

Joy K. Ward · Janis Antonovics · Richard B. Thomas
Boyd R. Strain

Is atmospheric CO₂ a selective agent on model C₃ annuals?

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Abstract Atmospheric CO₂ partial pressure ($p\text{CO}_2$) was as low as 18 Pa during the Pleistocene and is projected to increase from 36 to 70 Pa CO₂ before the end of the 21st century. High $p\text{CO}_2$ often increases the growth and reproduction of C₃ annuals, whereas low $p\text{CO}_2$ decreases growth and may reduce or prevent reproduction. Previous predictions regarding the effects of high and low $p\text{CO}_2$ on C₃ plants have rarely considered the effects of evolution. Knowledge of the potential for evolution of C₃ plants in response to CO₂ is important for predicting the degree to which plants may sequester atmospheric CO₂ in the future, and for understanding how plants may have functioned in response to low $p\text{CO}_2$ during the Pleistocene. Therefore, three studies using *Arabidopsis thaliana* as a model system for C₃ annuals were conducted: (1) a selection experiment to measure responses to selection for high seed number (a major component of fitness) at Pleistocene (20 Pa) and future (70 Pa) $p\text{CO}_2$ and to determine changes in development rate and biomass production during selection, (2) a growth experiment to determine if the effects of selection on final biomass were evident prior to reproduction, and (3) a reciprocal transplant experiment to test if $p\text{CO}_2$ was a selective agent on *Arabidopsis*. *Arabidopsis* showed significant positive responses to selection for high seed number at both 20 and 70 Pa CO₂ during the selection process. Furthermore, plants selected at 20 Pa CO₂

performed better than plants selected at 70 Pa CO₂ under low CO₂ conditions, indicating that low CO₂ acted as a selective agent on these annuals. However, plants selected at 70 Pa CO₂ did not have significantly higher seed production than plants selected at 20 Pa CO₂ when grown at high $p\text{CO}_2$. Nevertheless, there was some evidence that high CO₂ may also be a selective agent because changes in development rate and biomass production during selection occurred in opposite directions at low and high $p\text{CO}_2$. Plants selected at high $p\text{CO}_2$ showed no change or reductions in biomass relative to control plants due to a decrease in the length of the life cycle, as indicated by earlier initiation of flowering and senescence. In contrast, selection at low CO₂ resulted in an average 35% increase in biomass production, due to an increase in the length of the life cycle that resulted in a longer period for biomass accumulation before senescence. From the *Arabidopsis* model system we conclude that some C₃ annuals may have produced greater biomass in response to low $p\text{CO}_2$ during the Pleistocene relative to what has been predicted from studies exposing a single generation of C₃ plants to low $p\text{CO}_2$. Furthermore, C₃ annuals may exhibit evolutionary responses to high $p\text{CO}_2$ in the future that may result in developmental changes, but these are unlikely to increase biomass production. This series of studies shows that CO₂ may potentially act as a selective agent on C₃ annuals, producing changes in development rate and carbon accumulation that could not have been predicted from single-generation studies.

J.K. Ward (✉) · B.R. Strain
Department of Botany, Duke University,
Durham, NC 27708, USA

J. Antonovics
Department of Biology, University of Virginia,
Charlottesville, VA 22903, USA

R.B. Thomas
Department of Biology, West Virginia University,
Morgantown, WV 26506, USA

Present address:

J.K. Ward, Department of Biology, University of Utah,
257 South, 1400 East,
Salt Lake City, UT 84112-0840, USA,
e-mail: jward@biology.utah.edu,
Tel.: +1-801-5815927, Fax: +1-801-5814665

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Introduction

That the high atmospheric CO₂ partial pressure ($p\text{CO}_2$) projected for the future will increase the photosynthesis and growth of C₃ plants which comprise approximately 95% of higher plant species is now well established (Griffin and Seemann 1996). Over the last decade, much

emphasis has been placed on predicting the degree to which terrestrial ecosystems will sequester or release carbon under future scenarios of increasing atmospheric $p\text{CO}_2$ (Oechel et al. 1994; Hungate et al. 1997; DeLucia et al. 1999). However, predictions regarding the effects of high $p\text{CO}_2$ on the carbon uptake of C_3 plants have commonly assumed that plant responses will remain stable over long time scales, and relatively little attention has been focused on the effects of increasing CO_2 on plant evolution. Recent studies have demonstrated that C_3 plants exhibit genetic variation in response to high $p\text{CO}_2$ for photosynthesis (Curtis et al. 1996), stomatal characters (Case et al. 1998), growth (Zhang and Lechowicz 1995; Schmid et al. 1996), and reproduction (Curtis et al. 1994; Bazzaz et al. 1995; Ward and Strain 1997). A high level of genetic variation for responses to $p\text{CO}_2$ may result in rapid directional selection (Strain 1991), particularly in annuals, that may alter the capacity for carbon sequestration by some terrestrial ecosystems.

Vostok ice core data have provided a direct record of atmospheric CO_2 for the last 420,000 years (Petit et al. 1999). This record indicates that $p\text{CO}_2$ during the late Pleistocene ranged between 18 and 20 Pa during glacial maxima, which are among the lowest values predicted to have occurred during the last 600 million years (Berner 1991). Previous single-generation studies have reported a 56–92% reduction in the biomass of current C_3 plants in response to low $p\text{CO}_2$ of the Pleistocene compared to the current value, whereas C_4 species (that have a CO_2 -concentrating mechanism) were less negatively affected by low CO_2 (Polley et al. 1993; Dippery et al. 1995; Tissue et al. 1995; Cowling and Sage 1998). In addition, low CO_2 greatly reduced the survival of the C_3 annual *Arabidopsis thaliana* (J.K. Ward and B.R. Strain, unpublished data) and prevented reproduction in *Abutilon theophrasti* (Dippery et al. 1995). Consistent with these results from current genotypes, stable isotopic analyses of ancient plant material and soil carbonate layers have shown that there were regional shifts from species with the C_3 photosynthetic pathway to those with the C_4 pathway during low- CO_2 periods of the Pleistocene (Ehleringer et al. 1991; Cerling et al. 1993, 1998; Street-Perrott et al. 1997). It is clear, however, from pollen records and fossil evidence that C_3 species persisted and were abundant during the Pleistocene. Thus, it is likely that evolutionary responses may have occurred during the Pleistocene that ameliorated what are now observed as strong negative effects of low $p\text{CO}_2$ on plant growth and reproduction.

