PLANT ECOLOGY

Unexpected reversal of C_3 versus C_4 grass response to elevated CO_2 during a 20-year field experiment

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Theory predicts and evidence shows that plant species that use the C_4 photosynthetic pathway (C_4 species) are less responsive to elevated carbon dioxide (eCO_2) than species that use only the C_3 pathway (C_3 species). We document a reversal from this expected C_3-C_4 contrast. Over the first 12 years of a 20-year free-air CO_2 enrichment experiment with 88 C_3 or C_4 grassland plots, we found that biomass was markedly enhanced at eCO_2 relative to ambient CO_2 in C_3 but not C_4 plots, as expected. During the subsequent 8 years, the pattern reversed: Biomass was markedly enhanced at eCO_2 relative to ambient CO_2 in C_4 but not C_3 plots. Soil net nitrogen mineralization rates, an index of soil nitrogen supply, exhibited a similar shift: eCO_2 first enhanced but later depressed rates in C_3 plots, with the opposite true in C_4 plots, partially explaining the reversal of the eCO_2 biomass response. These findings challenge the current C_3-C_4 eCO_2 paradigm and show that even the best-supported short-term drivers of plant response to global change might not predict long-term results.

The idea that C_4 plants are less limited by ambient atmospheric CO_2 concentrations than C_3 plants, and will thus respond less to increasing CO_2 concentrations, has a long history (*1–3*) and is deeply embedded in models of past, present, and future vegetation-climate interactions (*2–7*). The hypothesis has proven useful, if not always entirely predictive, in describing C_3 and C_4 plant distributions (*2–4*, *8–11*) and biomass responses to environmental variation (*12–15*).

There is strong logic for this hypothesis. C_3 plants, which use the carboxylase enzyme RuBisCO (ribulose-1,5-bisphosphate carboxylase-oxygenase) to fix CO_2 from the air and obtain 3-carbon intermediate molecules as the first step in photosynthesis, lose a portion of their fixed CO_2 to oxidative photorespiration under present CO_2:O_2 ratios because RuBisCO is also an oxygenase (*1–3*). Thus, C_3 plants should exhibit increased leaf-level net photosynthesis as increasing CO_2:O_2 ratios reduce rates of photorespiration and increase rates of carboxylation (*1–3*). By contrast, in C_4 plants, a different enzyme (phosphoenolpyruvate carboxylase) with a high affinity for CO_2 and lacking oxygenase activity first incorporates CO_2 into a 4-carbon intermediate, which is then shuttled to specialized bundle sheath cells where CO_2 is released, resulting in locally high CO_2 concentrations. Here, RuBisCO catalyzes carboxylation, but with low rates of photorespiration because of the high CO_2:O_2 ratios in the bundle sheath cells. As a result, eCO_2 levels in air have little impact on photosynthetic rates for C_4 plants (*1–3*). Globally, most plants are C_3; graminoids are the only major group with substantial abundance of both C_3 and C_4 species (*2*, *16*). Given that C_4 grasslands may constitute one-fifth of global terrestrial net primary productivity (*16*), a better understanding of C_4 performance under rising CO_2 vis-à-vis C_3 grasses is needed for global ecology and for improved ecosystem and Earth system modeling (*5–7*, *17*). C_3 and C_4 grasses (and sedges) are also distinguished in terms of ecological success by different affinities for temperature, rainfall, and nutrient supply (as well as CO_2); as a consequence, they can cooccur or shift their relative abundances depending on the mix of conditions (*8–11*, *14*, *15*, *18–20*).

Experimental evidence has strongly supported the theoretical prediction that at current CO_2 levels, C_3 grasses are more CO_2-limited than C_4 species and thus will respond more to rising CO_2. Two influential meta-analyses (*12*, *13*) reported greater stimulation (by factors of 2 to 4) of aboveground biomass by eCO_2 for C_3 species than for C_4 species. One of those publications focused on published studies of graminoids, where plants were mostly grown in pots for less than a year (*12*), whereas the other focused on free-air CO_2 enrichment (FACE) field studies (mostly 3 years or shorter) with plants grown in the ground (*13*). Another more recent meta-analysis also showed much greater responses of biomass growth for C_3 than for C_4 plants (*21*). One of the few longer-term studies comparing C_3 and C_4 grasses under eCO_2 found that C_4 species were, surprisingly, apparent "winners" after 3 years of eCO_2 and warming (*20*). However, by 6 years of treatment, the situation had reversed, with C_3 species becoming more positively responsive to the simulated global changes and the dominant plant type, in accordance with expectations (*22*).

