

Indirect effects drive evolutionary responses to global change

Jennifer A. Lau^{1,5}, Ruth G. Shaw², Peter B. Reich^{3,4} and Peter Tiffin¹

¹Department of Plant Biology, University of Minnesota, St Paul, MN 55108, USA; ²Department of Ecology, Evolution and Behavior, University of Minnesota, St Paul, MN 55108, USA;

³Department of Forestry, University of Minnesota, St Paul, MN 55108, USA; ⁴Hawkesbury Institute for the Environment, University of Western Sydney, Penrith, NSW 2751, Australia;

⁵Present address: Kellogg Biological Station and Department of Plant Biology, Michigan State University, 3700 E Gull Lake Dr., Hickory Corners, MI 49060, USA

Summary

Author for correspondence:

Jennifer A. Lau

Tel: +1 269 671 2107

Email: jenlau@msu.edu

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- Anthropogenic environmental changes pose significant threats to plant and animal populations. These changes also may affect the evolution of natural populations either directly or indirectly by altering the outcome of species interactions that are important drivers of evolution. This latter indirect pathway may be especially important for evolutionary responses to elevated atmospheric CO₂ concentrations (eCO₂), which appear to have minimal direct effects on plant evolution but have large effects on interspecific interactions, such as competition.
- We manipulated competitive and CO₂ environments of experimental *Arabidopsis thaliana* populations to test whether eCO₂ alters evolutionary trajectories indirectly by altering selection imposed by competitors.
- We found that interspecific competition increased selection on growth traits, reduced heritabilities, and altered genetic covariances between traits and that the magnitude of these effects depended upon the CO₂ environment. Although eCO₂ had minimal direct effects on evolutionary processes, eCO₂ typically reduced the strength of selection imposed by competitors and, therefore, relaxed selection on plant traits when competitors were present.
- Our results indicate that global changes may affect plant evolution indirectly by altering competitive interactions and underscore the importance of conducting research in natural communities when attempting to predict population responses to global change.

Introduction

Manipulative experiments designed to characterize how environmental factors such as temperature, light or resource availability affect evolution almost always focus on the direct effects of the manipulation. This is particularly true of studies conducted in controlled environments such as chemostats, mesocosms, growth chambers and glasshouses in which the environment is much simpler than the conditions most organisms experience in nature. Study of direct effects can be powerful for identifying selective agents and for elucidating the genetic basis of phenotypic variation. However, exclusive focus on direct effects may be misleading if the goal of the research is to predict evolutionary responses in natural environments. In this case, partitioning direct effects from indirect effects that result when the environment affects species interactions provides much greater insight into selective agents and the genetic constraints that influence evolutionary responses to environmental change.

Ecological indirect effects may be particularly important for understanding how environmental changes that affect the resource environment influence plant evolution. Changes in resource supply, including CO₂, can alter the outcome of competitive interactions (Tilman, 1977; Brooker, 2006; Harpole & Tilman, 2006, 2007; Reich, 2009), and competition can be an important selective agent (Miller, 1995; Shaw *et al.*, 1995; Dorn

et al., 2000; Stanton *et al.*, 2004). Therefore, many of the ecological and evolutionary effects of changes in resource availability that have occurred since the Industrial Revolution may have come about through indirect pathways mediated by competitors rather than through direct effects. For example, elevated atmospheric CO₂ concentrations (eCO₂) typically increase plant growth directly by increasing carbon supply, but because taxa differ in magnitude of CO₂ response (reviewed in Poorter & Navas, 2003), some species may respond negatively to eCO₂ because of increased competition from species that benefit most from eCO₂ (reviewed in Brooker, 2006).

The increase in atmospheric CO₂ concentration is one of the most globally extensive and rapid anthropogenic environmental changes, and many studies have demonstrated large effects of eCO₂ on plant physiology, plant growth and reproduction, community structure and ecosystem functions (Bazzaz, 1990; Poorter & Navas, 2003; Niklaus & Körner, 2004; Reich *et al.*, 2006; van Groenigen *et al.*, 2011). The evolutionary consequences of eCO₂ also have received attention. In contrast to the strong ecological effects of CO₂, however, many evolutionary effects of CO₂ are weak in magnitude (reviewed in Leakey & Lau, 2012). For example, many studies fail to detect genotype × eCO₂ interactions (Zhang & Lechowicz, 1995; Luscher *et al.*, 1998; Volk & Körner, 2001; Veteli *et al.*, 2002; Wieneke *et al.*, 2004), suggesting that genotypes may commonly respond in similar ways to

rising CO₂ concentrations and that predicted future eCO₂ concentrations will not change either the rate of evolutionary change or which genotypes are favored by natural selection. Similarly, to our knowledge, no studies have documented adaptation to eCO₂ even though experimental evolution approaches have detected genetic differences in plant traits between populations that have evolved under ambient vs elevated CO₂ (Potvin & Tousignant, 1996) and even evidence for adaptation to subambient CO₂ concentrations (Ward *et al.*, 2000; Collins & Bell, 2004). Most tests of the evolutionary effects of eCO₂, however, have been conducted in simplistic growing environments where indirect effects resulting from altered species interactions cannot contribute to evolutionary responses.

