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## Legume presence increases photosynthesis and N concentrations of co-occurring non-fixers but does not modulate their responsiveness to carbon dioxide enrichment

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**Abstract** Legumes, with the ability to fix atmospheric nitrogen (N), may help alleviate the N limitations thought to constrain plant community response to elevated concentrations of atmospheric carbon dioxide (CO<sub>2</sub>). To address this issue we assessed: (1) the effects of the presence of the perennial grassland N<sub>2</sub> fixer, *Lupinus perennis*, on biomass accumulation and plant N concentrations of nine-species plots of differing plant composition; (2) leaf-level physiology of co-occurring non-fixing species (*Achillea millefolium*, *Agropyron repens*, *Koeleria cristata*) in these assemblages with and without *Lupinus*; (3) the effects of elevated CO<sub>2</sub> on *Lupinus* growth and symbiotic N<sub>2</sub> fixation in both monoculture and the nine-species assemblages; and (4) whether assemblages containing *Lupinus* exhibit larger physiological and growth responses to elevated CO<sub>2</sub> than those without. This study was part of a long-term grassland field experiment (BioCON) that controls atmospheric CO<sub>2</sub> at current ambient and elevated (560 μmol mol<sup>-1</sup>) concentrations using free-air CO<sub>2</sub> enrichment. Nine-species plots with *Lupinus* had 32% higher whole plot plant N concentrations and 26% higher total plant N pools than those without *Lupinus*, based on both above and belowground measurements. Co-occurring non-fixer leaf N concentrations increased 22% and mass-based net photosynthetic rates increased 41% in plots containing *Lupinus* compared to those without. With CO<sub>2</sub> enrich-

ment, *Lupinus* monocultures accumulated 32% more biomass and increased the proportion of N derived from fixation from 44% to 57%. In nine-species assemblages, *Lupinus* N derived from fixation increased similarly from 43% to 54%. Although *Lupinus* presence enhanced photosynthetic rates and leaf N concentrations of co-occurring non-fixers, and increased overall plant N pools, *Lupinus* presence did not facilitate stronger photosynthetic responses of non-fixing species or larger growth responses of overall plant communities to elevated CO<sub>2</sub>. Non-fixer leaf N concentrations declined similarly in response to elevated CO<sub>2</sub> with and without *Lupinus* present and the relationship between net photosynthesis and leaf N was not affected by *Lupinus* presence. Regardless of the presence or absence of *Lupinus*, CO<sub>2</sub> enrichment resulted in reduced leaf N concentrations and rates of net photosynthesis.

**Keywords** N fixation · Elevated CO<sub>2</sub> · Legume · Photosynthetic acclimation · Species interactions

### Introduction

Much of our knowledge concerning plant responses to elevated CO<sub>2</sub> relies on studies of individually grown plants; however, plant responses may differ when interacting with other species in a community (Bazzaz and McConnaughay 1992). Interspecific interactions may modify species responses to elevated CO<sub>2</sub> when grown in mixtures, just as differing species responses to elevated CO<sub>2</sub> may modify interspecific interactions (Bazzaz and McConnaughay 1992; Körner et al. 1996). Thus to predict how plant communities will respond under elevated CO<sub>2</sub>, we need to understand the ways in which the differential CO<sub>2</sub> responses of component species interact with each other and with the environment.

Plant responses to elevated CO<sub>2</sub> fundamentally involve photosynthesis (Drake et al. 1997) and can potentially lead to a suite of morphological and growth changes. Because tissue N is a major determinant of photosynthesis

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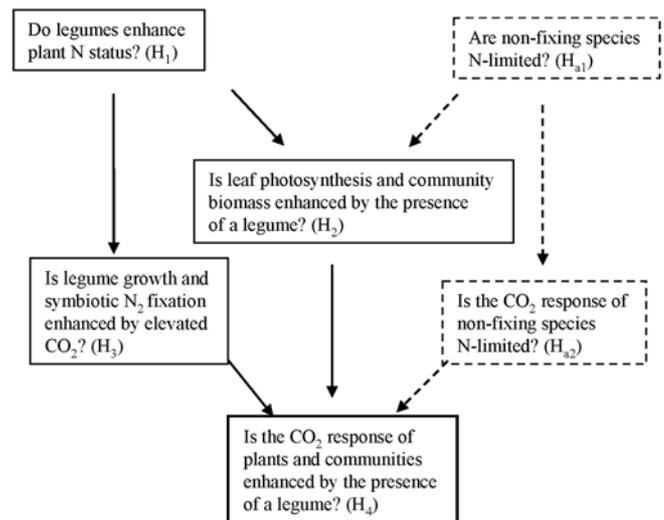
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(Reich et al. 1997), increased N inputs from stimulated  $N_2$  fixation may facilitate photosynthetic enhancements of non-fixing species under elevated  $CO_2$  in addition to providing more N for  $CO_2$ -induced increases in growth. The dependence of photosynthesis on nitrogenous compounds results in a general positive relationship between light-saturated photosynthetic rate ( $A$ ) and leaf N concentration (Reich et al. 1997; Peterson et al. 1999). Growth and physiological responses to  $CO_2$  enrichment or varied N availability have the potential to alter the  $A$ -N relationship relative to at ambient conditions, which would modulate a plant's ability to respond to the elevated  $CO_2$  concentrations and have important implications for predictive models of photosynthesis (Peterson et al. 1999).

A decreased sensitivity of plant growth to elevated  $CO_2$  has often but not always been linked to low nutrient availability (Díaz et al. 1993; Leadley and Körner 1996; Curtis and Wang 1998; Stöcklin and Körner 1999; Poorter and Pérez-Soba 2001; but see Lloyd and Farquhar 1996; Körner et al. 1997; Reich et al. 2001a, 2001b). Species differences in the ability to compete for potentially limiting resources such as N can have large effects on their responsiveness to elevated  $CO_2$  (Poorter 1993); therefore, legumes utilizing atmospheric  $N_2$  may be at a competitive advantage under elevated  $CO_2$  (Zanetti et al. 1996). Because symbiotic  $N_2$  fixation is an important source of N in many ecosystems, it may be especially important in nutrient poor grassland systems exposed to elevated  $CO_2$  (Soussana and Hartwig 1996; Zanetti et al. 1997, 1998). Furthermore, if legumes facilitate stronger and sustained  $CO_2$ -induced growth enhancements it would have implications on net carbon gain by plants and ecosystems as well as on the balance between  $CO_2$  fluxes to terrestrial and atmospheric sinks (Gifford 1992).