In sum, current physiological theory and short- and medium-term studies support the paradigm that C_4 species benefit much less from rising CO_2 than C_3 species. Given both theoretical and empirical support for the differences in eCO_2 response of C_3 and C_4 species, their differential responsiveness to eCO_2 comes as close to "accepted fact" as exists in ecology and, as such, is incorporated into many ecosystem and Earth system models (*3–6*, *8–13*). However, it is not known whether findings from short- and medium-term studies apply over ecologically realistic time frames (>10 years) in field settings where complex feedbacks might influence response to eCO_2. Understanding long-term responses of C_3 and C_4 species is especially germane given that the limited available evidence suggests strong nonlinearity of responses of ecosystems to eCO_2 over time (*23*).

Here, we report results from a long-term (20-year) FACE experiment in Minnesota, USA, that support the long-held paradigm for the early part of the experiment but reveal a gradual reversal to a much more positive response to eCO_2 by C_4 than by C_3 grasses. The study uses 88 plots that are components of several different but overlapping global change experiments within the BioCON project (*24*, *25*) and by themselves constitute a fully factorial 2 × 2 × 2 × 2 experiment of CO_2 levels (ambient or +180 parts per million), nitrogen levels (ambient or +4 g N m^{-2} $year^{-1}$), species richness (one or four species), and functional group identities (C_3 or C_4 grasses) (*26*). Eight species of temperate perennial grasses (four each of C_3 and C_4) were used in the study (*26*) and were equally weighted in the original plantings of those 88 plots; that is, there are equal numbers of replicated monocultures of all species, and the four-species plots contain all species within each functional group. Annually over 20 years, we sampled both aboveground and belowground (0 to 20 cm) biomass late in each growing season in every plot, and also made an independent measure of fine root production (0 to 20 cm). We also measured in situ soil net N mineralization in every plot for a 1-month period each year just prior to biomass sampling (*26*, *27*). Leaf-level net photosynthetic rates were measured midseason (*28*) for a subset of these eight grass species in 16 of the 20 years.

Over the 20-year experimental period, total biomass of C_4 grasses became increasingly enhanced by eCO_2 exposure, with the reverse true for C_3 grasses (Figs. 1 and 2 and figs. S1 and S2). During approximately the first 12 years (1998–2009), results were as expected (Fig. 1): C_3 plots averaged a 20% increase in total biomass (+136 g/m^2) at eCO_2 relative to ambient CO_2, in contrast to C_4 plots that averaged a 1% increase (+12 g/m^2). During the subsequent 8 years (2010–2017), the pattern reversed: C_3 plots averaged 2% less (–12 g/m^2) and C_4 plots 24% more (+233 g/m^2) biomass in eCO_2 than in ambient CO_2 (Fig. 1).

Repeated-measures analyses of variance (Table 1) support these conclusions, which are illustrated for successive 5-year periods in Fig. 2. Significant main effects on total biomass were found for N addition (higher biomass than at ambient N), species richness (higher biomass in plots with four species than in plots with one species), and functional group (higher biomass on average in

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C_4 plots than in C_3 plots) (Table 1). Additionally, on average across treatments, biomass of C_3 plots was originally greater than that of C_4 plots, but over time this ranking reversed (interaction of functional group × year, $P < 0.0001$; Table 1 and Fig. 1). Most germane was the significant functional group × year × CO_2 interaction ($P = 0.007$; Table 1), showing that C_3 and C_4 functional groups responded differently to eCO_2 over time (Fig. 2). For example, in each of the first two 5-year periods, C_3 grasses increased biomass under eCO_2 by ~20% (+140 g/m^2); this declined to a 10% enhancement (+40 g/m^2) in years 11 to 15 and a 2% decline in years 16 to 20 (–15 g/m^2). In contrast, under eCO_2, biomass of C_4 grasses was reduced by 2% (–23 g/m^2) in years 1 to 5, enhanced by ~7% (+60 g/m^2) in years 5 to 10 and 11 to 15, and enhanced by 31% (+298 g/m^2) in years 16 to 20. These different responses of functional groups to CO_2 and time were unaffected by N treatment ($P = 0.76$ for functional group × year × CO_2 × N interaction) and were slightly more pronounced in four-species plots than in one-species plots ($P = 0.048$ for functional group × year × CO_2 × species richness interaction) (Table 1 and fig. S3). Results were generally similar for aboveground and belowground biomass viewed separately, as well as for annual net primary production (estimated as the sum of annual aboveground biomass production and fine root production).