In previous studies, we detected little evidence that eCO₂ alters patterns of selection or expected responses to selection in *Arabidopsis thaliana* populations grown in the absence of competition (Lau *et al.*, 2007); however, other studies using similar approaches have found some evidence that eCO₂ alters patterns of natural selection and/or heritabilities when individuals are grown in competitive environments (Bazzaz *et al.*, 1995; Steinger *et al.*, 2007; Lau *et al.*, 2010). The differences across studies suggest that eCO₂ may have minimal direct effects on evolutionary processes, but potentially, larger indirect effects that result from eCO₂ altering interactions with other community members that are strong selective agents.

Here, we report on an experiment designed to characterize potential competitor-mediated indirect effects of increased atmospheric CO₂ concentration on the predicted evolution of experimental *Arabidopsis thaliana* populations. We investigated how eCO₂ affects interactions with competitors to alter: plant fitness; the expression of genetic variation (heritability); genetic trade-offs that can constrain or facilitate evolutionary responses; and natural selection on ecologically relevant plant traits. This study expands on previous work by our group that showed a surprising lack of direct effects of eCO₂ on predicted *A. thaliana* evolution (Lau *et al.*, 2007) and work that demonstrated that eCO₂ alters the effects of competition on *A. thaliana* fitness and selection on *A. thaliana* traits (Lau *et al.*, 2010). The rigorous quantitative genetics approach and large number of genotypes used in the current study, combined with experimental manipulation of both CO₂ and competitive environment, directly investigates the potential for competitor-mediated indirect effects of eCO₂ on all three components determining evolutionary responses: patterns of natural selection, heritabilities and genetic covariances.

Materials and Methods

Experimental design

This experiment was designed to test the potential for elevated CO₂ to indirectly affect plant evolution by altering competitive interactions. Accordingly, we used a highly variable synthetic population to increase statistical power for detecting differences in quantitative genetic parameters. Moreover, we imposed the competition treatments by addition of a species known to respond strongly to eCO₂. Specifically, we grew *Arabidopsis*

thaliana (L.) Heynh. individuals from 58 recombinant and two parental inbred lines (recombinant inbred lines, RILs) with and without competition by the C₃ grass *Bromus inermis* in either ambient (aCO₂) or elevated atmospheric CO₂ environments (eCO₂). We manipulated CO₂ and competition in a split-plot design in which CO₂ treatments were applied to whole plots, and competition treatments were randomly imposed on plants within each whole plot. The two CO₂ treatments (aCO₂ *c.* 378 μmol mol⁻¹ or eCO₂ *c.* 560 μmol mol⁻¹, the predicted concentration of atmospheric CO₂ in 2050 (IPCC, 2007)) were applied to six, 20-m diameter open-air rings (three rings per treatment), using FACE (www.biocon.umn.edu/; Reich *et al.*, 2001). The eCO₂ treatment was maintained by emitting concentrated CO₂ through vertically positioned pipes spaced around the perimeter of the ring during daylight hours throughout the course of the experiment. The control rings (aCO₂) were surrounded by similar apparatus, but the air emitted from these pipes was not enriched in CO₂. This experimental design for manipulating CO₂ concentration has only minor effects on microclimate and light conditions (Hendrey *et al.*, 1993).

We planted 12 replicates from each of 58 F8 RILs, plus the two parental accessions, of *A. thaliana* in each of four treatments (aCO₂/eCO₂ crossed with competition/no competition). Because some replicates failed to germinate, final sample sizes for most RILs ranged from 6 to 11 replicates per treatment. 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, Tajikistan, respectively (www.inra.fr/qlat/, Loudet *et al.*, 2002). These 58 RILs are a subset of those used by Lau *et al.* (2007) to investigate the direct evolutionary effects of eCO₂ and were chosen because they span the range of variation in fitness detected in that experiment. RIL collections are not natural populations and therefore require assumptions about the likelihood of similar alleles to those found in RIL parental lines segregating in nature. However, the use of an RIL population had three advantages over measuring selection on naturally occurring populations: we were able to compare selection on populations of the same genetic composition grown in two environments, so any observed differences between treatments cannot result from differences in trait variation among populations due to historically different selection regimes; the known genetic structure of the experimental population allowed us to estimate genetic variances and covariances; and because of recombination and a lack of selection during RIL propagation, genetic variation for quantitative traits in this and other RIL populations is generally large. Thus, the use of the RIL population likely increased power to detect differences in quantitative genetic parameters among treatments, and the high amount of phenotypic variation facilitated characterization of the pattern of selection over a wide range of trait values.