Niche complementarity or positive species interactions may contribute to greater productivity (Hector et al. 1999; Loreau and Hector 2001; Sala 2001) or greater responsiveness to elevated  $CO_2$  (Reich et al. 2001a) in high-diversity compared to species-poor communities.  $N_2$ -fixing trees (e.g., Vitousek and Walker 1989) and herbaceous legumes (e.g., Halvorson et al. 1991) increase N inputs and improve N availability in N-limited systems. In fact, intercropping legumes with non-fixing crops and applying legume residues have been methods used extensively to increase forage yield and reduce the dependence on external fertilizer inputs in managed cropping systems (e.g., Posler et al. 1993). In addition, experiments investigating the effects of species and functional group composition and diversity on ecosystem functioning have found that the presence of  $N_2$  fixers increases productivity (Vitousek and Walker 1989; Soussana and Hartwig 1996; Tilman et al. 1997; Hector et al. 1999).

This study took place within a long-term grassland field study that experimentally controls plant diversity in plots exposed to current ambient and elevated ( $560 \mu\text{mol mol}^{-1}$ )  $CO_2$  concentrations using free-air  $CO_2$  enrichment (FACE) (Reich et al. 2001a, 2001b). Our objective herein



**Fig. 1** Conceptual diagram outlining components of the hypothesis that the presence of legumes, with the ability to symbiotically fix atmospheric  $N_2$ , will enhance N status and hence the response of species and the community to elevated concentrations of  $CO_2$ . Hypotheses  $H_{a1}$  and  $H_{a2}$  (dashed boxes) are discussed, but not directly tested in the present study

was to assess the effect of legume presence on perennial grassland assemblages in an N-poor system under ambient and elevated  $CO_2$  concentrations. Figure 1 conceptually diagrams questions posed to assess the hypothesis that the presence of legumes will enhance N status and hence the response of co-occurring species and the community to elevated concentrations of  $CO_2$ . This overall hypothesis is based on two suppositions: (1) legumes enhance plant N status ( $H_1$ ); and (2) non-fixing species are N-limited ( $H_{a1}$ ). In evaluating the overall hypothesis, the following specific hypotheses were addressed: ( $H_1$ ) legume presence increases co-occurring plant N concentrations; ( $H_2$ ) the presence of a legume enhances leaf-level photosynthetic responses of co-occurring non-fixing species and community biomass accumulation; ( $H_3$ ) legume growth and symbiotic  $N_2$  fixation are enhanced under elevated  $CO_2$ ; and ( $H_4$ ) the presence of legumes in multi-species assemblages enhances plot- and leaf-level responses to elevated  $CO_2$ . Furthermore, we evaluate the effect of  $CO_2$  enrichment and legume presence specifically on the relationship between light-saturated photosynthesis and leaf N concentration to explore possible mechanisms of interacting species responses to  $CO_2$  enrichment.

## Materials and methods

### Research site, experimental design and the FACE system

This study took place within a larger experiment, BioCON (Biodiversity,  $CO_2$ , and N, <http://swan.lter.umn.edu/biocon/>), that is exploring the response of prairie grassland species to biodiversity,  $CO_2$  enrichment and simulated N deposition (Reich et al. 2001a, 2001b). BioCON is located at the Cedar Creek Natural

**Table 1** Sampling scheme for plot- and plant-level measurements taken in nine-species assemblages growing at ambient (368  $\mu\text{mol mol}^{-1}$ ) and elevated (560  $\mu\text{mol mol}^{-1}$ )  $\text{CO}_2$  concentration and with or without *Lupinus* present

Measurements	<i>Lupinus</i> presence	$\text{CO}_2$ treatment ( $\mu\text{mol mol}^{-1}$ )		Responses measured	Date measured
		368	560		
Plot level	Present	5	4	Total plot biomass Whole plant %N Total plot N	June and August 1998 and 1999 August 1998 and 1999 August 1998 and 1999
	Absent	4	4		
Plant level					
<i>Achillea</i>	Present	3	3		
	Absent	3	3		
<i>Agropyron</i>	Present	3	3	Leaf net photosynthesis Leaf N concentration	July 1999
	Absent	3	3		
<i>Koeleria</i>	Present	3	3		
	Absent	3	3		

History Area in east central Minnesota, USA (Lat. 45°N, Long. 93°W). Plots were arranged in six circular areas (rings) of 20-m diameter. Three rings received elevated atmospheric  $\text{CO}_2$  (560  $\mu\text{mol mol}^{-1}$ ) and three received ambient concentrations of  $\text{CO}_2$  (368  $\mu\text{mol mol}^{-1}$ ) using FACE technology (Lewin et al. 1994) during all daylight hours from 9 April to 16 October 1998 and 20 April to 9 November 1999 (Reich et al. 2001a, 2001b); 2x2 m plots were individually seeded in 1997 with fixed numbers of species selected at random from 16 perennial grassland species. For our study, we evaluated monoculture and nine-species plots as described below.

Our first objective was to evaluate the leaf-level responses of three focal species not capable of symbiotic  $\text{N}_2$  fixation and growing with and without the  $\text{N}_2$ -fixing legume *Lupinus perennis* L. The three focal non-fixers were: *Achillea millefolium* L. (forb), *Agropyron repens* (L.) Beauv. and *Koeleria cristata* Pers. ( $\text{C}_3$  grasses). Species hereafter are referred to by their genus. Since the nine-species plots were each composed of a unique set of randomly chosen plant species, we systematically chose those plots that had any of the three non-fixing focal species growing both with and without *Lupinus*. This resulted in a set of 17 plots (see Table 1). However, each  $\text{CO}_2$ /*Lupinus* presence treatment combination was replicated three times for each non-fixing focal species (for a total of 12 plots across all treatments for each species, Table 1), because in some cases *Achillea*, *Agropyron*, and/or *Koeleria* shared the same plot. See Table 1 for an outline of this sampling design. While these plots replicated the treatment combinations under examination, it is important to note that the combinations of the species in each nine-species plot were unique. In addition, all of these plots were growing on non-fertilized soil. The soils are entisols derived from a glacial outwash sand plain. They are 94% sand, classified in the Nymore sand series, acidic (pH =5.5), and N poor (total soil N =0.04%). Harvest data showed that plots without *Lupinus* had less than 1% of the aboveground biomass consisting of any other  $\text{N}_2$ -fixing species from the study combined. *Achillea*, *Agropyron*, and *Koeleria* were chosen to represent non-fixing species because: (1) they provided the greatest replication across plots with and without *Lupinus*, (2) represent two non-fixing plant functional groups (forbs and  $\text{C}_3$  grasses) with similar growing seasons, and (3) were significant components of the nine-species plots as a whole, collectively making up two-thirds of the total aboveground biomass on average.

