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Photosynthetic responses of 13 grassland species across 11 years of free-air CO_2 enrichment is modest, consistent and independent of N supply

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Abstract

If long-term responses of photosynthesis and leaf diffusive conductance to rising atmospheric carbon dioxide (CO_2) levels are similar or predictably different among species, functional types, and ecosystem types, general global models of elevated CO_2 effects can effectively be developed. To address this issue we measured gas exchange rates of 13 perennial grassland species from four functional groups across 11 years of long-term free-air CO_2 enrichment (eCO_2, + 180 ppm above ambient CO_2) in the BioCON experiment in Minnesota, USA. Eleven years of eCO_2 produced consistent but modest increases in leaf net photosynthetic rates of 10% on average compared with plants grown at ambient CO_2 concentrations across the 13 species. This eCO_2-induced enhancement did not depend on soil N treatment, is much less than the average across other longer-term studies, and represents strong acclimation (i.e. downregulation) as it is also much less than the instantaneous response to eCO_2. The legume and C3 nonlegume forb species were the most responsive among the functional groups (+ 13% in each), the C4 grasses the least responsive (+ 4%), and C3 grasses intermediate in their photosynthetic response to eCO_2 across years (+ 9%). Leaf stomatal conductance and nitrogen content declined comparably across species in eCO_2 compared with ambient CO_2 and to degrees corresponding to results from other studies. The significant acclimation of photosynthesis is explained in part by those eCO_2-induced decreases in leaf N content and stomatal conductance that reduce leaf photosynthetic capacity in plants grown under elevated compared with ambient CO_2 concentrations. Results of this study, probably the longest-term with the most species, suggest that carbon cycle models that assume and thereby simulate long-lived strong eCO_2 stimulation of photosynthesis (e.g. > 25%) for all of Earth's terrestrial ecosystems should be viewed with a great deal of caution.

Keywords: BioCON, Cedar Creek, CO_2 by N effects, elevated CO_2, functional groups, global change, grassland species, leaf-level physiology, photosynthesis, photosynthetic acclimation

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Introduction

Given the regulatory role of leaf photosynthesis and stomatal conductance in plant responses to atmospheric carbon dioxide (CO_2) concentrations, it is important to characterize the long-term consequences of rising CO_2 on these fundamental processes (Ainsworth & Rogers, 2007) and to determine the influence of other resources, such as soil nitrogen (N) (Körner, 2006; Crous *et al.*, 2010). This is especially so given that elevated CO_2-induced enhancement of productivity is considered likely to slow down climate warming by constraining rising atmospheric CO_2 concentrations, and that the future size and persistence of elevated CO_2-induced enhancement effects are among the largest uncertainties in global carbon cycle science (IPCC, 2007; Arneth *et al.*, 2010). However, with so few field studies of suitable duration (Ainsworth *et al.*, 2003; Naumburg *et al.*, 2003; Tricker *et al.*, 2005), and findings that show substantial variation in plant responses to eCO_2 that depend on species and functional group identity (Naumburg *et al.*, 2003; Ainsworth & Rogers, 2007; Crous *et al.*, 2010), questions concerning the magnitude and persistence of an elevated CO_2 (eCO_2)-induced stimulation of leaf photosynthesis remain unresolved. This is an important information gap for society, as knowing whether rising atmospheric CO_2 levels stimulate a long-term and persistent stimulation of photosynthesis and whether responses to eCO_2 are similar or predictably different among species, functional types, and ecosystem types is critical to the development of reliable coupled climate-carbon cycle models needed to predict future atmospheric CO_2 concentrations.

CO_2 and N likely interact at multiple levels in affecting plant responses to eCO_2 concentrations (Reich *et al.*,

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2006a, b). As CO_2 limitations on leaf net photosynthesis are eased under eCO_2, lack of available N in settings that are frequently N-limited may lead to greater constraints on the CO_2 response than when N is in ample supply (Hungate *et al*., 2003; Reich *et al*., 2006a, b). Few long-term field studies manipulate both CO_2 and N. To our knowledge only three such studies with plant communities have been conducted for more than 4 years, and all show at least some responses that differ depending on the levels of these treatments, but not always similarly among studies (Shaw *et al*., 2002; Schneider *et al*., 2004; Reich *et al*., 2006a, b). The results of these scarce long-term multifactorial experiments reveal the complexity with which global change factors interact to affect vegetation, and highlight the limits of our knowledge.

Recent reviews focusing on free-air CO_2 enrichment (FACE) studies have found that in plants grown under eCO_2, light-saturated photosynthesis increases an average of 26% (Nowak *et al*., 2004) or 31% across C3 species specifically (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007). The magnitude of eCO_2-induced stimulation of photosynthesis varies with system, species, functional groups, environmental conditions and the availability of other resources (Ainsworth & Rogers, 2007). For example, trees have shown the largest increases in net photosynthesis in response to growth at eCO_2 (an average of 45%) while grass species with the C4 photosynthetic pathway show little to no response. Additionally the eCO_2-induced stimulation of photosynthesis across species is generally greater (by 23% on average) in studies considered to have been made at high compared with low N (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007), which is consistent with greater productivity responses to eCO_2 at high compared with low *N* (Reich *et al*., 2006a, b). The responses of stomatal conductance to eCO_2 appear to be less variable across species and ecosystems, with reductions averaging 22% in leaves grown under eCO_2 compared with ambient CO_2 conditions (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007).

The 26–31% enhancement of photosynthesis reported (above) in response to long-term exposure to eCO_2 is approximately 20–25% less than would be predicted based on theoretical understanding of photosynthetic biochemistry and empirical observations of short-term (minutes) responses to increasing CO_2 concentrations (e.g., Farquhar & von Caemmerer, 1982; Lee *et al*., 2001). The magnitude of this long-term, leaf photosynthetic acclimation to eCO_2, which results from physiological adjustments that occur in response to growth at eCO_2, has also been found to vary depending on environmental factors such as nutrient and water availability and temperature. The acclimation of photosynthetic capacity to eCO_2 has been shown to be associated with concomitant decreases in leaf N (Lee *et al*., 2001; Ellsworth *et al*., 2004; Ainsworth & Long, 2005; Crous *et al*., 2010), reduced leaf enzyme content and activity (i.e. Rubisco), limited capacity for regeneration of the Rubisco substrate, ribulose bisphosphate (RuBP), reduced rates of electron transport (Rogers & Humphreys, 2000; Ainsworth *et al*., 2003), potential stomatal limitations on CO_2 uptake (Lee *et al*., 2001), and source-sink imbalances (Isopp *et al*., 2000; Rogers & Ainsworth, 2006).

The objective of this study was to evaluate the long-term (11 years) temporal patterns of leaf photosynthetic responses to free-air CO_2 enrichment ($\approx +180$ ppm above ambient CO_2) and increased soil N ($+4\,g\,N\,m^{-2}\,yr^{-1}$) in a long-term perennial grassland FACE experiment, BioCON, in Minnesota, USA. A companion study from BioCON (Crous *et al*., 2010) addressed some similar questions but with differences in approach that make the two studies highly complementary. Crous *et al*. (2010) studied seven of the BioCON species during a 1- to 4-year period using CO_2 response curves, allowing a greater quantification of photosynthetic parameters than in the current study. However, as none of the species could be studied comprehensively across the 4 years, Crous *et al*. (2010) were unable to assess patterns or consistency of responses over time. Thus, the present study expands on that prior work by examining 13 of the study species (providing better resolution of functional group responses) and by making measurements for 11 consecutive years.

