

CO₂ and nitrogen, but not population density, alter the size and C/N ratio of *Phytolacca americana* seeds

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Summary

1. Plants can provision seeds by optimizing seed size, number and nutrient content to maximize parental fitness. According to the McGinley–Charnov hypothesis, seed size should be determined by the ratio of carbon to nitrogen (C/N) available to the plant, with larger seed size correlating with larger C/N ratios and smaller absolute N content.
2. This hypothesis was tested by establishing monocultures of *Phytolacca americana* L. (Phytolaccaceae) at three population densities under ambient and elevated CO₂ environments, with two availabilities of soil N.
3. Elevated CO₂ reduced both seed size and N concentration while increasing the C/N ratio; high soil N availability produced the opposite result for N concentration and C/N ratio. Higher planting densities reduced plant biomass, but did not alter seed size.
4. In accordance with the McGinley–Charnov hypothesis, larger seeds had both larger C/N ratios and smaller N content. However, the increase in C/N ratio caused by elevated CO₂ corresponded with smaller seeds overall: elevated CO₂ reduced seed size, although the seed size–C/N relationship remained positive.
5. These results suggest an alternative mechanism to explain variation in seed size, and suggest that future climate conditions may alter seed quality and plant reproductive behaviour.

Key-words: density, elevated CO₂, McGinley–Charnov hypothesis, resource allocation, seed size

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Introduction

Variation in seed size is widespread in communities, populations, and even within individuals (Michaels *et al.* 1988). However, mean seed weight can be remarkably stable across populations for certain species, despite significant environmental heterogeneity which typically leads to variation in vegetative characters, and thus might reasonably be expected to create similar variation in reproductive characters (Harper *et al.* 1970). The absence of variation is perplexing, given that larger seeds tend to promote greater fitness (Black 1956; Dawson & Ehleringer 1991; Leishman & Westoby 1994; Mojonier & Raushner 1997) and produce more competitive plants in some species (Turnbull *et al.* 2004). Stabilizing selection for seeds of intermediate size is predicted if greater seed mass increases fitness and maternal plants make trade-offs between the size and number of seeds they produce (Smith & Fretwell 1974), although empirical evidence for such a trade-off is limited (Wolf *et al.* 1986;

Winn 1988; Ågren 1989). Other possible explanations for the predominance of intermediate seed sizes include selective forces acting independently on initial seed size (the size of seeds from which plants germinate) and maternal seed size (the size of seeds produced by plants, Mojonier 1998), or seed-caching and consumption by granivores (Vander Wall 2003).

Larger plants can also produce larger seeds if allocation to a large number of reproductive structures presents a rising cost as plants increase in size, constraining larger plants to invest relatively more resources in fewer seeds (Sakai & Sakai 1995). Such correlations have been found in *Blepharis indica*, a lignified desert annual in which reproduction represents a substantial resource investment (Narita 2003), and for outcrossing flowers of a summer annual, *Impatiens noli-tangere*, and a perennial, *Viola grypoceras* (Sakai & Sakai 1996).

Alternatively, seed size could be controlled by the balance of resources available to a plant, rather than by the balance between size and number. McGinley & Charnov (1988) proposed a model to evaluate optimal seed size with two resource pools, specifically carbon and nitrogen. This model suggests that optimal seed

size should correlate positively with the ratio of C to N pools available to a plant, which may be reflected by seed C/N ratio, and negatively with the absolute N content per seed. The model is based on the assumption that seed fitness is increased by additional investment in either of the two resources (C or N), but in a hyperbolic fashion. As C-based compounds compose the bulk of seed contents (Bewley & Black 1994), C content is tightly tied to seed mass. While the McGinley–Charnov model has been cited in studies of resource allocation to animal offspring (e.g. Trexler 1997), and has been acknowledged as one of several alternative explanations for seed size variation in contrast to the Smith–Fretwell one-resource model (Geritz *et al.* 1999), we are aware of no studies that have directly tested it by investigating C and N allocation in seeds.