Given the possibility that low $p\text{CO}_2$ of the Pleistocene and high $p\text{CO}_2$ predicted for the future may act as strong selective agents (Geber and Dawson 1993; Thomas and Jasienski 1996; Ward and Strain 1997), it is important to test these predictions directly to determine whether evolutionary responses to CO_2 may alter plant growth and function. *A. thaliana*, which is commonly used for classical genetic and molecular studies, is an ideal model system for investigating this issue for C_3 annuals. *Arabidopsis* has a short generation time and small stature that

allow for multiple-generation experiments at controlled CO_2 conditions. Furthermore, the availability of numerous field-collected genotypes from different latitudes and altitudes is crucial, because these genotypes may show variation for responses to $p\text{CO}_2$. For example, plants from high altitudes have been shown to respond differently to low and high $p\text{CO}_2$ compared to lowland plants due to possible adaptations to low $p\text{CO}_2$ at high altitudes (Billings et al. 1961; Körner and Diemer 1994; Ward and Strain 1997). Furthermore, examining the responses of genotypes from different regions rather than from small populations is particularly relevant to species such as *A. thaliana* and other annuals that exhibit a high degree of migration (which is often associated with railways and shipping routes), primarily because migration will affect the potential for selection. In addition, the life history and allocation strategy of *Arabidopsis* is common to numerous annuals that have a short generation time and allocate a high proportion of their resources to reproduction; thus, selection responses of *Arabidopsis* may provide valuable insights into the evolution of various annuals in response to changes in atmospheric CO_2 .

Here we report a selection experiment conducted for five generations using *A. thaliana* as a model system to begin determining if atmospheric CO_2 acts as a selective agent on C_3 annuals. We used two extremes in $p\text{CO}_2$, corresponding to the low $p\text{CO}_2$ of the Pleistocene (20 Pa) and high $p\text{CO}_2$ predicted for the future (70 Pa). Seeds used for selection were derived from random crosses between field-collected genotypes from different locations and altitudes, allowing for selection on variation resulting from recombination and segregation. We selected for high seed number, a major component of fitness (Primack and Antonovics 1981; Primack and Kang 1989), and measured changes in development rate and biomass production throughout selection. Following selection, we conducted a reciprocal transplant experiment to determine if *Arabidopsis* showed genetic adaptation to $p\text{CO}_2$.

Materials and methods

Research species and seed source

A. thaliana is an annual herb (C_3) belonging to the Brassicaceae that initiates flowering in response to long-day photoperiods (Napp-Zinn 1985) and produces pod-like fruits (siliques). *Arabidopsis* predominantly self-fertilizes in the field, and therefore wild genotypes are homozygous at the majority of loci (Estelle and Somerville 1986).

Genotypes of *A. thaliana* from different locations and altitudes (Table 1) were donated by the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, Columbus, Ohio). Genotypes originated from randomly chosen plants in field populations and were propagated under uniform conditions at the ABRC by self-fertilization for two generations. Seeds were conserved at the ABRC under dry, cool conditions (4°C) for 2 years. Prior to the selection experiment, random crosses were performed between the genotypes by manually pollinating emasculated flowers (prior to anthesis) to produce seven different hybrids (Table 1, Fig. 1). Pollen recipients were determined randomly and pollen donors were recipients from the previously chosen cross. Hybrid seeds

Table 1 Description of *Arabidopsis* genotypes including stock number (at the ABRC) and location, altitude, and local $p\text{CO}_2$ at the site of collection. Local $p\text{CO}_2$ was calculated as total atmospheric pressure multiplied by the CO_2 mixing ratio that remains constant with altitude (Sage and Reid 1992). These genotypes

Description of genotypes		Altitude (m)	Local $p\text{CO}_2$ (Pa)	Cross number						
Stock	Location			1	2	3	4	5	6	7
6187	Seattle, Wash., USA	0	35			R	D			
6084	Dartmouth, Devon, UK	0	35						R	
6064	Maidstone, Kent, UK	50	34.5							D
6073	Kent, UK	50	34.5				R	D		
6078	Sidmouth, Devon, UK	50	34.5					R	D	
3177	Columbia, Mo., USA	200	34		R	D				
6082	Chagford, Devon, UK	225	34	R	D					
902	Cape Verde Islands, Atlantic Ocean	1200	30.5	D						
6180	Pamiro-Alay, Tajikistan	3400	23							R

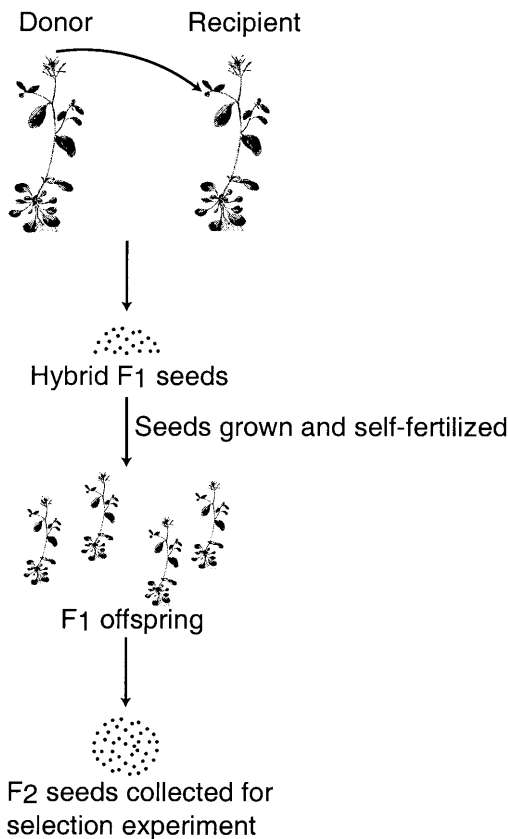


Fig. 1 Protocol for obtaining F_2 seeds used for the selection experiment

were then grown and self-fertilized for one generation under uniform conditions in a greenhouse to produce a large number of segregating F_2 seed progeny for the selection experiment (Fig. 1).

Growth conditions and measurements for the selection experiment

During the selection experiment, plants were grown for five generations in four CO_2 -controlled growth chambers at the Duke University Phytotron. Two chambers were controlled at 20 ± 1 Pa CO_2

were used to conduct controlled crosses, and F_2 seeds from these crosses were used for the selection experiment. The table indicates each cross number (1–7) along with the parental genotypes listed as either pollen donors (D) or recipients (R)

and two others at 70 ± 4 Pa CO_2 . The partial pressure of CO_2 was automatically monitored and controlled by infrared gas analysis and by continuous CO_2 injection. The 20-Pa chambers were scrubbed of excess CO_2 when necessary by passing chamber air over a hydrated lime/vermiculite mixture.