We explored several potential mechanisms for this long-term reversal of C_3 versus C_4 responsiveness to eCO_2, including a temporal switch in leaf-level photosynthetic response, differential CO_2 sensitivity associated with potential climate variation over the 20 years, and potential feedbacks from changing N cycle responses to eCO_2 over time. Measurements of light-saturated net photosynthesis were made for one to three C_3 and one to three C_4 grass species (mean of 2.2) in monocultures in 16 of the 20 years of the study, at all combinations of CO_2 and N treatment. There was no evidence of a shift over time in the enhancement of net photosynthesis as observed for biomass (no interactions of functional group × CO_2 × year; fig. S4). Moreover, there was no correspondence between years when eCO_2 enhancement of net photosynthesis was high and years when eCO_2 enhancement of biomass was high, in either functional group (compare Fig. 1 and fig. S4). Although we lack data for all species in all treatments in all years, the available data provide no evidence to suggest that the rank reversals of biomass responses to eCO_2 were driven by parallel rank reversals in leaf-level photosynthetic responses.

We then asked whether the shifting responsiveness of C_3 versus C_4 grass plots could be related to interannual variation in temperature or rainfall (*2–5*, *15*, *19*, *21*). Responses of C_3 and C_4 grasses did not depend on year-to-year variations in mean or lagged spring, summer, or growing-season daily average temperature. The only significant effect involved summer rainfall [May to July (MJJ)]: There was a significant ($P = 0.0264$) interaction of CO_2 × functional group × MJJ rainfall on the biomass response (table S1); C_4 grasses were slightly more responsive to eCO_2 when rainfall was higher, whereas C_3 grasses were more responsive in low rainfall. These results are inconsistent with C_3 and C_4 grass responses in many studies (*2–5*, *15*, *19*). However, MJJ rainfall was only weakly correlated with year, and the CO_2 × year × functional group interaction was significant in the model ($P = 0.0347$) even after accounting for differential responses to rainfall for the two functional

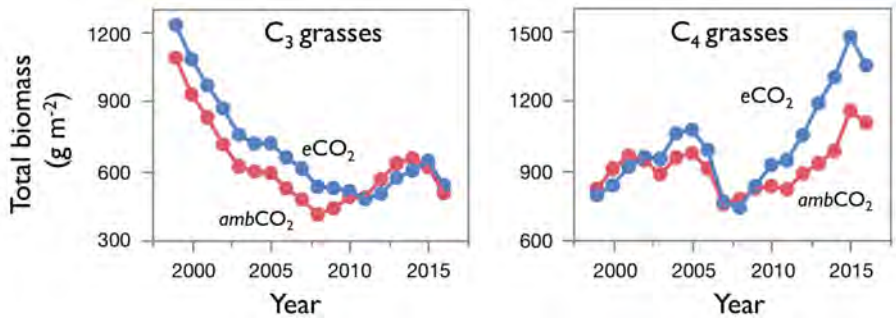

Fig. 1. Biomass over time of C_3 grasses and C_4 grasses at ambient and elevated CO_2. Total biomass (aboveground + 0 to 20 cm belowground) of plots comprising C_3 grasses and C_4 grasses in ambient CO_2 (red) and elevated CO_2 (blue) from 1998 to 2017. Data are shown as moving 3-year averages centered over the middle of each 3-year group. Each point represents data pooled across N treatments, and across monoculture and four-species plots (equally weighted), for each functional group ($n = 22$ plots for each functional group at each CO_2 level). See Table 1 for statistical analysis and fig. S1 for annual data and information on variation in response within treatments.

Table 1. Summary of repeated-measures analysis of variance of year, CO_2, N, species richness (SR), and C_3 versus C_4 functional group (FuncGroup) effects on total biomass and soil net N mineralization. Three-way or higher interactions involving treatments and two-way interactions or higher involving covariates shown only if significant. Five-way interactions were not tested. N mineralization data were missing for 2008 and 2017. Biomass was log-transformed prior to analysis. Year was a continuous term; Year and Year × Year terms were included in the model to assess linear and nonlinear changes over time involving CO_2 and functional groups. Significant terms ($P < 0.05$) in bold font.