For each replicate, 4–10 seeds of a given RIL were planted into a 164-ml conetainer (Ray Leach Conetainers™; Stuewe & Sons Inc., Corvallis, OR, USA) that had been filled with potting mix (Sunshine Mix #5; Sun Gro Horticulture Canada Ltd, Agawam, MA, USA) and bottom-watered until saturated. Following

planting, containers were placed in the dark at 4°C for 2 d to synchronize germination and then moved to a glasshouse where they remained until seeds germinated. After germination, plants were thinned to one *A. thaliana* seedling per pot. Two seeds of the competitor, *Bromus inermis*, were planted into replicates assigned to the competition treatment. *Bromus inermis* was chosen as a competitor because it is a common C₃ grass that shows relatively strong physiological responses to eCO₂ (Lee *et al.*, 2011). Competition treatments and RILs were randomly assigned to replicates within each ring, and all plants were moved to the field on 6 June 2006, c. 5 d after *A. thaliana* seedlings emerged. Containers were uniformly spaced within blocks located on the edge of each ring. When plants began to show signs of water limitation, we watered by placing all plants into tubs and bottom-watering so that all plants received similar amounts of water.

Plant measurements

We measured growth, phenology, and fitness traits on each plant. The first plants flowered on 13 June; after this date, we assessed flowering every other day for the remainder of the season. On 22 June, when most plants were just beginning to flower, we measured rosette diameter and visually estimated the proportion of leaf area damaged by herbivores. We harvested all plants after fruits on the main stem had matured and plants senesced (between 4 July and 21 July). After harvest, we recorded plant height, number of flowering stems and silique (fruit) number. The dry weights of the total aboveground portion of each *A. thaliana* plant and all competitors were obtained after drying tissue at 60°C. Fruit production is typically strongly correlated with estimated seed production in this species (Westerman & Lawrence, 1970; Mauricio & Rausher, 1997; Lau *et al.*, 2010), supporting the use of fruit number as an estimate of fecundity. All data presented in this article will be publicly available via Dryad and the Cedar Creek Ecosystem Science Reserve website www.cedarcreek.umn.edu.

Statistical analyses

Phenotypic effects, genetic variation and genotype-by-environment interactions We tested for effects of competition treatment, CO₂ environment, variation among RILs and variation in RIL response to competition and CO₂ treatments, with mixed model ANOVA on each trait (PROC MIXED; SAS Institute, 2001). In these analyses, CO₂ (the whole plot factor in the split plot design), competition, RIL and all interactions were included as fixed factors. Ring (CO₂) was included as a random factor. For the analyses of CO₂ and competition effects on fitness components (fruit production and aboveground biomass) both untransformed and natural log-transformed data were analyzed because we were interested in testing both additive and multiplicative models of the interactive effects of CO₂ and competition on mean fitness. To investigate how the size of the competitor plants affected the growth stimulation effects of CO₂ and genetic variation in this response, we performed an additional ANCOVA where we replaced the competition treatment term with competitor biomass (using only plants in the competition treatment). For

all analyses, the significance of random factors was determined with likelihood ratio tests.

Heritability and G-matrices We estimated the genetic and environmental components of variance of phenological (flowering date) and growth traits (rosette diameter and biomass) and the genetic covariance between these traits within each treatment using restricted maximum likelihood (REML) as implemented in the *nlme* program in Quercus (www.cbs.umn.edu/eeb/events/quercus.shtml; Shaw, 1987; Shaw & Shaw, 1994). To test for differences in G-matrices between treatments, we used likelihood ratio tests to compare models where all parameters were free to vary with models where genetic variance and/or covariance components were constrained to be equal across environments. In each analysis we first included all four environments (competition presence/absence by aCO₂/eCO₂). We then performed pairwise comparisons to compare G-matrices in: presence vs absence of competitors in aCO₂; presence vs absence of competitors in eCO₂; aCO₂ vs eCO₂ treatments in the absence of competitors; and aCO₂ vs eCO₂ in the presence of competitors.