A second objective was to evaluate *Lupinus*  $\text{N}_2$  fixation and growth responses to  $\text{CO}_2$  enrichment. To do this we used *Lupinus* plants growing in monocultures and in nine-species plots (described above) at ambient and elevated  $\text{CO}_2$  and on ambient and N-enriched soil (fertilized with 4 g  $\text{N m}^{-2} \text{ year}^{-1}$  applied as  $^{15}\text{N}$  enriched  $\text{NO}_3\text{NH}_4$  in May, June and July of 1998 and 1999). The N-enriched plots were required to calculate the amount of N derived from  $\text{N}_2$  fixation as described below.

#### Plot level data

For determination of biomass accumulation in June and August 1998 and 1999, a 10x100-cm strip was clipped at just above the soil surface; all matter was collected, sorted to live material and senesced litter by species and dried and weighed. Roots were sampled at 0–20 cm depth using three 5-cm diameter cores. In August of 1998 and 1999, subsamples were taken from biomass samples and ground and analyzed for tissue C and N using the NA1500 C-N Analyzer (Carlo-Erba Instruments, Milan, Italy) (Reich et al. 2001a, 2001b). Analyses of the plot level data for the nine-species assemblages included all species present.

#### Gas exchange and leaf nitrogen

During the 1999 growing season, in situ rates of leaf net photosynthesis ( $A$ ) of the non-fixing focal species, *Achillea*, *Agropyron*, and *Koeleria*, were measured using the CIRAS-1 portable infrared gas exchange system (PP Systems, Hitchin, UK) operated in open-configuration with controlled temperature,  $\text{CO}_2$  concentration, and vapor pressure. Measurements of a randomly chosen individual plant representing each focal species present in the plot were made on an upper fully expanded leaf on sunny days between 4 July and 2 August 1999 (6 days), typically between 0900 and 1500 hours local time. Rates were determined at or near light-saturating conditions (mean PAR  $\pm$  SE: 1,681 $\pm$ 13  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ), at 27.3 $\pm$ 0.1°C, near ambient humidity (mean leaf chamber vapor pressure deficit  $\pm$  SE: 1.66 $\pm$ 0.03 KPa), and near the  $\text{CO}_2$  concentrations under which the plants were grown (355 $\pm$ 1 or 545 $\pm$ 2  $\mu\text{mol mol}^{-1}$ ). In addition to measurements at a plant's growth  $\text{CO}_2$  concentration, photosynthetic rates of ambient  $\text{CO}_2$ -grown plants were also determined at an elevated measurement concentration of 560  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$  ( $A_{@560}$ ) by changing the  $\text{CO}_2$  concentration in the leaf chamber. This allowed the comparison of ambient and elevated  $\text{CO}_2$  grown plants at a common  $\text{CO}_2$  concentration, thus providing an estimate of the magnitude of photosynthetic acclimation to  $\text{CO}_2$  enrichment. Each plot was repeatedly subsampled four times (three for *Koeleria*), each on a separate, randomly chosen individual. The time of sampling was also random and on separate days, and averaged to provide an estimate of plot response to the treatment combinations (Table 1).

The projected areas of leaves used in gas exchange measurements were determined using digital image analysis program (WinRhizo 3.9, Regent Instruments, Quebec, Canada). Leaves were then oven-dried (65°C) to determine dry mass and then ground and analyzed for tissue nitrogen concentration as described in the previous section.

## Calculation of N derived from symbiotic N<sub>2</sub> fixation

Samples from aboveground tissue harvested on 1 June 1999, were finely ground and analyzed for <sup>15</sup>N atom % (Europa Scientific Integra isotope ratio mass spectrometer, University of California at Davis, Stable Isotope Facility, Davis, Calif.). Samples of the June harvest were used because *Lupinus* is a cool season legume, growing most actively in spring and early summer. N fertilized plots received 4 g N m<sup>-2</sup> year<sup>-1</sup> as NH<sub>4</sub>NO<sub>3</sub> in which the ammonium and nitrate was equally enriched with <sup>15</sup>N at 5.1% above background (Isotec, Miamisburg, Ohio; atom % <sup>15</sup>N of label = 0.38498%). Estimates of the amount of N derived by symbiotic N<sub>2</sub> fixation were calculated according to the following equations (Vose and Victoria 1986):

$$\%N_{\text{fertilizer}} = \left( \frac{a\%^{15}\text{N}_{\text{sample plant}} - a\%^{15}\text{N}_{\text{control}}}{a\%^{15}\text{N}_{\text{label}} - a\%^{15}\text{N}_{\text{control}}} \right) \times 100 \quad (1)$$

$$\text{mg } N_{\text{fertilizer}} (\text{g plant})^{-1} = \text{mg } N_{\text{total}} (\text{g plant})^{-1} \times \%N_{\text{fertilizer}} \times 0.01 \quad (2)$$

$$\text{mg } N_{\text{residual}} (\text{g plant})^{-1} = \text{mg } N_{\text{total}} (\text{g plant})^{-1} - \text{mg } N_{\text{fertilizer}} (\text{g plant})^{-1} \quad (3)$$

$$\text{mg } N_{\text{fixation}} (\text{g plant})^{-1} = \text{mg } N_{\text{residual}} (\text{g plant}_{\text{Lupinus}})^{-1} - \text{mg } N_{\text{residual}} (\text{g plant}_{\text{control}})^{-1} \quad (4)$$

$$\%N_{\text{fixation}} = \text{mg } N_{\text{fixation}} (\text{mg } N_{\text{total}})^{-1} \times 100 \quad (5)$$

$$\text{Total } N_{\text{fixation}} = \text{mg } N_{\text{fixation}} (\text{g plant})^{-1} \times \text{g plant}_{\text{Lupinus}} (\text{plot})^{-1}, \quad (6)$$

