Global Change Biology

Global Change Biology (2010) **16**, 2076–2088, doi: 10.1111/j.1365-2486.2009.02058.x

Maintenance of leaf N controls the photosynthetic CO_2 response of grassland species exposed to 9 years of free-air CO_2 enrichment

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Abstract

Determining underlying physiological patterns governing plant productivity and diversity in grasslands are critical to evaluate species responses to future environmental conditions of elevated CO_2 and nitrogen (N) deposition. In a 9-year experiment, N was added to monocultures of seven C_3 grassland species exposed to elevated atmospheric CO_2 (560 μmol CO_2 mol^{-1}) to evaluate how N addition affects CO_2 responsiveness in species of contrasting functional groups. Functional groups differed in their responses to elevated CO_2 and N treatments. Forb species exhibited strong down-regulation of leaf N_{mass} concentrations (−26%) and photosynthetic capacity (−28%) in response to elevated CO_2, especially at high N supply, whereas C_3 grasses did not. Hence, achieved photosynthetic performance was markedly enhanced for C_3 grasses (+68%) in elevated CO_2, but not significantly for forbs. Differences in access to soil resources between forbs and grasses may distinguish their responses to elevated CO_2 and N addition. Forbs had lesser root biomass, a lower distribution of biomass to roots, and lower specific root length than grasses. Maintenance of leaf N, possibly through increased root foraging in this nutrient-poor grassland, was necessary to sustain stimulation of photosynthesis under long-term elevated CO_2. Dilution of leaf N and associated photosynthetic down-regulation in forbs under elevated [CO_2], relative to the C_3 grasses, illustrates the potential for shifts in species composition and diversity in grassland ecosystems that have significant forb and grass components.

Keywords: C_3 grass species, carboxylation rate, FACE, free-air CO_2, Nitrogen, photosynthesis, species functional groups

Received 8 June 2009 and accepted 27 July 2009

Introduction

With increasing CO_2 emissions from human activities driving increases in mean global atmospheric [CO_2], there are concerns over the capacity of natural ecosystems to continue to serve as sinks for atmospheric CO_2 over decades to come (Canadell *et al*., 2007). During the 20th century, the sink capacity of native grasslands was related to climate, atmospheric CO_2, and nitrogen (N) deposition, but our understanding of the interactions among these factors and the mechanisms driving these interactions remains incomplete (Schimel *et al*., 2001). Because ecosystem C and N cycles are strongly coupled, interactive effects of elevated CO_2 and N availability are likely, potentially reducing the magnitude of photosynthetic enhancement under elevated CO_2 (McMurtrie & Comins, 1996; Rastetter *et al*., 1997; Luo *et al*., 2004). In addition, species-specific responses to environmental conditions influence the rate of C and N cycling in ecosystems and interactions between species and elevated CO_2 or N addition. Long-term field experiments in which multiple factors are manipulated simultaneously therefore provide an important tool for untangling ecological interactions (Hunter, 2001; Mikkelsen *et al*., 2008). Moreover, it is important to link

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physiological responses to whole plant biomass accumulation to understand the underlying controls and their effect on plant productivity in ecosystems under expected climatic and atmospheric change (Körner, 2003).

Plant N stocks and photosynthesis–leaf N relationships couple ecosystem C and N cycles (Lee *et al*., 2001, 2003; Ellsworth *et al*., 2004). Long-term elevated CO_2 typically causes a reduction in leaf N (Yin, 2002; Ainsworth & Long, 2005) and hence potentially in plant productivity per unit C availability, particularly when root N uptake is not enhanced to support increased growth demands in elevated CO_2 (Field *et al*., 1992; Luo *et al*., 1994). In contrast to the reduction of leaf N when [CO_2] is enriched, increased N supply to soils would be expected to increase leaf N (Field *et al*., 1992). Thus, leaf N and its impacts on leaf physiology can help us better understand the interactions between N availability and atmospheric CO_2 concentration that are critical to predicting how plant productivity and diversity are affected in an increasingly eutrophic biosphere (Vitousek, 1994).

Plant species can vary in their divergent responses to environmental change, including rising atmospheric [CO_2] and N addition (Zanetti *et al*., 1997; Joel *et al*., 2001; Lee *et al*., 2001; Poorter & Perez-Soba, 2001; Reich *et al*., 2004). Many grassland studies have found that increased plant growth under elevated CO_2 can only be sustained with sufficient N supply (Grünzweig & Körner, 2003; Lüscher *et al*., 2004; Schneider *et al*., 2004; Dukes *et al*., 2005; Reich *et al*., 2006a). However, such a response may not typify all species or functional groups (Poorter & Navas, 2003). Several studies have shown that forbs might be more sensitive to elevated CO_2 than other functional groups (Reich *et al*., 2001b; Teyssonneyre *et al*., 2002; Polley *et al*., 2003). Recent reports have shown that C_3 forbs in grasslands are negatively impacted by increased N deposition (Zavaleta *et al*., 2003b; Stevens *et al*., 2006; Clark & Tilman, 2008) but it is unclear how or why this functional group responds differently than other functional groups. A negative response to N addition in C_3 forbs could be related to water availability (Morgan *et al*., 2004), competition for light (Mohan *et al*., 2007) or soil resources, altered soil processes (Niklaus *et al*., 2003; West *et al*., 2006) or altered allocation between plant C pools (Poorter, 1993). Differential responses to elevated CO_2 and N deposition among plant groups can lead to changes in species composition and diversity, and the structure and function of ecosystems (Potvin *et al*., 2007).

Grasslands constitute 40% of global land area (Morgan *et al*., 2007) and are often relatively species-rich. Broad functional groups could be useful for capturing the aggregated responses of different types of species and their responses to changing environmental conditions (Zavaleta *et al*., 2003a). It has been hypothesized that a number of intrinsic physiological leaf traits, such as photosynthetic rates, specific leaf area (SLA) and foliar nitrogen, central to how species functional groups are depicted, also determine the response of species to elevated [CO_2] (Woodward & Cramer, 1996; Lavorel *et al*., 1997). These key functional traits shared by species in functional groups could be represented in models predicting community responses to environmental change (Suding *et al*., 2008).

