward *et al.*, 2000; Collins & Bell, 2004). That is, experimental populations selected under eCO_2 conditions do not have higher fitness than populations selected under ambient CO_2 (aCO_2) conditions when reared in eCO_2 environments. Nevertheless, some of these selection experiments have found that physiological and phenological traits have evolved in response to artificial selection in eCO_2 environments; after 1000 generations of growth under eCO_2, the unicellar alga, *Chlamydomonas reinhardtii,* showed changes suggestive of relaxed selection on photosynthetic efficiency (Collins & Bell, 2004), and five generations of selection on *Arabidopsis thaliana* seed production in eCO_2 vs subambient CO_2 environments resulted in differences in flowering time (Ward *et al.*, 2000). Because such experiments may impose stronger selection than populations typically experience in nature and focus primarily on the outcome of the evolutionary process, questions about the mechanisms underlying adaptive responses to environmental change remain. In the examples above, adaptation to eCO_2 environments could fail as a result of lack of genetic variation in CO_2 responsiveness, similarity of the intensity and direction of selection in aCO_2 and eCO_2 environments, or genetic constraints.

Here we report on the results of a large and statistically powerful experiment designed to predict evolutionary changes resulting from increased concentrations of atmospheric CO_2. We focus on ecologically important traits whose genetic basis is complex. We therefore use a quantitative genetic approach that allows us to predict the short-term evolutionary trajectory of populations grown in aCO_2 and eCO_2 environments. We consider all three components of evolution and use an experimental population of the model annual plant *A. thaliana* to estimate patterns of selection on growth, morphological, and phenological traits; heritabilities, which influence the rate of response to selection; and genetic covariances between traits, which may constrain the rate and direction of responses to selection. The advantage of this approach is that it allows for explicitly examining the mechanisms underlying evolutionary change and provides a basis for explaining why rising CO_2 concentrations may or may not affect evolution. Further, we compare the genetic relationship between fitness in aCO_2 vs eCO_2 treatments to assess directly differences in expected response to natural selection in the two CO_2 environments (Antonovics *et al.*, 1988). To accomplish these objectives, we collected data on traits of individual *A. thaliana* plants growing outdoors in a free-air CO_2 enrichment (FACE) facility. Making use of FACE allowed us to examine the effects of increased CO_2 in relatively natural field conditions, including natural amounts of light, rain, wind, and airborne pathogens.

Table 1 Studies detecting or not detecting statistically significant genotype × CO_2 environment interactions on plant biomass or fitness

Species	Trait	Method	No. of genotypes	References[b]
Studies detecting genotype × CO_2 environment interactions				
Abutilon theophrasti	Biomass, fruit biomass	GC	3	1
Arabidopsis thaliana	Biomass, fruit no., seed no.	GC	3–5	2–4
Betula alleghaniensis	Biomass[a]	GH	3	5
Bromus erectus	Biomass	GC	7	6
Gentianella germanica	Survival	OC	30	7
Pinus ponderosa	RGR	GC	4 pop.	8
Plantago lanceolata	Seed weight	GC	4	9
Populus tremuloides	Biomass, RGR	GH	6	10
Prosopis glandulosa	Biomass	GH	14	11
Studies not detecting Genotype × CO_2 environment interactions				
Arabidopsis thaliana	Biomass	GC	2	12
Arrhenatherum elatius	Biomass	F	9–14	13
Bromus erectus	Biomass	GH	14	14,15
Carex flacca	Biomass	GH	9	15
Dactylis glomerata	Biomass	F, GH	9–14	13,14
Festuca ovina	Biomass	GC, OC	5,18	6
Festuca pratensis	Biomass	F	9–14	13
Holcus lanatus	Biomass	F	9–14	13
Lolium multiflorum	Biomass	F	9–14	13
Lolium perenne	Biomass	F	9–14	13
Phlox drummondii	Biomass, seed no.	GC	4 pop.	16
Pinus ponderosa	Biomass	GC	4 pop.	8
Plantago lanceolata	Biomass	GC, OC	6,18	17,18
Populus tremuloides	Biomass	OC	6	19
Ranunculus friesianus	Biomass	F	9–14	13
Rhaphanus raphanistrum	Flower no., fruit no.	OC	5,36	20,21
Rumex acetosa	Biomass	F	9–14	13
Rumex obtusifolius	Biomass	F	9–14	13
Salix myrsinifolia	Biomass	GC	3,4	22,23
Sanguisorba minor	Biomass, fruit no.	GH	77	24
Trifolium pratense	Biomass	F	9–14	13
Trifolium repens	Biomass	F	9–14	13
Trisetum flavescens	Biomass	F	9–14	13

Listed are study species, the fitness trait measured (relative growth rate, RGR), the method of CO_2 manipulation (growth chambers, GC; glasshouse,GH; open top chambers in the field, OC; or field experiments, such as FACE, F), the number of genotypes, families, or populations included in the experiment, and the reference. Only studies comparing ambient vs elevated CO_2 environments are included.