Manipulations of atmospheric CO₂ concentration provide a way to examine the effects of increased C assimilation on plant reproduction, as well as to simulate potential future climate conditions. Doubling CO₂ increases leaf C/N ratio via reducing N content by a mean of 16% in C₃ plants (Cotrufo *et al.* 1998). In a meta-analysis of 79 species, Jablonski *et al.* (2002) found that doubling CO₂ generally increased seed C/N ratios and increased individual, but not total, seed mass in wild C₃ species. Furthermore, wild species exhibit small increases in seed weight in response to CO₂ enhancement (Garbutt & Bazzaz 1984; Ackerly & Bazzaz 1995; Farnsworth & Bazzaz 1995) relative to the large increases in seed weight of domesticated crops bred for maximum reproductive effort (Amthor 2001), although such crops have inconsistent responses to elevated CO₂ (Bai *et al.* 2003). Lending credence to the idiosyncratic responses of plants to CO₂, Leishman *et al.* (1999) found that elevated CO₂ affected the seeds of weedy C₃ plants by increasing C/N and altering seed weight not at all, positively, or negatively, and that the percentage germination of seeds produced under modified CO₂ did not change. Likewise, in a grassland community, grasses and forbs, but not legumes, increased their seed numbers in elevated CO₂, and across the whole community seeds were heavier with smaller N content (Thürig *et al.* 2003).

More available soil N can induce non-leguminous plants to produce seeds which are larger and contain more total N (Tungate *et al.* 2002). As predicted, variation in seed size was tightly correlated with N content in natural populations of *Purshia tridentata*, with larger seeds having increased N concentrations and containing more total N (Krannitz 1997). However, larger N pools can increase seed N concentration and content without increasing seed weight (Kinugasa *et al.* 2003).

Harper *et al.* (1970) noted that seed size can be remarkably consistent within species, even across many-fold differences in planting density which directly affect whole plant size. If plants balance seed size and seed number, it is conceivable that smaller plants resulting from high density sacrifice seed size to maintain a seed number optimal for parental fitness. Harper *et al.* (1970) proposed that such constancy is most pronounced in

pure stands of a species, and that interspecific competition at varying densities allows for greater variation in seed size due to varied resources at the times of seed number determination and seed-filling. According to the McGinley–Charnov hypothesis, however, higher planting density of a single species should modify the relative C and N pools, reducing the seed C/N ratio and decreasing seed size. This was supported by the finding that *Chamaesyce hirta* grown at high density produced smaller seeds containing more protein (Snell 1976). In contrast, in experimental monocultures of *Avena sativa*, higher planting density led to the production of fewer, heavier seeds with increased N content (Peterson & Rendig 2003).

Here we present results from an investigation into seed properties of *Phytolacca americana* L. (Phytolaccaceae), a common C₃ perennial, grown in varying conditions of atmospheric CO₂, soil N, and density. The experimental treatments were intended to manipulate C and N pools available to the plant, to test the McGinley–Charnov hypothesis directly.

Materials and methods

STUDY ORGANISM

Phytolacca americana, commonly known as pokeweed, is a polycarpic perennial herb common to much of the eastern USA, ranging from Quebec and Ontario south to north-eastern Mexico (Caulkins & Wyatt 1990). It is often abundant in open, disturbed habitats, particularly in forest edges and canopy gaps (Sauer 1952; Caulkins & Wyatt 1990; Wilson & Shure 1993). *Phytolacca americana* was selected for this experiment because it is a typical edge species, is widespread, and produces copious seeds. This experiment was conducted on a single species due to limitations in facility space. Clearly, species may exhibit unique responses when grown under similar experimental conditions, and thus a broader range of species would help avoid the pitfalls of generalizing from species-specific responses. However, we believe *P. americana*, as a typical C₃ ruderal, can represent the responses of a substantial number of terrestrial plants. Furthermore, at a time when forest fragmentation is increasing, the responses of this edge species are relevant to investigations of community dynamics.

In an investigation at Harvard University's Concord Field Station, MA, USA, we found that first-year plants have a mean height of 98 cm, with a mean total dry biomass of 28 g and a rooting depth of 15–25 cm, depending on soil conditions (J.-S.H., personal observation). The plant is a predominantly autogamous species (Armesto *et al.* 1983). Fruits are fleshy and bird-dispersed (Thompson & Willson 1978; McDonnell *et al.* 1984). Nitrogen content is a critical determinant of bird frugivory, exhibiting complex patterns at large geographical scales (Hampe 2003), and determining the quantity of seeds consumed by certain species (Witmer 1998).