This study aims to provide insight into mechanisms that drive species responses to atmospheric change for two contrasting functional groups under a combination of elevated CO_2 and N addition. This is particularly important in light of recent reports of species losses in grassland ecosystems under climatic and atmospheric change (Joel *et al*., 2001; Zavaleta *et al*., 2003a; Suding *et al*., 2005). Our goal was to examine how trait differences between C_3 forbs and C_3 grasses yield different responses to the combination of elevated CO_2 and N addition. We investigated physiological mechanisms underlying species responses to both elevated CO_2 and N deposition, and the multiple interactions between these environmental factors and species. Functional group responses were also examined to assess whether these groupings could represent species responses within their respective functional group. We studied C_3 grass and forb species across the sixth to ninth years of elevated [CO_2] exposure and chronically low levels of N addition in a nutrient-poor prairie grassland in Minnesota, USA to address the following hypotheses:

H.1. Long-term reductions in foliar N under elevated CO_2 are reflected in declining photosynthetic capacity such that the instantaneous CO_2 enhancement effect is offset by photosynthetic down-regulation. This would result in little or no enhancement of realized photosynthetic rates in elevated CO_2 in a nutrient-poor grassland.

H.2. Nutrient addition can compensate for reduced foliar N under elevated CO_2 such that photosynthetic capacity of C_3 grassland species remains unchanged or increased with CO_2 enrichment.

We examined these hypotheses for multiple C_3 grassland species in a long-term grassland free-air CO_2 enrichment (FACE) experiment where atmospheric [CO_2] and soil N were manipulated (Reich *et al*., 2001a). Collectively, these hypotheses are used to explain differences in species performance in elevated CO_2 and N addition, and test for commonality of species and functional group responses, thereby

improving our capacity to generalize global change responses in grassland ecosystems.

Materials and methods

Site description and experimental design

The BioCON (Biodiversity, CO_2 and N) FACE experiment is part of the U.S. National Science Foundation Long-term Ecological Research network and is located in central Minnesota, USA (45°24′13.5″N, 93°11′08″W). The site is located in a humid continental climate on glacial outwash comprised of loamy sand soils with low nutrient availability (Grigal *et al*., 1976). The mean annual precipitation is 660 mm yr^{-1} and the mean maximum July temperature is 28.3 °C.

The BioCON FACE experiment consists of six circular plots of 20 m diameter, three of which control atmospheric [CO_2] to 560 μmol mol^{-1} while three plots remain at ambient [CO_2]. Daytime exposure of plots to elevated [CO_2] proceeds continuously from the beginning of the growing season in April until the end of the growing season in October. One-minute average [CO_2] in FACE rings were within 10% of the target concentration >95% of the time during the years of this study. The plants were planted in 1997, with the first season of CO_2 fumigation in 1998.

A subset of plots from the complete FACE experiment (see Reich *et al*., 2001a, b) was used for the analyses here, specifically the 56 2 m × 2 m plots within the six FACE rings with monocultures of our seven target C_3 grass or nonleguminous forb species. Monocultures were used to assess species responses rather than mixtures since the emphasis was on independent species responses to the treatment factors. Among these plots, soil N addition treatments had been randomly assigned in two replicates in a split-plot design since the start of the experiment in 1998. N addition consisted of 4 g N m^{-2} yr^{-1} in the form of solid ammonium nitrate applied each year across May, June and July. There were eight monoculture subplots of each of the seven species equally divided across the four combinations of CO_2 and N-addition treatments. Above-, belowground and total biomass of these plots were determined each year in June by harvest of a subsample area of the main plot (Reich *et al*., 2001b). Belowground harvests were conducted by means of three 5-cm diameter cores to 20 cm depth. Fine roots were defined as <2 mm diameter and were separated manually from the larger roots.

The species chosen for this study were four C_3 grasses: *Poa pratensis* L., *Koeleria cristata* Pers., *Bromus inermis* Leyss. and *Agropyron repens* L. and three forb species: *Solidago rigida* L. and *Anemone cylindrica* A. Gray and *Achillea millefolium* L. These species are referred to in figures by a combination of the first three letters of the genus and the first two letters of the species name.

Gas exchange and leaf nitrogen

Measurements in this study were made during the sixth through ninth growing seasons of the experiment (2003–2006) to assess the long-term effects of elevated CO_2 and nitrogen additions and potential interactions between them. Species composition, biomass and physiological responses to CO_2 and N were relatively stable at this stage of the experiment. Gas exchange measurements were conducted with a portable infrared gas analyzer system (LiCOR 6400, Li-Cor Inc., Lincoln NE, USA) during the main portion of the season when each species was active (May–June of each growing season). To assess instantaneous and long-term (up to 9 years) effects of elevated CO_2 on photosynthetic capacity, photosynthetic CO_2 response curves (A–C_i) were measured on leaves of each plant species with a minimum of seven different CO_2 concentrations between 60 and 1500 μmol mol^{-1}, using saturating light conditions (photon flux density of 1800 μmol m^{-2} s^{-1}) and controlled temperatures (leaf temperatures of 28–30 °C) in the leaf cuvette. Per species, plants in monoculture plots were measured with two replicates for each CO_2 and N treatment. All grass measurements were from the topmost fully expanded leaf adjacent to the flag leaf to ensure similar leaf ages. Leaves were collected and placed on ice after each A–C_i response curve to determine projected leaf area in the chamber (IMAGE J v1.37, National Institutes of Health, Bethesda, MD, USA). In the laboratory, leaves were dried at 70 °C, weighed, and finely ground. A subsample was analyzed for total nitrogen and carbon content using an elemental analyzer (Carlo-Erba Strumentazione, Milan, Italy) with appropriate reference standards for herbaceous leaves in each analysis run (National Institute of Standards and Technology, Boulder, CO, USA).