[a]G × E only detected under competition.

[b]References: 1, Bazzaz *et al*. (1995); 2, Norton *et al*. (1995); 3, Andalo *et al*. (2001); 4, Bidart-Bouzat *et al*. (2004); 5, Wayne & Bazzaz (1997); 6, Leadley & Stocklin (1996); 7, Fischer *et al*. (1997); 8, Callaway *et al*. (1994); 9, Wulff & Alexander (1985); 10, Lindroth *et al*. (2001); 11, Polley *et al*. (2006); 12, Zhang & Lechowicz (1995); 13, Luscher *et al*. (1998); 14, Roumet *et al*. (2002); 15, Volk & Körner, 2001); 16, Garbutt & Bazzaz (1984); 17, Fajer *et al*. (1992); 18, Klus *et al*. (2001); 19, Zak *et al*. (2000); 20, Curtis *et al*. (1994); 21, Case *et al*. (1998); 22, Julkunen-Tiitto *et al*. (1993); 23, Veteli *et al*. (2002); 24, Wieneke *et al*. (2004).

Materials and Methods

Experimental design

Seven to 18 individuals were grown from each of 162 eighth-generation recombinant inbred lines (RILs), plus the two parental accessions, of *Arabidopsis thaliana* (L.) Heynh. in each of two atmospheric CO_2 environments: ambient (aCO_2, *c.* 368 μmol mol^{-1}) or elevated (eCO_2, *c.* 560 μmol mol^{-1}), the predicted concentration of atmospheric CO_2 in 2050 (Houghton *et al*., 2001). The RILs were generated from a cross between two divergent *A. thaliana* accessions, Bay-0 (ARBC reference CS954) and Shahdara (CS929), collected from fallow-land near Bayreuth, Germany, and from the Pamiro-Alay mountains in Tadjikistan, respectively (Loudet *et al*., 2002). When, as in this case, the parental accessions are genetically divergent, the recombination that occurs during the production of RILs generates many genetic combinations that differ from those of the parents. Thus, the range of variation in quantitative traits can greatly exceed that of the

parents (transgressive segregation), and genetic variation in the RIL population is high even for traits for which the parental genotypes are phenotypically similar. Accordingly, because the RILs were propagated without selection, the 164 lines used here are expected to represent a broader range of genetic and phenotypic variation than would be present in a highly selfing, natural population of *A. thaliana*. This high amount of variation is evident in all traits studied, with variation in genotypic means frequently spanning six standard deviations, even when the two parental phenotypes are near the mean of the distribution. This increased variation, as well as the large size of the study (5260 plants), affords considerable statistical power to detect genotypic effects in response to the CO_2 environments and to detect nonlinear relationships between fitness and trait variation. For these reasons, the use of an RIL population in estimating patterns of selection and expected responses to selection does not suffer from the limited allelic diversity present in an RIL population, which can be a problem for identifying the loci that contribute to phenotypic variation.

The CO_2 treatments were part of an ongoing FACE experiment at Cedar Creek Natural History Area, Minnesota, USA (http://biocon.fr.umn.edu) (Reich *et al.*, 2001). In this experiment, the two CO_2 treatments (elevated and ambient) are applied to six 20-m-diameter open-air rings (three rings per treatment). The eCO_2 treatment is maintained by blowing concentrated CO_2 through vertically positioned pipes spaced at approx. 2 m intervals around the perimeter of the ring. The control rings (aCO_2) are surrounded by the same pipe structure, but the air blown through these pipes is not enriched in CO_2. The CO_2 treatments were applied during daylight hours over the course of the entire experiment, with CO_2 concentrations monitored and adjusted every 4 s. Manipulating atmospheric CO_2 concentrations in natural field environments in this way has only minor effects on microclimate or light conditions (Hendrey *et al.*, 1993) and effectively maintains CO_2 concentrations close to target values: 92% of 5 min averages in the eCO_2 rings deviated from the target concentration by < 5% (D. Bahauddin, pers. comm.).

The 36 individuals from each line were grown in two blocks (three replicates per block), within each of the three rings, within each of the two CO_2 environments (final sample sizes, seven to 18 individuals per line per CO_2 treatment). Individuals were randomly assigned to a location within each block. Four to 10 seeds of the appropriate line were planted into a 164 ml Conetainer™ (Ray Leach Conetainers, Stuewe & Sons Inc., Corvallis, OR, USA) that had been filled with relatively low nutrient potting mix (Sunshine Mix #5; Sun Gro Horticulture Canada Ltd, Alberta, Canada) and bottom-watered until saturated. Following planting, Conetainers were placed in a dark 4°C cold-room for 4 d to synchronize germination and then moved to a glasshouse where they remained until plants germinated. The germinants were thinned so that only the centermost plant in each pot remained. All plants were moved to the field on 22 May 2005, approx. 5–7 d after germination, where they were exposed to natural conditions (light, water, and nutrients were not manipulated). On 11 June, plants were sprayed with the generalist insecticide Sevin to control an outbreak of the crucifer-specialist *Plutella xylostella* (diamondback moth). All plants were harvested on 27–30 June when flowering had ceased, the majority of plants had begun to senesce, and fruits were beginning to dehisce.