We used the genetic and environmental variances obtained from Quercus to calculate broad sense heritabilities ($H^2 = V_g/V_p$) for each trait in each competition × CO₂ treatment. Broad sense heritabilities are upper bound estimates of the amount of heritable variation because they confound additive genetic effects with dominance and maternal environmental effects (Falconer & Mackay, 1996; Lynch & Walsh, 1998) but may be more relevant for predicting short-term evolutionary change for organisms with high selfing rates, such as *A. thaliana* (Roughgarden, 1979). It is important to note that in this study heritabilities are estimated from a synthetic RIL mapping population rather than a natural interbreeding population; however, we expect that genetic and phenotypic variances of natural populations would respond similarly to the eCO₂ and competition treatments.

Patterns of selection We used phenotypic selection analyses (PSA) to estimate selection gradients (β) as the partial regression coefficients from regressions of relative fitness on standardized traits (flowering date, rosette diameter and biomass). Relative fitness was estimated as individual fruit number divided by mean fruit number in that treatment, and all traits were standardized by their within-treatment standard deviation to allow for comparisons with other traits and species with differing phenotypic distributions (Lande & Arnold, 1983; Arnold & Wade, 1984). Although data differed significantly from a normal distribution ($P < 0.0001$), these deviations were relatively minor as evidenced by the high Shapiro–Wilk statistic (0.993) and visual examination of normal probability plots. Moreover, data were not transformed for these selection analyses because data transformations may produce biased fitness estimates (Stanton & Thiede, 2005). Additional regression models that included the squared trait values were used to estimate nonlinear selection coefficients which are suggestive of stabilizing or disruptive selection. Following standard conventions, the presented nonlinear selection coefficients are the doubled quadratic regression coefficients (Lande & Arnold, 1983; Stinchcombe *et al.*, 2008).

We tested for between-treatment differences in linear selection gradients with ANCOVA, where plant traits, competition treatment, CO₂ treatment and all interactions were included as predictor variables, and relative fitness (individual fruit number divided by mean fruit number in that treatment) was included as the response variable. As already described, all traits were standardized by their within-treatment standard deviation. Significant interactions between traits and competition treatments provide evidence that the competitive environment alters natural selection. Likewise, significant interactions between traits and CO₂ treatments provide evidence that patterns of selection differ across CO₂ environments. Ring (CO₂) was included as a random factor. We tested for differences in nonlinear (stabilizing) selection coefficients across treatments with models that included both linear and quadratic trait values and interactions with competition and CO₂ treatments.

Because of concerns that standardizing and relativizing fitness within treatments can bias tests for between treatment differences in selection when the distribution of plant traits is affected by experimental treatments (Stanton & Thiede, 2005), we also performed phenotypic selection analyses and ANCOVA on trait values and fitness that were standardized/relativized across all treatments. Selection coefficients and ANCOVA results from these analyses were very similar to results from analyses using more traditional within-treatment standardization approaches. Therefore, we present results from analyses using the more traditional within-treatment standardization approach in the main text and report results from analyses on traits and fitness standardized across treatments in Supporting Information Tables S1–S5.

Phenotypic selection analyses can be biased by environmental variation that affects both fitness and phenotype (Mitchell-Olds & Shaw, 1987; Rausher, 1992; Stinchcombe *et al.*, 2002). Therefore, we also used REML as implemented in Quercus (Shaw & Shaw, 1994) to estimate predicted evolutionary responses (i.e. Δz , the predicted change in trait values in units of standard deviations after one generation of selection) directly as the genetic covariance between trait and fitness (Price, 1970). The REML analyses account for sampling variance of genotypic effects and 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). As a result, the REML analyses are not vulnerable to the criticisms of Hadfield *et al.* (2010), such as biased estimates of selection coefficients, confounding of genetic and environmental effects, and erroneously high levels of statistical significance. For these analyses, traits and fitness were standardized/relativized within treatments. We used REML approaches to test for effects of competition and CO₂ treatments on predicted evolutionary responses by using likelihood ratio tests to compare the log-likelihood of a model that constrained fitness-trait covariances to be equal in each environment to that of a model that allowed these parameters to differ between environments. Then, we tested whether predicted evolutionary responses differ between aCO₂ and eCO₂ environments when competitors are absent (direct effects of eCO₂) with *a priori* contrasts comparing the log-likelihood of a model that constrained fitness-trait

covariances to be equal in the aCO₂ and eCO₂ environments in the absence of competition to a model where fitness-trait covariances were free to vary. To test whether predicted evolutionary responses differ between aCO₂ and eCO₂ treatments in the presence of competitors (direct and indirect effects of eCO₂), similar log-likelihood ratio tests were conducted. Finally, we tested whether genetic covariances between each trait and fitness were significantly different from zero by comparing the likelihoods of models where genetic covariances between traits and fitness were constrained to zero with models in which these parameters were free to vary. We also used the REML analyses to estimate cross-environment genetic correlations in fitness (fruit production). Cross-environment genetic correlations for fitness that approach one indicate that the same genotypes are favored by natural selection in both environments.