where  $\%N_{\text{fertilizer}}$  is the percent of plant N coming from the applied fertilizer and  $a\%^{15}\text{N}$  is the atom % <sup>15</sup>N in the *Lupinus* or *Achillea* sample tissue or the label applied as fertilizer. The  $a\%^{15}\text{N}_{\text{control}}$  values used in Eq. 1 for nine-species assemblages were the mean <sup>15</sup>N concentrations of ambient and elevated CO<sub>2</sub> grown control *Achillea* plants (grown without N addition or *Lupinus*), which were 0.3694 and 0.3674%, respectively. For the monocultures, the  $a\%^{15}\text{N}_{\text{control}}$  values were 0.3672% and 0.3668% in ambient and elevated CO<sub>2</sub>, respectively.  $N_{\text{residual}}$  (Eq. 3) represents the amount of N derived from sources other than the labeled fertilizer, which for *Achillea* was exclusively the N originally available in the soil. Assuming that the  $N_{\text{residual}}$  in *Achillea* estimates the amount of N in *Lupinus* derived from the soil, the difference in residual N between the N<sub>2</sub> fixer and the non-fixer (Eq. 4) is an estimate of the amount of N derived from symbiotic N<sub>2</sub> fixation ( $N_{\text{fixation}}$ ). This approach integrates symbiotic N<sub>2</sub> fixation with whole plant growth over the growth interval, and although it has nontrivial limitations, it is thought to be the most valid integrated measurement of N<sub>2</sub> fixation for plants grown in soil (Vose and Victoria 1986). One important assumption is that the legume and its control plant assimilate fertilizer and soil N in the same proportion (Vose and Victoria 1986). *Achillea* was chosen as the reference plant because of similar growth patterns, growing period, and rooting depths and distribution, therefore providing a control from which to calculate yield-independent estimates of the proportions of soil vs. fertilizer N uptake in *Lupinus* (Lee 2001). In addition, the potentially negative effect of added fertilizer N on N<sub>2</sub> fixation can be a source of error inherent in the <sup>15</sup>N dilution method (Vose and Victoria 1986). However, results from a companion study on *Lupinus* demonstrated that N addition treatments greater than +4 g m<sup>-2</sup> year<sup>-1</sup> were required to negatively affect N<sub>2</sub> fixation or biomass distributed to nodules in *Lupinus* in these soils (Lee 2001). Nonetheless, it should be noted that the effect of added N on N<sub>2</sub> fixation might lead to underestimates of the absolute amount of N derived from fixation.

## Data analysis

The experimental design consisted of a split-plot arrangement of treatments in a randomized design with CO<sub>2</sub> treatment as the whole-plot factor, which is replicated three times among the six rings. The subplot factors of N addition and *Lupinus* treatments were randomly assigned to individual plots within each ring. For data collected in June and August of 1998 and 1999, repeated measures ANOVA was used to determine if responses to the treatment combinations varied with season or year. There were no significant time × treatment interactions with the exception of whole plant %N (time × *Lupinus* presence,  $P=0.05$ ) in which the magnitude of the positive effect of *Lupinus* presence on % N was greater in 1999 than 1998. Therefore, ANOVA results on plot averages over time are presented. All treatment effects were considered fixed. Using  $F$ -tests, the effect of CO<sub>2</sub> (1  $df$ ) was tested against the random effect of ring nested within CO<sub>2</sub> (4  $df$ ). In nine-species plots, the main effects of *Lupinus* presence (1  $df$ ) and interactions between CO<sub>2</sub> and *Lupinus* presence were tested against the residual error. In *Lupinus* monocultures, the main effects of N treatment (1  $df$ ) and interactions between CO<sub>2</sub> and N were tested against the residual error. To test the effect of CO<sub>2</sub> and *Lupinus* treatment on the relationship between light-saturated photosynthesis (mass-based, nmol g<sup>-1</sup> s<sup>-1</sup>,  $A_{m,@560}$ ) and leaf N concentration (%), we examined data across species using “separate slopes” analysis of covariance. We tested whether the slopes of regression lines varied among CO<sub>2</sub> or *Lupinus* treatments. If they did not differ significantly, “same slopes” analyses were used to test for equality of intercepts between the CO<sub>2</sub> or *Lupinus* treatment regression lines. All analyses were conducted with statistical analysis software (JMP Version 3.2.6, SAS Institute, Cary, N.C.).

## Results

### Did *Lupinus* enhance plant N status (H<sub>1</sub>)?

Under ambient CO<sub>2</sub>, *Lupinus* derived almost half of its N from symbiotic N<sub>2</sub> fixation in both monoculture and nine-species plots (Table 1). For *Lupinus* monocultures, *Lupinus* N<sub>2</sub> fixation was estimated to contribute 4.3 g N m<sup>-2</sup> to the total plot N pool (Table 2) and given the relative abundance of *Lupinus* in nine-species plots (Table 2), likely contributed markedly to the N cycle in those plots also, supporting H<sub>1</sub> (Fig. 1). The 4 g N m<sup>-2</sup> year<sup>-1</sup> of fertilizer N applied to N enriched plots did not significantly affect the proportion of N derived from fixation (data not shown).

In addition to increases in *Lupinus* N<sub>2</sub> fixation, whole-plot plant N concentration was 32% greater ( $P<0.0001$ , Table 3), and leaf N concentrations of the individual non-fixing species were 22% greater ( $P<0.0001$ , Fig. 2) in plots containing *Lupinus* compared to those without, also supporting H<sub>1</sub>. This increase in N concentration was associated with an overall 26% increase in the plot total plant N pool ( $P=0.06$ , Table 3).

### Is photosynthesis of non-fixing species and plot biomass enhanced by *Lupinus* presence (H<sub>2</sub>)?

Nine-species plots containing *Lupinus* had similar total biomass (g m<sup>-2</sup>) as those without ( $P=0.72$ , Table 3). However, for all three non-fixers, and at both CO<sub>2</sub> levels,

**Table 2** Nitrogen derived from symbiotic N<sub>2</sub> fixation and standing plot biomass for *Lupinus* grown in monoculture and nine-species assemblages at ambient (368 μmol mol<sup>-1</sup>) and elevated (560 μmol mol<sup>-1</sup>) concentrations of atmospheric CO<sub>2</sub>. Shown are least squares means (± SE) and ANOVA probabilities (*P*>*F*) of the main effect

Response		Growth CO <sub>2</sub> concentration (μmol mol <sup>-1</sup> )		ANOVA <i>P</i> > <i>F</i> (%change)
		368	560	
<i>Lupinus</i> in monoculture plots	Proportion of N derived from fixation	0.44±0.04	0.57±0.04	<b>0.09</b> (+29%)
	Plot total fixed N (gN m <sup>-2</sup> )	4.3±0.5	9.6±0.6	<b>0.01</b> (+121%)
	Plot total biomass (g m <sup>-2</sup> )	479.0±32.7	659.2±34.5	<b>0.04</b> (+38%)
<i>Lupinus</i> in nine-species plots	Proportion of N derived from fixation	0.43±0.03	0.54±0.03	<b>0.05</b> (+26%)
	<i>Lupinus</i> aboveground biomass proportion	0.16±0.03	0.25±0.04	0.15 (+54%)