The growth medium consisted of a 1:1:1 (v/v) mixture of gravel, Turface, and vermiculite. Approximately 20 seeds from the appropriate seed source were planted on the surface of the medium in each 750-ml plastic pot and were covered with a thin layer (1 mm) of “metro-mix 220” (vermiculite, sphagnum, perlite, Scotts-Sierra, Horticultural Products, Marysville, Ohio) to prevent seed movement. Seeds were exposed to a dark, cold treatment of 4°C for 4 days after planting to break dormancy. Afterwards, light/dark periods were 14/10 h with corresponding air temperatures of $25/18^\circ\text{C}$. Using sodium vapor and metal halide lamps, the photosynthetic photon flux density during the light period was maintained at $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the level of rosette leaves. Relative humidity was approximately 60% during the light period and 90% during the dark period. Prior to seedling emergence, pots were watered to saturation with deionized water twice each day. Following emergence, pots were watered to saturation with half-strength modified Hoagland’s solution (Downs and Hellmers 1978) each morning and with deionized water each afternoon. At 6 days after emergence, pots were thinned to the plant closest to the center of each pot.

The number of days required by each plant to reach the first flower was recorded. Plants were harvested after 62 days of growth (excluding time in the cold treatment), at which point they had begun senescing in all treatments. The number of siliques per plant was determined by counting all intact siliques and central partitions of siliques that persisted after seed maturity. Total seed number was calculated as the total number of siliques per plant multiplied by the mean number of seeds per silique (determined from 15 randomly chosen siliques per plant). Plant material was oven dried (65°C) for 48 h and total mass was determined.

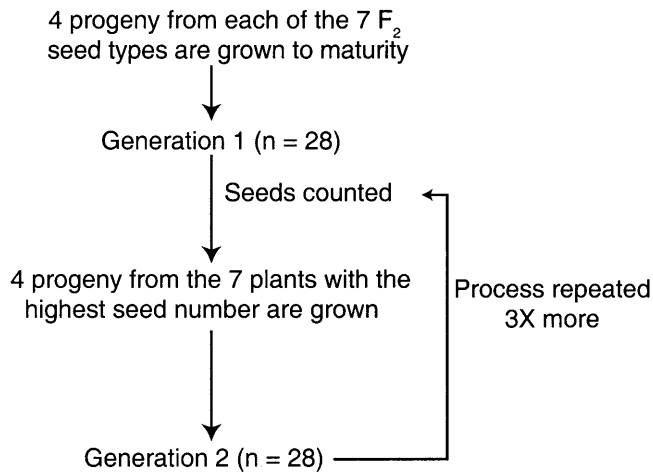
Design and statistical analysis of the selection experiment

Within each of the four CO_2 -controlled chambers (two at 20 Pa and two at 70 Pa), three independent populations were maintained during each generation: two selection populations and one control population (Table 2). Initial populations comprised 28 plants (following thinning) developed from four progeny from each of the seven F_2 seed types (Table 1, Fig. 2). Following the first generation, four random progeny from each of the seven plants producing the highest total seed number (25% truncation) within each selection population were grown for the next generation (Fig. 2). Within

Table 2 Design of the selection experiment. During every generation, each of the four CO₂-controlled chambers contained three independent populations (two selection populations and one control population) with 28 plants per population. The selection procedure was carried out for five generations

	Chamber			
	1	2	3	4
<i>p</i> CO ₂ (Pa)	70	70	20	20
Populations	Selection 1 Selection 2 Control C1,2	Selection 3 Selection 4 Control C3,4	Selection 5 Selection 6 Control C5,6	Selection 7 Selection 8 Control C7,8

Selection Populations



Control Populations

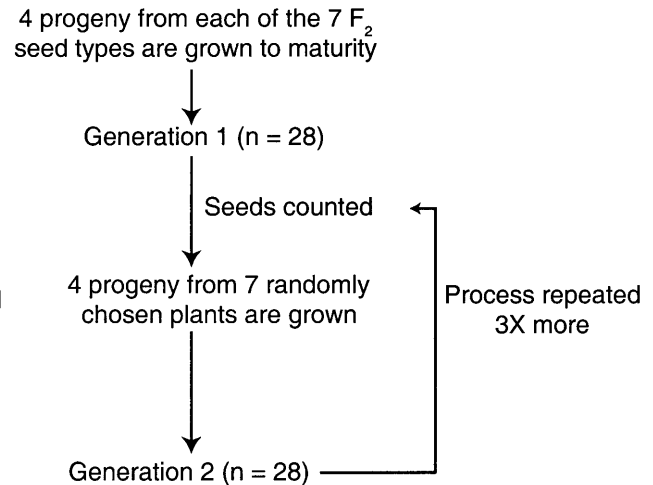


Fig. 2 Protocol for producing populations that underwent selection for high seed number and control populations that underwent random selection

each control population, four random progeny from each of seven randomly chosen plants were grown for the next generation (Falconer 1981; Fig. 2). This process was repeated through four more generations. By this design, the size of each population remained at 28 plants in each generation. An internal control (randomly selected population) within each chamber was necessary to account for possible differences in the environment of the growth chambers between generations (Falconer 1981). The average value of CO₂ in the current atmosphere of 36 Pa was not a relevant baseline control, however, because: (1) genotypes from different altitudes may be adapted to different *p*CO₂, (2) it is unlikely that plants are adapted to the current *p*CO₂ because CO₂ has risen very rapidly during this century, and (3) plants have been exposed to lower *p*CO₂ than the current value for most of the past 1 million years (Barnola et al. 1987; Jouzel et al. 1993; Petit et al. 1999) which may have influenced the genetic composition of current populations.

Outcrossing has been shown to occur at a very low frequency (0.001%) in *Arabidopsis* grown under laboratory conditions (Kranz and Kirchheim 1987), and therefore it can be assumed that plants predominantly self-fertilized throughout the selection experiment. This was advantageous because reproductive output was dependent on internal plant processes as opposed to external factors such as hand pollinations that may result in limiting pollen loads. Furthermore, self-fertilization prevented cross-pollinations from occurring between independent selection and control populations. At the beginning of the experiment, we decided that the selection process would be terminated following the fifth generation because plants would be homozygous at the majority of loci following several rounds of self-fertilization (98.4% of loci estimated to be homozygous by the end of selection), and therefore, the potential for further change due to selection would be minimal.

Analyses of variance (ANOVAs) were performed on data from individual plants for total seed number, time to first flower, and total mass at each generation in order to assess the effects of *p*CO₂, selection regime (selection versus control), the *p*CO₂ × selection regime interaction, and the nested effects of chamber(*p*CO₂) and population(chamber, selection regime) (JMP 3.1, 1995, SAS Institute, Cary, N.C.). The chamber(*p*CO₂) variation was used as the error term for the *p*CO₂ effect, and the other terms were tested over the residual variation. Data for total mass were log_e-transformed to meet the assumptions of ANOVA. The effect of *p*CO₂ on the proportion of offspring from the original seven crosses (Table 1) represented at the fifth generation of selection was analyzed with a likelihood ratio chi-square test (JMP 3.1). Effects were considered significant at the *P* < 0.05 level.