Plant measurements

Growth, phenological, and fitness traits, were measured, as well as damage from herbivores. On 31 May, the number of leaves were counted, rosette diameter was measured to the nearest 1 mm, and the number of leaves with evidence of *Phyllotreta striolata* (flea beetle) damage were recorded. On 8 June, when plants were just beginning to flower, we measured rosette diameter and visually estimated the proportion of leaf area damaged by *Plutella xylostella*. Plants began flowering on 6 June, and we assessed flowering every other day for the remainder of the season. From half of the plants from each line in each ring, we collected a single fully expanded leaf at the time of flowering to estimate specific leaf area (SLA), calculated as the area (cm^2) of a fresh leaf (measured using SCION image analysis software; Scion Corporation, Frederick, MD, USA) divided by leaf dry weight (g). After harvest, we recorded plant height, number of flowering stems, and silique (fruit) number. Fruit number is highly correlated with seed production and is a good estimate of lifetime fitness in this species (Westerman & Lawrence, 1970; Mauricio & Rausher, 1997). The vast majority of plants survived to reproduction (> 97%); those that did not survive were assigned zero values for fruit production. The dry weights of the total above-ground portion of each plant and of leaves used to calculate SLA were obtained after drying tissue at 60°C.

Statistical analyses

Phenotypic effects, genetic variation, and genotype × environment interactions Separate mixed-model nested ANOVAs were performed on each trait, using PROC MIXED (SAS Institute) to test for significant effects of CO_2 environment, variation among RILs, and variation in RIL response to CO_2 environment. In these analyses CO_2, RIL, and their interaction were included as fixed factors. Ring(CO_2) and block(CO_2 ring) were included as random factors. Significant RIL terms were interpreted as evidence for genetic variation, and the $CO_2 \times$ RIL term provides a test for a genotype × environment interaction (i.e. genetically variable plasticity to CO_2 environment). Significance of random factors was determined with likelihood ratio tests.

Because we measured several traits on each individual, we corrected for multiple comparisons, using a table-wise sequential Bonferroni method. Because harvesting a leaf may have

influenced later season growth and morphological traits, we also included the leaf removal treatment as a fixed factor in the analyses of height, stem number, biomass, and fruit number. We included 'counter' in the fruit number analysis as a fixed factor because researchers differed in fruit counts. While these two factors explained substantial variation in response variables, removing leaf and counter from the analyses did not qualitatively change any results. Late-flowering individuals that did not fully complete their life cycle over the course of the experiment were removed from the analyses of late season growth and fitness traits.

Heritability and genetic covariance The genetic variance of each trait and the genetic covariance between each pair of traits within each environment were estimated using restricted maximum likelihood (REML) as implemented in the *nf3* program in Quercus (available from http://www.cbs.umn.edu/eeb/events/quercus.shtml) (Shaw, 1987; Shaw & Shaw, 1994). To test for differences in G-matrices between aCO_2 and eCO_2 treatments, log-likelihood ratio tests were used to compare models where all parameters were free to vary with models where genetic variance-covariance components were constrained to be equal across environments. We also used the genetic and environmental variances obtained from Quercus to calculate broad-sense heritabilities ($H^2 = V_g/V_p$, i.e. the proportion of total phenotypic variation that results from genetic variation) for each trait in each CO_2 treatment. Broad-sense heritabilities confound additive genetic effects with dominance effects and are upper-bound estimates of the amount of heritable variation (Falconer & Mackay, 1996; Lynch & Walsh, 1998). However, for organisms with high selfing rates, such as *A. thaliana*, broad-sense heritabilities may be more relevant for predicting short-term evolutionary change than narrow-sense heritabilities (Roughgarden, 1979). The genetic design of the experiment also confounds maternal effects with genetic effects, but this contribution is expected to be minor because maternal effects tend to diminish by adulthood (Roach & Wulff, 1987).

Patterns of selection Patterns of selection within each CO_2 environment were characterized and tested for between-environment differences at both phenotypic and genotypic levels (Robertson, 1966; Price, 1970; Lande & Arnold, 1983). In the phenotypic selection analysis, individual relative fitness was the response variable, and the morphological traits (above-ground biomass, stem number, rosette size, height, and SLA), phenological traits (flowering date), and resistance to herbivory were predictor variables. Because phenotypic analyses can be biased by microenvironmental variation that affects both fitness and the traits of interest (Mitchell-Olds & Shaw, 1987; Rausher, 1992; Stinchcombe *et al.*, 2002), REML as implemented in Quercus (Shaw & Shaw, 1994) was used to estimate the genetic covariance between relative fitness and the traits. The REML analyses account for variance around genotypic means and further differentiate between genetic and environmental covariances by including all individuals in the analysis and incorporating within-family covariances into likelihood estimations (Shaw, 1987; Shaw & Shaw, 1994).