Results

Phenotypic effects of competition

As expected, competition reduced plant growth, and eCO₂ increased plant growth (Table S1). Additionally, proportional increases in both biomass and fruit number due to eCO₂ were greater when competitors were present (significant CO₂ × competition interaction on ln-transformed biomass ($F_{1,1636} = 14.58$, $P < 0.0001$) and fruit production ($F_{1,1630} = 16.69$, $P < 0.0001$)). Reciprocally, fitness effects of competition were reduced in eCO₂ environments; for example, increasing competitor biomass by 0.1 g reduced *A. thaliana* fruit production by an average of 43% in aCO₂ but only by 21% in eCO₂. Although competitors were, on average, 0.13 g larger in the eCO₂ environment, the net effect was for eCO₂ to slightly reduce the fitness effects of competition. Survival was high in all experimental treatments (> 97.8%).

Effects of competition and CO₂ on the expression of genetic variation

We found strong evidence for genetic variation for almost all measured traits (significant RIL effects), but rather weak evidence that genotypes respond differently to competition or CO₂ treatments (few significant RIL × competition or RIL × CO₂ effects after correcting for multiple comparisons; Table S2). A notable exception is the significant CO₂ × RIL interaction on fruit production, which suggests that eCO₂ alters the relative fitness advantages of the different RILs. This significant interaction appears to be due to both crossing reaction norms and changes in relative fitness rankings across CO₂ environments. Genetic correlations in fruit production between CO₂ treatments were significant and high (pairwise r_g between treatments ranged from 0.70 to 0.99), indicating that genotypes that had high fitness in aCO₂ also tended to have high fitness in eCO₂, but additional analyses on ranked fitness data indicate that changes in which genotypes are most favored by natural selection also contributed to the significant genotype × CO₂ interaction ($F_{59,1639} = 1.45$, $P = 0.015$).

Genetic variances and covariances between traits (**G**-matrices, Table S3) differed significantly among the four competition × CO₂

environments ($\chi^2 = 44.40$, $P < 0.044$, Table 1). This result was driven primarily by the effect of competition in aCO₂; we detected significant differences in **G**-matrices between competitive environments in aCO₂ ($\chi^2 = 35.91$, $P < 0.0001$), but no evidence that **G**-matrices differed between competitive environments in eCO₂ ($\chi^2 = 12.98$, $P > 0.22$; Table 1). Additional analyses revealed that the significant difference in **G** was largely due to the effect of competition on genetic variances (Table 1). Genetic variances of the growth traits (rosette size and biomass) were reduced when competitors were present under both aCO₂ ($\chi^2 = 22.31$, $P < 0.0002$) and eCO₂ ($\chi^2 = 13.09$, $P < 0.011$; Tables 1, S3),

Table 1 Likelihood ratio tests comparing **G** matrices and components of **G** between *Arabidopsis thaliana* populations grown in four environments

Model	χ^2	df	<i>P</i>
Equal G			
$H_0 =$ All G equal	44.40	30	0.044
$H_1 =$ G equal between aCO ₂ vs eCO ₂ in absence of competition	8.06	10	0.623
$H_2 =$ G equal between aCO ₂ vs eCO ₂ in presence of competition	14.03	10	0.172
$H_3 =$ G equal between competition vs no competition in aCO ₂	35.91	10	<0.0001
$H_4 =$ G equal between competition vs no competition in eCO ₂	12.98	10	0.225
Equal covariances between traits and fitness (Δz)			
$H_0 =$ Δz do not differ between treatments	19.17	9	0.024
$H_1 =$ Δz equal between aCO ₂ vs eCO ₂ in absence of competition	3.24	3	0.356
$H_2 =$ Δz equal between aCO ₂ vs eCO ₂ in presence of competition	7.27	3	0.064
$H_3 =$ Δz equal between competition vs no competition in aCO ₂	8.41	3	0.038
$H_4 =$ Δz equal between competition vs no competition in eCO ₂	3.45	3	0.327
Equal genetic variances			
$H_0 = \sigma^2_G$ do not differ between treatments	35.11	12	0.0005
$H_1 = \sigma^2_G$ equal between aCO ₂ vs eCO ₂ in absence of competition	4.46	4	0.35
$H_2 = \sigma^2_G$ equal between aCO ₂ vs eCO ₂ in presence of competition	9.59	4	0.048
$H_3 = \sigma^2_G$ equal between competition vs no competition in aCO ₂	22.31	4	0.0002
$H_4 = \sigma^2_G$ equal between competition vs no competition in eCO ₂	13.09	4	0.011
Equal covariances			
$H_0 = \sigma_G(z_1, z_2)$ do not differ between treatments	10.95	9	ns
$H_1 = \sigma_G(z_1, z_2)$ equal between aCO ₂ vs eCO ₂ in absence of competition	1.38	3	0.71
$H_2 = \sigma_G(z_1, z_2)$ equal between aCO ₂ vs eCO ₂ in presence of competition	3.92	3	0.27
$H_3 = \sigma_G(z_1, z_2)$ equal between competition vs no competition in aCO ₂	8.19	3	0.043
$H_4 = \sigma_G(z_1, z_2)$ equal between competition vs no competition in eCO ₂	1.93	3	0.59