Our goal was to quantify leaf photosynthetic responses to long-term eCO_2 of 13 grassland species representing four functional groups of plants while considering the potentially interactive effects of soil N availability and time. To our knowledge, eCO_2-induced changes in photosynthesis were documented over a longer term and for more species than in any other study, thus providing insights into important long-term ecosystem responses. The responses of other related variables (such as leaf N, leaf stomatal conductance and water and N-use efficiencies) of plants grown under long-term treatments of CO_2 and N were also investigated to collectively address the following questions: (1) To what degree does leaf-level physiology respond to eCO_2, do responses converge or diverge over 11 years, and how do the responses of these native/naturalized species in this N-limited grassland compare with other species/systems?; (2) Is the eCO_2-induced stimulation in photosynthesis greater with an increase in soil N supply or does an interaction between CO_2 concentration and soil N availability develop over time (as feedbacks might lead to N limitations)?; (3) Do species respond in predictably different ways that can

be related to their unique functional attributes such as C4 photosynthesis or symbiotic N_2 fixation?

Materials and methods

Research site

The study site is located at the Cedar Creek Ecosystem Science Reserve in east central Minnesota, USA (latitude 45°N, longitude 93°W). The soils are sandy, derived from a glacial outwash sandplain, and plant growth is nitrogen-limited generally at the site and specifically in this experiment (Reich *et al*., 2001, 2006a). Cedar Creek has a continental climate with cold winters (mean January temperature = −11 °C), warm summers (mean July temperature = 22 °C), and mean annual precipitation totaling 660 mm yr^{-1}. The average daily maximum temperature and average precipitation for days before measurements (including day of measurement) and for each growing season (April–September) are presented in Table 1.

Experimental design and the FACE system

The overall experiment, referred to as BioCON (Biodiversity, CO_2, and Nitrogen effects on ecosystem functioning, http://www.biocon.umn.edu), was established in 1997 on secondary successional grassland after removing previous vegetation (*Reich et al*., 2001). The study site consists of six circular areas (20-m diameter), each containing sixty-one 2 × 2 m plots. The main experiment uses 296 plots and has treatments arranged in factorial combination of CO_2 concentration (ambient and ambient + 180 μmol mol^{-1}), soil N supply [ambient (low) or enriched (+ 4 g N m^{-2} yr^{-1})], and species richness (planted with 1, 4, 9 or 16 species). For this study we sampled only in monocultures. Each species in monoculture is replicated twice for every CO_2 × N level. The design consisted of a split-plot arrangement of treatments in a randomized design with CO_2 treatment as the whole-plot factor, which is replicated three times among the six rings. The subplot factor of soil N treatment was randomly assigned to individual plots among the six rings. CO_2 was applied using free-air CO_2 enrichment (FACE) technology (*Lewin et al*., 1994) during all daylight hours during the growing season (early April to early November on average). Across all years, 93% of 1-minute CO_2 concentration averages were within 10% of the target value. The high N plots were amended with 4 g N m^{-2} yr^{-1}, as ammonium nitrate (NH_4NO_3) in solid form, divided evenly among three applications each year (May, June and July). Monoculture plots of 13 species, representing four functional classifications of plants based on similarities in physiology and growth forms: C_3 grasses, C_4 grasses, legumes, and nonlegume forbs, were chosen for leaf-level physiological measurements in this study. See Table 1 for specifics concerning measurements dates and average conditions. Not all 13 species were measured each year due to time or instrument constraints, changing persistence in plots, or rare pest issues in a few cases. The 13 species include: C_3 grasses: *Agropyron repens* (L.) Beauv., *Bromus inermis* Leyss., *Koeleria cristata* Pers.; C_4 grasses: *Andropogon*

Table 1 Range of dates, number of days and species measured each year in monoculture plots. Weather data include total precipitation in May-June, average amount of precipitation for the 3-days prior and average daily maximum temperature the week before and including the day of measurements. Not all species were measured each year, and number of measurement days varied due to variable weather

Year	*N*	Date range	Total days measured	Total precipitation (May–June, mm)	Average amount of precipitation 3 days before measurement (mm)	Average maximum air temp week before measurement (°C)	C3 grasses			C4 grasses			Nonlegume forbs			Legumes			
							Agropyron	*Bromus*	*Koeleria*	*Andropogon*	*Schizachyrium*	*Sorghastrum*	*Achillea*	*Anemone*	*Solidago*	*Amorpha*	*Lespedeza*	*Lupinus*	*Petalostemum*
1998	331	6/26–7/25	12	245	13	28	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	
1999	379	6/12–7/23	15	302	10	27	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓
2000	352	6/12–7/21	15	253	12	26	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		
2001	405	5/30–8/14	21	304	7	26	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2002	244	5/23–8/8	17	382	14	25	✓	✓		✓		✓	✓		✓		✓	✓	✓
2003	305	6/9–7/18	14	327	3	26	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2004	214	5/26–7/22	5	339	2	23	✓		✓	✓		✓		✓	✓		✓	✓	✓
2005	141	5/31–8/10	8	311	3	27			✓	✓		✓			✓		✓	✓	
2006	266	5/17–7/12	14	111	2	25	✓	✓		✓	✓	✓	✓		✓	✓	✓	✓	✓
2007	168	5/17–6/27	10	140	3	27	✓	✓		✓	✓				✓		✓	✓	
2008	168	6/16–7/19	4	181	6	24	✓	✓		✓		✓			✓		✓	✓	

gerardii Vit., *Schizachyrium scoparium* (Mich.) Nash, *Sorghastrum nutans* (L.) Nash; nonlegume forbs: *Achillea millefolium* L., *Anemone cylindrica* A. Gray, *Solidago rigida* L.; and legumes: *Lupinus perennis* L., *Lespedeza capitata* Mich., *Amorpha canescens* Pursh, *Petalostemum villosum* Nutt. [synonym *Dalea villosa* (Nutt.) Spreng]. Species hereafter are referred to by their genus (Table 1).

Gas exchange and leaf nitrogen

In situ rates of leaf net photosynthesis (A) were measured using CIRAS-1 portable infrared gas exchange systems (PP Systems, Hitchin, UK) operated in open-configuration with controlled temperature, CO_2 concentration, and vapor pressure. Measurements were made on an upper fully expanded leaf of an individual plant representing each monoculture plot, typically between 09:00 and 15:00 local time. Since leaf traits vary with age, all measures were made using leaves of similar ontogenetic stage. We used upper fully expanded young to mid-aged leaves, which correspond to the period when many leaf traits are relatively stable (Reich *et al*., 1991). Gas exchange rates of individual leaves of each species were measured on sunny days between 17 May and 14 August, depending on the year (Table 1). Each of two replicate plots representing each of the four $CO_2 \times N$ treatment combinations was sampled three to four times each year. These multiple samplings of each plot were measured on separate days, for separate plants, at random time points, and were averaged for each replicate plot to incorporate day to day and within day variation in environmental conditions. We checked for any systematic time of day effect by looking for interactions between treatment and time of day, using hourly bins. There was no such interaction for net photosynthetic rates but there was an interaction between time of day $\times$ CO_2 for stomatal conductance. However, this explained a very small fraction of the variance compared with the main effect of CO_2 (data not shown) and all treatment combinations were well represented in each hourly bin; moreover, at every measurement hour, there was a strong negative impact of eCO_2 on g_s and the presented results show the average of those. Rates were determined at or near light-saturating conditions [mean photosynthetically active radiation $\pm$ SE: $1665 \pm 6\,\mu mol\,m^{-2}\,s^{-1}$, across all measurements], at $25.0 \pm 0.04\,^{\circ}C$, near ambient humidity [mean chamber vapor pressure deficit (VPD) $\pm$ SE: 1.43 ± 0.01], and at approximately the CO_2 concentrations under which the plants were grown (ambient or ambient $+180\,\mu mol\,mol^{-1}$).