For both analyses, selection differentials and selection gradients were estimated. Selection differentials provide an estimate of the net selection resulting from selection acting directly on each trait plus any selection acting on correlated traits and were estimated by performing separate univariate analyses on each trait (Robertson, 1966; Price, 1970). Selection gradients provide estimates of the strength of selection acting directly on the trait while accounting for selection on correlated traits included in the analysis (Lande & Arnold, 1983). Similar analyses were also performed on RIL best linear unbiased predictions (BLUPs) (genotypic selection analysis, Rausher, 1992). Results from the analyses using BLUPs were qualitatively similar to those from the REML analysis and are presented in Supplementary Material (Table S1).

Preliminary selection analyses revealed that quadratic terms and interactions between predictor variables (nonlinear selection) were small in magnitude relative to the directional selection coefficients, were seldom significant, and improved model fit only slightly. Preliminary analyses also revealed that results were robust to the traits included in the multiple-regression model (i.e. directional selection gradients obtained from a model that included all traits were similar to those obtained from a reduced model that included only biomass, flowering date, rosette size, and SLA). For simplicity, only linear selection differentials and gradients from the four-trait model are presented (quadratic and interaction terms are presented in Table S1).

For all analyses, relative fitness was calculated as individual fruit production divided by mean fruit production in that CO_2 environment, and all predictor traits were standardized by their standard deviations within the relevant CO_2 environment to allow for comparison between CO_2 environments and between traits measured on different scales (Lande & Arnold, 1983; Arnold & Wade, 1984). In the phenotypic selection analyses, CO_2 treatment was included in the model as a fixed factor; significant $CO_2 \times$ trait interactions indicate that patterns of selection differ between CO_2 environments. Ring(CO_2) and block(ring CO_2) were included as random factors. Fruit counter was also included in the model as a fixed factor.

In the REML analysis, fruit counter and block were included as fixed factors. Differences in patterns of selection between CO_2 environments were tested by comparing twice the difference in log-likelihoods of a model with identical selection gradients (or differentials) in both environments, with a model that allowed these parameters to differ between environments to a χ^2 distribution (log-likelihood ratio tests). Similarly, we tested whether selection differentials and gradients were significantly different from zero by comparing the likelihoods of models where the genetic covariances

Table 2 Least-square means (± 1 SE) for each trait in ambient (aCO_2) and elevated (eCO_2) CO_2 environments of *Arabidopsis thaliana* plants

Trait	aCO_2	eCO_2
Leaf number	3.64 ± 0.19	3.79 ± 0.19
May rosette diameter (mm)	14.41 ± 0.45	15.63 ± 0.45
June rosette diameter (mm)	**43.13 ± 1.63**	**51.00 ± 1.63**
SLA (Specific leaf area, $cm^2\ g^{-1}$)	**181.81 ± 2.6**	**156.25 ± 1.9**
Flowering date (days postgermination)	33.33 ± 0.21	33.12 ± 0.21
Phylotretta damage	0.09 ± 0.06	0.03 ± 0.04
Plutella leaf damage	0.60 ± 0.12	0.47 ± 0.12
Plant height (cm)	24.75 ± 0.64	28.96 ± 0.64
Stem number	6.25 ± 0.17	6.92 ± 0.17
Above-ground biomass (g)	**0.28 ± 0.01**	**0.40 ± 0.01**
Fruit number	**115.79 ± 4.27**	**139.03 ± 4.27**

Values shown in bold differ significantly ($P < 0.05$, post-Bonferroni correction) between CO_2 environments.

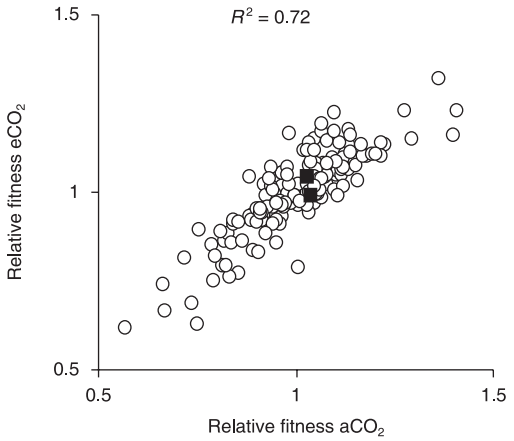

Fig. 1 Relationship between relative fitness (fruit number) under ambient (aCO_2) vs elevated (eCO_2) CO_2 conditions in *Arabidopsis thaliana* plants. Each data point is the best linear unbiased prediction for relative fitness for one recombinant inbred line. The black squares correspond to the two parental accessions.

between the traits and fitness were constrained to zero with models in which these parameters were free to vary.