aCO₂, ambient atmospheric CO₂; eCO₂, elevated atmospheric CO₂; Δz , predicted change in population mean trait value across one generation; σ^2_G , genetic variance; $\sigma_G(z_1, z_2)$, genetic covariance.

resulting in lower heritabilities in the competitive compared to the noncompetitive environment for most traits (Table S4). We detected little evidence that between-trait covariances (excluding fitness, see next section) differed among treatments, although we did detect evidence that competition affected covariances in the aCO₂ environment ($\chi^2 = 8.19$, $P < 0.043$; Table 1).

Effects of competition and CO₂ on selection and predicted evolutionary responses

The phenotypic selection gradient analysis showed that selection favored earlier flowering, smaller rosette diameters and larger total aboveground biomass in all environments (Table 2), and ANCOVA indicated that competition and CO₂ treatments influenced the strength of selection on these traits (Table S5). Selection for earlier flowering was 50% stronger in the presence of competitors than in their absence (ANCOVA: flowering date \times competition interaction on relative fitness $F_{1,1932} = 9.24$, $P = 0.0024$). CO₂ affected the strength of selection for increased biomass and reduced rosette size, but these effects depended on the presence of competitors (biomass \times CO₂ \times competition interaction $F_{1,1932} = 67.44$, $P < 0.0001$; rosette diameter \times CO₂ \times competition interaction $F_{1,1932} = 6.31$, $P = 0.012$). In no case was the effect of CO₂ on selection gradients detected in the absence of competition (all $P > 0.13$); however, when competitors were present, selection for increased biomass was 48% weaker in eCO₂ than aCO₂ ($F_{1,918} = 105.50$, $P < 0.001$) and selection for smaller rosette diameters also tended to be weaker under eCO₂ ($F_{1,918} = 2.97$, $P = 0.085$; Table 2). Although significant nonlinear selection gradients were detected for biomass and flowering date in some treatments, nonlinear gradients were much weaker than linear selection gradients (Table S5), suggesting that selection was predominantly directional over the range of trait values observed in this study. Stabilizing selection on biomass was, however, significantly weaker in the eCO₂ environments than in the aCO₂ environments (biomass² \times CO₂; $F_{1,1920} = 32.89$, $P < 0.0001$).

Similar to results from the phenotypic selection gradient analysis, the multivariate REML analyses indicated that predicted evolutionary responses differed among environments ($\chi^2 = 19.17$, $P < 0.024$; Table 1), largely because of differences in predicted evolutionary responses between competitive environments in aCO₂ ($\chi^2 = 8.41$, $P < 0.038$; Table 1). For example, competition increased the magnitude of the predicted evolutionary change in flowering date by 84% in aCO₂ compared to 43% in eCO₂ (Table 2). We also detected weak evidence for differences between predicted evolutionary responses in aCO₂ vs eCO₂ treatments, but only when competitors were present (competitors present: $\chi^2 = 7.27$, $P < 0.064$; competitors absent: $\chi^2 = 3.24$, $P < 0.36$).

Discussion

Direct and indirect evolutionary effects of CO₂

Elevated atmospheric CO₂ concentrations consistently alter plant phenotypes (Körner, 2003; Poorter & Navas, 2003; Tonsor & Scheiner, 2007). Our results are consistent with these findings:

Table 2 Phenotypic selection analysis (PSA) gradients on *Arabidopsis thaliana* populations in each environment and predicted evolutionary responses (covariance between trait and fitness) as estimated from the restricted maximum likelihood (REML) analysis

Trait	PSA selection gradient				REML evolutionary response			
	No competition		Competition		No competition		Competition	
	aCO ₂	eCO ₂	aCO ₂	eCO ₂	aCO ₂	eCO ₂	aCO ₂	eCO ₂
Biomass	0.18 (0.012)	0.19 (0.015)	0.50 (0.017)	0.27 (0.015)	0.005 (0.011)	0.020 (0.015)	-0.007 (0.014)	0.024 (0.015)
Flowering date ¹	-0.09 (0.010)	-0.07 (0.012)	-0.13 (0.014)	-0.11 (0.013)	-0.032 (0.016)	-0.035 (0.021)	-0.059 (0.028)	-0.050 (0.023)
June rosette size	-0.08 (0.012)	-0.11 (0.015)	-0.11 (0.017)	-0.07 (0.015)	-0.005 (0.010)	-0.015 (0.014)	-0.010 (0.016)	0.009 (0.014)

aCO₂, ambient atmospheric CO₂; eCO₂, elevated atmospheric CO₂.

