Decade-long soil nitrogen constraint on the CO_2 fertilization of plant biomass

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The stimulation of plant growth by elevated CO_2 concentration has been widely observed. Such fertilization, and associated carbon storage, could dampen future increases in atmospheric CO_2 levels and associated climate warming[1]. However, the CO_2 fertilization of plant biomass may be sensitive to nitrogen supply[2-4]. Herein we show that in the latest decade of a long-term perennial grassland experiment, low ambient soil nitrogen availability constrained the positive response of plant biomass to elevated CO_2, a result not seen in the first years (1998–2000) of the study. From 2001 to 2010, elevated CO_2 stimulated plant biomass half as much under ambient as under enriched nitrogen supply, an effect mirrored over this period by more positive effects of elevated CO_2 on soil nitrogen supply (net nitrogen mineralization) and plant nitrogen status under enriched than ambient nitrogen supply. The results did not strongly support either the progressive nitrogen limitation hypothesis, or the alternative hypothesis of priming of soil nitrogen release by elevated CO_2. As nitrogen limitation to productivity is widespread, persistent nitrogen constraints on terrestrial responses to rising CO_2 are probably pervasive. Further incorporation of such interactions into Earth system models is recommended to better predict future CO_2 fertilization effects and impacts on the global carbon cycle.

Continued emissions of CO_2 from fossil-fuel combustion and deforestation are likely to further increase atmospheric CO_2 concentrations ($[CO_2]$) and the temperature of the Earth[1]. However, the stimulation of plant biomass accumulation by elevated $[CO_2]$—the CO_2 fertilization effect—could increase ecosystem carbon (C) storage and thus dampen the future rate of increase in $[CO_2]$ and associated climate warming[1]. Uncertainty regarding the magnitude of this biomass fertilization makes it the largest unknown for terrestrial feedbacks to the C cycle–climate system[1]. One important aspect of this uncertainty is whether limited soil nitrogen (N) availability restricts CO_2-induced biomass enhancement and related C sequestration[2-19]. Several published studies on plant biomass and productivity under contrasting CO_2 and N supply suggest an N-limitation constraint on CO_2 fertilization[3-6], as noted in the last Intergovernmental Panel on Climate Change report[1]. Nevertheless, nutrient limitations were not incorporated into the coupled climate–C cycle models used in that report. This may have been due in part to the very low number and limited range of ecologically realistic long-term experiments of atmospheric CO_2 and N supply together. Herein we report on long-term (13 year) results of one such experiment, which is one of only three published, well-replicated long-term (>5 years) open-air $CO_2 \times N$ experiments[4,5,19].

Long-term CO_2-fertilization-related feedbacks to the C cycle depend in part on the degree of enhancement of terrestrial C uptake and biomass accumulation, whether this is influenced by availability of other resources, and whether enhanced C uptake over time influences the supply of other resources (for example, N) in ways that alter plant C exchange[13-18]. This paper focuses on elucidating the long-term impact of CO_2 fertilization on grassland C cycling as affected by N addition and the effects of CO_2 on soil N cycling.

Low N availability might limit the enhancement of biomass accumulation in response to elevated CO_2 in several ways, consistent with multiple-resource-limitation theory that suggests that as any one factor becomes less limiting to production, others should become increasingly co-limiting. At elevated CO_2, low soil N availability could limit leaf area and thus light interception, or limit photosynthetic capacity or other aspects of physiological N use. Furthermore, low N could influence the impacts of elevated CO_2 on N cycling in ways that lead to reduced C uptake, although previous evidence about the effects of elevated CO_2 on N cycling are remarkably varied. For example, soil N availability has been observed to decrease, remain stable or increase under elevated CO_2 (refs 4,5,13,14,17,18,20–23). These conflicting results are consistent with two opposing hypotheses. One hypothesis (progressive N limitation) posits that under elevated CO_2, higher C/N ratios in live and dead plants slow rates of decomposition and promote N immobilization, leading to lower rates of N cycling[3,17]. The other hypothesis (priming) suggests that increased inputs of labile C to soils caused by elevated CO_2 (refs 16,20–27) stimulate net N mineralization (the transformation of N from organic to inorganic forms) and hence plant N supply over time[25]. It is possible that both sets of processes occur simultaneously, but to differing degrees, among ecosystems, leading to the large variability in observed responses of N supply to elevated CO_2.