Results

Phenotypic effects, genetic variation, and genotype × environment interactions

Elevated CO_2 significantly increased plant growth and reproduction and tended to decrease the amount of herbivore damage incurred by plants (Table 2). In addition, evidence for significant genetic variation (significant RIL effects) was detected for all measured traits (Table 3), indicating that each of the traits may respond to selection. However, very few genotype × environment interactions were detected; significant $CO_2 \times$ RIL effects only were detected for leaf number and plant height (Table 3), and the cross-environment genetic correlations of even these traits were high (leaf number $r = 0.85$, height $r = 0.98$). The absence of $CO_2 \times$ RIL interactions for most traits suggests that genotypes exhibited similar relative trait values in both environments. Furthermore, no evidence was detected that the number of fruits produced by the genotypes was affected differentially by CO_2, suggesting that genotypes had similar fitness ranks in the two CO_2 environments and that increases in atmospheric CO_2 concentrations will not change which genotypes are favored by natural selection (Fig. 1). Also consistent with this was a high across-environment genetic correlation in RIL fruit production ($r = 0.98$).

Heritability and genetic covariance

While we detected genetic variation for all traits examined, broad-sense heritabilities appeared to differ only slightly between aCO_2 and eCO_2 environments (Table 4). Similarly, genetic variance/covariance matrices (G matrices) did not differ significantly across environments (d.f. = 15, $\chi^2 = 17.1$, $P = 0.31$), yielding no indication that CO_2 environment affected the expression of genetic variation or the covariances among traits, which can limit or facilitate evolutionary responses (Table 5). Therefore, changes in the rate at which this population would respond to selection are not expected with increasing CO_2 concentrations.

Natural selection on plant traits and effects of CO_2 on patterns of selection

In both CO_2 environments, we detected evidence for directional selection on many traits, with selection favoring genotypes that were larger (i.e. more stems and greater above-ground biomass), flowered earlier and had thinner leaves (higher SLA) (Table 6, Fig. 2). Multiple regression analyses, which measure the direct selection acting on each trait, also revealed evidence for selection favoring early flowering genotypes with larger above-ground biomass (Table 6). Few significant quadratic or interactive selection gradients were detected, and they were typically small in magnitude relative to the directional selection coefficients (Table S1). Therefore, selection is primarily directional across the range of phenotypic variation included in this population, and selection on one trait does not depend on the values of other traits.

While strong selection on many traits was detected, no convincing evidence was found that the CO_2 environment altered patterns of selection. The genetic analyses via REML detected no difference between CO_2 environments in selection gradients ($P > 0.31$), which measure direct selection on each trait. While the more powerful phenotypic selection analysis

Table 3 *F*-values and statistical significance of the effects of CO_2, recombinant inbred lines (RILs), and their interaction, and χ^2 values for random effects on *Arabidopsis thaliana* plants

Source	Leaf number	May diameter	June diameter	SLA	Flowering date	*Phylotretta* damage	*Plutella* damage	Height	Stem number	Biomass	Fruit number
CO_2	0.35	3.66	11.71**	33.87***	0.47	0.76	0.58	20.85*	7.71*	30.76***	32.83**
RIL	4.39****	6.97****	7.64****	5.93****	61.99****	1.75****	3.73****	17.82****	11.86****	6.33****	12.19****
$CO_2 \times$ RIL	1.30**	0.98	0.93	1.03	1.04	1.07	1.12	1.40***	1.12	0.94	1.14
Random effects											
Ring(CO_2)	224****	183****	214****	2.6	140****	249****	744****	324****	69.3****	164****	57.2****
Block(CO_2 ring)	62.5****	17.4****	469****	25.2****	6.8**	1420****	1118****	524****	30.7****	460****	28.5****

Numerator degrees of freedom (d.f.) = 1 for CO_2 and ranged from 159 to 163 for the RIL and $CO_2 \times$ RIL terms. Denominator d.f. ranged from 4 to 10 for CO_2 effects and from 4006 to 4919 for terms including RIL, with the exception of specific leaf area (SLA), for which d.f. = 1838. Models for height, branch number, biomass, and fruit number included whether a leaf was removed and which researcher counted fruits (results not shown).

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Table 4 Broad-sense heritabilities (H^2) for each *Arabidopsis thaliana* trait in ambient (aCO_2) and elevated (eCO_2) CO_2 environments

Trait	aCO_2	eCO_2
Fitness (fruit production)	0.29	0.31
Biomass	0.18	0.15
Flowering date	0.51	0.52
June rosette size	0.18	0.18
SLA	0.12	0.09
Height	0.39	0.44
Stem number	0.32	0.39
May rosette size	0.15	0.16
May leaf number	0.12	0.10
Phylostretta damage	0.02	0.03
Plutella damage	0.08	0.07

SLA, specific leaf area.

Heritabilities were calculated from the variance components estimated by restricted maximum likelihood (REML) ($H^2 = V_g/V_p$).