The projected areas of leaves used in gas exchange measurements were determined using a digital image analysis program (WINRHIZO 3.9, Regent Instruments, QC, Canada, SCION IMAGE or IMAGEJ, National Institutes of Health, Bethesda, MD, USA). Leaves were then oven-dried ($65\,^{\circ}C$) to determine dry mass and specific leaf area (SLA, $cm^{-2}\,g^{-1}$). Each leaf used in gas exchange measurements was ground and analyzed for tissue nitrogen concentration with a C-N Analyzer (NA1500, Carlo-Erba Instruments or ECS 4010, COSTECH Analytical Technologies Inc., Valencia, CA, USA). Intrinsic instantaneous water-use efficiency (A_{area}/g_s) and photosynthetic N-use efficiency (PNUE, A_{area}/N_{area}) were derived from gas exchange and tissue N data.

Data analysis

For most of the results presented, we use a repeated measures analysis of variance (ANOVA), in which the main effect of functional group (3 df) was tested against the random effect of species nested within functional group (since not all of the same species were measured in each year, Table 1). All treatment effects were considered fixed. Using F-tests, the effects of CO_2 (1 df) was always tested against the random effect of ring nested within CO_2 (4 df). The main effects of N (1 df) and all interactions among CO_2, N and functional group were tested against the random effect of plot nested within these treatments. The main effect of year (10 df) and all remaining interactions were tested against the residual error. As the philosophy of the experiment was to provide a broad examination of responses across and within functional groups many species were sampled, reducing power to assess individual species. Hence we focus little on comparing individual species, and do so within the context of the overall full repeated measures ANOVA (see Table 2).

In all cases, data from three or four individual leaves from different plants, each measured on a separate day, were averaged for each replicate plot to provide an estimate of plot-scale response to the treatment combinations within each year. Therefore, plot was the experimental unit used in repeated measures ANOVA including year in the model. Data from the first 2 of the 11 years presented were used in a prior publication (Lee *et al*., 2001); they are included herein in new, different, and expanded analyses. All data were checked whether they fit the normality and homoscedasticity assumptions of ANOVA and when clearly necessary, data were ln transformed. All analyses were conducted with statistical analysis software (JMP Version 7, 2007, SAS Institute Inc., Cary, NC, USA).

Results

Eleven years of free-air carbon dioxide enrichment (eCO_2) produced modest increases in light-saturated leaf net photosynthetic rates of 10% on average across 13 grassland species compared with plants grown at ambient CO_2 concentrations (CO_2 effect $P = 0.0091$ and 0.0539, on area and mass bases of expression, respectively, Fig 1a and b, Table 2). The average cross-species responses to eCO_2 were remarkably consistent across time (year $\times$ CO_2 $P > 0.97$, Fig. 1a and b, Table 2); the 95% CI for the stimulation by eCO_2 ranged by 7–14%. The responses of the legume and C3 nonlegume forb species were significant ($P < 0.05$) and the greatest among the functional groups (+13% in each), the C4 grasses the least responsive (+4%), and C3 grasses intermediate in their photosynthetic response to eCO_2 across years (+9%) (Fig. 2a). This variation in the

Table 2 Repeated measures ANOVA probabilities ($P < F$) for treatment (CO_2, N, functional group) main effects and interactions on leaf-level traits of 13 grassland species grown at ambient/elevated CO_2 levels (+ 180 ppm) and ambient/enriched N treatments (+ 4 g N m^{-2} y^{-1}) over 11 seasons

Effect	Leaf net photosynthesis		Stomatal conductance*, (g_w mmol m^{-2} s^{-1})	Instantaneous water use efficiency* (mmol CO_2 mol H_2O^{-1})	Leaf N content (g N m^{-2})	Leaf N concentration (%)	Photosynthetic N-use efficiency (μmol CO_2 g N^{-1} s^{-1})	Specific leaf area (SLA, cm^2 g^{-1})
	A_{area} (μmol m^{-2} s^{-1})	A_{mass} (nmol g^{-1} s^{-1})						
CO_2	0.0091 (+10)†	0.0539 (+9)	0.0002 (−22)	<0.0001 (+40)	0.0015 (−11)	0.0004 (−13)	0.0022 (+21)	0.8797 (Ø)
N	0.0612 (+4)	0.0181 (+6)	0.1103 (−2)	0.0048 (+5)	<0.0001 (+10)	<0.0001 (+10)	0.0192 (−5)	0.6393 (+1)
$CO_2 \times N$	0.8446	0.5205	0.5028	0.2030	0.0138	0.0177	0.6111	0.3598
fxgroup	0.4287	0.2715	0.1401	0.0036	0.0041	0.0079	0.0006	0.2198
fxgroup × CO_2	0.2882	0.7746	0.3437	0.1180	0.0051	<0.0001	0.4222	0.0360
fxgroup × N	0.0028	<0.0001	0.0250	0.0800	0.1772	<0.0001	0.5366	0.1696
fxgroup × CO_2 × N	0.4175	0.6327	0.2333	0.5563	0.7408	0.6169	0.9154	0.3157
yr × CO_2	0.9811	0.9711	0.2226	0.1372	0.1557	0.7940	0.6446	0.1024
yr × N	0.4794	0.4201	0.3418	0.9633	0.0051	0.0133	0.7591	0.9501
yr × fxgroup	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	< <0.0001
yr × fxgroup × CO_2	0.0423	0.2389	0.0569	0.0241	0.9225	0.9473	0.5189	0.8612
yr × fxgroup × N	0.5992	0.5493	0.4623	0.7318	0.3976	0.9003	0.8371	0.9667
yr × CO_2 × N	0.5936	0.7048	0.9772	0.6095	0.6763	0.7340	0.4915	0.9487

*ln-transformed; $P < 0.10$ are underlined.
†Refers to the percent change due to the main effect of treatment [(elevated-ambient)/ambient × 100].

response to CO_2 among functional groupings was not statistically significant (functional group × CO_2, $P = 0.2882$, Table 2), due in part to the considerable variation in magnitude of enhancement responses of species within functional groups (Fig. 2a).

Increased soil N availability modestly increased leaf net photosynthesis (4–6%) across species and years (N effect, $P = 0.0612$ and 0.0181, on area and mass bases, respectively, Table 2, Fig. 2b). This response varied among functional groups (fxgroup × N, $P < 0.0028$) and was driven predominantly by the positive effect of N on photosynthesis in C4 grasses (+ 17%) compared with little to no effect on the other functional groups (Fig. 2b, Table 2). Furthermore, the enriched soil N treatment did not affect photosynthetic responses to eCO_2 ($CO_2 \times N$, $P > 0.52$, Table 2, Fig. 3). However, significant stimulation of photosynthesis by eCO_2 was noted only at elevated (and not ambient) N treatment in the most recent 2 years (Fig. 3).