**Table 3** Plot-level plant N concentration, total plant N pool and plot biomass of nine-species plots grown at CO<sub>2</sub> concentrations of 368 and 560 μmol mol<sup>-1</sup> and in the absence and presence of *Lupinus*. Shown are least squares means (± SE) and ANOVA probabilities (*P*>*F*) for CO<sub>2</sub> and *Lupinus* presence main effects and their interactions. *P*≤0.10 are bold-faced. Data were analyzed on

Response	<i>Lupinus</i> presence	Growth CO <sub>2</sub> concentration (μmol mol <sup>-1</sup> )		ANOVA <i>P</i> > <i>F</i> (% change)		
		368	560	CO <sub>2</sub>	<i>Lupinus</i> presence	CO <sub>2</sub> × <i>Lupinus</i>
Whole plant N (%)	Absent	0.8±0.04	0.7±0.04	<b>0.006</b> (-14%)	<b>&lt;0.0001</b> (+32%)	0.45
	Present	1.1±0.03	0.9±0.04			
Plot total plant N pool (g m <sup>-2</sup> )	Absent	9.7±1.2	9.7±1.2	0.70 (-3%)	<b>0.06</b> (+26%)	0.78
	Present	12.6±1.1	11.9±1.2			
Total plot biomass (g m <sup>-2</sup> )	Absent	1,187±101	1,368±101	0.29 (+13%)	0.72 (-3%)	0.81
	Present	1,175±90	1,306±101			

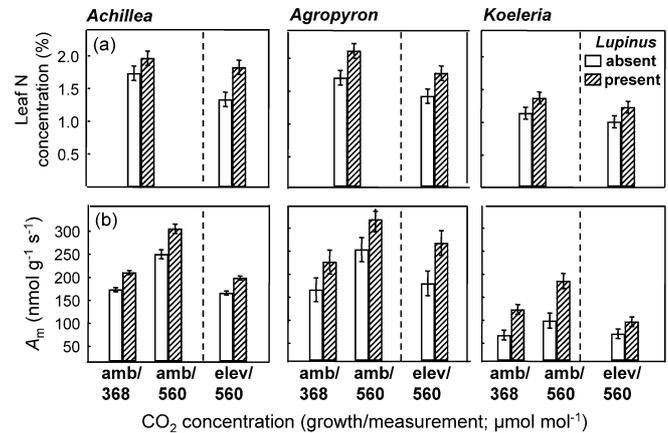
mean rates of leaf net photosynthesis increased by 41% and 24%, on mass ( $A_m$ , nmol g<sup>-1</sup> s<sup>-1</sup>, Fig. 2b) and area (μmol m<sup>-2</sup> s<sup>-1</sup>, data not shown) bases respectively, when grown with compared to without *Lupinus* (*P*<0.002). While leaf-level photosynthesis was enhanced by the presence of a legume, community biomass was not, therefore, H<sub>2</sub> is only partially supported.

Is *Lupinus* growth and symbiotic N<sub>2</sub> fixation enhanced by elevated CO<sub>2</sub> (H<sub>3</sub>)?

In support of H<sub>3</sub>, the proportion of *Lupinus* N derived from symbiotic N<sub>2</sub> fixation increased from 44% to 57% in monoculture and from 43% to 54% in nine-species plots when grown under elevated compared to ambient CO<sub>2</sub> (Table 2). In monoculture, the increase in the proportion of N derived from fixation, adjusted by the increase in total plot biomass, resulted in a doubling of N fixed per plot (g fixed N m<sup>-2</sup>) under elevated compared to ambient CO<sub>2</sub> (*P*=0.01, Table 2). Enrichment of plots with 4 g m<sup>-2</sup> year<sup>-1</sup> fertilizer N did not have a significant effect on the proportion of N derived from fixation or plot total biomass and only marginally reduced the relative proportion *Lupinus* aboveground biomass from 24% to 17% in nine-species assemblages compared to ambient N plots (*P*=0.06, data not shown).

of CO<sub>2</sub> treatment (N treatment and CO<sub>2</sub> × N interaction were not significant, *P*>0.20, and therefore, not shown). *P*≤0.10 are bold-faced. Repeated measures ANOVA did not result in any significant interactions with time for these responses

plot averages across harvests in 1998 and 1999 (*n*=17 plots). Repeated measures ANOVA resulted in one significant interaction with time for these responses: whole plant N (%) time × *Lupinus* presence *P*=0.05, in which the response to *Lupinus* presence was positive in both years but strongest in 1999. *Lupinus* are shown beside *P*-value. *P*≤0.10 are bold-faced



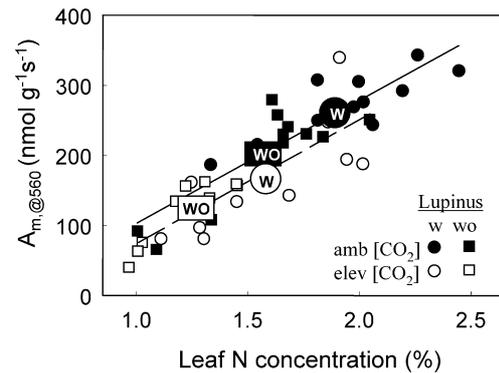
**Fig. 2** a Mean leaf N concentration (%) and b mean mass-based rates of leaf net photosynthesis ( $A_m$ , nmol g<sup>-1</sup> s<sup>-1</sup>) of three non-fixing species (*Achillea*, *Agropyron*, *Koeleria*) grown in nine-species assemblages at ambient (368 μmol mol<sup>-1</sup>) and elevated (560 μmol mol<sup>-1</sup>) CO<sub>2</sub> concentrations and in the absence (clear bars) and presence (shaded bars) of *Lupinus*.  $A_m$  was measured at the CO<sub>2</sub> concentrations under which plants were grown: ambient (amb/368 μmol mol<sup>-1</sup>) and elevated (elev/560 μmol mol<sup>-1</sup>), and ambient grown plants were also measured at elevated CO<sub>2</sub> concentrations (amb/560 μmol mol<sup>-1</sup>). Shown are least squares means (±SE). Significant (*P*<0.05) ANOVA probabilities: a CO<sub>2</sub> *P*=0.02, *Lupinus* presence *P*=<0.0001; b  $A_{m@growthCO_2}$ : *Lupinus* presence *P*<0.0001,  $A_{m@560}$ : CO<sub>2</sub> *P*=0.009, *Lupinus* presence *P*<0.0001, *Lupinus* presence × species *P*=0.05, species × CO<sub>2</sub> × *Lupinus* presence *P*=0.03

Is the CO<sub>2</sub> response of plants and communities enhanced by the presence of *Lupinus* (H<sub>4</sub>)?