Table 5 Additive genetic variance-covariance matrices (G) of populations of *Arabidopsis thaliana* plants reared under ambient and elevated CO_2 environments (the two G matrices do not significantly differ at $P > 0.31$)

	Fitness	Rosette size	Biomass	Flowering date	SLA
Ambient CO_2					
Fitness	0.039	0.014	0.045	–0.113	0.026
Rosette size		0.156	0.125	–0.047	–0.051
Biomass			0.163	–0.137	–0.010
Flowering date				0.531	–0.066
SLA					0.120
Elevated CO_2					
Fitness	0.040	0.010	0.044	–0.124	0.018
Rosette size		0.155	0.096	–0.044	–0.038
Biomass			0.133	–0.150	–0.010
Flowering date				0.549	–0.060
SLA					0.089

SLA, specific leaf area.

suggested that selection gradients for biomass ($F_{1,2068} = 10.99$, $P = 0.0009$) and June rosette size ($F_{1,2068} = 4.68$, $P = 0.03$) differed across CO_2 treatments, selection gradients in the two environments were similar in magnitude and never differed in direction (Table 6). Phenotypic selection estimates must be interpreted with caution because of the potential for environmental covariances between traits to bias selection measures.

Selection differentials include selection acting directly on a trait plus any selection acting on correlated traits. The REML analyses revealed a significant difference between the magnitudes of the genetic selection differentials in aCO_2 vs eCO_2 treatments for May rosette size (d.f. = 1, $\chi^2 = 3.95$, $P = 0.05$) and leaf number (d.f. = 1, $\chi^2 = 8.0$, $P = 0.005$); however, these differences were not significant after a Bonferroni

	Selection differentials				Gradients			
	PSA		REML		PSA		REML	
Trait	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
Biomass	**0.25**	**0.25**	**0.04**	**0.04**	**0.30**	**0.24**	**0.05**	**0.04**
Flowering date	**–0.21**	**–0.21**	**–0.12**	**–0.13**	**–0.11**	**–0.12**	**–0.11**	**–0.12**
June rosette size	**0.16**	**0.15**	0.01	0.01	**–0.12**	**–0.08**	0.01	0.01
SLA	0.00	0.00	**0.03**	**0.02**	0.01	0.01	**0.03**	**0.02**
Height	**0.09**	**0.07**	–0.00	0.00				
Stem number	**0.15**	**0.14**	**0.06**	**0.07**				
May rosette size	**0.15**	**0.12**	**0.02**	0.00				
Leaf number	**0.15**	**0.13**	**0.01**	–0.01				
Phylotreta damage	**–0.03**	–0.01	–0.00	0.00				
Plutella damage	0.02	0.00	**0.02**	**0.02**				

Table 6 Selection differentials and selection gradients in elevated (eCO_2) vs ambient (aCO_2) CO_2 environments, calculated using phenotypic (PSA) and the restricted maximum likelihood (REML) analyses

Selection differentials and gradients that significantly differ from 0 ($P < 0.05$, after Bonferroni correction) are indicated in bold.
SLA, specific leaf area.

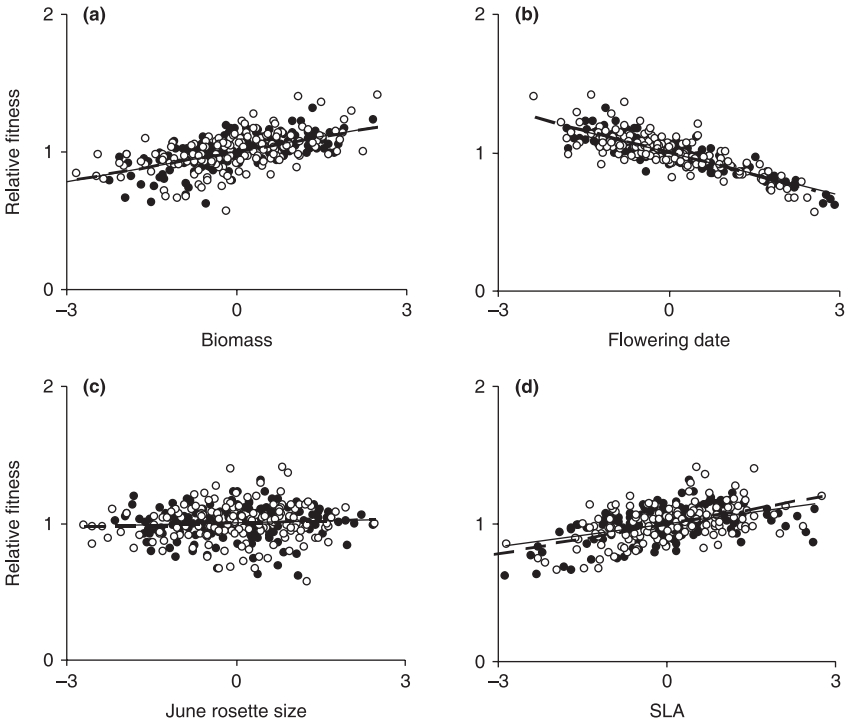

Fig. 2 Relationship in *Arabidopsis thaliana* between relative fitness and standardized values of (a) biomass, (b) flowering date, (c) rosette size, and (d) specific leaf area (SLA) under ambient (aCO_2, open circles, dashed lines) and elevated (eCO_2, filled circles, solid line) conditions. Each data point is the fitness and trait best linear unbiased prediction for one recombinant inbred line.