Experiment to determine the effects of selection prior to the initiation of reproduction

A second experiment was conducted to determine if the effects of selection were evident on biomass production at an early stage of development. Following the fifth generation of selection, seeds were collected from the 7 plants (out of 28) producing the highest seed number within each selection population and from 7 randomly chosen plants within each control population. An equal number of seeds from each of the 7 selected plants from each population was randomly mixed. Seeds were planted in six replicate pots for each selection and control population and were exposed to a dark, cold treatment of 4°C for 4 days after planting to break dormancy. Pots with seeds derived from all selection and control populations at 70 Pa CO₂ (six pots each for selection populations 1, 2, 3, 4, and control populations C1,2 and C3,4) were grown in a single 70-Pa CO₂-controlled chamber, and seeds derived from all selection and control populations at 20 Pa CO₂ (six pots each for selection populations 5, 6, 7, 8 and control populations C5,6 and C7,8) were grown in a 20-Pa CO₂-controlled chamber. All other growth conditions were similar to the selection experiment.

Plants were harvested after 21–23 days of growth at 70 Pa CO₂ and after 25–27 days of growth at 20 Pa CO₂ which corresponded to a developmental stage prior to the initiation of reproduction in both selection and control populations. Plant material was separated into rosette leaves and roots. Leaf area was measured using a LI-3100 leaf area meter (Li-Cor, Lincoln, Neb.), and biomass of leaves and roots was determined following oven drying at 65°C. Data were analyzed using one-way ANOVA with selection regime as the main effect to test for differences between plants derived from selection and control populations within each of the CO₂ treatments. Data from the 20-Pa and 70-Pa CO₂ treatments were analyzed using separate ANOVAs because harvests were necessarily conducted at different time periods (to control for stage of development), and maternal effects may have varied between CO₂ treatments.

Experiment to determine if plants exhibited adaptive responses to pCO₂

The third experiment was a reciprocal transplant to test if *Arabidopsis* exhibited genetic adaptation to pCO₂. This was accomplished by first collecting seeds from plants selected for high seed number at 20 and 70 Pa CO₂, growing them under common conditions, at both 20 and 70 Pa CO₂, and determining if resulting plants had greater seed production at their selection pCO₂ compared to the seed production of plants selected at a different pCO₂.

Seeds were collected randomly from the seven plants producing the highest seed number from each of the eight populations selected for high seed number at 20 and 70 Pa CO₂. Seeds from each of these plants were then planted in separate pots (56 pots total, one pot for each of the seven selected plants from each of the eight selection populations) and were exposed to the same dark, cold treatment as during selection to break dormancy. Following the cold treatment, all seeds were developed within the same 35-Pa CO₂-controlled chamber in order to reduce possible environmental maternal effects associated with the selection process (Andalo et al. 1996, 1998). At reproductive maturity, seeds were collected from each of the 56 plants developed under similar conditions. These were then planted in separate pots and exposed to the same cold treatment as during selection. Pots were then divided equally between a 70-Pa and 20-Pa CO₂-controlled chamber such that sev-

en offspring derived from each of the eight selection populations were grown at both 20 and 70 Pa CO₂. All other growth conditions were similar to the selection experiment. Plants were harvested after 62 days of growth (same generation time as selection), and total seed number per plant (selected character) was determined as during selection. Data for total seed number were analyzed using two-way ANOVA (log_e-transformed to meet the assumptions of ANOVA) to test the interaction between selection pCO₂ and growth pCO₂. The interaction term indicated if the selection pCO₂ affected responses to the growth pCO₂, which would demonstrate whether pCO₂ was a selective agent on the *Arabidopsis* populations. Multiple comparisons of means were conducted using Tukey-Kramer tests.

Results

Selection experiment

At the first generation (prior to the first round of selection, see Fig. 2), there were no significant effects of selection regime or the selection regime×pCO₂ interaction on total seed number, days to first flower, and total mass, indicating that there were no initial differences between selection and control populations (Table 3, Figs. 3, 4, and 5). At the third through fifth generations of selection, however, there was a significant effect of selection regime on total seed number (Table 3, data for generation 4 not shown), whereby selection populations produced significantly more seed than control populations (Fig. 3). Furthermore, relative increases in total seed number between control and selection populations were similar at both 20 Pa and 70 Pa CO₂ (non-significant pCO₂×selection interaction; Table 3).

The selection regime×pCO₂ interaction term was significant for days to first flower at generations 3–5 (Table

Table 3 Summary statistics for the effects of pCO₂, chamber(pCO₂), selection regime, population(chamber, selection), and the pCO₂×selection interaction for total seed number (selected

character), days to first flower, and total mass at the first (prior to selection), third, and fifth generations of selection

	Total seed number			Days to first flower			Total mass		
	MS	df	P-value	MS	df	P-value	MS	df	P-value
Generation 1									
pCO ₂	1.3×10 ¹¹	1	0.015	518.5	1	0.0049	305.3	1	0.0017
Chamber(pCO ₂)	2.7×10 ⁹	2	0.025	15.4	2	0.81	1.17	2	0.25
Selection	4.1×10 ⁷	1	0.81	18.7	1	0.61	0.26	1	0.58
Population(chamber, selection)	5.6×10 ⁸	4	0.53	68.9	4	0.44	0.99	4	0.32
pCO ₂ ×selection	3.4×10 ⁸	1	0.49	53.7	1	0.39	0.98	1	0.28
Generation 3									
pCO ₂	2.8×10 ¹¹	1	0.025	2436.5	1	0.067	406.6	1	0.030
Chamber(pCO ₂)	8.5×10 ⁹	2	0.0001	214.2	2	0.0023	14.9	2	0.0001
Selection	1.4×10 ¹⁰	1	0.0001	12.5	1	0.55	0.013	1	0.89
Population(chamber, selection)	2.2×10 ⁸	4	0.82	81.7	4	0.053	0.68	4	0.46
pCO ₂ ×Selection	9.0×10 ⁸	1	0.22	357.9	1	0.0014	9.42	1	0.0005
Generation 5									
pCO ₂	2.7×10 ¹¹	1	0.014	3240.9	1	0.060	325.9	1	0.055
Chamber(pCO ₂)	5.0×10 ⁹	2	0.0011	263.7	2	0.0008	23.1	2	0.0001
Selection	1.5×10 ¹⁰	1	0.0001	26.0	1	0.40	0.82	1	0.30
Population(chamber, selection)	5.1×10 ⁸	4	0.58	42.2	4	0.32	0.91	4	0.32
pCO ₂ ×selection	3.1×10 ⁸	1	0.51	634.2	1	0.0001	22.1	1	0.0001