Selection gradients and predicted responses that significantly differ from 0 ($P < 0.05$, after Bonferroni correction) are indicated in bold. Standard errors are shown in parentheses.

¹Likelihood ratio tests could not be obtained for flowering date because constrained models did not converge. Instead, confidence intervals were estimated from the asymptotic variance-covariance matrix of estimates. Thus, for flowering date, bold values indicate 95% confidence intervals that do not include 0.

plants grown in eCO₂ were larger and produced more fruits than plants grown in aCO₂. These ecological effects were not, however, accompanied by evolutionary effects when competitors were absent. In the absence of competitors, eCO₂ had little effect on the expression of genetic variation or patterns of selection.

The lack of strong evidence for direct effects of eCO₂ on evolutionary trajectories is consistent with results of an experiment by Lau *et al.* (2007), which was conducted the previous summer and used 163 RILs sampled from the same population from which the 58 RILs used in this study were drawn. It is also consistent with a second experiment using 19 accessions collected from diverse habitats in the native range of *A. thaliana* (Lau *et al.*, 2010). Both of those previous studies found that the direct effects of eCO₂ on patterns of natural selection are small in magnitude and in few cases statistically significant, despite high statistical power for detecting even small differences in selection. Combined with the findings presented here, results from these three studies conducted in two different years on two different synthetic *A. thaliana* populations suggest that this result is general and robust to yearly variation in environmental conditions. Similarly, Bazzaz *et al.* (1995), investigating CO₂ responses of three genotypes of a temperate forest tree (*Betula alleghensis*) and eight genotypes of an annual plant (*Abutilon theophrasti*), detected little evidence that higher atmospheric CO₂ concentrations alter evolutionary processes when plants were grown in the absence of competitors. The agreement between the current study and these previous results suggest that the findings that eCO₂ will have minor direct effects on evolutionary trajectories is applicable to a broad range of genotypes, plant species and may be robust to yearly variation in temperature and water availability. Nevertheless, there are examples showing direct effects of eCO₂ on plant evolution; Tonsor & Scheiner (2007) found that eCO₂ relaxed selection on carbon assimilation rates (A_c) when plants were grown in growth chambers in the absence of competition, herbivory and other biotic interactions that could drive indirect effects. Selection on A_c monotonically decreased from strongly positive to weakly negative across a CO₂ gradient ranging from 250 ppm (subambient) to 710 ppm, likely because as CO₂ supply becomes less limiting, CO₂ becomes a less important influence on plant

fitness. Even in this case, however, the trade-offs driving the observed shift in selection across CO₂ concentrations may be even more important in the face of competition and other biotic stressors.

Competition-mediated indirect effects

Although eCO₂ had minimal direct effects on predicted evolution, eCO₂ significantly altered predicted evolutionary trajectories indirectly by altering the effects competitors had on selection and heritability. When plants were grown in the presence of competition, selection on biomass and other size measures was significantly weaker in the eCO₂ than aCO₂ environment. Other studies have also detected stronger effects of CO₂ on selection on plant traits when competitors were present (Bazzaz *et al.*, 1995; Steinger *et al.*, 2007; Lau *et al.*, 2010), suggesting that the evolutionary effects of eCO₂ may be commonly mediated by competitors. In addition to effects on directional selection, stabilizing selection on late season growth traits was typically weaker under eCO₂ than aCO₂. Together, these results suggest that eCO₂ weakens the selective effects of competition and that selection acting on *A. thaliana* growth traits will be relaxed in future eCO₂ conditions.

A major strength of our approach was that we estimated heritabilities and genetic covariances (in addition to selection gradients) to understand how elevated CO₂ alters the three main components determining evolutionary responses. For example, a previous study by our group also detected relaxed selection on growth traits in eCO₂ when plants were grown with competitors (Lau *et al.*, 2010). However, because this prior study lacked the replication and family structure required for estimating effects of competition and CO₂ on heritabilities and genetic covariances, evolutionary responses could not be predicted. Here, we found that eCO₂ not only affected patterns of natural selection, but also altered heritabilities in the presence of competition. Heritabilities for growth traits were typically higher under eCO₂, especially when competitors were present, even though selection on growth traits was relaxed under eCO₂. As a result, the difference in predicted evolutionary response between CO₂ environments was smaller in magnitude than CO₂ effects on selection alone would

suggest. Very few studies investigating the effects of global change on predicted evolutionary processes investigate all components of the evolutionary process (but see Etterson, 2004). However, because the magnitude of evolutionary response is directly related to both selection and genetic variances and covariances, it is important to examine effects on both patterns of selection and the expression of genetic variation when investigating the net evolutionary effects of global change.