correction was applied. No other significant differences in selection between CO_2 treatments were detected with the REML analysis (all $P > 0.18$). Similarly, the more powerful phenotypic selection analysis suggested that the magnitude of selection on height ($F_{1,4637} = 4.45$, $P = 0.03$), leaf number ($F_{1,4640} = 4.94$, $P = 0.03$), and May rosette size ($F_{1,4641} = 4.38$, $P = 0.04$) may differ between CO_2 environments, but these differences also were not significant after correcting for multiple comparisons with a sequential Bonferroni correction. Furthermore, in all analyses, the differences in the estimates of selection between the two CO_2 treatments were small (< 0.03, Table 6), suggesting that CO_2 has, at most, very subtle effects on patterns of selection. Our capability of detecting even very weak differences in selection between CO_2 treatments attests to the unusually powerful scale and design of this study. The very close similarity in selection, however, argues for strongly similar evolutionary responses in aCO_2 and eCO_2 environments.

Interestingly, for eight of the 10 traits, the point estimates of the selection differentials obtained from the phenotypic selection analyses were substantially greater than those obtained from the REML analyses (Table 6). These differences likely result from high environmental covariances between many traits and fitness, causing biased selection estimates in the phenotypic analysis.

Discussion

Increasing atmospheric CO_2 concentrations and related changes in global temperature and precipitation patterns are expected to impact plant growth, community dynamics, and ecosystem function. If increasing CO_2 concentrations also alter patterns of natural selection or other components of the evolutionary process, then the effects of eCO_2 on plant communities may be ameliorated or exacerbated by genetic changes that occur within plant populations (Geber & Dawson, 1993; Bazzaz *et al.*, 1995; Curtis *et al.*, 1996; Thomas & Jasienski, 1996; Yoshida *et al.*, 2003). In a statistically powerful experiment using the model vascular plant *A. thaliana* grown in a relatively natural environment, little evidence was detected that increasing CO_2 concentrations will alter the short-term evolutionary trajectories of ecologically important traits. In particular, we detected no significant differences between aCO_2 and eCO_2 treatments in the magnitude or direction of selection gradients, heritabilities, or genetic covariances between traits. Selection differentials were also very similar across CO_2 treatments and did not differ significantly, with two exceptions: both the phenotypic selection analyses and the REML analyses indicated that eCO_2 may affect selection on leaf number and May rosette size. Although these results may be indicative of changes in selection regimes between CO_2 environments, selection on both of these traits was very weak and the differences in the magnitude of selection were slight (0.02); therefore, the change in selection with increasing CO_2 concentration would result in only minor differences in plant phenotypes. For example, the smaller selection differential for May rosette size under eCO_2 would result in only a 0.1 mm difference in rosette size, after 10 generations of selection. These results reinforce those obtained in other studies measuring intensities of selection under aCO_2 and eCO_2 environments: both Steinger *et al.* (2007) and Bazzaz *et al.* (1995) show only minor differences in selection on biomass between eCO_2 and aCO_2 treatments.

We also detected little evidence for genetic variation in plastic responses to CO_2. Considering fitness, in particular, we found that the same genotypes favored under current CO_2 concentrations were favored under eCO_2 conditions, as indicated by the cross-environment genetic correlation for fitness approaching 1 ($r = 0.98$). The G matrix also remained remarkably constant across environments, indicating that trade-offs that may contribute to genotypic differences in fitness will persist with rising CO_2 concentrations. In short, evidence for eCO_2 to alter predicted evolutionary trajectories was lacking despite highly significant estimates of selection, heritability, and genetic covariance within each of the separate CO_2 environments.

While our results suggest that eCO_2 will have little impact on the evolution of a variety of ecologically important traits, we did not measure selection on all traits thought to be important to CO_2 responsiveness (e.g. stomatal density or photosynthetic rates). However, the genotype $\times$ CO_2 environment interaction for fitness, the most direct assessment of difference in selection between environments, was not detectable, despite the large scale of the experiment. Thus it does not support the inference that rising CO_2 concentrations will alter which genotypes are favored by natural selection. Therefore, it is not expected that selection on unmeasured traits will differ across CO_2 conditions, unless under the unlikely scenario where genotypes differ in plasticity and patterns of selection differ between CO_2 environments in a manner that exactly counteracts these differences so as not to result in a genotype $\times$ environment interaction on fitness.

The lack of genotype $\times$ CO_2 interaction in our study contrasts with results from four of the five other studies investigating $G \times CO_2$ interactions in *A. thaliana* (Table 1). While four studies detected significant $G \times CO_2$ interactions on fitness components, in one case, the interaction resulted entirely from a strong response of only one accession (Norton *et al.*, 1995), and in a second example, the $G \times E$ interaction appeared to be driven primarily by a subambient CO_2 treatment rather than the elevated CO_2 treatment (Ward & Strain, 1997). Additionally, most studies were performed in growth chambers, often with limited replication. In the field, increased environmental variation may overwhelm any genotypic effects that are minor in magnitude.