The effects of CO_2 and N treatments on leaf N content (g N m^{-2}) and concentration (%N) were also generally consistent across time and species (Fig 1e and f), but did vary somewhat among species and functional groups (Table 2, Fig. 4). Leaf N content and concentration were on average 11% and 13% lower, respectively, in eCO_2-grown compared with ambient CO_2 grown plants (CO_2 effect $P < 0.0015$, Table 2, Fig 1e and f). For leaf N concentration, these responses were greater for the C3 functional groups compared with the C4 grasses (fxgroup × CO_2 $P < 0.0051$, Fig. 4a, Table 2). Furthermore, these eCO_2 responses depended on N treatment in that the eCO_2-induced reduction in leaf N was greater in plants grown under increased compared with ambient soil N supply (e.g., −14% compared with −9% on an area basis, $CO_2 \times N$, $P = 0.0138$, Table 2). The overall effect of soil N enrichment was to increase leaf N concentration by 10% across years (N effect, $P < 0.0001$, Table 2) but only in the nonlegume functional groups with an average 16% increase in response to N enrichment compared with no effect of N enrichment on the legumes species (fxgroup × N, $P < 0.0001$, Table 2, Fig. 4b).

SLA was not significantly affected by CO_2 or N treatments (Table 2, Fig. 1g). This explains why leaf net photosynthesis and leaf N calculated on area and mass bases responded similarly to CO_2 and N treatments (Table 2, Fig. 1).

The CO_2 effects on leaf net photosynthesis and leaf N concentrations collectively resulted in greater instantaneous PNUE across years (+21% on average, CO_2 effect $P = 0.0022$, Fig. 1h, Table 2). PNUE of the C4 grasses were the least responsive to eCO_2 compared with other functional groups (Fig. 4c, Table 2). Enriched N treatments generally reduced PNUE across

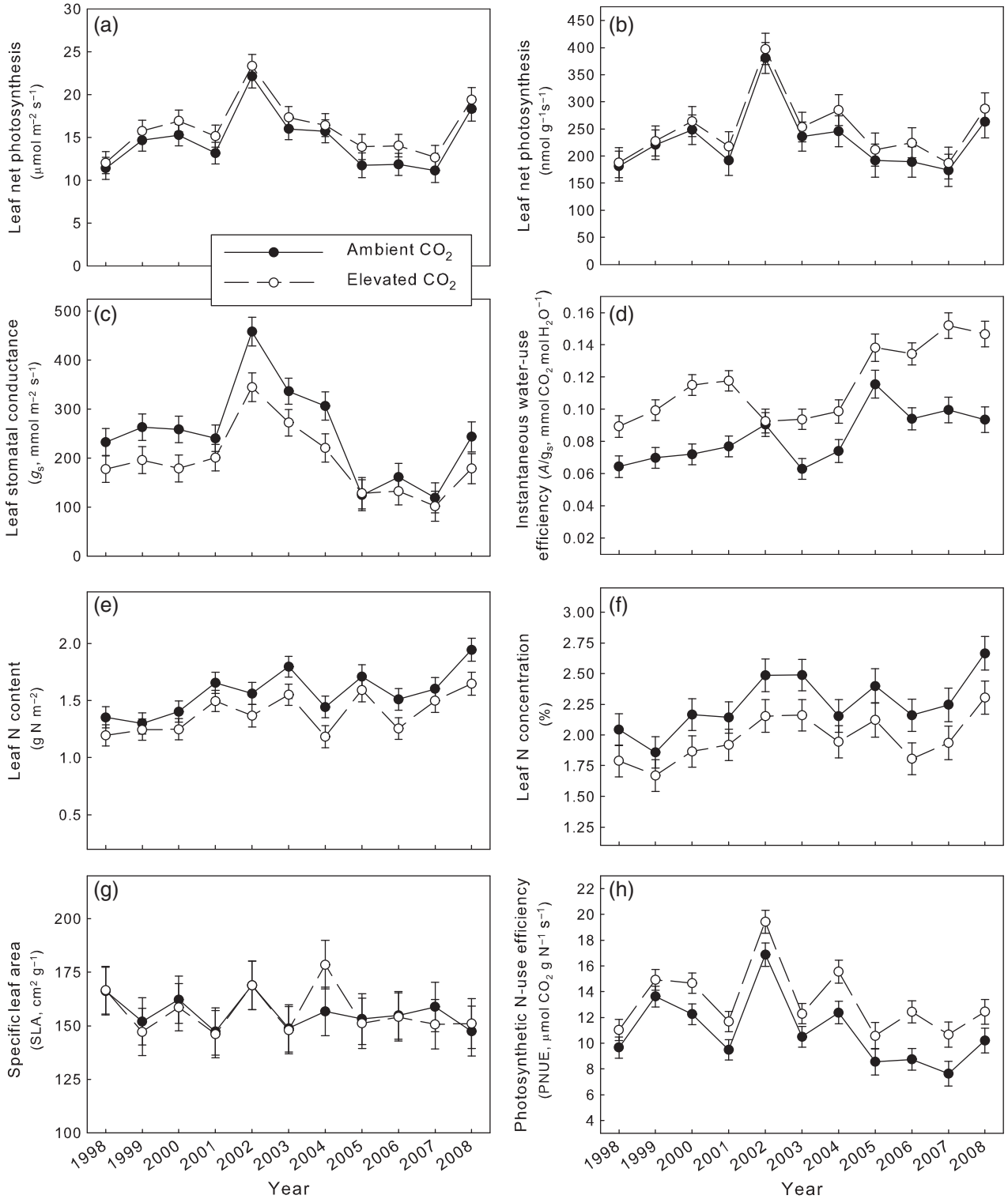

Fig. 1 Leaf net photosynthesis and related parameters of 13 species grown and measured across 11 years at elevated (+ 180 ppm above ambient) compared with ambient CO_2 concentrations. Leaf net photosynthesis on an (a) area-basis (A_{area}, μmol $CO_2\,m^{-2}\,s^{-1}$) and (b) mass-basis (A_{mass}, nmol $g^{-1}\,s^{-1}$); (c) leaf stomatal conductance (g_s, mmol $H_2O\,m^{-2}\,s^{-1}$); (d) instantaneous water-use efficiency (A/g_s, mmol CO_2 mol H_2O^{-1}); (e) leaf N content (g N m^{-2}); (f) leaf N concentration (%N); (g) specific leaf area (SLA, $cm^2\,g^{-1}$); and (h) photosynthetic N-use efficiency (PNUE, μmol $CO_2\,g\,N^{-1}\,s^{-1}$). Data are pooled across 13 grassland species (see Table 1) and N treatments and shown are least squares means ± SE from repeated measures ANOVA. In all cases, the CO_2 treatment × year interactions were not significant, see Table 2 for complete statistics.