Both of these suggested effects of elevated CO_2 on N cycling are likely to be stronger under lower than higher N supply. However, whether one set of mechanisms should dominate at low N supply is unclear. It is plausible that priming effects should be greatest under conditions of highest C/N stoichiometry (elevated CO_2 under ambient N supply in our study) because plants should produce the most labile C fluxes to soils under such conditions; but higher C/N under such conditions could also lead to greater N immobilization and thus reduced N cycling in soil.

In our experiment (called BioCON) we established 296 field plots planted with different numbers (1, 4, 9 or 16 species) and combinations of perennial grassland species under ambient and elevated ($+180\,\mu mol\,mol^{-1}$) atmospheric CO_2 and with either ambient or enriched soil N supply (enriched with $4\,g\,N\,m^{-2}\,yr^{-1}$ as

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Table 1 | Repeated-measures analysis of variance of CO_2, N and period effects on plant biomass, total plant N pools and soil N mineralization.

Effect Whole model	Total biomass (g m^2) $R^2 = 0.548$, $P < 0.0001$, $n = 7,499$		Total plant N pool (g m^2) $R^2 = 0.48$, $P < 0.0001$, $n = 3,478$		Net N mineralization $R^2 = 0.17$, $P < 0.0001$, $n = 3,464$	
	F	$P > F$	F	$P > F$	F	$P > F$
CO_2	39.41	0.0002	6.45	0.039	0.30	0.609
N	44.94	<0.0001	68.20	<0.0001	1.66	0.198
Period	293.6	<0.0001	41.09	<0.0001	0.59	0.442
$CO_2 \times$ period	0.79	0.376	10.97	0.0009	3.93	0.0476
$N \times$ period	9.69	0.0019	4.45	0.0349	2.76	0.0968
$CO_2 \times N$	0.55	0.458	0.12	0.733	0.21	0.644
$CO_2 \times N \times$ period	4.49	0.034	3.13	0.077	3.56	0.0593
Diversity	154.55	<0.0001	98.53	<0.0001	13.55	<0.0001

Effects of year, CO_2, N and diversity, and all interactions, on total biomass (g m^{-2}; above-ground plus 0–20 cm fine roots), total plant N pools (g N m^{-2}) and net N mineralization (mg kg^{-1} d^{-1}) rate. The period compares years 1–3 (1998–2000) versus 4–13 (2001–2010) of the experiment. Diversity was a significant main effect but it did not influence the $CO_2 \times N$ interaction ($CO_2 \times N\times$ diversity, $P \geq 0.6$) or the period $\times CO_2 \times N$ interaction ($CO_2 \times N \times$ year $\times$ diversity, $P \geq 0.6$) for any of the three dependent variables. Thus, for brevity, interaction terms including diversity effects are not shown, and the effects of period, CO_2 and N are shown pooled across diversity treatments in Figs 1– 3. For biomass, two collections were made annually, and the season (June versus August) was included in the model. Neither a main effect of season nor significant interactions of season $\times CO_2$ or N were noted; hence, these are not shown in the table.

NH_4NO_3) (see Methods). Over the course of the experiment there was no detectable movement of N from N-enriched to ambient-N plots ($P > 0.5$), on the basis of comparison of total plant N across the range of borders shared with N-enriched plots (range from 0 to 4). The contrasting high versus low levels of N supply in our study ($\approx 3–4$ versus $\approx 7–8$ g N m^{-2} yr^{-1} when including background net N mineralization rates plus added N) are broadly representative of cross-ecosystem variation in natural soil N supply, which ranges from lower to much higher than in either of our treatments. Atmospheric N (wet) deposition adds another ≈ 0.6 g N m^{-2} yr^{-1} to all plots at the experimental site[28].

A previous report from BioCON showed that from 2001 to 2003 the biomass fertilization response to CO_2 was smaller under ambient than enriched N, in contrast to results from 1998 to 2000, when biomass response to CO_2 was independent of soil N supply[5]. Given these dynamic responses early in the experiment, the long-term record is valuable in assessing $CO_2 \times N$ responses beyond the initial stage of ecosystem development, and is the focus of this study. Furthermore, interannual variation in biomass productivity and its response to CO_2, N or their interaction, was high and unrelated to climate variability (see Supplementary Fig. S1). Examination across longer periods (such as the most recent decade) may therefore provide a broader view of average responses.