Elevated CO<sub>2</sub> did not stimulate biomass accumulation in nine-species assemblages more in the presence than absence of *Lupinus* (Table 3). Whole plant %N was 14% lower on average ( $P=0.006$ ) while the total plant N pool remained similar in elevated compared to ambient CO<sub>2</sub> and neither of these responses were influenced by the presence or absence of *Lupinus*. Although the proportion of N derived from fixation increased in *Lupinus* grown in nine-species plots at elevated CO<sub>2</sub>, and *Lupinus* presence enhanced leaf and plot N concentrations and total plot plant N pool, *Lupinus* did not facilitate greater standing biomass accumulation under elevated CO<sub>2</sub>, contradicting H<sub>4</sub> (Table 3).

In nine-species plots, the leaf net photosynthetic rates ( $A_m$ ) of the three non-fixing focal species grown and measured at elevated CO<sub>2</sub> were not different than in plants grown and measured at ambient CO<sub>2</sub> ( $P=0.82$ ), and the presence of *Lupinus* had no effect on this response to CO<sub>2</sub> (Fig. 2b), also contradicting H<sub>4</sub>. Photosynthetic rates of ambient CO<sub>2</sub> grown plants were also measured at 560  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> to compare ambient and elevated grown plants at a common CO<sub>2</sub> concentration ( $A_{m@560}$ ), which can reveal evidence for biochemical down regulation of photosynthesis in response to CO<sub>2</sub> enrichment.  $A_{m@560}$  was 28% lower in elevated compared to ambient CO<sub>2</sub>-grown plants ( $P=0.009$ , Fig. 2b). Hence, marked photosynthetic acclimation to elevated CO<sub>2</sub> occurred in all three non-fixing species. This acclimation did not consistently depend on *Lupinus* presence. However, the significant interaction between *Lupinus* presence, species identity, and CO<sub>2</sub> treatment ( $P=0.04$ , Fig. 2b) suggests that species varied in the magnitude of acclimation depending on whether *Lupinus* was present or absent.

The relationship between leaf net photosynthesis of ambient and elevated CO<sub>2</sub> grown plants and leaf N concentration is shown in Fig. 3. The higher photosynthetic rates of plants grown in the presence of *Lupinus* were associated with increased leaf %N (Fig. 3). *Lupinus* presence did not alter the slope of the  $A_{m@560}$ -%N regression relationship ( $P=0.60$ ), but rather data points representing plants grown in the presence of *Lupinus* were shifted upwards along the general  $A_{m@560}$ -%N relationship. This suggests that the stimulation in  $A_{m@560}$  could be attributed to the higher leaf %N in plants grown with compared to without *Lupinus* (Figs. 2, 3). In contrast, the intercept of the  $A_{m@560}$ -%N relationship representing elevated CO<sub>2</sub>-grown plants was significantly lower than that representing ambient CO<sub>2</sub> grown plants (CO<sub>2</sub> effect  $P=0.05$ ). Therefore, the lower photosynthetic rates of elevated CO<sub>2</sub> grown plants were associated with both decreased %N (i.e., moving down the slope) and with lower photosynthetic rates at a given leaf %N (Fig. 3).



**Fig. 3** Relationship between leaf net photosynthesis measured at a common CO<sub>2</sub> concentration of 560  $\mu\text{mol mol}^{-1}$  ( $A_{m@560}$ ,  $\text{nmol g}^{-1} \text{s}^{-1}$ ) and leaf N concentration (%) for foliage of three non-fixing species (*Achillea*, *Agropyron*, *Koeleria*) grown in nine-species assemblages in the presence (*black circles*, *clear circles*) and absence (*black squares*, *clear squares*) of *Lupinus* or at ambient (*black circles*, *black squares*, 368  $\mu\text{mol mol}^{-1}$ ) and elevated (*clear circles*, *clear squares*, 560  $\mu\text{mol mol}^{-1}$ ) concentrations of CO<sub>2</sub>. Shown are plot averages of each of the three species. Separate regression lines are shown only where the elevation of the treatment lines was significantly different. ANOVA probabilities ( $P>F$ ) for the main effects are: *Lupinus* presence,  $P=0.60$ ; and CO<sub>2</sub> treatment,  $P=0.05$  (ambient CO<sub>2</sub>:  $r^2=0.83$ ,  $P<0.0001$ , elevated CO<sub>2</sub>:  $r^2=0.72$ ,  $P<0.0001$ ). *Extra large symbols* represent the data centroids of each treatment combination

## Discussion

In these experimental grassland assemblages, the presence of the N<sub>2</sub> fixer *Lupinus* enhanced overall plant N status, as well as leaf-level photosynthetic rates and N concentrations of individual co-occurring non-fixers within the community. Enhanced photosynthesis occurred via enhanced leaf N concentrations when grown in the presence of *Lupinus* and as a result of the strong dependence of photosynthesis on leaf %N in these species across these treatments (Fig. 3). Studies from other systems have also shown that the presence of N<sub>2</sub>-fixing species enhances the growth of co-occurring species and overall productivity (Vitousek and Walker 1989; Soussana and Hartwig 1996; Hector et al. 1999). This is often explained by an N feedback mechanism in which N<sub>2</sub> fixers modulate the N status of the plant-soil system to meet the changing demands for N of plants and ecosystems (Hartwig 1998). The presence of N<sub>2</sub> fixers, therefore, could facilitate relatively stronger growth responses of communities experiencing an increased N demand due to elevated CO<sub>2</sub>-induced stimulation of growth. Nevertheless, in our study the physiological and growth responses to CO<sub>2</sub> enrichment of these communities, and sampled component species, were not modulated by the presence of *Lupinus*.