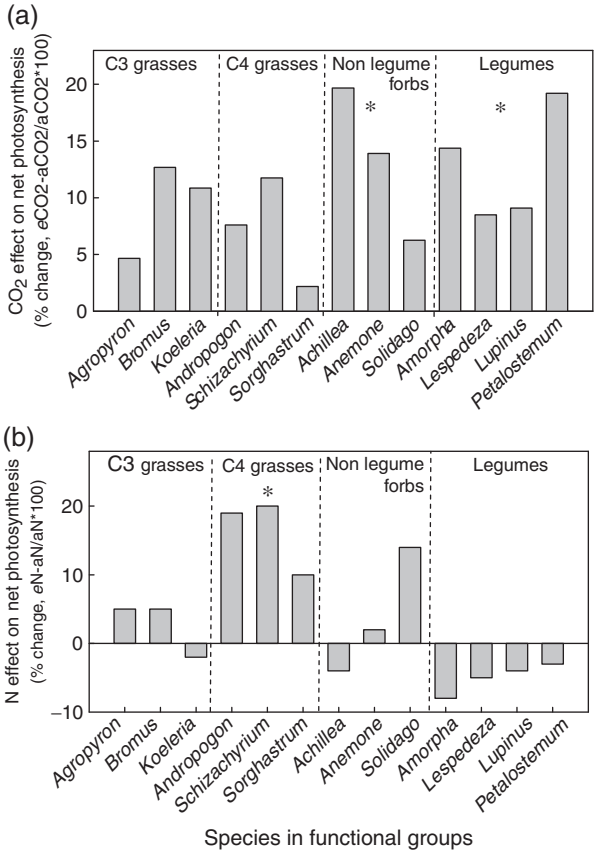

Fig. 2 Leaf net photosynthetic responses ($\mu mol\, CO_2\, m^{-2}\, s^{-1}$) of 13 grassland species (arranged by functional group) grown in monoculture plots under factorial combination of elevated (+180 ppm above ambient) and ambient CO_2 concentrations and N enriched ($+4\, g\, N\, m^{-2}\, yr^{-1}$) and ambient N treatments. (a) CO_2 effect (%) on leaf net photosynthesis [($eCO_2 - aCO_2$)/$aCO_2 \times 100$] of each species pooled across N treatments and years measured; (b) N effect (%) on leaf net photosynthesis [($eN - aN$)/$aN \times 100$] of each species pooled across CO_2 treatments and years. *Significant functional group responses to eCO_2 and N enrichment (Student's t post hoc tests $P < 0.05$, see Table 2 for complete statistics).

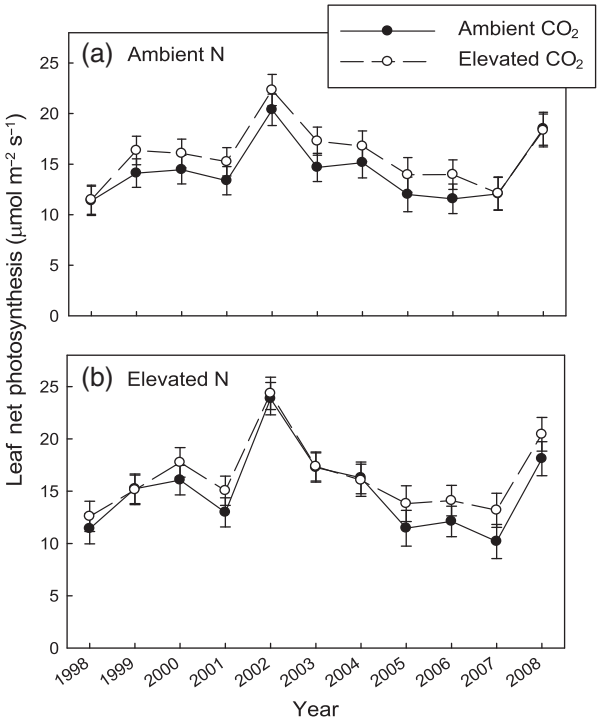

Fig. 3 Leaf net photosynthesis ($\mu mol\, CO_2\, m^{-2}\, s^{-1}$) of grassland species grown and measured across 11 years at elevated (+180 ppm above ambient) compared with ambient CO_2 concentrations. Data are pooled across 13 species (see Table 1), shown are least squares means ± SE, and the CO_2 effect is shown separately for (a) ambient N and (b) elevated N-treated plants. $CO_2 \times N$ treatment interaction $P = 0.8446$, see Table 1 for complete statistics.

functional groups (−5% on average, N effect $P = 0.0192$, Table 2, Fig. 4d).

Other notable effects of CO_2 and N on leaf level physiology include a consistent decrease in stomatal conductance (g_s) in plants under elevated compared with ambient CO_2 across years (−22%, 95% CI range from 14% to 27%; CO_2 effect $P = 0.0002$, $CO_2 \times$ year $P = 0.2226$, Table 2, Fig. 1c). This response was relatively consistent across functional groups (Fig. 5a). Stomatal conductance was not affected by N enrichment but for the C3 grasses in which rates were lower in N enriched compared with ambient N plots for most of the species in this group (Fig. 5b). The eCO_2-induced decreases in stomatal conductance, coupled with modest increases in net photosynthesis, translated into significant increases in instantaneous water-use efficiencies (A/g_s) of 40% across functional groups grown in elevated compared with ambient CO_2 (CO_2 effect $P < 0.0001$) and 5% in N enrichment compared with ambient N (N effect $P = 0.0048$) with the C3 grasses and nonlegume forbs showing significant responses to N treatment (Table 2, Fig. 5c and d). The variable responses of net photosynthesis and g_s to N treatments resulted in a marginal increase in A/g_s across some functional groups under N enrichment compared with ambient N (Fig. 5d).

We also explored the role of eCO_2-induced decreases in leaf N and stomatal conductance in the acclimation of leaf net photosynthesis to eCO_2. Relationships between mass-based net photosynthetic rates ($nmol\, m^{-2}\, s^{-1}$, A_{mass}) and leaf N concentration (%N) – indicative of biochemical processes – are shown in Fig. 6 and relationships between area-based net photosynthetic rates ($\mu mol\, m^{-2}\, s^{-1}$, A_{area}) and stomatal conductance to water vapor ($mmol\, m^{-2}\, s^{-1}$, g_s) – indicative of surficial flux processes – are shown in Fig. 7; each by elevated