EXPERIMENTAL DESIGN AND GROWTH
CONDITIONS

Mature seeds of *P. americana* were collected from several individuals of a population in Lexington, MA, USA in October 1999 and stored dry at 4 °C for 6 months. On 29 June 2000, seeds were sown into 53 × 40 × 20 cm plastic tubs (Consolidated Plastics Company, Inc., Twinsburg, OH, USA) filled with Pro-Mix general-purpose growing medium (Premier Horticultural Co., Red Hill, PA, USA). The background N availability in this soil was 70–150 mg l⁻¹ NO₃-N (equivalent to 100–222 kg N ha⁻¹). When seeds were sown, 9 g Osmocote controlled-release fertilizer (N:P:K = 14:14:14%; Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) were applied to each tub. The fertilizer was assumed to release evenly over the 4 month experimental period. Six holes were drilled into the bottom of each tub to provide drainage.

Three planting densities, two nutrient levels, and two CO₂ concentrations (3 density × 2 nutrient × 2 CO₂) were applied in a complete factorial design. Tubs were randomly assigned to one of the 12 treatment combinations. Each treatment had six replicates (*n* = 72). Two CO₂ concentrations (370 or 700 μmol CO₂ mol⁻¹) simulated atmospheric CO₂ concentrations of the present and projected future conditions within the next century. For the density treatments, 20, 100 and 500 seeds were sown per tub for the low, medium and high density, and produced a mean of 8, 23 and 108 plants per tub at harvest (realized density = 44, 126 and 592 plants m⁻²). These densities were typical of seedlings of *P. americana* growing in open habitats and forest edges (J.-S.H., personal observation). No thinning was carried out during the experiment. Nutrient conditions were established by supplementing half of the tubs with an additional 25 g Osmocote at planting. Thus the high-nutrient treatment received 36 g fertilizer per tub (equivalent to 276 kg N ha⁻¹), while the low-nutrient treatment received 9 g (69 kg N ha⁻¹). These nutrient treatments were based on those used for a nutrient-saturation experiment in a hardwood forest (Magill *et al.* 2004), but were increased to account for the larger nutrient demands of *P. americana*.

Tubs were randomly assigned to six separately controlled chambers of an environmentally controlled glasshouse at Harvard University (Cambridge, MA, USA). CO₂ concentration was maintained at 370 ± 50 μmol CO₂ mol⁻¹ in three chambers, and at 700 ± 50 μmol CO₂ mol⁻¹ in the other chambers. The temperature in all chambers was 25 °C from 08:00 to 20:00 h and 19 °C overnight. Lighting was provided by natural sunlight filtered through the roof of the glasshouse, which reduced irradiance by ≈ 28%.

Tubs were arranged on one bench to form a population in each chamber, forming a rectangle of 3 × 4 tubs. For the first 4 weeks, positions of the tubs within each chamber were re-randomized once a week to reduce potential variation in growing conditions. In the fifth

week, when the canopy closed, a shade cloth wall was established around each population to eliminate edge effects. The height of the shade cloth wall was set level with the top of the canopy and adjusted as the canopy height increased. Plants were watered daily throughout the experiment.

Other results from this study have been reported previously He & Bazzaz (2003); the methods of the two studies are identical, with the exception that results for the two nutrient treatments were combined in that study for ease of analysis.

HARVESTING AND CHEMICAL ANALYSIS

Plants began to flower on 28 August 2000, beginning with plants from the elevated CO₂ chambers. Plants reached the mature seed stage by 15–22 November, and all 72 tubs were then harvested. Roots were removed from the soil and hand-washed to remove soil particles, and fruits were collected from each plant. All biomass samples were dried to a constant weight at 65 °C and weighed (Acculab Lt-320, Danvers, MA, USA). The total number of seeds produced per plant was determined by multiplying the mean number of seeds produced per 1 g random fruits on that plant by the dry mass of fruits on that plant. Mean seed mass was determined by dividing seed weight, measured on an electronic semi-analytical balance (Sartorius AG, Goettingen, Germany) by the seed number in 1 g of a random sample of fruits on each plant. Seed coat and endosperm were not distinguished in measurements of seed mass or C/N.

Dried seeds from each plant were ground *en masse* by mortar and pestle. Total C and N contents were determined on 5–6 mg of the homogeneous ground material of each sample using an elemental analyzer (CHNS/O Elemental Analyzer, Perkin-Elmer, Boston, MA, USA) at Peking University, Beijing, China.