Physiological variables were fitted from the A–C_i response curves using the Farquhar photosynthesis model (Farquhar *et al*., 1980) according to the procedure laid out in Ellsworth *et al*. (2004). To evaluate changes in photosynthetic capacity and assess potential down-regulation of photosynthesis, we analyzed the variables maximum carboxylation rate (V_{cmax}) and the maximum electron transport rate (J_{max}) as well as the measured net photosynthesis in current growth conditions (either ambient or elevated [CO_2]) (A_{net}) and net photosynthesis at a common CO_2 level of 365 μmol μmol^{-1}. Comparing photosynthesis at a common measurement CO_2 level allows for assessing the long-term effects of elevated [CO_2] and N on intrinsic photosynthetic capacity

(Lee *et al*., 2001; Ellsworth *et al*., 2004; Ainsworth & Rogers, 2007). Net photosynthesis at a common CO_2 level was analyzed both on a mass basis (A_{m365}) and area basis (A_{a365}), concurrent with leaf N expressed on a mass basis (N_{mass}) and on an area basis (N_{area}). A slight increase in LMA (leaf mass per area) was observed in elevated CO_2 ($P = 0.07$). Despite this, results were generally similar whether expressed on mass or area bases. We also analyzed net photosynthesis at a common CO_2 level of 560 μmol mol^{-1} corresponding to the CO_2 concentration of the elevated CO_2 treatment, however, those results strongly paralleled the results for A_{a365} and hence are not shown. These variables help to evaluate the basic physiological mechanisms behind changes in plant growth and productivity in long-term elevated CO_2 and N addition, and facilitate the comparison of those mechanisms in different C_3 species.

Statistical analyses

Since we are interested in the long-term effects of elevated CO_2 and N addition rather than interannual variation, species averages were from photosynthetic CO_2 response curves conducted across the four years when biomass responses to treatments were constant. Averaging across years resulted in similar sample sizes for each species per treatment combination, and represented average responses of each species to long-term elevated CO_2 and N addition. Seasonal variation was minimized because the same species were measured during the same time of the year across years, just before when species achieved peak biomass. Year effects were tested via a full factorial three-way ANOVA using [CO_2], N and year as main effects. There was no significant year effect for any variables of interest or any significant interactions of [CO_2] and N with year. All further analyses of variance described below were conducted on variables averaged across years by species, plot, CO_2 and N treatment.

The BioCON experiment was designed as a split-plot with N addition nested within atmospheric CO_2 treatment (Reich *et al*., 2001b). Treatment effects were assessed using the appropriate whole-plot random effect of atmospheric CO_2 or within-plot error variances against the residual error in the *F*-test. The whole-plot random effect was not significant ($P > 0.1$) in any case. Since our goal was to investigate species within functional groups responses to the experimental treatments, as well as responses of functional groups themselves, we conducted ANOVA using main effects CO_2 level, N level, and Functional group and Species identity within functional group [denoted Spp(FunctGr) in Tables 1 and 2] to test for effects and interactions in the experiment (Table 1). The statistical significance of the functional group factor as well as the interactions involving this factor were assessed using the Spp(Funct Gr) term in the denominator of the *F*-test. Post-hoc Tukey's tests were used to examine differences among the different species. Because species responded differently to elevated CO_2 (Table 1), we further analyzed differences

Table 1 *P*-values, whole-model error mean squares (MS) and goodness of fit for an ANOVA with CO_2 treatment (CO_2), N addition treatment (N), Functional Group (Funct gr) and species within functional group [Spp(Funct gr)] as main effects, including degrees of freedom (df), for the following variables: maximum carboxylation rate (V_{cmax}), maximum electron transport rate (J_{max}), net photosynthesis in respective growth conditions e.g., either ambient or elevated [CO_2] (A_{net}), net photosynthesis at a common CO_2 level of 365 μmol mol^{-1} on an area basis (A_{a365}) and mass basis (A_{m365}) and foliage N on a mass basis (N_{mass}) and area basis (N_{area})

		P-values						
Source	df	V_{cmax}	J_{max}	A_{net}	A_{a365}	A_{m365}	N_{mass}	N_{area}*
CO_2	1	–[1]	–	<0.0001	–	–	<0.0001	–
N	1	0.016	0.023	0.067	–	–	<0.0001	<0.0001
$CO_2 \times N$	1	–	–	–	0.043	0.001	0.021	–
Funct gr	1	–	–	–	–	–	0.042	–
Spp (Funct gr)	5	<0.0001	0.0002	<0.0001	<0.0001	<0.0001	0.003	<0.0001
$CO_2 \times$ Funct gr	1	0.011	0.068	0.001	0.006	0.006	0.010	–
N × Funct gr	1	–	–	–	–	0.015	–	–
$CO_2 \times$ Spp(Funct gr)	5	0.039	0.060	–	–	0.021	0.005	–
N × Spp(Funct gr)	5	0.016	0.008	–	–	–	0.008	–
$CO_2 \times N \times$ Funct gr	1	0.022	0.016	0.081	0.059	0.001	–	–
$CO_2 \times N \times$ Spp(Funct gr)	5	–	–	–	–	–	–	–
Error MS	35–38	128.0	398.7	8.69	7.44	1465.0	3.02	0.03
Whole model R^2		0.76	0.73	0.81	0.69	0.81	0.85	0.84

[1]– Denotes that results were not significant ($P > 0.1$).

*Transformation used to meet normality assumption: Log(N_{area}−0.2).

between ambient and elevated CO_2 for each species separately, including the whole-plot random effect.

Species and functional group responses to elevated CO_2 were further explored for N-addition plots because there were significant $CO_2 \times N$ interactions (alone or in combination with functional group) for all key metrics (see Table 1), with the largest differences in responses seen in the N-addition plots. All statistical analyses were conducted in JMP 5.0.1 software, SAS Institute, Cary, NC, USA.

Results

Since the BioCON FACE experiment was designed with [CO_2] and N as the two central experimentally manipulated factors, we first focus on the main and interactive effects of these factors. We then present species and functional group effects as well as higher-order interactions of elevated CO_2 and N with these factors.

Effects of elevated CO_2 and N treatments on leaf nitrogen and photosynthesis across species

A number of photosynthetic and nitrogen-related traits varied significantly with CO_2 treatment, N addition treatment and their interaction across all seven grassland species (Table 1). As expected, foliar N concentration was increased 23% with N addition across all species (both area- and mass-based N, $P<0.0001$, Table 1, Fig. 1). Long-term elevated CO_2 exposure significantly decreased foliar N on a mass basis (-11%, $P<0.0001$, Table 1) more than on an area basis (N.S. in Table 1). However, there was a significant $CO_2 \times N$ interaction in N_{mass} ($P=0.021$, Table 1) because there was a much larger decline in N_{mass} due to elevated CO_2 treatment at high than at low N levels. There were similar trends for N_{area} to those for N_{mass}, but $CO_2 \times N$ was not statistically significant for this parameter ($P>0.10$, Fig. 1b).