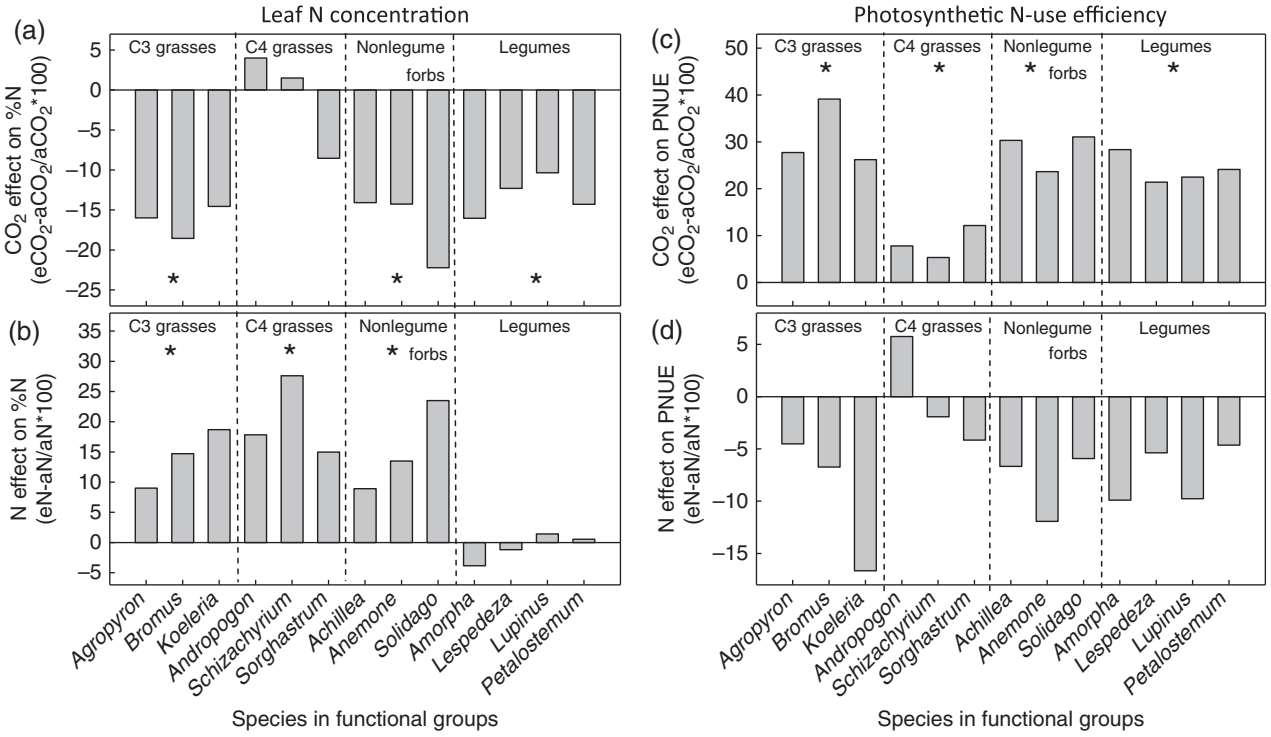

Fig. 4 CO_2 and N treatment effects (%, [elevated−ambient]/ambient × 100) on leaf N concentration (%N) and photosynthetic N-use efficiency (PNUE, μmol $CO_2 gN^{-1}s^{-1}$) by species (arranged by functional group). (a) CO_2 effect on leaf N concentration, (b) N effect on leaf N concentration, (c) CO_2 effect on PNUE, and (d) N effect on PNUE. *Significant functional group responses to eCO_2 and N enrichment (Student's t post hoc tests $P < 0.05$, see Table 2 for complete statistics).

compared with ambient CO_2 treatments. For any given leaf N concentration, A_{mass} was slightly higher in eCO_2 compared with ambient CO_2 grown plants (Fig. 6). However, realized A_{mass} in eCO_2 would have been higher if not for the concomitant eCO_2-induced decline in leaf %N in all functional groups except the C4 grasses (Fig. 6a–d). This is illustrated by comparing (for ambient N plants only for simplicity) the large squares (representing what A_{mass} of eCO_2 grown plants would have been at the average leaf %N value without down-regulation of leaf %N in eCO_2) with the large circle (the observed A_{mass} of eCO_2 grown plants at the observed mean leaf %N in eCO_2). While this estimated effect of eCO_2-induced reductions in leaf N on A_{mass} was rather strong in the C3 grasses and nonlegume forbs (Fig. 6a and c), this effect was less for the legumes (Fig. 6d) and nonexistent for the C4 grasses (Fig. 6b)]. Without down-regulation of leaf % N in plants grown at eCO_2, A_{mass} would have been stimulated by 18.5% instead of the 8.1% observed across functional groups in the ambient N treatment. Therefore, reduced leaf %N in eCO_2 grown plants appears to contribute to the constrained A_{mass} response to eCO_2. Similarly, eCO_2 induced reductions in g_s were correlated with the limited eCO_2-induced simulation of A_{area} (Fig. 7). This is illustrated by comparing the large squares (representing an estimation of A_{area} in eCO_2 without the observed eCO_2-induced decreases in g_s) with the large circle (the observed A_{area} of eCO_2 grown plants at the observed mean leaf g_s in eCO_2). Without eCO_2-induced reductions in g_s, A_{area} would have been stimulated by 22.0% instead of the 9.5% observed. This phenomenon was generally similar across functional groups (Fig. 7a–d).

Discussion

The observed average stimulation of photosynthesis of ≈ 10% over 13 species (11% over the ten C3 species and 4% over the three C4 species) and 11 years was markedly less than one would predict based on the Farquhar model of photosynthesis under conditions where there is no photosynthetic acclimation to growth in elevated atmospheric CO_2 (Farquhar *et al.*, 1980). Theory based on simple CO_2 diffusion into C3 leaves suggests that a photosynthetic enhancement of 55% is expected with an average 200 ppm enrichment in CO_2 and no photosynthetic acclimation (Katul *et al.*, 2000), and consistent with this, we observed a mean short-term photosynthetic enhancement of 54% for the C3 species in this study (Lee *et al.*, 2001). However, some degree of

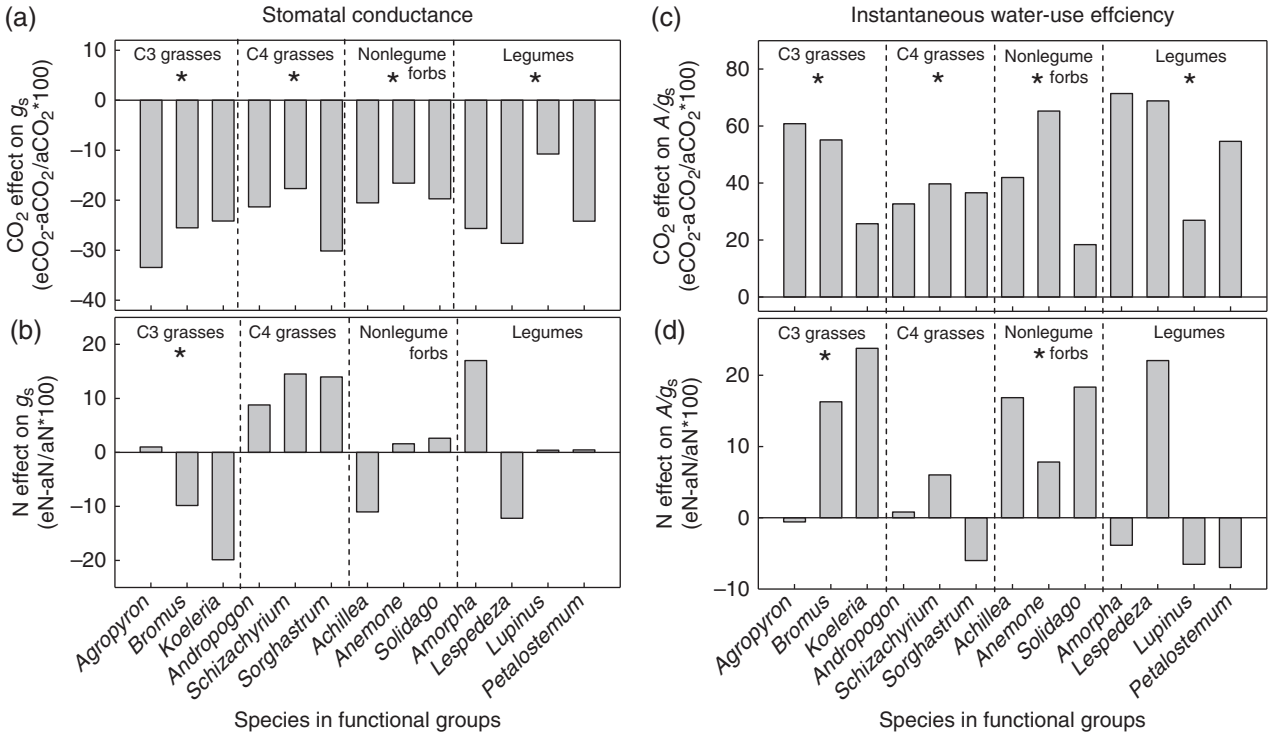

Fig. 5 CO_2 and N treatment effects (%, [elevated−ambient]/ambient × 100) on leaf stomatal conductance (g_s, mmol m^{-2} s^{-1}) and instantaneous water-use efficiency (A/g_s, mmol CO_2/mol H_2O) by species (arranged by functional group). (a) CO_2 effect on g_s, (b) N effect on g_s, (c) CO_2 effect on A/g_s, and (d) N effect on A/g_s. *Significant functional group responses to eCO_2 and N enrichment (Student's t post hoc tests $P<0.05$, see Table 1 for complete statistics).