Fig. 3 Total seed number for plants in selection populations (1–8; selection for high seed number) and corresponding control populations (C1,2; C3,4; C5,6; C7,8; random selection) during five generations of selection within two growth chambers controlled at 70 Pa CO₂ (a,b) and two chambers controlled at 20 Pa CO₂ (c,d). A cross above a generation indicates a significant effect of selection (selection for high seed number versus random selection). Symbols are means±SE (*n*=23–28). Note that the scale of the y-axis differs between 20 Pa and 70 Pa CO₂

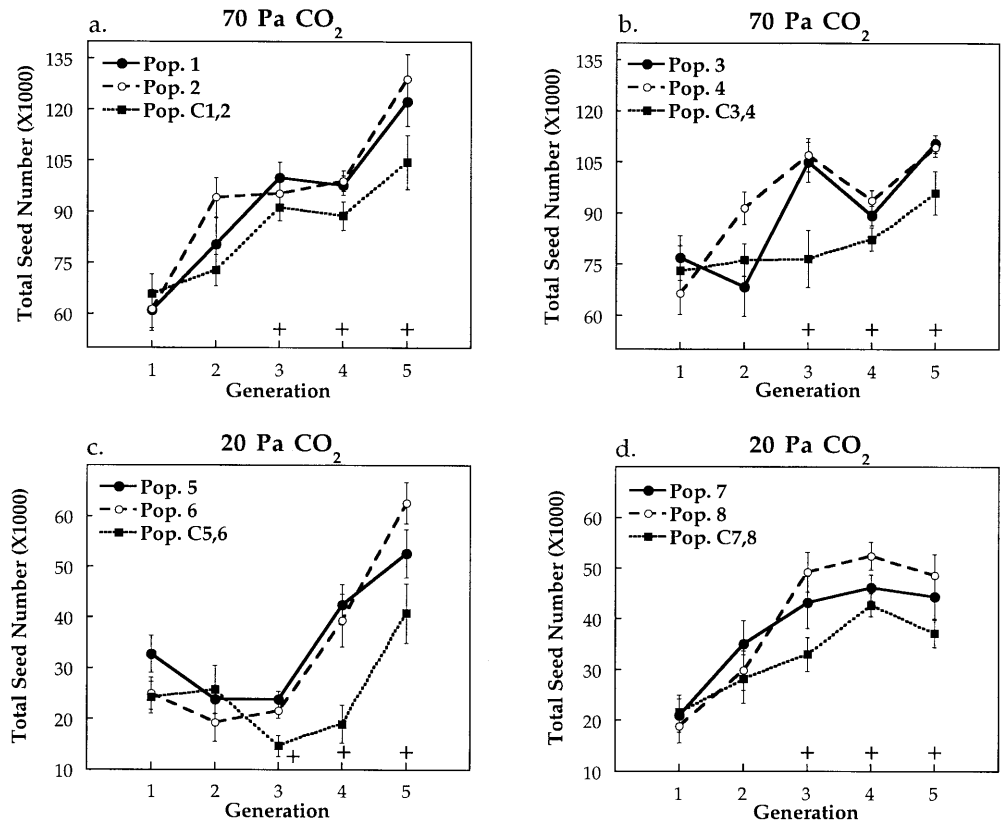


Fig. 4 Time to first flower for plants in selection populations (1–8; selection for high seed number) and corresponding control populations (C1,2; C3,4; C5,6; C7,8; random selection) during five generations of selection within two growth chambers controlled at 70 Pa CO₂ (a,b) and two chambers controlled at 20 Pa CO₂ (c,d). An asterisk above a generation indicates a significant interaction of *p*CO₂×selection. Symbols are means±SE (*n*=23–28). Note that the scale of the y-axis differs between 20 Pa and 70 Pa CO₂

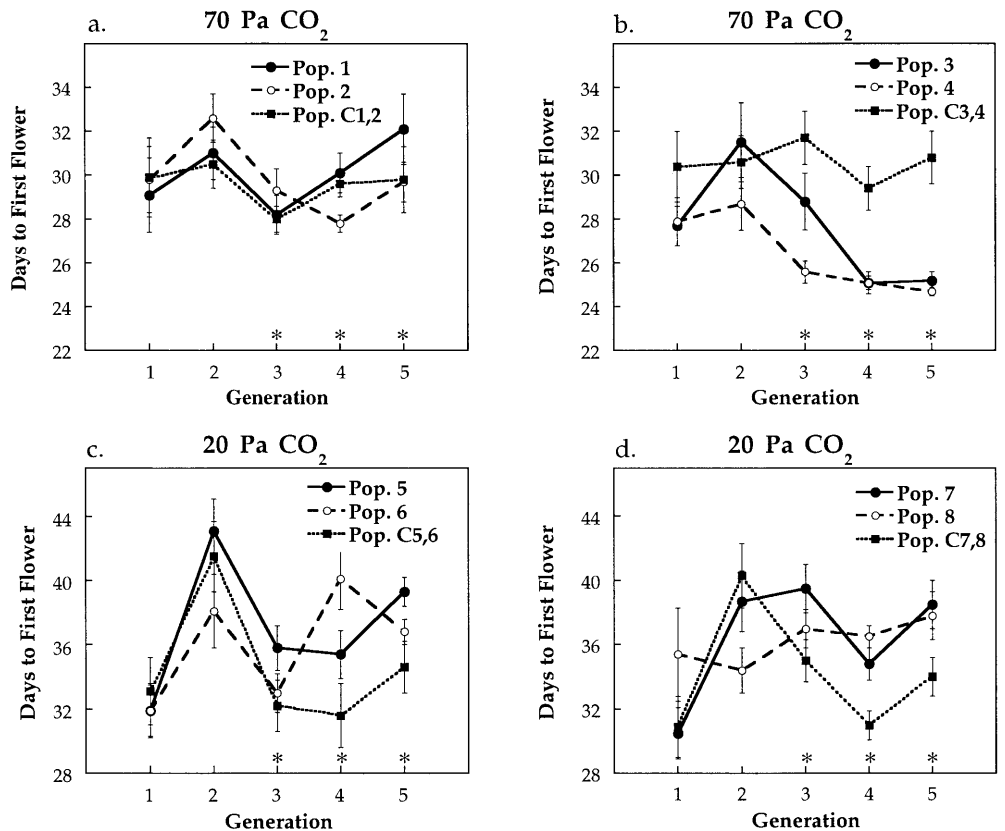
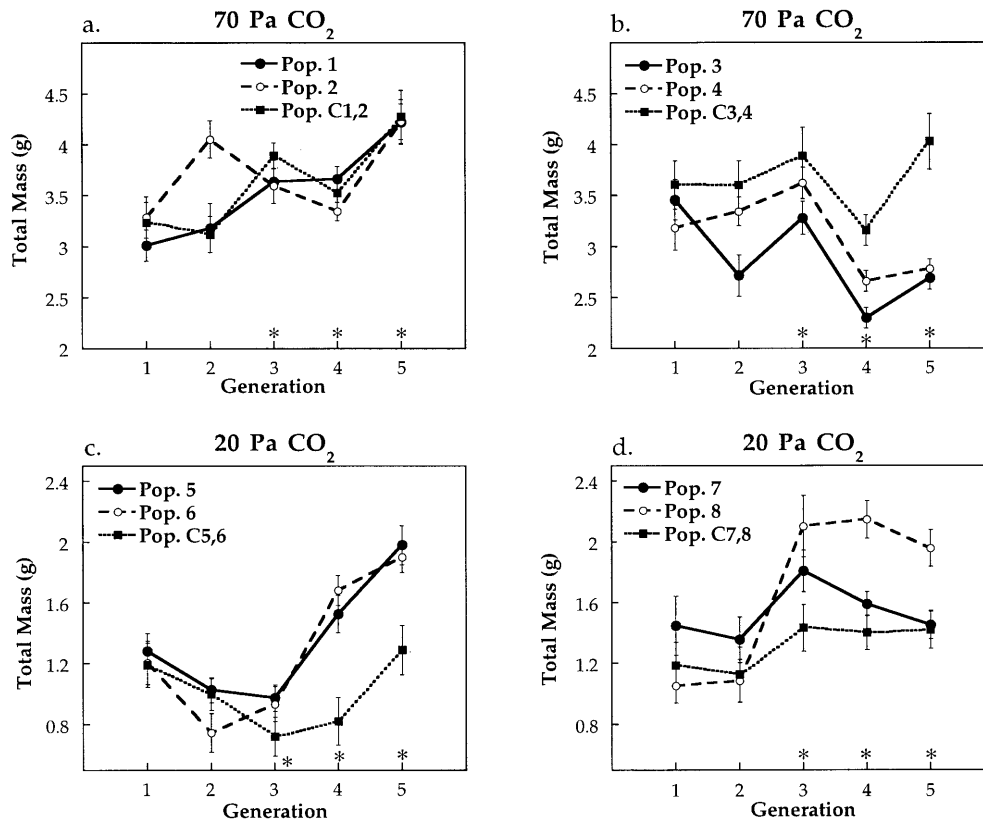