With a $+200\,\mu mol\,mol^{-1}$ enrichment in [CO_2], there was a significant enhancement in realized net photosynthesis ($+41\%$ response in A_{net}, $P<0.0001$, Table 1) across all species and functional groups. In contrast, net photosynthesis in growth [CO_2] conditions (A_{net}) responded weakly to N addition ($+8\%$, $P=0.067$, Table 1). There was no significant $CO_2 \times N$ interaction for A_{net} across species. The long-term CO_2 treatment had no significant main effect on photosynthesis at a common CO_2 level (A_{365}), but showed a significant $CO_2 \times N$ interaction (Table 1; Fig. 1c and d). Both area- (A_{a365}) and mass-based (A_{m365}) photosynthesis at a common measurement [CO_2] showed CO_2 treatment-induced down-regulation under added N but not under ambient N.

As with A_{a365} and A_{m365}, V_{cmax} and J_{max}, did not differ significantly between CO_2 treatments when pooled across the different species (Table 1), in these cases because species or functional groups differed in their response to elevated CO_2 or elevated CO_2 and N. Both V_{cmax} and J_{max} increased significantly with N

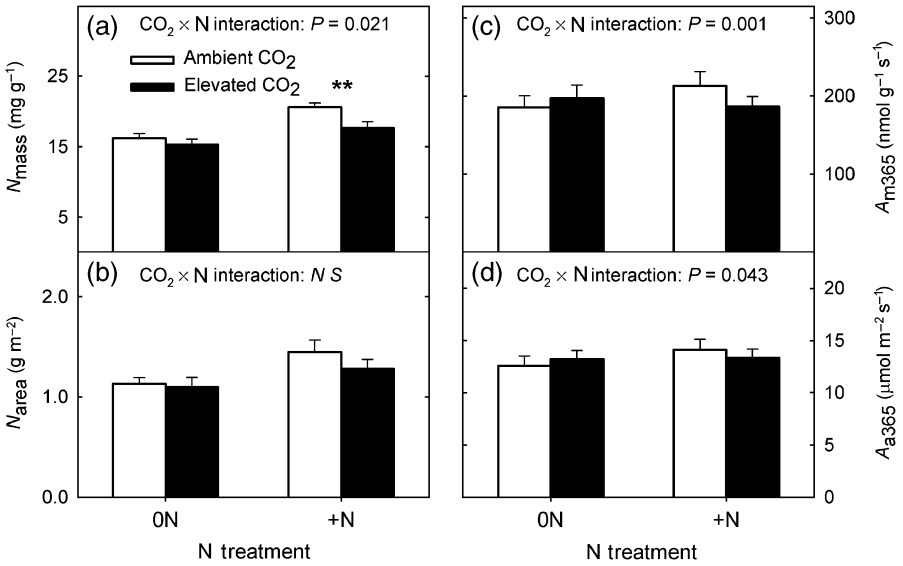

Fig. 1 Effects of elevated CO_2 and N addition treatments on (a) foliar N on a mass basis (N_{mass}), (b) foliar N on an area basis (N_{area}), (c) photosynthesis at a common CO_2 level on a mass basis, A_{m365} and (d), photosynthesis at a common CO_2 level on an area basis, A_{a365} across seven grassland species. Means in ambient CO_2 (open bars) and elevated CO_2 treatment (black bars) are shown. 0N denotes unamended plots and +N denotes N-addition plots. Sample sizes associated with the means and standard error bars in this figure varied between 6 and 8. **Represents a significant *t*-test within N-treatment of $P<0.01$.

addition (+11% and +10%, respectively, Table 1) across species.

Species effects and higher-order interactions

Species differed significantly in all measures of photosynthetic capacity and leaf N ($P \leq 0.003$, Table 1). All grass species had higher N_{mass} values than forb species, resulting in a significant functional group difference ($P = 0.042$). Across CO_2 and N treatments, species ranked similarly in V_{cmax}, J_{max}, A_{a365} and A_{net}. *S. rigida* had consistently the highest photosynthetic capacity, and *A. millefolium*, *B. inermis* and *P. pratensis* always represented the lowest three values (in descending order).

For some variables, there were significant treatment × species interactions (Table 1). There were several CO_2 × species interactions, because only *Solidago* and *Bromus* showed significantly reduced V_{cmax}, J_{max} or N_{mass} in elevated CO_2. The same physiological variables also showed significant N × Species interactions (Table 1). Species consistently responded to N addition with a significant increase in N_{mass} (11–45% increase, $P < 0.04$), except *Anemone*. For V_{cmax} and J_{max}, only *Poa* and *Anemone* showed a significant increase with N-addition.

These species differences were often consistent with functional group differences in response to elevated CO_2. Forbs reduced photosynthetic capacity and leaf N in elevated CO_2 by at least 15% ($P = 0.042$), whereas grasses did not show significant reductions in elevated CO_2. Moreover, there were significant three-way interactions of $CO_2 \times N \times$ Functional group for variables reflecting photosynthetic capacity: V_{cmax} ($P = 0.022$), J_{max} ($P = 0.016$), A_{m365} ($P = 0.001$) and A_{a365} ($P = 0.059$) (Table 1). These measures of photosynthetic capacity