acclimation routinely occurs in plants grown under eCO_2 (Urban, 2003; Ainsworth & Rogers, 2007). Photosynthetic acclimation to eCO_2 is thought to occur due to a variety of adjustments, including, but not limited to, biochemical downregulation (such as shifts in leaf N concentration or Rubisco activity, e.g. Reich *et al*., 2006a, b; Crous *et al*., 2010) and stomatal adjustments that decrease conductance to water vapor and change operating internal CO_2 concentrations (e.g. Nowak *et al*., 2004; Ainsworth & Rogers, 2007).

The results of our study are consistent with these ideas, as species in this long-term study showed significant reductions in both leaf %N and stomatal conductance, also noted in earlier reports from this study (Lee *et al*., 2001; Ellsworth *et al*., 2004; Crous *et al*., 2010). However, as instantaneous stimulation of photosynthesis by eCO_2 was more than 50% for these species (Lee *et al*., 2001; T.D. Lee, unpublished data) and stimulation estimated without downregulation of leaf %N or eCO_2-induced reductions in g_s were only 18.5% and 22%, respectively, the negative impacts of reduced leaf %N and g_s on A do not fully explain the degree of photosynthetic acclimation to eCO_2 seen in this study.

The nitrogen contribution to the downregulation of photosynthetic enhancement associated with growth at eCO_2 can result from acclimation of carboxylation capacity as a result of reduced ribulose bisphosphate carboxylase (Rubisco) concentrations and activity and substrate regeneration (Long *et al*., 2004; Ainsworth & Rogers, 2007). Elevated N supply has been hypothesized to alleviate this. However, in our study, the response to CO_2 did not depend on N treatment, in contrast to average results of other studies (Ainsworth & Rogers, 2007). Nonetheless, decreases in leaf N appear to contribute to decreased photosynthetic capacity.

Functional groupings of species based on similar physiology and growth forms are often useful in summarizing results across studies and species in meta-analyses (Long *et al*., 2004; Ainsworth & Long, 2005; Ainsworth & Rogers, 2007). However, we found weak to modest evidence that species respond in predictably different ways based on the species and functional groupings in this study. This was due to in part to considerable variation in response among species within functional groupings. However, in some instances, species within functional groups did respond similarly. For example, the effect of the elevated N treatment on photosynthesis was more positive across the C4 species than C3 species and was negative for leaf N content in legumes compared with nonlegume species. Similarly,

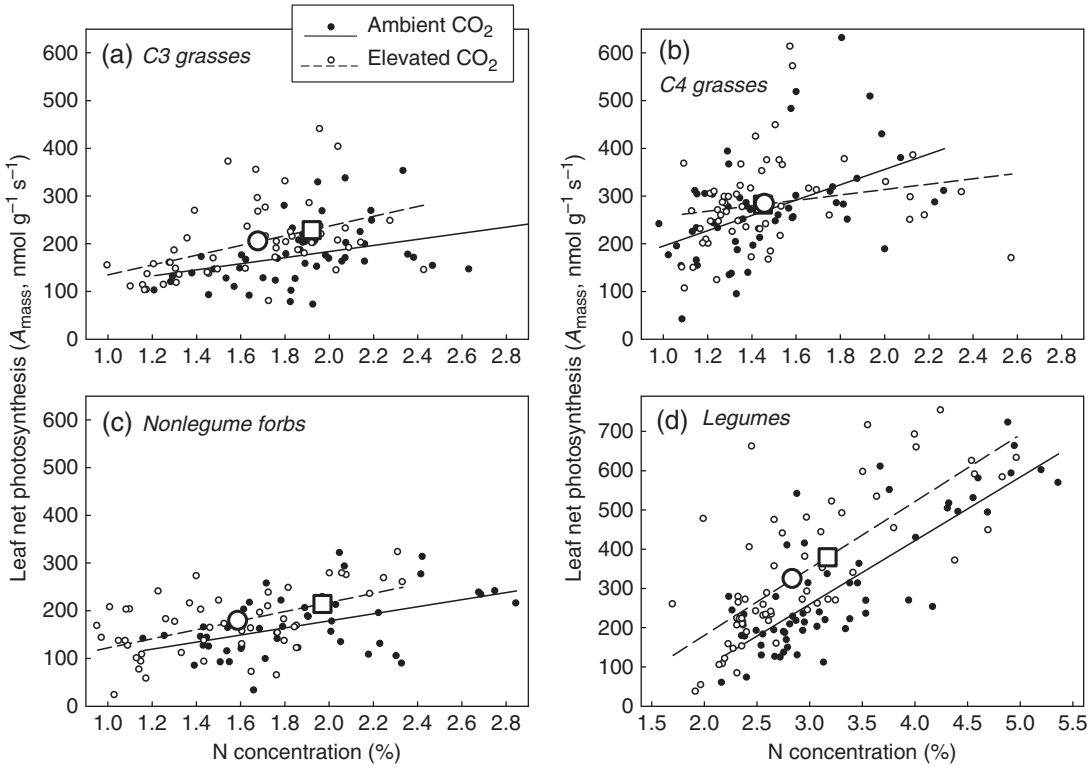

Fig. 6 Relationships between leaf net photosynthesis (nmol g^{-1} s^{-2}, A_{mass}) and leaf N concentration (%N) grouped by CO_2 treatment. Shown are plot averages of each species measured in each year in the ambient N treatment only. Along the A_{mass}-%N relationship for elevated CO_2 plants (open circles, dashed lines), the larger symbols represent A_{mass} when calculated with the mean %N value for leaves grown at elevated CO_2 (large open circle) or the mean %N value for leaves grown at ambient CO_2 (i.e. what net photosynthetic rates would be without the eCO_2 induced change in N; large open square). (a) C3 grasses ($n = 104$), (b) C4 grasses ($n = 112$), (c) nonlegume forbs ($n = 96$), (d) legumes ($n = 124$).

the effect of CO_2 on net photosynthesis was greater in forbs and legumes than in grasses and the effect of CO_2 on leaf N was significant in C3 but not C4 species. A related study of leaf-level CO_2 responses at BioCON (Crous *et al*., 2010) found more systematic differences between C3 grasses and forbs than in this present study. Such differences may have resulted from differences in taxa and years studied, with the present study having much higher replication of species and measurements, over 11 vs. 1 to 4 years of observation. The conclusion – that functional groupings do represent some systematic differences in response, but not consistently enough to be predictive – is similar to that from studies of biomass and root : shoot partitioning responses (Reich *et al*., 2001).