Fig. 5 Total mass for plants in selection populations (1–8; selection for high seed number) and corresponding control populations (C1,2; C3,4; C5,6; C7,8; random selection) during five generations of selection within two growth chambers controlled at 70 Pa CO₂ (a,b) and two chambers controlled at 20 Pa CO₂ (c,d). An asterisk above a generation indicates a significant interaction of $p\text{CO}_2 \times \text{selection}$. Symbols are means \pm SE ($n=23\text{--}28$). Note that the scale of the y-axis differs between 20 Pa and 70 Pa CO₂



3; data for generation 4 not shown), indicating that the effects of selection for high seed number on days to first flower varied between CO₂ treatments (Fig. 4). At 70 Pa CO₂, for example, selection populations either showed no differences in time to first flower (populations 1 and 2) or flowered earlier (by as much as 6 days, populations 3 and 4) than control populations (Fig. 4). At 20 Pa CO₂, however, all selection populations flowered later than control populations at generations 3–5. Furthermore, although not quantified, visual assessment of the plants indicated that selected plants in populations 3 and 4 at 70 Pa CO₂ began senescing earlier than corresponding control plants, and plants in all selection populations at 20 Pa CO₂ began senescing later than plants in control populations.

The selection regime \times $p\text{CO}_2$ interaction term was significant for total mass (dry biomass) at generations 3–5 (Table 3; data for generation 4 not shown), indicating that the effects of selection for high seed number on total mass varied between the 20 and 70 Pa CO₂ treatments (Fig. 5). At 70 Pa CO₂, the total mass of selection populations was either similar (populations 1 and 2) or lower (populations 3 and 4) than control populations (Fig. 5). At 20 Pa CO₂, however, selection for high seed number resulted in an average 35% increase in total mass in selection populations relative to control populations by the fifth generation, due to higher total mass in three out of the four selection populations compared with control populations.

The CO₂ treatment had a significant effect on the types of crosses (Table 1) that were represented at the

fifth generation of selection (likelihood ratio chi-square test, $P=0.0001$). Overall, offspring derived from cross 7 were the most highly represented in populations selected for high seed number at 70 Pa CO₂ and were present in three out of the four selection populations by the end of selection (Table 4). At 20 Pa CO₂, cross 7 was also the most highly represented cross due to a high occurrence in selection populations 5 and 6. Offspring from cross 6, however, were most highly represented in selection populations 7 and 8 at 20 Pa CO₂, but did not occur in selection populations at 70 Pa CO₂ (Table 4). Offspring from crosses 3 and 4 that involved a common parent (Table 1) were also highly represented at 70 Pa CO₂.

Experiment to determine the effects of selection prior to the initiation of reproduction

Because the selection process resulted in changes in biomass at the end of the life cycle, biomass production of plants from selection and control populations was then measured earlier in the life cycle (prior to the initiation of reproduction). This study indicated if changes in final biomass production during selection for high seed number may have been due to changes in development rate (time to reproduce and length of the life cycle) or some other factor such as differences in carbon accumulation rate early in the life cycle. The results indicated that before the onset of reproduction, there were no significant differences between plants from selection and control

Table 4 The representation of offspring (in percent) derived from each cross (Table 1) within selection and control (C) populations at the fifth generation of selection (top value) and the average total

seed number for plants of that specific cross (*in parentheses*). *Blank cells* indicate 0% representation

Population	CO ₂ (Pa)	Cross number (see Table 1)						
		1	2	3	4	5	6	7
1	70			54% (118,745)				46% (126,589)
2	70				100% (129,087)			
C1,2	70	31% (123,522)	15% (80,126)	31% (111,833)		23% (85,739)		
3	70				29% (111,639)			71% (109,857)
4	70							100% (109,307)
C3,4	70	26% (108,181)	15% (103,076)	15% (106,974)			44% (82,923)	
5	20					26% (59,263)	15% (53,065)	59% (49,657)
6	20	12% (57,206)						88% (63,411)
C5,6	20	36% (46,524)				64% (37,637)		
7	20		57% (52,154)				43% (33,958)	
8	20				17% (30,311)		83% (52,263)	
C7,8	20			36% (38,077)		28% (26,577)	36% (44,111)	

populations at 70 Pa CO₂ for leaf area ($P=0.61$), total mass ($P=0.80$), and components of total mass (data not shown). Likewise, plants from selection and control populations at 20 Pa CO₂ did not differ for leaf area ($P=0.44$), total mass ($P=0.27$), and components of total mass prior to reproduction (data not shown). These results, along with the results that selection affected time to senescence, indicate that changes in final biomass between selection and control populations at both 20 and 70 Pa CO₂ were likely due to changes in the length of the life cycle rather than differences in the carbon accumulation rate prior to reproduction.