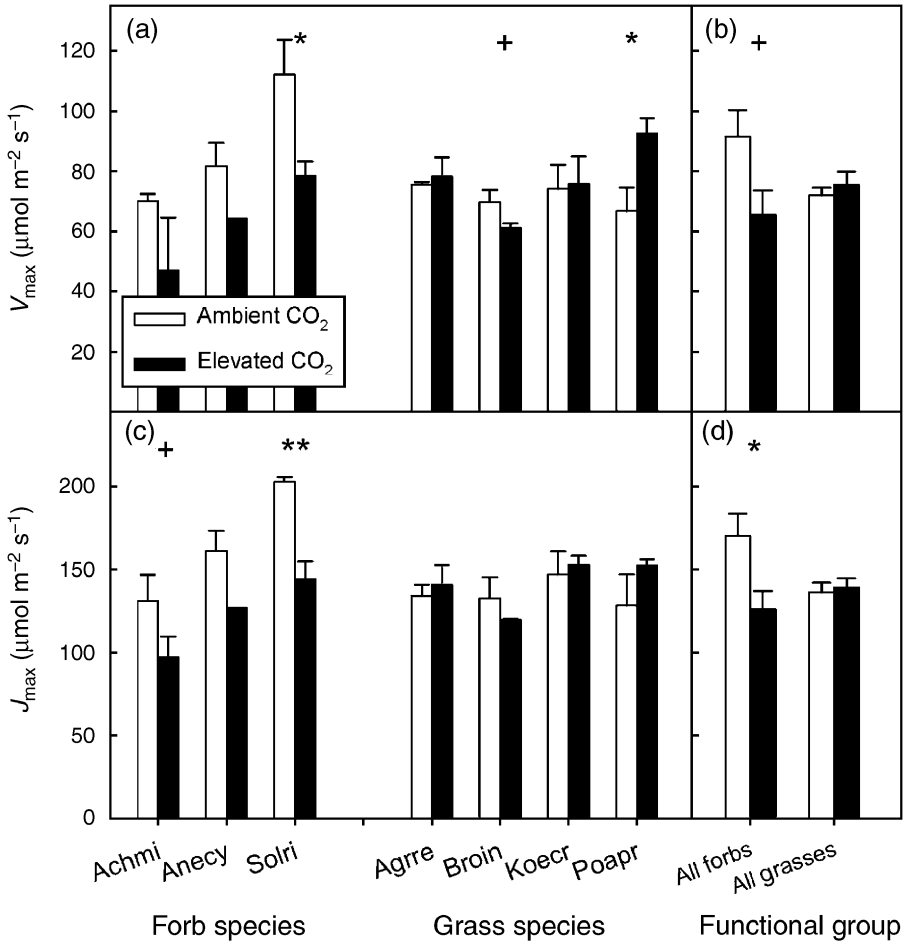

Fig. 2 Species-specific responses and standard error bars (left panels) to elevated CO_2 in N-addition plots for maximum carboxylation rate, V_{cmax} (a, b) and maximum electron transport rate, J_{max} (c, d). The aggregate functional groups responses of V_{cmax} and J_{max} to elevated CO_2 are shown at right in (b) and (d). Open bars represent the ambient CO_2 treatment and black bars are the elevated CO_2 treatment. Significant differences between CO_2 treatments within each species or functional group are represented by $^{+}P < 0.1$, $^{*}P < 0.05$, and $^{**}P < 0.01$. Samples sizes ranged from one to three for species effects (a, c) and six to eight for functional group effects (b, d).

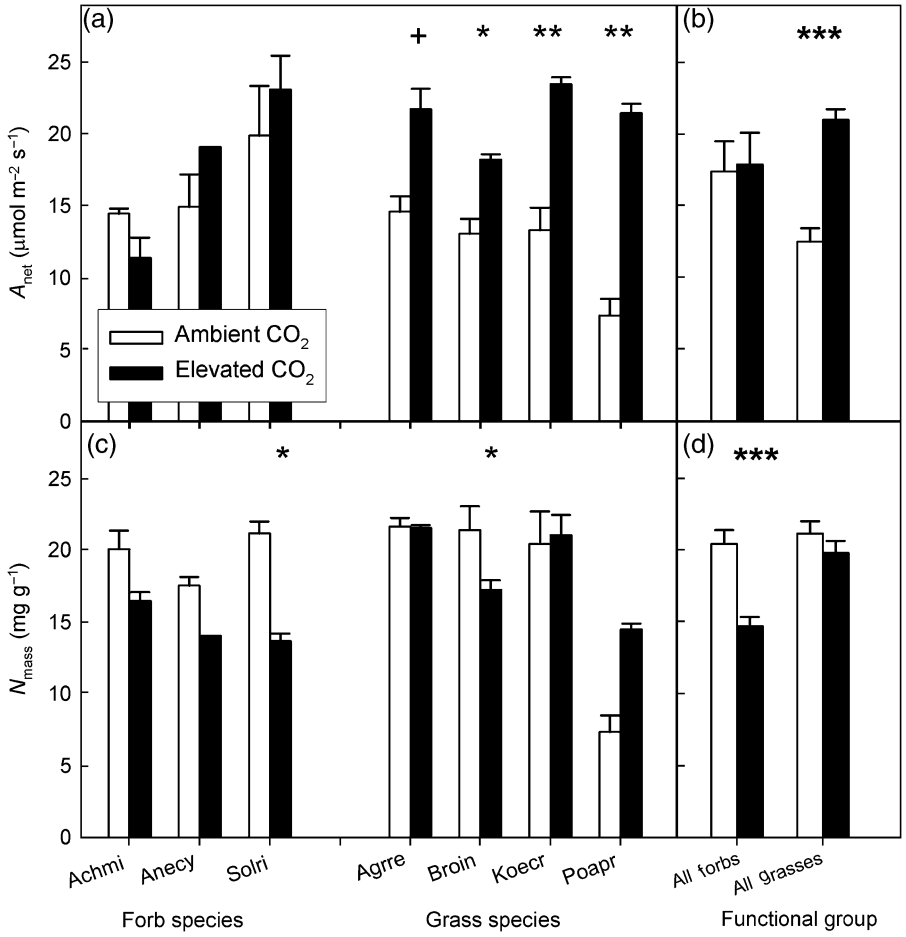

Fig. 3 Species-specific responses and standard error bars (left panels) to elevated CO_2 in N-addition plots for net photosynthesis in respective growth conditions, A_{net} (a, b) and mass-based foliage nitrogen concentration, N_{mass} (c, d). The aggregate functional groups responses of A_{net} and N_{mass} to elevated CO_2 are shown at right in (b) and (d). Open bars represent the ambient CO_2 treatment and black bars are the elevated CO_2 treatment. Significant differences between CO_2 treatments within species or functional group are represented by $^{+}P<0.1$, $*P<0.05$, and $**P<0.01$. Samples sizes ranged from one to three for species effects (a, c) and six to eight for functional group effects (b, d).

were generally reduced by elevated CO_2 more in forbs than grasses (Table 1, two-way interactions). Moreover, these reductions were more pronounced in N addition treatments (Table 1: three-way interactions, Fig. S1 in Supporting information), and hence were examined in more detail. Also, examining the CO_2 responses of different functional groups in the N-addition plots provides insight into $CO_2 \times$ functional group interactions that are difficult to visualize as three-way interactions with N.