Overall for 13 grassland species over 11 years, photosynthetic stimulation by eCO_2 was consistent but small, with no evidence of convergence or divergence over time. In the few other long-term (5–10 years) studies, there is also little evidence that the CO_2 response changes over time (e.g. Ainsworth *et al*., 2003). However, the eCO_2-induced enhancement of photosynthetic rates of 10% (compared with rates in plants grown and measured at ambient CO_2) was much smaller than the average 31% (Ainsworth & Rogers, 2007) or $26 \pm 5\%$ (Nowak *et al*., 2004) reported in more recent meta-analyses of FACE studies. The results of our study are important for at least three reasons.

First, by providing information for 13 species over an 11-year period, our study expands the peer-reviewed literature in both taxonomic and temporal ways. Given the importance of developing robust models of long-term CO_2 enrichment effects on productivity, and associated carbon sequestration (Jackson & Schlesinger, 2004), long-term information is of paramount importance. The generally consistent and persistent stimulation of photosynthesis by eCO_2 would suggest such long-term models might be possible and simpler to develop (than if complex temporal responses were observed). However, given the complex ways in which the eCO_2 response can vary over time due to myriad potential interactions among different components of ecosystems, it would be unwise to assume that the stability of the long-term photosynthetic enhancement

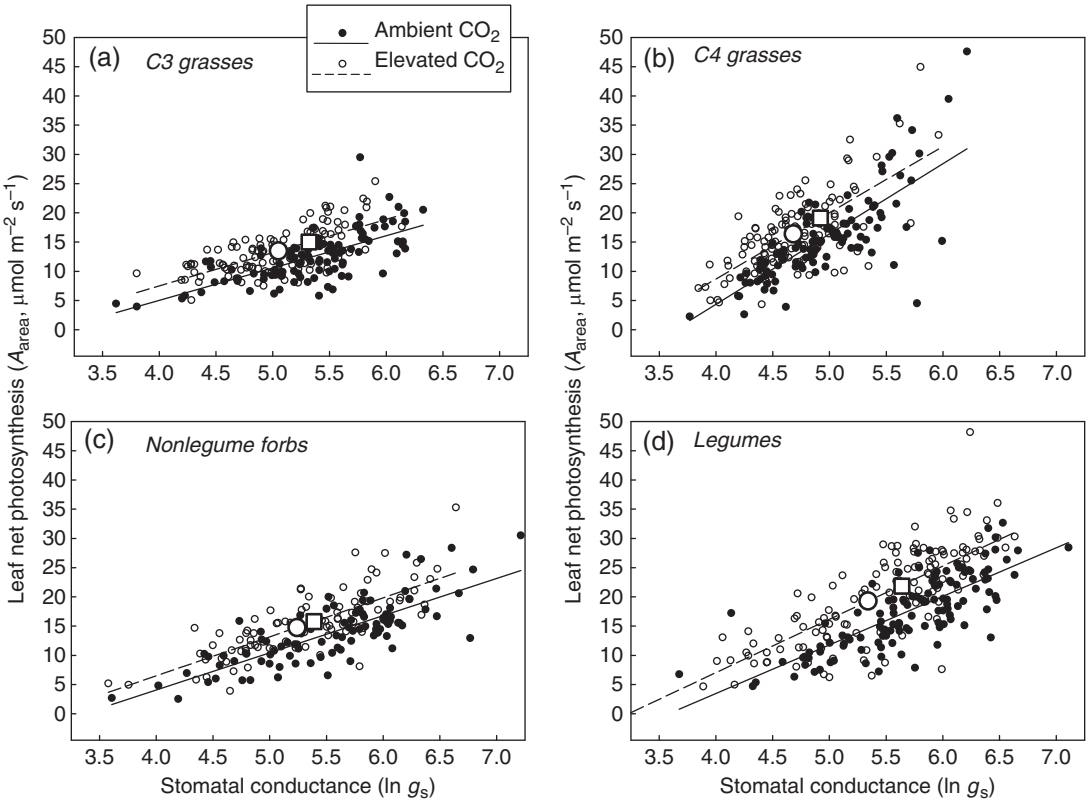

Fig. 7 Relationships between leaf net photosynthesis (A_{area}, $\mu mol\ m^{-2}\ s^{-1}$) and stomatal conductance (ln transformed g_s, $mmol\ m^{-2}\ s^{-1}$) grouped by CO_2 treatment. Shown are plot averages of each species measured in each year and in each N treatment. Along the A_{area} −ln g_s relationship for elevated CO_2 plants (open circles, dashed lines), the larger symbols represent A_{area} when calculated with the mean ln g_s value for leaves grown at elevated CO_2 (large open circle) or the mean ln g_s value for leaves grown at ambient CO_2 (i.e. what net photosynthetic rates would be without the eCO_2 induced change in g_s, large open square). (a) C3 grasses ($n = 208$), (b) C4 grasses ($n = 224$), (c) nonlegume forbs ($n = 192$), and (d) legumes ($n = 248$).

observed in BioCON would occur regularly in other species and ecosystems.

Second, the consistently lesser stimulation of photosynthesis in BioCON than in other experiments is both important and puzzling. It is important for the very reason of its divergence from other studies across a larger set of species and longer time frame than in any other study; and puzzling when one considers the comparable magnitude of responses of the related leaf-level parameters, leaf N content and stomatal conductance to water vapor, to those in meta-analyses (e.g. Ainsworth & Rogers, 2007). Without these results from BioCON, we might assume that eCO_2-induced increases in photosynthesis of 26–32% might be the norm, and therefore be used to constrain ecosystem scale and global carbon cycle models. Until we know what fraction of species and ecosystem types globally show such limited responses as in BioCON, the assumption of long-lived strong stimulation of photosynthesis (e.g. >25%) for all of Earth's terrestrial ecosystems should be made with a great deal of caution.

Third, as one of only three long-term (>5 years) $CO_2 \times N$ manipulations (and the only one with a continuous record of photosynthesis) the results are valuable given the potential importance of N limitations to CO_2 responses (Hungate *et al.*, 2003; Reich *et al.*, 2006a, b) and the consequences for climate predictions that rely on carbon cycle models. However, the lack of $CO_2 \times N$ interactions observed at the level of leaf photosynthesis is in marked contrast to the temporal development of a strong and persistent N limitation to eCO_2-induced stimulation of biomass at this site (Reich *et al.*, 2006a) that persists until the present (data not shown). Despite the critical importance of photosynthesis in regulating the acquisition of new carbon, processes such as leaf area development, root–shoot interactions, biomass turnover rates, biogeochemical feedbacks, and many others, can lead to complex interactions at the system level even with none at the leaf level. Complex $CO_2 \times N$ interactions are possible at multiple scales in any system, and the results from this experiment for leaf photosynthesis provide a beginning for what *should*

be the development of a large data base sufficient to arrive at a comprehensive synthetic understanding, not only of $CO_2 \times N$ interaction on photosynthesis, but of the links among leaf scale to ecosystem scale responses to $CO_2 \times N$. Unfortunately however, to our knowledge, as no other long-term ongoing experiment (>4 years) manipulating CO_2 and N in a perennial ecosystem exists, we are unlikely to arrive at such understanding in the foreseeable future.

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