DATA ANALYSIS

Seed mass was used as a measure of seed size. Differences between mean seed size, total N concentration (percentage dry mass), and C/N mass ratio among CO₂, N and density treatments were tested using a mixed general linear model (GLM) procedure, employing type III sums of squares, in the SAS statistical software package ver. 8.01 (SAS Institute 1999, Cary, NC, USA). In the split-plot analysis, CO₂ treatments were analyzed as the main effect, with density and N as subplot treatments (Potvin 2001). Additionally, CO₂ was analyzed as a fixed effect. Individual plant biomass was natural log-transformed to meet assumptions of normality. The influence of density on mean individual plant biomass and seed size, as well as the correlations between seed C/N ratio and seed N content with seed size, were all tested with the GLM procedure as above. Significant results were explored using Scheffé *post hoc* tests.

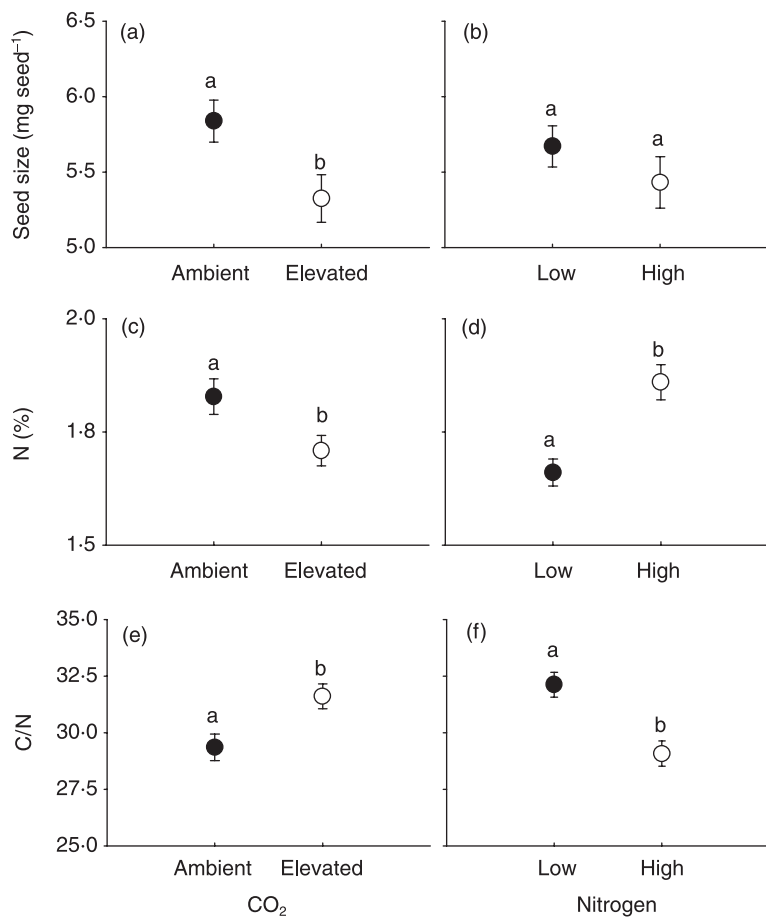


Fig. 1. Effects of elevated atmospheric CO₂ and nitrogen treatments on *Phytolacca americana* seed size (a,b); seed N concentration (c,d); and seed C/N ratio (e,f) (mean ± 1 SE, *n* = 18). Letters represent results of Scheffé *post hoc* analyses. Closed circles = ambient CO₂; open circles = elevated CO₂.

Results

SEED SIZE

Seed size varied as C and N pools were modified. A doubling of atmospheric CO₂ reduced seed size by 8.8% (Fig. 1a), while increased soil N availability had no detectable effect on seed size (Table 1; Fig. 1b). Across the three planting densities, mean individual biomass was reduced by a factor of 10 at high density (Fig. 2a).