Across all 13 years of the experiment, both CO_2 and N generally stimulated biomass accumulation ($P < 0.001$, Table 1 and Supplementary Fig. S1), on average by 13% and 20% respectively, but the response to elevated CO_2 differed with N supply level in early (first 3 years) versus later (next 10 years) periods ($P = 0.034$ for the $CO_2 \times N \times$ period interaction; Table 1 and Fig. 1). In the first 3 years of the experiment, the stimulation of total plant biomass by elevated CO_2 averaged ≈ 90 g m^{-2} (11%), and was no less at ambient than at enriched N supply. In the most recent 10 years, however, elevated CO_2 stimulated plant biomass by only half as much under ambient than enriched N supply (73 versus 139 g m^{-2} respectively, Table 1 and Fig. 1). This difference in response across time was similar if the first two years were compared with the subsequent eleven.

The mechanisms responsible for the different responses of biomass accumulation to CO_2 and N supply early versus later in the study could involve temporal heterogeneity in plant physiological acclimation, or in soil and plant processes that influence N availability and use. Leaf gas exchange responses were stable over time and showed no evidence of long-term $CO_2 \times N$ interaction on photosynthetic C acquisition[29], so dynamics in leaf scale physiology
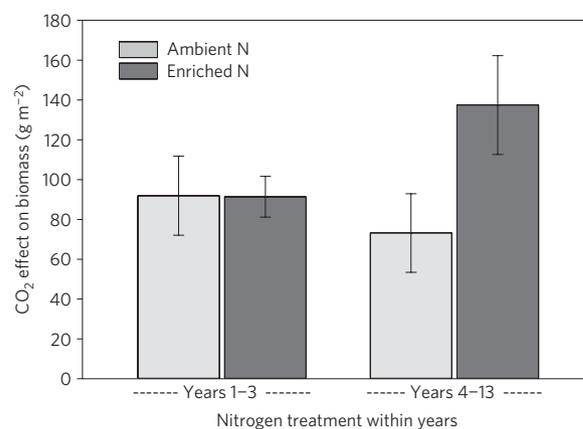

Figure 1 | Effects of CO_2 on total plant biomass. The relative effect of elevated CO_2 on mean plant biomass (g m^{-2}; above-ground plus 0–20 cm depth fine-root biomass measured twice per year) at ambient and enriched N levels for two periods, early in the experiment (1998–2000) or during the following 10 years (2001–2010). The relative effect was calculated as (annual mean biomass at elevated CO_2–annual mean biomass at ambient CO_2) at each N treatment level. One standard error of the among-year means is shown. As diversity did not influence $CO_2 \times N$ interactions (Table 1, no significant three-way interaction), data are shown pooled across diversity treatments.

cannot explain the shifting $CO_2 \times N$ effects on biomass. In contrast, the change in biomass response to CO_2 at contrasting N supply in early versus later years of the study (Fig. 1) was mirrored by similar patterns for soil net N mineralization and plant N pools (Figs 2 and 3); with high probability (97%, 95% and 93% for biomass, net N mineralization and plant N pools, respectively) that the dependence of the response to elevated CO_2 on N supply differed early versus late in the experiment (on the basis of P values for the three-way interaction of $CO_2 \times N \times$ period, Table 1).

During the first three years of the study, net N mineralization (an index of plant N supply) was lower under elevated than ambient CO_2 at both N supply treatment levels. Furthermore, the inhibitory impact of CO_2 on net N mineralization was larger at enriched than at ambient N and corresponded to a negative effect of CO_2 on total plant N pools under enriched, but not ambient, N (Table 1, Figs 2 and 3). These data, showing more depressive effects of CO_2 on N availability under enriched compared with ambient N, could
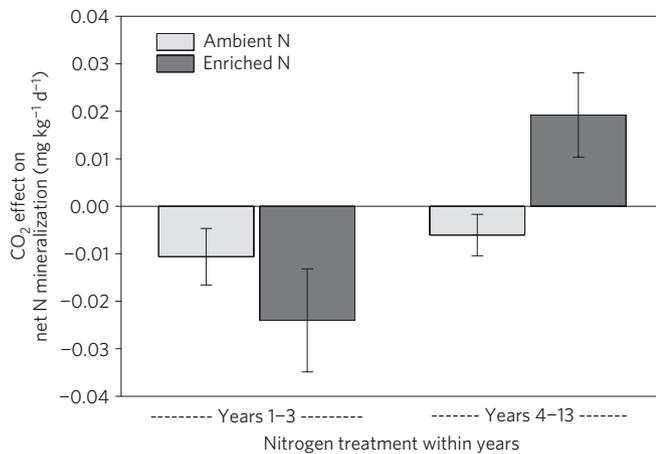
Figure 2 | Effects of CO_2 on soil net N mineralization rate. The relative effect of elevated CO_2 on mean daily soil net N mineralization rate ($mg\,kg^{-1}\,d^{-1}$; averaged for one mid-growing season month per year) at ambient and enriched N levels for two periods, early in the experiment (1998–2000) or during the following 10 years (2001–2010). The relative effect was calculated as (annual mean at elevated CO_2–annual mean at ambient CO_2) at each N treatment level. One standard error of the among-year means is shown. As diversity did not influence $CO_2 \times N$ interactions (Table 1, no significant three-way interaction), data are shown pooled across diversity treatments.