Effect	Total biomass (g m^{-2})		Net N mineralization (mg kg^{-1} day^{-1})	
Whole model	$R^2 = 0.609$, $P < 0.0001$, $n = 1760$		$R^2 = 0.086$, $P < 0.0001$, $n = 1582$	
	F	*P* > *F*	*F*	*P* > *F*
Year	**158.95**	**<0.0001**	0.31	0.5787
CO_2	2.92	0.1521	0.75	0.4020
N	**13.14**	**0.0005**	**12.19**	**0.0008**
SR	**10.11**	**0.0022**	0.01	0.9187
FuncGroup	**42.32**	**<0.0001**	**5.52**	**0.0195**
Year × CO_2	1.96	0.1614	0.39	0.5327
Year × N	2.26	0.1326	2.08	0.1493
Year × SR	**23.64**	**<0.0001**	0.00	0.96865
Year × FuncGroup	**296.56**	**<0.0001**	1.46	0.2269
CO_2 × N	0.01	0.9342	0.67	0.4170
CO_2 × SR	0.01	0.9265	0.36	0.5505
CO_2 × FuncGroup	0.02	0.8965	0.49	0.4853
N × SR	1.42	0.2379	0.73	0.3953
N × FuncGroup	0.48	0.4885	**5.48**	**0.0221**
SR × FuncGroup	0.40	0.5314	0.56	0.4574
Year × CO_2 × FuncGroup	**7.24**	**0.0072**	**3.99**	**0.0461**
Year × CO_2 × FuncGroup × SR	**3.89**	**0.0486**	1.16	0.2827
Year × Year	**104.34**	**<0.0001**	**20.87**	**<0.0001**
Year × Year × CO_2	0.23	0.6325	0.42	0.5189
Year × Year × CO_2 × FuncGroup	0.08	0.7709	0.27	0.6034

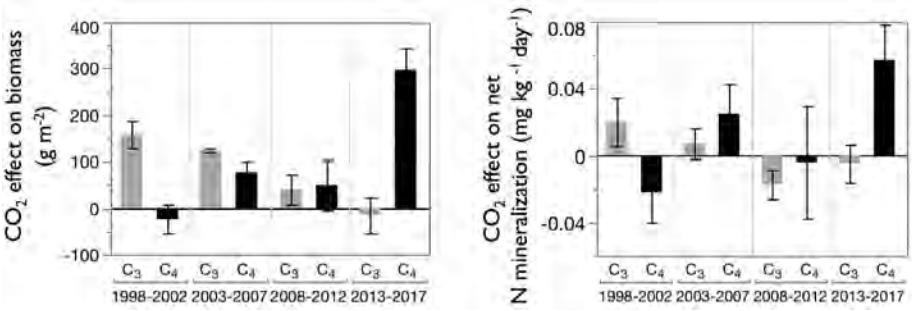

Fig. 2. Elevated CO_2 effect on total biomass and soil net N mineralization rates in plots comprising C_3 grasses and C_4 grasses. Left: Mean annual difference in biomass (mean biomass in eCO_2 − mean biomass in ambient CO_2) for C_3 and C_4 plots for four time periods during the study (1998–2002, 2003–2007, 2008–2012, and 2013–2017). Right: Mean difference in mean soil N mineralization rate (eCO_2 − ambient CO_2) for C_3 and C_4 plots for the same four time periods. Each bar represents data pooled across N treatments, and across monoculture and four-species plots (equally weighted), for each functional group (n = 22 plots for each functional group at each CO_2 level). Error bars represent SE among years.

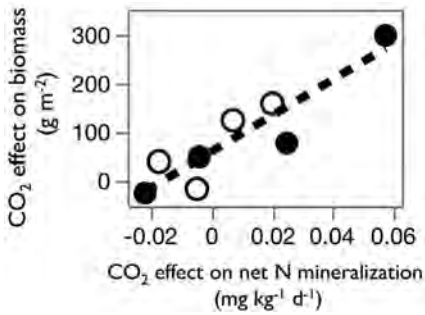

Fig. 3. Correspondence between biomass and net N mineralization responses to elevated CO_2. Relationship of biomass response to eCO_2 (effect size = biomass in eCO_2 − biomass in ambient CO_2) versus net N mineralization response to eCO_2 (defined similarly) in plots comprising C_3 grasses (open circles) and C_4 grasses (solid circles). R^2 = 0.82, P = 0.0018. Each data point represents effect sizes for each functional group for the four 5-year periods during the study (as in Fig. 2), based on the average biomass and net N mineralization across years in each period at each CO_2 level.

groups by including rainfall and rainfall interactions in the model (table S1). Thus, the reversal of responsiveness of C_3 and C_4 plots to eCO_2 over time was not explained by interannual variation in precipitation.