Experiment to determine if plants exhibited adaptive responses to $p\text{CO}_2$

To evaluate whether plants exhibited genetic adaptation to $p\text{CO}_2$, a reciprocal transplant experiment was conducted in which plants derived from all selection populations were grown at both low and high $p\text{CO}_2$. We detected a significant interaction between selection $p\text{CO}_2$ and growth $p\text{CO}_2$ (Fig. 6; $P=0.0015$), indicating that selection history had an effect on responses to the growth $p\text{CO}_2$. However, individual comparison of means indicated that this significant interaction was mainly driven by the result that plants selected at 20 Pa CO₂ had sig-

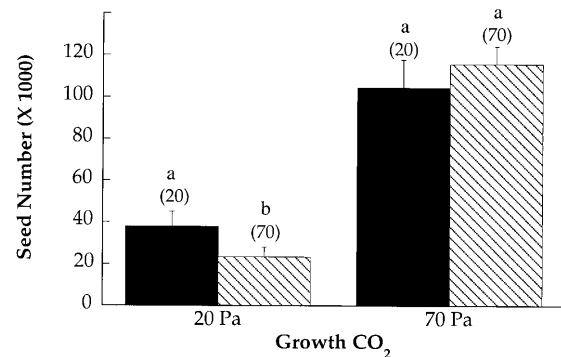


Fig. 6 Total seed number of plants selected for high seed number at 20 and 70 Pa CO₂ when grown at both CO₂ treatments following one generation of growth under common conditions. The values in parentheses indicate the selection $p\text{CO}_2$. Different letters within the same growth $p\text{CO}_2$ indicate significant differences at the $P<0.05$ level. Bars are means+SE ($n=28$)

nificantly greater seed production when grown at 20 Pa CO₂ compared to plants selected at 70 Pa CO₂ (Fig. 6). There was not a significant difference, however, between plants selected at 70 Pa CO₂ and those selected at 20 Pa CO₂ when both were grown at 70 Pa CO₂ (Fig. 6).

Discussion

These studies using *A. thaliana* as a model system indicate that the low $p\text{CO}_2$ that occurred during the Pleistocene could have been a strong selective agent on some C_3 annuals. At 20 Pa CO_2 , there were significant positive responses to selection for high seed number, a major component of fitness, among selection populations that were derived from nine original field-collected genotypes. Furthermore, plants from populations selected at 20 Pa CO_2 had higher seed production than those selected at 70 Pa CO_2 when both were grown at 20 Pa CO_2 . Because genetic adaptation to a specific environment may be defined as higher performance under the conditions of selection compared to other conditions (Ågren and Schemske 1993; Tousseignant and Potvin 1996), this result indicates that plants selected at 20 Pa CO_2 exhibited true adaptive responses to low $p\text{CO}_2$. Thus, the results of this research will begin to shed light on possible mechanisms by which C_3 annuals may have evolved in response to low $p\text{CO}_2$ during the Pleistocene.

This selection study indicates that plant growth may have been greater during the Pleistocene than has been predicted from single-generation experiments that do not consider the effects of evolution at low $p\text{CO}_2$ (Polley et al. 1993; Dippery et al. 1995; Tissue et al. 1995; Ward and Strain 1997; Cowling and Sage 1998). Selection for high seed number at 20 Pa CO_2 resulted in an average 35% increase in total mass among replicate selection populations relative to control populations at the fifth generation. The increased biomass was attributed to an extended period of productivity in which plants selected at 20 Pa CO_2 for high seed number flowered later and began senescing later than control plants, resulting in a longer period for accumulation of biomass. Furthermore, selection and control populations had similar leaf area and total biomass prior to the initiation of reproduction, indicating that the rate of carbon accumulation was similar at least during early stages of growth. However, it is also possible that selection and control plants may have varied in the rate of respiration and photosynthesis during later stages of development, which may have contributed to differences in final biomass between selection and control populations at senescence. In support of this idea, Delgado et al. (1994) found that populations of tobacco (*Nicotiana tabacum*; derived from haploid plants produced from mutagen-treated anthers) screened for survival near the CO_2 compensation point (6 Pa) had higher biomass production under current field conditions compared to their parent cultivar. The increased biomass among low- CO_2 -selected plants was attributed to decreased dark respiration and increased CO_2 assimilation in older leaves. In this study, the longer life cycle and greater biomass that accrued in *Arabidopsis* as a result of selection at 20 Pa CO_2 suggests how some C_3 annuals may have evolved during periods of low $p\text{CO}_2$, and begins to explain how annuals were able to accumulate enough resources for reproduction under severe carbon limitations. This mechanism may have been operating so

long as the growing season was of sufficient duration for maturation of fruits during the cooler glacial periods (Petit et al. 1999).

Plants selected at the predicted future $p\text{CO}_2$ also exhibited significant positive responses to selection for high seed number during the selection process. However, in the reciprocal transplant experiment, they did not have significantly higher seed production (only a trend) compared to plants selected at 20 Pa CO_2 when grown at 70 Pa CO_2 . This result may have been due to several possible factors. The first possibility is that plants selected at 70 Pa CO_2 did not undergo genetic adaptation to high $p\text{CO}_2$. However, this seems unlikely, because during selection for high seed number, changes in biomass production and development rate between selection populations and control populations often occurred in opposite directions between the 20- and 70-Pa CO_2 treatments, despite high replicate variation at 70 Pa CO_2 . This result indicates that genetic change during selection was primarily in response to the CO_2 treatment. On the other hand, it is more likely that plants selected at 20 Pa CO_2 may have acquired greater plasticity for responses to $p\text{CO}_2$ or may have become more efficient for uptake and use of CO_2 , resulting in similar seed production between plants selected at 20 and 70 Pa CO_2 when grown at 70 Pa CO_2 . Selection for high seed number at 20 Pa CO_2 appeared to concomitantly favor genes that induced later flowering which may have been associated with greater plasticity for responses to $p\text{CO}_2$. In support of this notion, Zhang and Lechowicz (1994) found that later onset of flowering (which is highly heritable) among genotypes of *A. thaliana* was highly correlated with increased fitness and greater phenotypic plasticity for responses to nutrient resources. There is also evidence from past studies indicating that evolutionary responses to low $p\text{CO}_2$ may result in more efficient assimilation and use of CO_2 . For example, Körner and Diemer (1994) found that alpine plants that evolved under conditions of low $p\text{CO}_2$ had higher carbon gain when transferred to elevated CO_2 above the current value compared with lowland plants of the same genera (mainly *Ranunculus* and *Geum*). Furthermore, Billings et al. (1961) showed that genotypes of *Oxyria digyna* originating from high altitudes and low $p\text{CO}_2$ had higher assimilation of CO_2 than genotypes from sea level when measured at both low and high CO_2 levels. It is also noteworthy that although $p\text{CO}_2$ affected the types of crosses represented at the fifth generation of selection in the present study, offspring from cross 7 were highly represented in populations selected at both 20 and 70 Pa CO_2 , possibly because this cross involved a high-altitude genotype which may have contributed to high performance at both low and high $p\text{CO}_2$ (Ward and Strain 1997).