Figure 3 | Effects of CO_2 on total plant N pools. The relative effect of elevated CO_2 on total plant N pools ($g\,N\,m^{-2}$; measured once per year, no 2010 data available) at ambient and enriched N levels for two periods, early in the experiment (1998–2000) or during the following nine years (2001–2009). The relative effect was calculated as (annual mean at elevated CO_2–annual mean at ambient CO_2) at each N treatment level. One standard error of the among-year means is also shown. As diversity did not influence $CO_2 \times N$ interactions (Table 1, no significant three-way interaction), data are shown pooled across diversity treatments.

help explain why enriched N treatment did not stimulate CO_2 fertilization of biomass during the first three years.

In contrast, during the next 10 years, the effect of elevated CO_2 on net N mineralization and plant N pools became more positive under enriched than ambient N (Table 1 and Figs 2 and 3 and Supplementary Fig. S1), consistent with the more positive biomass response to elevated CO_2 under enriched N during this period. The patterns in net N mineralization over time could result in part from initial immobilization of N into living tissues and decomposing organic matter, because of high biomass (especially in the first year) and greater labile C inputs under elevated CO_2 (ref. 27). After three years, the sign of the elevated CO_2 effect on net N mineralization reversed under the enriched N treatment, perhaps as senesced tissues began turning over and releasing N during decomposition processes. Collectively, these results suggest that the more positive biomass response to elevated CO_2 at higher than lower N supply during the most recent 10-year-period is probably related, at least in part, to more positive impacts of CO_2 on N availability at high than low N supply.

The temporal pattern in $CO_2 \times N$ interactions for biomass and N cycling were independent of plant species richness (Table 1). Although species richness had a large main effect on biomass[30], the $CO_2 \times N \times$ period interaction was not influenced by richness (Table 1). These interactions were also independent of composition. We focus here on the potential role of N-fixing legume species (evaluating pure legume plots separately from all others); given that N-fixing legumes might play a key role in responses under contrasting C and N availabilities. However, the patterns in Figs 1 and 3 (all plots) were similar for the subset of plots for which legumes were 25% or less of the initial seed pool (Supplementary Fig. S2). Moreover, although pure legume plots, as expected, had higher net N mineralization rates than all other plots (data not shown), from 2001 to 2010 the pure legume plots showed more positive effects of elevated CO_2 on biomass, net N mineralization rates and total plant N pools under enriched than ambient N (Supplementary Fig. S2), just as did the population of plots with low legume dominance (Supplementary Fig. S2). These results indicate that high legume dominance shifted plots
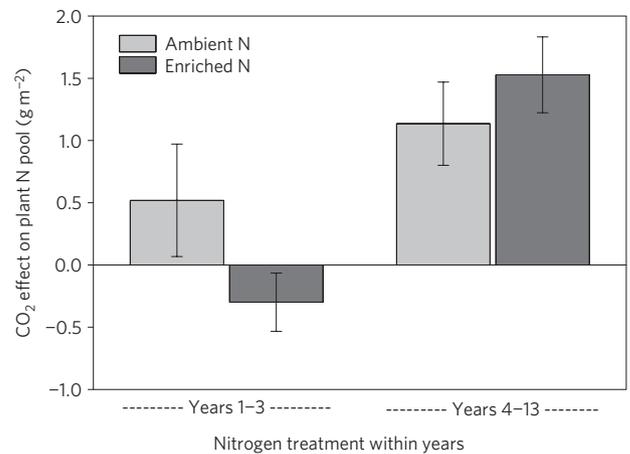
under both ambient and enriched N treatments to higher N cycling conditions, but did not cause or eliminate the interactive effects of $CO_2 \times N$ on plant biomass.