Nine-species assemblages containing *Lupinus* had significantly greater plant N concentrations and greater total plant N pools than those without. Furthermore, elevated CO<sub>2</sub> enhanced both *Lupinus* dry matter accumulation and the amount and proportion of N derived from symbiotic N<sub>2</sub> fixation, leading to more fixed N apparently

entering the system under elevated compared to ambient CO<sub>2</sub> concentrations (H<sub>1</sub>, H<sub>3</sub>). N<sub>2</sub>-fixing species often show increases in the amount of N derived from symbiotic N<sub>2</sub> fixation that coincide with increases in plant growth at elevated CO<sub>2</sub> (Soussana and Hartwig 1996; Zanetti et al. 1996, 1998; Zanetti and Harwig 1997). However, for N<sub>2</sub> fixers to facilitate plant and community responses to elevated CO<sub>2</sub> by enhancing the N status of the vegetation, the CO<sub>2</sub> response of non-fixing species must be in fact N-limited (H<sub>a1</sub>). Numerous studies have demonstrated increases in productivity with N addition, and evidence from this and companion studies found that biomass accumulation, plant tissue and soil N concentrations, and net photosynthetic rates were in most cases enhanced when N was added to the soil (Lee et al. 2001; Lee 2001; Reich et al. 2001a, 2001b). This suggests that individuals and assemblages of *Achillea*, *Agropyron*, and *Koeleria* were constrained by the low supply of N, supporting H<sub>a1</sub>. Furthermore, it is logical to hypothesize that the response to elevated CO<sub>2</sub> of the non-fixing species in our study was constrained by the inherently low availability of N (H<sub>a2</sub>). Similarly, modest growth responses to CO<sub>2</sub> enrichment in some species and systems have been attributed to other nutrients, mainly N, becoming limiting (Díaz et al. 1993; Leadley and Körner 1996; Zanetti and Hartwig 1997; Curtis and Wang 1998; Stöcklin and Körner 1999; Poorter and Pérez-Soba 2001).

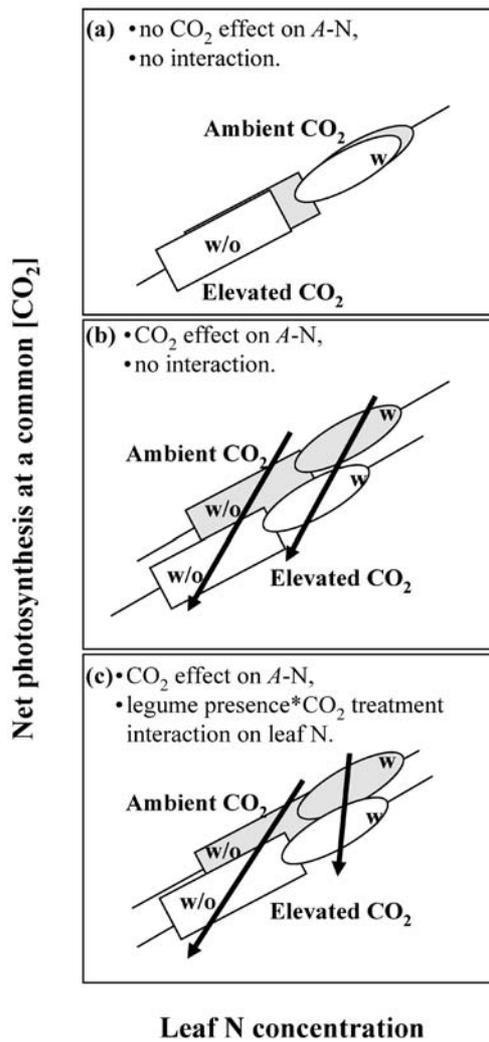
The interactive effects of CO<sub>2</sub> and any environmental factor depend in part on the magnitude of the effect of that factor on growth at ambient CO<sub>2</sub> concentrations (Poorter and Pérez-Soba 2001). In the overall BioCON experiment, the addition of 4 g N m<sup>-2</sup> year<sup>-1</sup> positively affected nearly all parameters measured in the greater BioCON experiment, however, these species did not demonstrate larger elevated CO<sub>2</sub>-induced increases in biomass accumulation in the N-enriched compared to the ambient soil N treatments (Reich et al. 2001a, 2001b). Several other studies have also found that despite significant effects on plant growth, nutrient addition did not enable greater responsiveness to elevated CO<sub>2</sub> (Chiariello and Field 1996; Lloyd and Farquhar 1996; Körner et al. 1997; Reich et al. 2001a, 2001b). But a noteworthy number of studies found increasing magnitudes of growth stimulation in response to elevated CO<sub>2</sub> as N supply increased (Hebeisen et al. 1997a; Wand et al. 1999; Poorter and Pérez-Soba 2001). The lack of agreement across studies, and the fact that the CO<sub>2</sub> response of some species appears to be independent of N supply, represents an ongoing major uncertainty in CO<sub>2</sub> research and suggests that mechanisms independent of N limitation may be important in determining the ability to respond to increased CO<sub>2</sub> concentrations or may reflect species-specific differences in response to CO<sub>2</sub> concentration and their interactions with the nutrient status of each respective system.

The overall biomass responses of nine-species assemblages, as well as the leaf-level responses of component non-fixing species to elevated CO<sub>2</sub>, were not influenced by *Lupinus* presence and are consistent with the lack of an N effect on the CO<sub>2</sub> response in the larger study

(described above). Zanetti et al. (1997) found that leaf N concentrations of *Lolium perenne* in managed pastures declined less in response to elevated CO<sub>2</sub> and had greater total plant N yield when grown in mixtures with *Trifolium repens* than without; however, the biomass of *Lolium* grown in mixtures did not respond to increased CO<sub>2</sub> due to a CO<sub>2</sub>-induced increase in the competitive ability of *Trifolium* (Hebeisen et al. 1997b). In the present study, the proportion of *Lupinus* aboveground biomass with CO<sub>2</sub> enrichment increased despite no effect on total plot biomass. The proportion of *Lupinus* also declined with added N. Both of these responses corroborate the hypothesis that legume competitive ability is enhanced by CO<sub>2</sub> enrichment and/or low N conditions.

*Lupinus* presence may result in a shift in species composition that does not necessarily result in greater overall biomass accumulation. Niklaus et al. (2001) found that initial positive responses of plants communities to elevated CO<sub>2</sub> concentrations were attributable to responses of certain component species. However, these species were selected against overtime such that the positive effects of elevated CO<sub>2</sub> on plant biomass diminished. In addition, Chiariello and Field (1996) found an added nutrient treatment affected aboveground production in every species, but since the grasses and forb responses were opposite in direction the main result was a changed species composition. Evaluating species changes in their proportion of the total plot aboveground biomass in the present, however, did not reveal evidence of a shifting species composition in response to the presence of *Lupinus* or to elevated CO<sub>2</sub> concentrations in these nine-species assemblages during the time of this study (data not shown). However, a change in species composition may be difficult to detect considering the limited replication and high variability due to the unique species composition of each plot. Furthermore, *Lupinus* has a disproportionately low fraction of its standing biomass located belowground compared to all other species in the overall experiment (Reich et al. 2001b). Whether this represents low *Lupinus* root biomass production or high fine root turnover is unknown, but it emphasizes the difficulty in interpreting biomass differences. The failure of our data to support H<sub>4</sub> lies in the fact that *Lupinus* presence alone did not lead to greater overall biomass accumulation and the lack of significant interactions between plant N status and leaf, plant, or plot-level CO<sub>2</sub> responses.