Elevated CO_2 responses of functional groups under N addition

Under added N conditions, the response of photosynthetic capacity to elevated CO_2 varied among functional groups (significant $CO_2 \times$ Functional group interactions). The three variables that best reflect photosynthetic capacity (V_{cmax}, J_{max} and A_{m365}) were all reduced in all forb species in response to elevated CO_2 (by $>25\%$), whereas grasses showed no change in these variables in elevated CO_2 (Fig. 2). These effects were generally consistent among species within each group (Fig. 2a) and hence represent functional group differences (Fig. 2b and d). *Poa*, *Koeleria* and *Agropyron* did not show down-regulation of photosynthetic capacity in elevated CO_2 ($V_{cmax} = +39\%$, $+2\%$ and $+4\%$ enhancement respectively) under N addition (Fig. 2a) while *Bromus* showed a -10% change in V_{cmax} (Fig. 2a). In contrast, *Achillea*, *Anemone* and *Solidago* all had lower V_{cmax} in elevated CO_2 ($P<0.1$ across all forb species; -33%, -21%, -30%, respectively) in N addition plots

Table 2 *P*-values, whole-model error mean squares (MS) and goodness of fit for an ANOVA with CO_2 treatment (CO_2), N addition treatment (N), Functional Group (Funct gr) and species within functional group [Spp(Funct gr)] as main effects, including degrees of freedom (df) for total root biomass and root mass fraction across years 2003–2006

		P-values		
Source	df	Total root biomass (0–20 cm) ($g\,m^{-2}$)	Root mass fraction ($g\,g^{-1}$)	Total fine root biomass (0–20 cm) ($g\,m^{-2}$)
CO_2	1	–[1]	–	–
N	1	0.001	0.046	0.001
$CO_2 \times N$	1	–	–	–
Funct gr	1	0.028	–	0.050
Spp (Funct gr)	5	<0.0001	<0.0001	<0.0001
$CO_2 \times$ Funct gr	1	–	0.027	0.089
N × Funct gr	1	0.032	–	–
$CO_2 \times$ Spp(Funct gr)	5	–	–	–
N × Spp(Funct gr)	5	–	–	–
$CO_2 \times N \times$ Funct gr	1	–	–	–
$CO_2 \times N \times$ Spp(Funct gr)	5	–	–	–
Error MS	23	12.7	0.016	145.0
Whole Model R^2		0.93	0.83	0.93

All variables were transformed to meet normality assumptions for ANOVA: a square root transformation was used for the total root biomass and root mass fraction ratio was power transformed, fine root biomass was log and power transformed.
[1]– Denotes that results were not significant ($P > 0.1$).

(Fig. 2a). In the N-enriched treatment, the larger magnitude of down-regulation in forbs vs. grasses resulted in no significant enhancement of net photosynthesis in elevated CO_2 for forbs in contrast to a sizeable enhancement for the grasses (Fig. 3b).

We found similar trends in N_{mass} to those for photosynthetic capacity. A significant $CO_2 \times$ functional group interaction on leaf N_{mass} ($P = 0.01$) showed that elevated CO_2 negatively affected the leaf N concentration in forbs but not in grasses (Table 1). Under added N, N_{mass} in forb leaves was 26% lower in elevated CO_2 ($P = 0.0004$) compared with ambient CO_2 (range among species within this group of −18% to −35%; Fig. 3c and d). In contrast, there was no consistent CO_2 effect on N_{mass} among grass species (Fig. 3c and d), though *Bromus* did in fact show a decrease of 19% ($P = 0.013$). Thus, leaf N concentrations were reduced strongly in forbs when exposed to elevated CO_2, whereas grasses were more generally able to maintain leaf N concentrations in elevated CO_2.

Root biomass allocation patterns in forb and grass species

In an attempt to gain perspective on the observed elevated CO_2 responses in N-addition plots, we examined biomass allocation patterns in the seven species in this study because non N-fixing species gain most required N from the soil. Root mass fraction (total root biomass/total biomass) was calculated for each species and treatment across the sixth to ninth growing season of elevated CO_2 exposure (Table 2). Although there were no significant CO_2 treatment differences in total root biomass, fine root biomass and root mass fraction, all increased in N-addition plots compared with unamended plots across species ($P = 0.0009$, 0.0008 and 0.046, respectively, Table 2). In addition, there was a strong functional group effect in which C_3 grasses showed higher total root biomass (Fig. 4a, $P = 0.028$, Table 2) and fine root biomass ($P = 0.050$, Fig. 4b) compared with the forb species, and this was especially pronounced in N-addition plots (Table 2, Fig. 4a and b). Root mass fraction also showed a significant interaction between elevated CO_2 and functional group ($P = 0.027$, Table 2). Whereas root mass fraction was no different between forbs and grasses in ambient CO_2, forbs decreased root mass fraction significantly (−28%) in response to elevated CO_2 and grasses did not (Fig. 4c). Grasses always had higher fractional distribution of biomass to roots compared with forbs (Fig. 4d), and these patterns remained regardless of elevated CO_2 treatments or N addition.