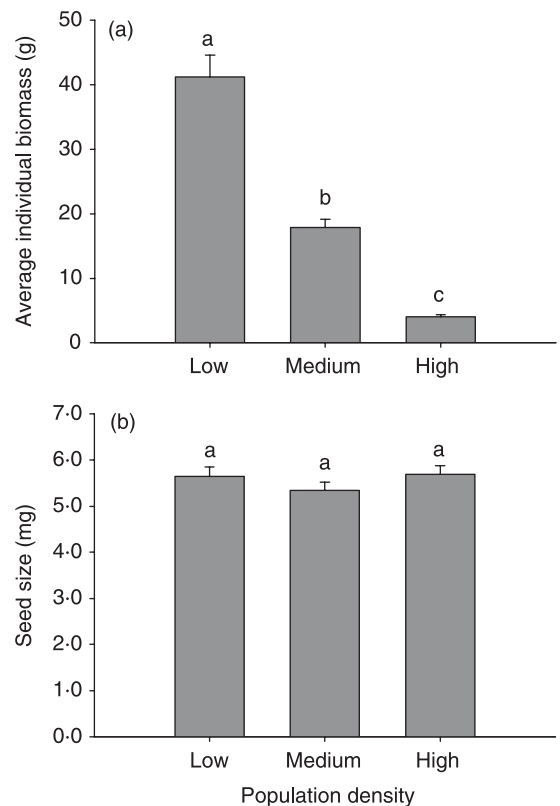


Fig. 2. Increasing density of artificial communities of *Phytolacca americana* decreased mean individual biomass (a) but did not influence seed size (b) over three planting densities (44, 126 and 592 plants m⁻² at harvest) (mean ± 1 SE, *n* = 24).

However, seed size was unaffected by density (Fig. 2b). There were no interactive effects between any of the growth conditions on seed size.

SEED QUALITY

The N and C contents of *P. americana* seeds were strongly influenced by environmental conditions, but not density (Table 1). Elevated CO₂ reduced N content in seeds by 6.5%, while raising the C/N ratio by 7.7% (Fig. 1c,e). Conversely, addition of soil N increased seed N concentration by 12.0% while decreasing C/N ratio by 9.5% (Fig. 1d,f).

Table 1. Summary of split-plot ANOVA results for effects of CO₂ and nitrogen treatments on *Phytolacca americana* seed size, nitrogen content and C/N ratio

Treatment	Seed size			N			C/N		
	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
CO ₂	1	9.84	0.033	1	14.10	0.018	1	15.75	0.016
Main plot error	4	–	–	4	–	–	4	–	–
Chamber	4	0.59	0.668	4	0.042	0.791	4	0.55	0.697
N	1	0.96	0.330	1	16.29	<0.001	1	14.23	<0.001
Density	2	1.24	0.294	2	1.49	0.231	2	1.09	0.341
CO ₂ × N	1	0.14	0.711	1	0.03	0.858	1	0.86	0.335
Subplot error	107	–	–	109	–	–	106	–	–

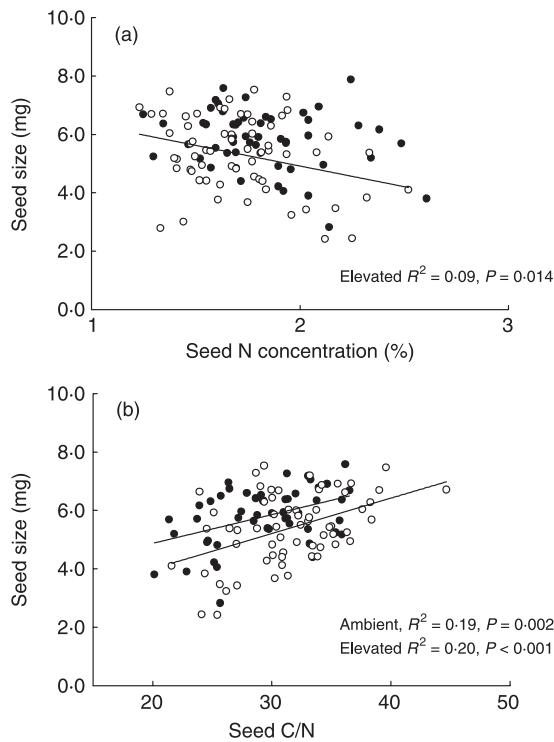


Fig. 3. Nitrogen concentration in *Phytolacca americana* seeds, with variation caused by modifications of atmospheric CO₂, soil N, and planting density, negatively correlates with seed size at elevated, but not ambient, CO₂ concentrations (a). Conversely, seed C/N ratio positively correlates with seed size (b) ($n = 36$). Closed circles, bold line = ambient CO₂; open circles, thin line = elevated CO₂.