To our knowledge, this is the first study that examines leaf-level physiology to investigate the effects of legume presence on co-occurring species in multi-species assemblages. To help explain the lack of enhanced CO<sub>2</sub> responsiveness in plants co-occurring with *Lupinus*, despite increased leaf N concentrations, we present hypothetical models in Fig. 4. These models demonstrate possible ways in which growth at ambient or elevated CO<sub>2</sub> concentrations, and with or without a legume, could affect the N concentration of leaves and the relationship between net photosynthesis ( $A_{m@560}$ ) and leaf N concentration in co-occurring non-fixers when measured at a



**Fig. 4a-c** Hypothetical models depicting possible modes of effect for legume presence on the net photosynthesis-leaf N concentration (A-N) relationship of plants grown at ambient (*shaded shapes*) compared to elevated (*open shapes*) CO<sub>2</sub> concentrations. **a** Depicts the null model, no CO<sub>2</sub> effect on A-N relationship and no legume presence × CO<sub>2</sub> treatment interaction, **b** a CO<sub>2</sub> effect on A-N relationship but no legume presence × CO<sub>2</sub> treatment interaction, and **c** a CO<sub>2</sub> effect on A-N relationship coupled with a legume presence × CO<sub>2</sub> treatment interaction

common CO<sub>2</sub> concentration. For clarity, and since our data showed no effect of *Lupinus* presence on the A-N relationship (Fig. 3), we limit the hypothetical models to those in which the presence of a legume does affect leaf N but does not affect the A-N relationship. It is also important to note that factors that alter the magnitude of acclimation (i.e., down-regulation, which is demonstrated in Fig. 4) in fact modulate the degree of enhancement due to elevated CO<sub>2</sub> when plants are compared across their growth environments.

Panel A depicts the null hypothesis in which elevated CO<sub>2</sub> concentration does not affect the A-N relationship, and in which legume presence affects leaf N similarly under elevated and ambient CO<sub>2</sub> treatments (no interac-

tion) (Fig. 4a). The effects of CO<sub>2</sub> and legume presence on leaf N merely shift leaves up and down a common slope. Panel B also demonstrates no interaction between CO<sub>2</sub> treatment and legume presence on leaf N; however, growth at elevated CO<sub>2</sub> results in a downward shift of the intercept of the A-N relationship such that A is lower at any given N in elevated compared to ambient CO<sub>2</sub>. Legume presence simply results in rates further up the generalized A-N relationship, and similarly in ambient and elevated CO<sub>2</sub> treatments. Thus in this case, the main effects of CO<sub>2</sub> treatment and legume presence are additive. Panel C shows the same elevated CO<sub>2</sub>-induced downward shift in the intercept of the A-N relationship. However, in addition to this CO<sub>2</sub> treatment effect, legume presence reduces the magnitude of the common elevated CO<sub>2</sub>-induced decline in leaf N such that leaf N declines less under elevated CO<sub>2</sub> when grown with compared to without a legume (non-parallel arrows). This would support higher photosynthetic rates under elevated CO<sub>2</sub> than would otherwise occur (i.e., resulting in less down-regulation of A than occurs without a legume).

How do our results compare to the hypothetical scenarios in Fig. 4? In our study, leaf N concentrations declined similarly under elevated CO<sub>2</sub> with and without *Lupinus* present (arrows are parallel) and there was no change in the slope of the A-N relationship due to CO<sub>2</sub> treatment (Fig. 3). Hence, there was not a significant legume presence × CO<sub>2</sub> treatment interaction on photosynthetic rates of the non-fixers, which would be required to support the hypothesis that the CO<sub>2</sub> response of leaf photosynthesis in non-fixers is enhanced by the presence of legumes. Therefore, these data best fit the model in panel B. The responses of net photosynthetic rates and leaf N to CO<sub>2</sub> and *Lupinus* presence treatments were strictly additive and statistically independent. Hence, the lack of stimulation of the CO<sub>2</sub> response of photosynthesis by the presence of *Lupinus* results from the lack of interaction between *Lupinus* presence and the reduction in leaf %N due to CO<sub>2</sub> enrichment.

In conclusion, this study demonstrated that the presence of a legume in N-poor nine-species grassland assemblages enhanced plant N concentration, which stimulated leaf-level net photosynthesis of co-occurring non-fixing species. In addition, elevated CO<sub>2</sub> stimulated legume growth and symbiotic N<sub>2</sub> fixation. However, the enhancements in N status, owing to the legume, did not lead to greater biomass or photosynthetic responses to elevated compared to ambient CO<sub>2</sub>. In other words, we did not find evidence that the CO<sub>2</sub> response of species in this system is limited by N, but rather that the effects of elevated CO<sub>2</sub> concentrations, combined with the effects of legume presence, are merely additive. It remains unclear why some studies show clear CO<sub>2</sub> by N interactions while others fail to support this. Perhaps it is incorrect to think that there should be consistency of such responses if interactions in key attributes, such as leaf %N or leaf area, among CO<sub>2</sub> and N levels, can vary with species, situation or resource levels. Our lack of evidence for a CO<sub>2</sub> by N interaction may be due to CO<sub>2</sub> responses being indepen-

dent of leaf N in this system. Alternatively, a CO<sub>2</sub> by leaf N interaction could exist at the tissue or plant-scale, in another system or in this system under alternative treatment levels, but complex system-level interactions might modulate system-scale responses. In our case, these could include increased competitive pressure from *Lupinus*, changes in microbial communities, or altered soil N cycling under elevated compared to ambient CO<sub>2</sub>, with the result that the interaction either does not occur or is not detectable under our present conditions. Our data suggest that mechanisms independent of N limitation may be important in determining the ability to respond to increased CO<sub>2</sub> concentrations. Further research into the roles of species-species and species-environment interactions in community responses to elevated atmospheric CO<sub>2</sub> concentrations will help clarify the variety of mechanisms underlying ecosystem response to global change.

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