The results of this study show that after an initial 3-year phase following experiment establishment, ambient N supply limited potential biomass accumulation in response to elevated CO_2 over the subsequent decade, with associated shifts over time in N cycling probably playing a role. Ambient N supply did not preclude long-term stimulation of plant productivity by elevated CO_2, but the stimulation at ambient N was only half of that under enriched N. The results of our study are not entirely consistent with either the progressive N limitation hypothesis or the CO_2 priming hypothesis. According to the former, soil N availability should be gradually reduced over time by elevated CO_2, and thus the CO_2 fertilization effect should also diminish over time. There was no evidence that elevated CO_2 suppressed N cycling in the later years of the study, other than perhaps very modestly at ambient N supply, and elevated CO_2 effects became more positive with time rather than more negative. The CO_2 priming hypothesis suggests that labile C inputs stimulate soil N mineralization, thus increasing N supply to plants. Some data are consistent with this—in the past decade, elevated CO_2 increased plant N pools at both N supply rates and net N mineralization in the enriched N treatment. However, multiple resource limitation theory suggests that stimulation of labile C inputs by elevated CO_2 should be greater at low N supply, as plants will allocate more C to growth under high N supply. Thus, we expected that any priming effect on net N mineralization by elevated CO_2 similarly should be greater at low rather than high N supply, which was not observed.

How do our results on biomass fertilization by elevated CO_2 compare with previous reports on this topic? These decadal-scale results support previous experimental studies demonstrating a smaller response of biomass production to elevated CO_2 under low than high N supply, including in a managed pasture, a forest plantation and a natural wetland before compositional change[3,4,10,11]; but such differences have not always been observed[6,13,19]. However, studies that did not show a different response to CO_2 at low versus high N supply often have been in systems showing little or no CO_2 response at all[6,19], probably owing to other limitations

or harsh environmental conditions, and thus may be of minimal help in understanding ecosystems that do show CO_2 fertilization. Furthermore, most studies that manipulated both CO_2 and N have been short (three years or less). Short-term experiments may in some cases have a limited value for understanding interactions over longer timescales, because of potential biogeochemical and/or plant compositional shifts that occur in ecosystems over time[10,30] that might alter long-term responses to any given environmental factor.

To our knowledge, the experiment (BioCON) reported herein is one of only three published, well-replicated long-term (>5 years) open-air $CO_2 \times$ N experiments[4,5,19]. The other two showed strikingly differing results from each other. In a water- and phosphorus-limited annual grassland in California, elevated CO_2 did not stimulate biomass production under either moderate N addition (7 g N m^{-2} yr^{-1}) or ambient N supply[19], whereas in a managed pasture with high N requirement in Switzerland, there was a large and persistent elevated CO_2 fertilization of biomass under very high N fertilization (56 g N m^{-2} yr^{-1}) but none under lower (but still high) N fertilization (14 g N m^{-2} yr^{-1}; ref. 4). The latter result is consistent with our BioCON results in showing that low N availability can limit the CO_2 fertilization of biomass production. Similarly, a long-term replicated CO_2 experiment in a pine plantation, with an unreplicated $CO_2 \times$ N factorial extension, also suggests that low N availability can limit the CO_2 fertilization of biomass production[3,11].

The results of our research and related studies[3,4,11] suggest that widespread soil N limitations will constrain the capacity of terrestrial ecosystems to acquire (and incorporate in biomass) the extra CO_2 emitted by human activities in the future. How this translates into total net ecosystem C storage also depends on whether elevated CO_2 alters the mean residence time of C in plants and soils, and whether this differs at different soil N supply. Early results from BioCON about soil C storage indicate little effect of elevated CO_2 so far, probably because the time needed for such effects to occur and be detectable is long relative to impacts on biomass accumulation. Resampling of soils in the future will enable us to better address this aspect of the terrestrial C cycle within the BioCON context.