Potential mechanisms underlying indirect effects

Determining how species interactions influence evolutionary responses to environmental change is imperative for understanding population responses to global change in natural communities. Species interactions are likely to mediate evolutionary responses through several processes. First, global changes may alter the intensity or likelihood of interactions between species (Tylianakis *et al.*, 2008). For example, in this study, eCO₂ reduced the strength of competition. As a result, competition became a weaker agent of selection in predicted future eCO₂ environments. Although it is unlikely that *Arabidopsis* and *Bromus* compete directly for CO₂, eCO₂ likely reduced competition for other limiting resources, such as water. Increased WUE (water use efficiency) is often observed in high CO₂ conditions, which may reduce competition for this resource. Similarly, numerous other studies have shown that eCO₂ can influence both the amount and type of herbivory on plants (reviewed in Bezemer & Jones, 1998; Stiling & Cornelissen, 2007). Because herbivores can impose strong selection on plant growth and defense traits, eCO₂ may alter selection on these same traits by altering the likelihood of interactions with a diverse array of enemies.

Second, global changes may alter evolutionary processes by altering the expression of plant traits. Similar to the minimal direct effects of eCO₂ documented in this study, Vannette & Hunter (2011) found few differences among *Asclepias syriaca* genotypes in growth or reproductive trait responses to eCO₂. However, genotypes differed in the magnitude of reduction in expression of chemical defense traits under eCO₂. If reduced defense trait expression increases herbivory and if herbivores are strong selective agents, then elevated CO₂ may change genotypic fitness rankings when herbivores are abundant.

Finally, because other species in the community are also responding to global changes, the presence of other community members may either heighten or reduce the fitness consequences of global change for focal populations. For example, drought stress caused significant changes to the community structure of the belowground microbial community associating with experimental *Brassica rapa* populations, and these changes to the microbial community mitigated the negative fitness effects of drought stress on *B. rapa*. When plants were grown with a 'drought-adapted' microbial community, drought stress reduced *B. rapa* fruit production by < 21%, whereas drought stress reduced plant fitness by over 58% when plants were grown in association with a microbial community adapted to wetter soils (Lau & Lennon, 2012). In this example, because other community members (belowground microbes) mitigate the fitness consequences of

environmental change, the selective role of that environmental change may be reduced, making evolutionary responses to the environmental change less likely. Regardless of the underlying mechanism, because species interactions may influence evolutionary responses to anthropogenic environmental changes, evolutionary responses likely will differ across natural biological communities that differ in composition.

Conclusions

This study adds to a growing number of studies that have identified stronger indirect than direct effects of rising atmospheric CO₂ concentrations on predicted evolution (Bazzaz *et al.*, 1995; Steinger *et al.*, 2007; Lau & Tiffin, 2009; Lau *et al.*, 2010). Similarly, investigations of soil fertility (Snaydon & Davies, 1972) and exotic species (Lau, 2008) also have documented that environmental perturbations that have large ecological effects can have minimal direct but substantial indirect effects on evolutionary trajectories. Many of the evolutionary responses in *Anthoxanthum odoratum* morphology to the soil fertility manipulations in the long-running Park Grass Experiment, for example, appear to be more closely associated with changes in the competitive environment (average stand height and/or productivity) than the direct result of changes in soil nutrient concentrations (Snaydon & Davies, 1972). The potential importance of indirect effects highlights the need to conduct empirical research on the evolutionary impacts of ongoing global climate change, as well as other environmental factors, in ecologically realistic environments. As our results indicate, manipulating single abiotic factors in less complex environments may give incomplete or false estimates of the ecological and evolutionary effects of these types of environmental changes on some populations.

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Supporting Information

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

Table S1 Mean trait values in the presence and absence of competitors in each CO₂ environment

Table S2 Results from ANOVA testing the effects of CO₂, the presence of competitors and plant genotype (RIL) on trait values

Table S3 Additive genetic variance–covariance matrices (**G**) for fitness (fruit number), plus the three focal traits (flowering date, rosette diameter and aboveground biomass) in each of the four competition × CO₂ treatments

Table S4 Estimated broad-sense heritabilities ($H^2 = V_g/V_p$) for each trait in each environment

Table S5 Estimated directional (β) and nonlinear (γ) selection gradients in each environment and results from ANCOVA testing for differences in β across environments

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