We also considered soil processes that might have played a role in the shifting responses of the C_3 and C_4 assemblages. Soil N supply has shaped the dynamics of biomass response to eCO_2 in the wider BioCON experiment (including the 9- and 16-species mixtures) because, as predicted by multiple resource limitation theory, responses to eCO_2 were greater when N supply levels were high [e.g., (*27*)]. Hence, we asked what role soil N availability (using soil net N mineralization as an index) might play here. The response of soil net N mineralization to eCO_2 changed over time (Table 1, Fig. 2, and figs. S2 and S5), mirroring responses of biomass for the C_3 and C_4 groups (Table 1). There was a significant (P = 0.046) interaction of CO_2 × year × functional group: The response of net N mineralization to eCO_2 in C_4 grass plots became more positive over time, whereas that of C_3 grass plots became more negative (Table 1 and Fig. 2).

Moreover, relationships between biomass and soil net N mineralization rate, in concert with the shifts in the response of net N mineralization to eCO_2 over time, help to explain the shifting biomass response to eCO_2 of both functional groups. Biomass and its response to eCO_2 were both positively related to net N mineralization rate: Across years, biomass in both functional groups increased with net N mineralization (P = 0.0031; table S1), more so at eCO_2 than at ambient CO_2 (interaction of CO_2 × net N mineralization, P = 0.024; table S1), and more so in C_4 than in C_3 plots (interaction of CO_2 × functional group, P = 0.038; table S1). Given that biomass is positively related to net N mineralization, and that the net N mineralization response to eCO_2 was increasingly positive over time in C_4 grass plots and increasingly negative in C_3 grass plots, shifting soil N biogeochemistry partially explains the shifting biomass responses to eCO_2.

These effects can be illustrated by the significant positive linear relationship between the eCO_2 enhancement of biomass and the eCO_2 enhancement of net N mineralization for the four 5-year periods of the study (Fig. 3): In periods when net N mineralization rates were higher under eCO_2 than under ambient CO_2, biomass tended to be higher in eCO_2 as well (Fig. 3 and table S1). These results are consistent with prior results showing that response to eCO_2 in this ecosystem is partially contingent on N supply (*27*), with greater N availability tending to promote greater eCO_2 response. Overall, 20 years of observations in this FACE experiment suggest that the opposite directional responses of net N mineralization to eCO_2 over time in C_3 versus C_4 grass plots (Table 1 and Fig. 2) may have contributed to the reversal of C_3 and C_4 biomass responses to eCO_2 over time. Why these soil N cycling responses played out in this fashion remains an open question, however.

Models that simulate future carbon cycling responses at ecosystem, regional, and global scales assume differing sensitivities of C_3 versus C_4 species to CO_2 based on differences in their photosynthetic physiology (*5*, *6*, *8*–*11*, *17*). Although those assumptions have major impacts on vegetation dynamics under varying climate and CO_2 scenarios (*8*–*11*, *29*, *30*), they do not match up well with the dynamic results of this long-term study. Our results thus serve as a reminder that even the best-predicted short-term ecosystem responses to global change can yield mid-term (decades) to long-term (centuries) surprises, as complex responses and interactions may occur over time. Determining whether the mid- to long-term responses demonstrated here are themselves broadly predictable represents a major unmet challenge for experimental and observational studies.

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P.B.R. wrote the first draft; and all authors jointly revised the manuscript. **Competing interests:** None. **Data and materials availability:** The data reported in this paper are available at the Environmental Data Initiative (EDI) (net nitrogen mineralization, DOI 10.6073/pasta/2ac4677a929290462877fd0df375ffa4; net nitrogen mineralization, DOI 10.6073/pasta/2ac4677a929290462877fd0df375ffa4; aboveground biomass, DOI 10.6073/pasta/8524be9f00b40a9e71b73a8ba2dc9ed0; belowground biomass, DOI 10.6073/pasta/c00662959002e588597bd77e0c7dbdbb). All other data needed to evaluate the conclusions in the paper are present in the paper or the supplementary materials.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S5
Table S1
References (*31*, *32*)

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