CORRELATIVE RELATIONSHIPS

We found significant relationships between C and N content in seeds and seed size. As C/N ratio increased, mean seed size increased significantly (Fig. 3a; $R^2 = 0.19$, $P = 0.002$ for ambient CO₂; $R^2 = 0.20$, $P < 0.001$ for elevated CO₂). Likewise, as seed N content increased, mean seed size decreased, albeit significantly only under elevated CO₂ (Fig. 3b, $R^2 = 0.07$, $P = 0.067$ for ambient CO₂; $R^2 = 0.09$, $P = 0.014$ for elevated CO₂). Although the elevated CO₂ treatment reduced seed size and increased C/N ratio overall, the correlation between seed size and seed C/N remained positive (Fig. 3a).

Discussion

Altered atmospheric CO₂ concentration and soil N availability altered the size and concentrations of C and N in *P. americana* seeds. The results from this species provide only limited support for the McGinley–Charnov hypothesis that C/N ratio can contribute to the control of seed size, with size increasing as C/N ratio rises. However, we found that modifying the C pool available to the plant via elevated CO₂ reduced seed size, while preserving the positive relationship between seed size and C/N ratio.

MODIFIED RESOURCE POOLS

Seed C/N varied positively with seed size under both altered atmospheric CO₂ and soil N (Table 1). These treatments were designed to modify the relative C and N pools available to plants to test the McGinley–Charnov model. Constant or decreasing seed size has been observed in some undomesticated non-leguminous plants, including *Abutilon theophrasti*, *Datura stramonium*, and *Aegilops peregrina*, under elevated atmospheric CO₂ (Garbutt & Bazzaz 1984; Grünzweig & Körner 2001). However, total seed weight and reproductive allocation typically increase under elevated CO₂ (Jablonski *et al.* 2002). Furthermore, increased atmospheric CO₂ concentrations increase C/N ratios in the leaves of most C₃ plants (Curtis & Wang 1998). It is thus reasonable to expect that the enhanced supply of C via increased atmospheric CO₂ concentrations would lead to larger seeds with the larger C/N ratios typical of wild C₃ species. In this experiment we were surprised to find that *P. americana* plants grown under elevated CO₂ produced smaller seeds with greater C/N. While the mechanisms underlying this change are unclear, modifying soil N availabilities independently of atmospheric CO₂ concentration allowed further analysis of the relationship between C/N and seed size.

According to the McGinley–Charnov model, seed sizes optimal for parental fitness should correlate positively with the ratio of the C and N pools available to offspring for investment, and seed size and absolute seed N content should correlate negatively. While such relationships may intuitively appear to be autocorrelated, as C typically forms the bulk of a seed's mass, concurrent changes in N concentration could conceivably negate or reverse such trends. Thus a test of the McGinley–Charnov hypothesis would require direct measurements of seed size, C and N. In this study, when all seeds from both CO₂ treatments and N treatments were pooled, we found evidence to support this model, in that seed size increased with increasing C/N, and decreased with N concentration. However, although the overall relationship between seed size and C/N remained positive, elevated CO₂ shifted the slope of the relationship by reducing seed size and raising seed C/N. Thus seed size was reduced under conditions of elevated CO₂, but by varying soil N we observed that the C/N–seed size relationship remained positive and linear.

While the shifts we observed in C/N ratio can be explained by the altered availability of C and N via elevated atmospheric CO₂ and soil N, the positive relationship between seed C/N and seed size, as well as the reduction in seed size under elevated CO₂, require further analysis. The positive relationship between seed size and seed C/N, along with the negative relationship between N content and seed size, suggests that decreasing the relative amount of N available to plants did not restrict increases in seed size; there appeared to be no restraining condition requiring a minimum amount of N to be invested in a seed per unit C. Conversely, greater

C availability via elevated CO₂ increased C/N ratios, but overall seeds were smaller, contrasting with some of the predictions of the McGinley–Charnov model. CO₂ elevation may modify the mechanisms determining seed size and C/N ratio, but did not alter the positive relationship between the two characters.