Finding similar patterns of selection, genetic variance, and genetic covariance in aCO_2 and eCO_2 environments is surprising for at least two reasons. First, several previous studies have suggested that evolutionary responses to rising CO_2 concentrations are likely (reviewed in Ward & Kelly, 2004). However, only 11 out of 39 experiments testing for genotypic effects of eCO_2 on growth or fitness have detected genotypic variation in response to eCO_2 (Table 1). Therefore, the preponderance of evidence appears consistent with the results from this study in suggesting that eCO_2 will not directly alter which genotypes are favored by natural selection. The second reason that the negligible effect of eCO_2 on plant evolution is surprising is that eCO_2 had large phenotypic effects. Elevated CO_2 increased biomass by 40%, increased fruit production by 20%, and reduced specific leaf area by 15%. Even if CO_2 *per se* does not alter patterns of selection, these large phenotypic effects might be expected to influence resource allocation and plant development, potentially changing patterns of selection, genetic variation, or evolutionary constraints. Instead, our data suggest that selection acting on a multitude of growth traits is linear across a wide range of

phenotypic variation and that the genetic constraints that influence evolutionary responses to selection appear to be little affected by either CO_2 or the growth differences that occur when plants are reared under eCO_2 vs aCO_2. Together these results suggest that selective surfaces may be constrained across a large range of phenotypic trait values and demonstrate that environmental changes that have dramatic impacts on plant growth and morphology, community dynamics, and ecosystem functioning will not necessarily influence evolutionary trajectories.

Because our study population was composed of RILs generated from crosses between genetically diverged natural populations, we expected to maximize the opportunity to detect genetic variation in response to CO_2. Yet, we detected genetic variation in all traits measured, with the notable exception of CO_2 responsiveness. The low amount of genetic variation in CO_2 responsiveness may reflect historically low amounts of variation in atmospheric CO_2 concentrations across natural environments. There is little spatial variation in CO_2 concentrations at fine or coarse scales, and atmospheric CO_2 concentrations fluctuated temporally only over very long timescales before the industrial age. Temporal and spatial variation in selection, combined with genotype × environment interactions (i.e. different genotypes favored in different environments), may contribute to the maintenance of genetic variation in natural populations (Gillespie & Turelli, 1989; Turelli & Barton, 2004). Although few other environmental variables are either as spatially uniform or as temporally predictable as atmospheric CO_2 concentrations, genetic variation in fitness responses to other entirely novel environmental conditions, such as insecticide or heavy metal contamination (Bradshaw, 1991; Alhiyaly *et al.*, 1993; Macnair, 1997), is present in some populations and lacking in others (reviewed in Blows & Hoffmann, 2005).

While we employed FACE technology to grow plants under more natural environmental conditions than most previous studies investigating the potential for evolutionary responses to eCO_2, this experiment was conducted in a less complex environment than plants experience in nature. If many of the effects of eCO_2 on plant evolution are indirect (Thomas & Jasienski, 1996), increased concentrations of atmospheric CO_2 may impact evolutionary trajectories when plants experience competition, greater herbivore damage, natural soil environments, or abiotic stress (e.g. drought or heat stress). For example, Bazzaz *et al.* (1995) showed that genetic variation, and thus the predicted evolutionary response, of *Abutilon theophrasti* biomass production was threefold higher under eCO_2 than under aCO_2, but only when plants were grown in competitive environments. Similarly, other studies have documented significant shifts in genotypic ranks in growth or fitness only when plants were grown at high density (Bazzaz *et al.*, 1995); however, other studies have demonstrated the opposite pattern, only observing genetic variation in responsiveness to CO_2 in the absence of competition (Steinger *et al.*, 1997). Interestingly, more pronounced evolutionary impacts of eCO_2 in complex than in simple ecological environments would be the opposite of the phenotypic effects of eCO_2 on plant growth and fitness, which tend to be greater in simple environments (reviewed in Ainsworth & Long, 2005).

Regardless of environmental complexity, the results of this study indicate that patterns of natural selection and quantitative genetic parameters are robust to large increases in CO_2 concentration and that eCO_2 itself will have minimal impact on the evolutionary trajectory of this *A. thaliana* population. Our study therefore suggests that the biotic changes that occur in response to eCO_2 will be primarily, if not entirely, ecological. It remains to be determined, however, whether this finding generalizes to other plant populations growing in biotically more realistic environments.

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Supplementary Material

The following supplementary material is available for this article:

Table S1 *F*-statistics, χ^2 values for random factors, and parameter estimates for directional (β) and quadratic (γ) selection gradients in aCO_2 and eCO_2 environments estimated from the phenotypic selection analyses, as well as from genotypic selection analyses on RIL BLUPs.

This material is available as part of the online article from:
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