Discussion

We investigated differential responses in photosynthetic capacity of C_3 species from two grassland functional groups to elevated atmospheric CO_2 and N addition to understand their long-term responses to global eutro-

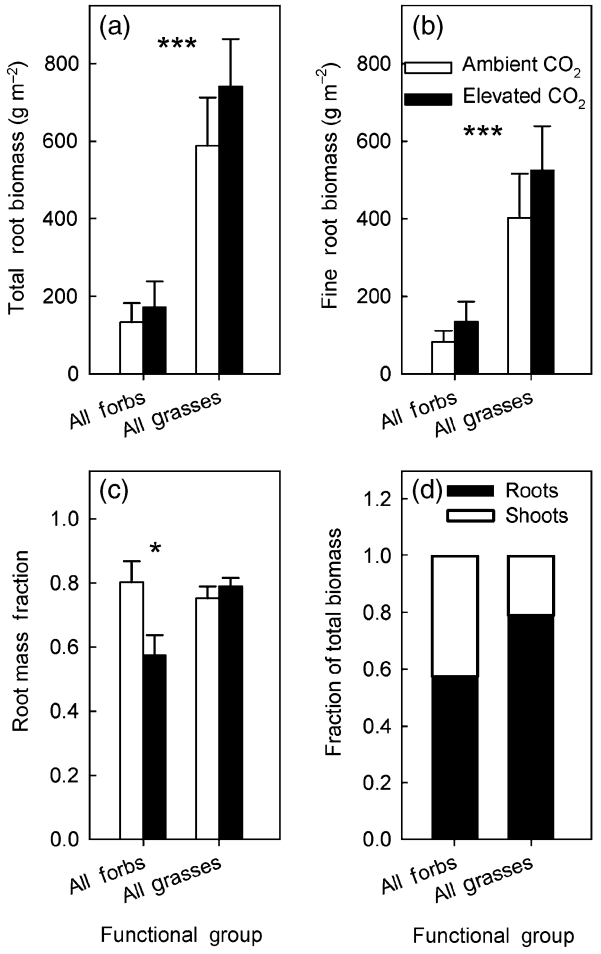

Fig. 4 Means with standard error of total root biomass (a), total fine root biomass (b) and root mass fraction (c) for each functional group (e.g. all C_3 grasses and all non-leguminous forbs) in N-addition plots in ambient [CO_2] (open bars) and elevated [CO_2] (black bars). Stars indicate significant differences between functional groups: $^*P<0.05$ and $^{***}P<0.001$. Sample sizes were $n=6$ for forbs and $n=8$ for grasses. The fraction of total biomass (d) is shown across N-addition and elevated CO_2 treatment because there were no differences in allocation with regard to those treatments within each functional group.

phication. Such differential responses could be ecologically important if they influence species productivity and subsequent species dynamics (Joel *et al*., 2001; Zavaleta *et al*., 2003a; Niklaus *et al*., 2007). C_3 forb responses to elevated CO_2 have been compared previously with those of grasses in a number of experiments (Knapp *et al*., 1996; Anderson *et al*., 2001; Morgan *et al*., 2001). However, few of these experiments have examined such responses under combinations of elevated CO_2 and enhanced N supply, or for time periods longer than a few years. In our study, after 6–9 years of elevated CO_2 exposure, significant $CO_2 \times N$ interactions were observed across species for photosynthetic capacity (A_{m365}) and leaf nitrogen (N_{mass}). These interactions indicated that elevated CO_2 induced stronger down-regulation of photosynthetic capacity and its related variables when N availability was higher (Fig. 1). Moreover, we observed strong differences in photosynthetic responses to elevated CO_2 between functional groups, and these were especially pronounced under higher N supply. Forbs showed strong and consistent photosynthetic down-regulation that eliminated the elevated CO_2 fertilization effect on photosynthesis. In contrast, C_3 grasses maintained a substantial photosynthetic stimulation even after 9 years of elevated CO_2 exposure.

In our study, reduced rates of carboxylation and electron transport, and less realized photosynthetic enhancement in elevated CO_2, correlated with a reduction in leaf N concentration in elevated CO_2. In species or functional groups in which reductions in leaf N were observed under elevated CO_2, photosynthetic down-regulation followed. The leaf N responses to long-term elevated CO_2 appear to drive down-regulation of photosynthetic capacity. Because most nitrogen is invested in photosynthetic components, the CO_2-induced reduction in N_{mass} resulted in no significant CO_2-;induced enhancement of realized photosynthesis in forb species receiving N addition. Based on these results, we conclude that our first hypothesis – that down-regulation is of a similar magnitude as, and therefore can eliminate, the CO_2 fertilization effect – was supported. However, it was only true for one functional group, the C_3 forbs, and was not supported at all for the C_3 grasses. In contrast, the second hypothesis – that the down-regulatory process would be ameliorated by enhanced N supply – was surprisingly countered by the data, in opposition to general support for enhanced productivity responses to elevated CO_2 with greater N supply (Reich *et al*., 2006b). Our observation of less photosynthetic enhancement from elevated CO_2 under N enrichment than ambient N supply was observed in forbs but not in grasses.

Despite the photosynthetic down-regulation responses to elevated CO_2 reported here, positive biomass responses (and predominantly for roots) to elevated CO_2 and N addition were reported early in this experiment by Reich *et al*. (2001a, b) in C_3 species, including both C_3 grasses and perennial forbs. However, the present study provides evidence of a strong increase in root biomass in response to N addition for the grasses but not for the forb species (Table 2). Even though these species share the same photosynthetic pathway, they have different growth forms and rooting patterns which have implications for resource uptake and allocation. It has also been argued that responses to N deposition and

N addition to soils are different between these species groups (Stevens *et al*., 2006), consistent with our results. Our results showing reduced photosynthesis in forbs in response to elevated CO_2 and N addition (Fig. 3) are also consistent with Reich *et al*. (2004) where forbs showed a 12% biomass reduction to N addition but grasses showed larger biomass increases (by 20%). The total plant N pool increased strongly in grasses and there was no response in forbs, suggesting forbs did not take up the additional N supply (Reich *et al*., 2004). Our findings are also consistent with species-specific data, in which two of the three forb species (*Achillea* and *Anemone*) showed greater biomass enhancement due to elevated CO_2 in ambient N than in N-addition plots across a larger set of grassland species and species mixtures (data not shown).

Although stimulation of photosynthesis in elevated CO_2 is still possible with reduced leaf N concentrations (Ainsworth & Long, 2005), this N-redistributing mechanism likely does not provide all plant growth demands for N in elevated CO_2 (BassiriRad *et al*., 2001; Hungate *et al*., 2003). This may even be the case when additional N is supplied, if the plants cannot take advantage of the additional resources. Plants may not be able to take up the additional N due to increased immobilization of N in elevated CO_2 (de Graaff *et al*., 2006; Finzi *et al*., 2006; Holmes *et al*., 2006; Hungate *et al*., 2006; Knops *et al*., 2007), lack of mycorrhizal colonization of roots (Hartnett & Wilson, 1999), or increased N leaching in N addition plots (Hobbie, 1992; Dijkstra *et al*., 2007). Leaching of dissolved inorganic nitrogen was especially apparent in forb and legume monocultures at our site (Dijkstra *et al*., 2007).