This study provides evidence that evolutionary responses to high $p\text{CO}_2$ may not result in further increases in biomass accumulation among some C_3 annuals relative to results from single-generation studies. In contrast to responses at 20 Pa CO_2 , plants in selection populations at 70 Pa CO_2 exhibited either similar or lower final

biomass production relative to control populations. Lower final biomass in some selection populations was a result of a shorter life cycle and a shorter period for biomass accumulation, as indicated by more rapid initiation of reproduction and earlier senescence in selection populations relative to control populations. Furthermore, plants from selection populations had similar leaf area and biomass before the initiation of reproduction, indicating that the rate of carbon accumulation was similar early in the life cycle. Thus, modifications in development rate associated with evolution at high $p\text{CO}_2$ may constrain the potential for increased biomass production following long-term exposure to high $p\text{CO}_2$. In support of these results with the *Arabidopsis* model system, Cook et al. (1998) investigated the long-term effects of high $p\text{CO}_2$ on perennial *Nardus stricta* growing near an elevated- CO_2 spring in Iceland, which may have been emitting high $p\text{CO}_2$ (similar to predicted future levels) for up to 2500 years. Similar to our results with *Arabidopsis*, the authors discovered that plants near the spring senesced earlier and had lower above-ground growth than control plants far from the spring. However, reduced growth in *N. stricta* was in part attributed to lower photosynthetic capacity and reduced Rubisco content and activity in plants near the vent relative to plants farther from it. These responses to high $p\text{CO}_2$ may have been a result of genetic adaptation to very long term exposure to high $p\text{CO}_2$, and may provide some of the first insights into the mechanisms by which perennials may evolve in response to high $p\text{CO}_2$ in the future.

One obvious trend among the data presented in this selection experiment is that control populations tended to show increased seed number from early to later generations. There are four possible explanations for this trend. First, selection plants may have outcrossed with control plants. This possibility is unlikely, however, because past *Arabidopsis* studies (Kranz and Kirchheim 1987) indicate that outcrossing is very uncommon in this species. Second, the growth chambers may have provided improved growth conditions over time. However, our measurements of the growth chamber environment indicate that there were no improvements in the growth conditions throughout the selection process. Third, accumulation of maternal effects related to the CO_2 treatments may have increased seed quality and resulted in more vigorous seedlings and adults. This is also unlikely, particularly because past studies have indicated that high CO_2 induces negative maternal effects that reduce early seedling growth in *Arabidopsis* (Andalo et al. 1998). Finally, inadvertent selection may have occurred if there was mortality prior to the thinning procedure that was conducted 6 days after emergence. Such early mortality would have eliminated poor performers from the pool from which the individual nearest the center of the pot was chosen. Although mortality within the first 6 days after emergence was not measured in this experiment, our unpublished studies (J.K. Ward and B.R. Strain) have shown that mortality can be high at this stage of development, particularly at low CO_2 . Mortality after the

thinning process was relatively low and averaged 0% of control populations at 70 Pa CO_2 and 9% of control populations at 20 Pa CO_2 per generation. Therefore, we suggest that inadvertent selection resulting from mortality before thinning is the most likely reason for increased seed number with generation in control populations. Furthermore, it should be noted that without this possible inadvertent selection on control populations, the difference between selection and control populations would have been greater, suggesting an even stronger response to selection than is demonstrated in this study.

The present study provides some of the first evidence from artificial selection that $p\text{CO}_2$ alone may act as a selective agent on C_3 annuals. It will be important in future research to determine if other aspects of global change will also interact with $p\text{CO}_2$ to affect the evolution of C_3 annuals. Previous selection experiments have already been conducted to evaluate the effects of both high $p\text{CO}_2$ and high temperature on selection responses of C_3 species (very little is known about evolutionary responses to low $p\text{CO}_2$). Although these experiments were not designed to determine the independent effects of high $p\text{CO}_2$, they provide valuable insights into the interactive effects of two aspects of global change on the evolution of C_3 species. For example, Maxon Smith (1977) did not find a positive response to selection for increased yield of lettuce (*Lactuca sativa*) grown at very high $p\text{CO}_2$ (3×current level) and high temperature (minimum 18.3°C) for eight generations. Furthermore, Tousignant and Potvin (1996; see also Potvin and Tousignant 1996) selected *Brassica juncea* for high siliqua mass over seven generations. The two selection treatments included (1) gradual increases in $p\text{CO}_2$ and temperature above the current level coupled with heat shocks to simulate future conditions and (2) the current atmospheric $p\text{CO}_2$ with constant temperature as a control. Despite selection for increased siliqua biomass, the biomass of siliques decreased in both treatments during the selection process, and decreased to a greater extent in the future environment than in the control environment. The authors concluded that *B. juncea* did not show genetic adaptation to the treatment simulating future conditions, and they attributed the lack of a positive response to selection to inbreeding depression that may have been induced by the stressful future environment (Potvin and Tousignant 1996). Taken together, these studies suggest that high temperature may reduce the potential for evolution at high $p\text{CO}_2$. Furthermore, other studies have indicated that high $p\text{CO}_2$ may indirectly produce novel selection regimes that may result in shifts in the genetic composition of C_3 plants. For example, Bazzaz et al. (1995) demonstrated that high $p\text{CO}_2$ may intensify intraspecific competition and result in selection for genotypes with greater competitive ability for resources unrelated to CO_2 .

In summary, low $p\text{CO}_2$ was found to be a strong selective agent and high $p\text{CO}_2$ was a possible, but less pronounced, selective agent on the *Arabidopsis* model system. Selection at low $p\text{CO}_2$ produced plants with higher biomass production compared to control plants

due to an extension in the length of the life cycle. At high $p\text{CO}_2$, selected plants exhibited no change in biomass production or produced lower biomass relative to control plants that was related to a reduction in the length of the life cycle. From the *Arabidopsis* model system comes the conclusion that some C_3 annuals grown at the low $p\text{CO}_2$ of the Pleistocene may accumulate greater biomass throughout the life cycle than has been predicted from single-generation studies that do not consider the effects of evolution. Furthermore, C_3 annuals may exhibit evolutionary responses to the future predicted high $p\text{CO}_2$, but may not accumulate greater biomass production following evolution, as has been suggested in previous single-generation studies. Moreover, this research points out the need for more evolutionary work at both low and high $p\text{CO}_2$ in order to better predict the responses of plants to global change over geologic and contemporary time scales.

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