Our results for 2001–2010 are consistent with the idea that ambient N availability will dampen the capacity of land ecosystems to sequester large amounts of C in the coming century[2], below that predicted by models that assume no N constraint on CO_2 fertilization, as some recent models are also beginning to suggest[8,9,12]. Although widespread incorporation of such N constraints into models is recommended, it will first require the development of a robust understanding of the long-term CO_2 fertilization impacts on a range of systems and under a range of N supply rates. Although our study suggests that caution is necessary in assuming strong CO_2 fertilization effects in a world with chronic soil nutrient limitation, more long-term studies such as BioCON will be needed to adequately inform the development of realistic Earth system models. Unfortunately, funding for such studies is scarce, suggesting that this key knowledge gap will not be filled anytime soon.

Methods

In the BioCON experiment we planted 296 field plots (each 2 m × 2 m) with different numbers and combinations of perennial grassland species under ambient and elevated atmospheric CO_2 and with either ambient or enriched soil N supply[5,27,29]. A total of 16 native or naturalized species were used in the study, four each from four functional groups (legumes, non-leguminous forbs, C_3 grasses and C_4 grasses). As the results shown in this report were unaffected by plant composition or diversity (see below), the results are generally pooled across such sources of variation. The plots were arranged in six circular 20-m diameter rings at the Cedar Creek Ecosystem Science Reserve in central Minnesota. In three elevated-CO_2 rings, a free-air CO_2 enrichment system[3–5] was used during each growing season to elevate the CO_2 concentration by 180 μmol mol^{-1} above

ambient. Three ambient CO_2 rings were treated identically but without additional CO_2. Half of the plots in each ring received N amendments of 4 g N m^{-2} yr^{-1} applied as NH_4NO_3 on three dates each year. The treatments were arranged in a complete factorial combination of two levels of atmospheric CO_2 (ambient and elevated), four levels of plant species richness (1, 4, 9 and 16) and two levels of N (ambient and enriched with added N; refs 5,27,29). There were initially 32, 15, 15 and 12 plots with 1, 4, 9 and 16 species, respectively, at each of the four contrasting CO_2 and N levels. Six of the 15 plots planted with nine species in each $CO_2 \times$ N level were used for a complementary experiment beginning in 2007 (rainfall was halved) and these were no longer included as part of the present study after that time. The 16 study species included four C_4 grasses (*Andropogon gerardii*, *Bouteloua gracilis*, *Schizachyrium scoparium* and *Sorghastrum nutans*), four C_3 grasses (*Agropyron repens*, *Bromus inermis*, *Koeleria cristata* and *Poa pratensis*), four N-fixing legumes (*Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis* and *Petalostemum villosum*) and four non-leguminous forb species (*Achillea millefolium*, *Anemone cylindrica*, *Asclepias tuberosa* and *Solidago rigida*).

Each year (1998–2010) in every plot we assessed both above- and below-ground plant biomass (in both June and August) and the plant C and N concentrations of those samples (only for August)[5,27]. Above-ground biomass was collected by clipping a single 10 cm × 100 cm strip just above the soil surface. Below-ground fine-root biomass was sampled at 0–20 cm depth in three cores 5 cm in diameter. Soil net N mineralization rates were measured *in situ* each year in each plot by using a semi-open core, one-month incubation[5] beginning in late June. Net N mineralization is the net transformation of N from organic to inorganic forms and is considered to represent the availability of N to plants in systems such as this where plants obtain most N from inorganic forms. Net N mineralization data from 2008 were compromised, probably by contamination, and were not used. At the time of these analyses, tissue N data were available only for 2001–2009. On the basis of shifts in response noted in the early years of the experiment[5] and the goal of examining long-term responses of an ecosystem beyond the establishment phase, we examined results for the 1998–2010 period divided into the first three years and the subsequent 10 years, and used a repeated-measures analysis of variance to test for the main effects of CO_2, N and diversity and their interactions, and whether these differed with period. The results were similar if instead we compared the first 2 to the next 11 years. Analyses were run for all plots (Fig. 1 and Table 1), and as well for plots divided into pure legume plots ($n = 32$) and all others ($n = 264$), which are dominated or solely made up of non-legumes. To further test for a potential influence of legume contribution to composition, we included the relative abundance of legumes in mixed plots as a covariate in the full model (shown in Table 1), but this did not alter the model output, suggesting that the observed responses were not driven by legume effects.

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Author contributions

P.B.R. designed, orchestrated, coordinated and managed the implementation of the experiment; conducted the data analyses; and wrote the manuscript. S.H. assisted with all of these except the study design.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.B.R.

Competing financial interests

The authors declare no competing financial interests.