We observed a strong increase in leaf N and a weaker increase in net photosynthesis to N addition across species. Grasses responded to N addition with a 15% increase in net photosynthesis whereas forbs did not respond to N addition. This is consistent with a study at the same site that found that soil solution N concentration was close to zero underneath grass monocultures, whereas it was about 60% underneath forb species (Reich *et al*., 2004), indicating that grasses forage strongly for available soil N whereas forb monocultures do not.

If plant N demand exceeds N supply, then the stimulated growth response in elevated CO_2 is likely not sustainable (Luo *et al*., 2004; Gill *et al*., 2006) as indicated by a recent review of $CO_2 \times N$ interactions in long-term field studies (Reich *et al*., 2006b). Increased fine root growth is a potential way to access more soil nitrogen in elevated CO_2 (BassiriRad *et al*., 2001). Fine root biomass as well as specific root length was significantly smaller in forbs compared with grasses grown under a combination of elevated CO_2 and N addition (Craine *et al*., 2002). Typical interpretations of specific root length would suggest that forbs do not have as much root absorption or N acquisition capacity as grasses. Smaller root biomass combined with smaller specific root length suggests that forbs might not be able to exploit the soil as efficiently as grasses do. Forbs have a greater fraction of biomass in aboveground components ($>50\%$) whereas in grasses more than 75% of total biomass is found in the roots. Therefore, differences in access to soil resources due to different root morphology, root biomass distribution, and total root biomass between forbs and grasses (Fig. 4d) likely affected the CO_2 responsiveness in these functional groups, in particular the ability to maintain leaf N and avoid down-regulation of photosynthetic capacity by grasses but not by forbs.

Another way to increase soil exploitation is via mycorrhizal symbiosis. In a grassland study, Hartnett & Wilson (1999) observed large increases in forb biomass with mycorrizal symbiosis, compared with no increase in biomass with mycorrhizae in C_3 grasses. While mycorrhizal colonization is generally increased in elevated CO_2 (Gamper *et al*., 2004), many studies have clearly found lower mycorrhizal colonization in N-fertilized plots (Högberg *et al*., 2003; Johnson *et al*., 2003; Blanke *et al*., 2005; Egerton-Warburton *et al*., 2007). Therefore, it is possible that these antagonistic effects may be more detrimental to forbs than grasses, accounting for the reductions in leaf N in forbs and down-regulation of photosynthesis that was observed. Grasses, which are less dependent on mycorrhizal symbiosis due to their large root systems (Wilson & Hartnett, 1998; Craine *et al*., 2002), may be able to maintain their leaf N and photosynthetic enhancement in elevated CO_2 and N-added plots. Different root morphology as well as reliance on mycorrhizal colonization between C_3 grasses and forbs may provide insight into relationships between plant community structure, species diversity and ecosystem functioning in species-diverse grasslands (van der Heijden *et al*., 2006).

Resource differentiation is a mechanism underlying niche complementarity (Tilman, 1986) which helps maintain biodiversity because species with similar resource requirements access resources differently in space or time. The biodiversity of grasslands may not be maintained in conditions of elevated CO_2 and N deposition. Although earlier studies found increased forb biomass in response to elevated CO_2 (Leadley *et al*., 1999; Teyssonneyre *et al*., 2002; Polley *et al*., 2003), reduced biomass and relative abundance in forbs has been found in elevated CO_2 (Reich *et al*., 2001b; Zavaleta *et al*., 2003b; Niklaus & Korner, 2004) and with N

deposition (Stevens *et al*., 2006; Clark & Tilman, 2008). The significant three-way interaction in this study (Table 1) shows that forbs were negatively affected in elevated CO_2 compared with grasses, and this was exacerbated in high N conditions.

Zavaleta *et al*. (2003b) reported reduced species richness at the Jasper Ridge grassland site with a combination of elevated CO_2 and N deposition, due largely to poor performance of the forbs. Thus, if these kinds of shifts in the competitive balance of grasses and forbs commonly occur, it may lead to less diverse grasslands dominated by graminoids in the elevated [CO_2] of the future. Lower grassland diversity could also be a consequence of reduced association with mycorrhizae in the forbs grown in high N conditions, inducing a shift towards more C_3 dominated grasslands (Egerton-Warburton *et al*., 2007). However, empirical evidence for such shifts is still sparse (but see Thomas *et al*., 2004) and other grasslands might respond differently due to resource limitations other than nitrogen, particularly in low-rainfall zones (Morgan *et al*., 2004).

Although species responses to elevated CO_2 and N addition treatments in this study were individualistic, there were also strong functional group responses (Figs 2 and 3). Our goal was to examine whether and how differences between forbs and C_3 grasses yield different functional group responses to a combination of elevated CO_2 and N addition. Our results suggest that differences in resource acquisition might drive differences in CO_2 responsiveness in these temperate grassland species. Therefore, functional group responses to climate change perturbations could be useful for modeling responses and feedbacks to ecosystem C-cycling, even when not predictive of species-specific responses (Zavaleta *et al*., 2003b). That species and functional group traits like leaf N and functional group photosynthetic characteristics and whole-plant responses may be related to community-level responses argues for further work evaluating mechanistic links between ecophysiological and community level processes in order to predict the direction and magnitude of environmental change to ecosystem functioning and composition (Suding *et al*., 2008).

Acknowledgements

Many thanks to Dan Bahauddin for technical support during fieldwork. S. Patterson, K. Sargeant and J. Mascaro are thanked for field and lab assistance and the many interns at BioCON are also recognized for their crucial role in maintaining the plots. BioCON was supported by NSF LTER (DEB 0080382) and Biocomplexity (0322057) program awards.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Functional group responses and standard error bars to elevated CO_2 in ambient N plots (Natural N, left panels) and N-addition plots (Amended N, right panels) for for maximum carboxylation rate, V_{cmax} and maximum electron transport rate, J_{max}.net photosynthesis in respective growth conditions, A_{net} and mass-based foliage nitrogen concentration, N_{mass}. Significant differences between CO_2 treatments within species or functional group are represented by $^{+}$ for $P<0.1$, * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$.

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