The quality of seeds produced under elevated CO₂ conditions has been evaluated, but few studies have examined interactive effects of both N and CO₂ enrichment on seed quality. Smaller seeds produced from *Bromus madritensis* in elevated CO₂ during parental growth produced seedlings with smaller leaves, which were delayed in development and had smaller roots (Huxman *et al.* 1999). However, Steinger *et al.* (2000) found that although elevated CO₂ increased seed mass, the CO₂ conditions during parental growth did not significantly affect seedling size. Steinger *et al.* (2000) argued that the advantage of increased seed mass at elevated CO₂ could have been offset by the reduced N concentration. Although we did not directly test seed quality in *P. americana*, the reduced seed size due to both elevated CO₂ and elevated soil N, and high C/N values under elevated CO₂, suggest that such environmental conditions can limit the growth of the resulting plants. We speculate that the high C/N, coupled with smaller seed mass in our experiment, could reduce germination rates and produce smaller offspring. However, relationships between seed mass and germination rates are difficult to predict (Milberg *et al.* 1996) and this speculation remains to be tested empirically.

A wide variety of seed-size responses to elevated CO₂ have been reported in earlier studies. For example, our analysis of this single species contrasts in part with the general findings of Jablonski *et al.* (2002), who found that wild, non-legume C₃ species such as *P. americana* generally respond to increased CO₂ with limited increases in seed size. A similar result was found in perennial C₃ and C₄ grasses, which exhibited no changes in seed-head production after being exposed to two seasons of CO₂ enrichment (Hunt *et al.* 1996). In contrast, seed production of the perennial *Plantago lanceolata* was reduced when it was grown in elevated CO₂ on soils that had developed in naturally high atmospheric CO₂ concentrations (Edwards *et al.* 2003), as well as when grown in a glasshouse (Fajer *et al.* 1991). However, seed number increased significantly and seed weight tended to increase in two other perennial grassland species, *Anthoxanthum odoratum* and *Trifolium repens*, grown in free-air CO₂ enrichment sites (Edwards *et al.* 2001).

Perennial species exhibit a wide variety of seed-size responses to elevated CO₂, while seed C/N ratios have not been measured with sufficient frequency to corroborate our findings of higher C/N along with smaller seeds. Additionally, comparisons between the above-mentioned studies may be confounded by differences in N availability across the experiments, reducing our ability to separate CO₂ effects from N effects when interpreting the results.

MODIFIED PLANTING DENSITY

Increased planting density in our experiment did not modify either seed size or C/N, despite dramatically reducing plant size. This discrepancy suggests mechanistic differences by which plant size and seed size are determined. Density is an important determinant of plant growth and reproduction (Harper 1977), potentially reducing individual plant biomass in densely populated habitats due to competition for resources (Grace & Tilman 1990). Several plant reproductive characters, such as allocation to seeds, decrease as plant density increases (Harper 1977; Bazzaz *et al.* 1992; Casper & Cahill 1998). In *P. americana*, He & Bazzaz (2003) observed that the interaction between CO₂ and density on reproductive allocation was not significant at the population level; however, at the individual level the effects of elevated CO₂ on reproductive allocation were density-dependent. In that experiment, elevated CO₂ decreased the reproductive mass per unit vegetative mass (reproductive allocation) at low density, but increased it at high density. Additionally CO₂ increased seed number while decreasing seed size, but those differences vanished at high density.

Other studies have found that planting density directly modifies the chemical composition of seeds. High density reduced seed size and increased seed N content in *C. hirta* (Snell 1976), while density treatments increased both seed size and absolute N content in *A. sativa* (Peterson & Rendig 2003). Here we found that, while the mean individual plant biomass decreased by a factor of 10 over three density treatments, seed size and C/N ratio remained virtually unchanged. This consistency in seed size across a gradient of increasing density, even while seed number, total reproductive allocation and total biomass decreased (He & Bazzaz 2003), suggests that *P. americana* can maintain constant seed biomass across a wide range of intraspecific competition intensities. Additionally, as population density did not alter seed C or N concentrations, either relatively or absolutely, this suggests either that (even in high-density stands of *P. americana*) soil N availability remained sufficient for seed provisioning, or that in high-density conditions *P. americana* individuals sacrificed vegetative biomass to maintain both constant seed size and constant C/N ratio.

Conclusions

In conclusion, our results provide some evidence that future changes in atmospheric CO₂ composition could influence seed size, potentially affecting seed quality and competitiveness, as well as altering fruit characteristics critical for dispersal by frugivores. However, these results come from analysis of only a single species, examined under highly favorable glasshouse conditions. Care must be taken in making inferences from these data. Further examination of the role that resource pool dynamics play in regulating seed size is warranted

and could reveal species-specific or functional group-specific variation in these patterns.

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