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Nitrogen inputs and losses in response to chronic CO_2 exposure in a subtropical oak woodland

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Abstract. Rising atmospheric CO_2 concentrations may alter the nitrogen (N) content of ecosystems by changing N inputs and N losses, but responses vary in field experiments, possibly because multiple mechanisms are at play. We measured N fixation and N losses in a subtropical oak woodland exposed to 11 years of elevated atmospheric CO_2 concentrations. We also explored the role of herbivory, carbon limitation, and competition for light or nutrients in shaping the response of N fixation to elevated CO_2. Elevated CO_2 did not significantly alter gaseous N losses, but lower recovery and deeper distribution in the soil of a long-term ^{15}N tracer indicated that elevated CO_2 increased leaching losses. Elevated CO_2 had no effect on nonsymbiotic N fixation, and had a transient effect on symbiotic N fixation by the dominant legume. Elevated CO_2 tended to reduce soil and plant concentrations of iron, molybdenum, phosphorus, and vanadium, nutrients essential for N fixation. Competition for nutrients and herbivory likely contributed to the declining response of N fixation to elevated CO_2. These results indicate that positive responses of N fixation to elevated CO_2 may be transient and that chronic exposure to elevated CO_2 can increase N leaching. Models that assume increased fixation or reduced N losses with elevated CO_2 may overestimate future N accumulation in the biosphere.

1 Introduction

Nitrogen (N) is the element most frequently limiting to plant growth (LeBauer and Treseder, 2008). Nitrogen inputs and losses from terrestrial ecosystems determine ecosystem N pool size, and in turn influence the potential for carbon (C) uptake when plant growth is N limited. Carbon uptake and storage are thus sensitive to the balance of N inputs and losses (Pepper et al., 2007; Gerber et al., 2010; Esser et al., 2011). Here, we synthesize the effects of 11 years of chronic exposure to increased CO_2 concentrations on N inputs and losses from a subtropical oak woodland.

Nitrogen fixation is the major biological pathway through which the biosphere accumulates N. Nitrogen fixation has a high demand for reducing power to break the triple covalent bond shared by the two atoms in the N_2 molecule (Benemann and Valentine, 1972). Symbioses between bacteria capable of N fixation and photosynthetic organisms have evolved

multiple times, likely an adaptive pairing because of the high energetic cost of N fixation and N being frequently limiting to plant growth (Sprent, 1985). Increased photosynthesis with rising concentrations of atmospheric CO_2 has been postulated to shunt more labile C from the plant to bacterial root symbionts, increasing rates of N fixation. In some cases, elevated CO_2 has been found to disproportionately increase the growth of N-fixing plants (Norby and Sigal, 1989; Arnone and Gordon, 1990; Hartwig et al., 2000; Soussana and Hartwig, 1996; Zanetti et al., 1996; Hebeisen et al., 1997; Feng et al., 2004) and increase N fixation, though this potential is not always realized under field conditions (Schäppi and Korner, 1997; Arnone, 1999; Hoosbeek et al., 2011).

Some symbiotic (i.e., free-living) heterotrophic bacteria can also fix N. Elevated CO_2 can increase the amount of C that plants produce belowground through root growth, turnover, and exudation (Drake et al., 2011; Hagedorn et al., 2013; Lagomarsino et al., 2013) and thereby alleviate C limitation of nonsymbiotic N fixation. This may explain why elevated atmospheric CO_2 has been found to stimulate nonsymbiotic N fixation by soil bacteria in a salt marsh (Dakora and Drake, 2000) and a rice paddy soil (Hoque et al., 2001). However, in a temperate pine forest and desert soil, elevated CO_2 had no effect on nonsymbiotic N_2 fixation (Hofmockel and Schlesinger, 2007; Billings et al., 2003). Thus, effects of elevated CO_2 on nonsymbiotic N_2 fixation are equivocal.

N-fixing organisms require high concentrations of iron (Fe), phosphate (P), and molybdenum (Mo), or in some instances vanadium (V) (Williams, 2002). (Smith, 1992). Responses of N-fixation to elevated CO_2 can be limited by availability of these nutrients (Niklaus et al., 1998;Jin et al., 2012). The response of N fixation to elevated CO_2 across multiple studies was only significant when non-N nutrients were added as fertilizer; without nutrient amendments, the effect of CO_2 on N fixation was negligible and not significant (van Groenigen et al., 2006). Elevated CO_2 often increases plant growth and element accumulation (Luo et al., 2006), including in the subtropical woodland studied here (Duval et al., 2013). Therefore, increased element uptake by non-fixing plants could restrict nutrient availability for N-fixing organisms, potentially limiting their response to elevated CO_2.

Because of the high energy requirements of N fixation, shading by the canopy can limit the growth of N-fixing plants (Gutschick, 1987; Vitousek et al., 2002). Therefore, if elevated CO_2 promotes growth of the dominant species, enhancing its leaf area, the growth of N-fixing plants could be suppressed. Herbivory could also influence the response of N-fixing plants to elevated CO_2. Herbivores often prefer the tissue of N-fixing plants to that of other plants because N-fixing plants have a higher protein content than non-fixing plants (Ritchie and Tilman, 1995; Hulme, 1994, 1996). For this reason, factors promoting the growth of N-fixing plants could in turn stimulate selective herbivory.

With the exception of episodic losses during disturbance, N losses from terrestrial ecosystems occur primarily as gaseous products of nitrification and denitrification (NO, N_2O, and N_2) and through leaching of NO_3^- and organic N. Elevated CO_2 can alter N losses if input of labile C to the rhizosphere enhances denitrification rates (Smart et al., 1997; Robinson and Conroy, 1999; Baggs et al., 2003a, b), or by altering soil water content because of reduced evapotranspiration (Hungate et al., 1997a; Arnone and Bohlen, 1998; Robinson and Conroy, 1999). Elevated CO_2 could also reduce ammonium availability to nitrifiers, suppressing nitrification and N losses through gaseous fluxes (Hungate et al., 1997b) and NO_3^- leaching (Torbert et al., 1998). Across studies conducted to date, elevated CO_2 has been found to increase N_2O efflux from terrestrial ecosystems (van Groenigen et al., 2011); effects on N leaching have not been synthesized.

During the first 6 years of the experimental treatment of the subtropical woodland studied here, elevated CO_2 increased N_2 fixation during the first year of treatment, but the response subsequently disappeared (Hungate et al., 2004). Here, we extend this record to include symbiotic N_2 fixation during the full 11 years of the CO_2 experiment, and we also assess responses of nonsymbiotic N_2 fixation to elevated CO_2. We also investigate possible mechanisms shaping the responses to CO_2, testing the hypotheses that selective herbivory, light competition, and changes in nutrient availability modulate the response of N_2 fixation to elevated CO_2. We also report new data on rates of N gas losses and tracer ^{15}N recovery in deep soil to assess effects of CO_2 on N leaching.

2 Materials and methods

2.1 Site description

This work was conducted at the Smithsonian Environmental Research Center's elevated CO_2 experiment at Kennedy Space Center, Cape Canaveral, Florida, USA (28°38′ N, 80°42′ W). The experiment consisted of 16 open-top chambers, each 2.5 m high with an octagonal surface area of 9.42 m^2. Eight chambers were maintained at ambient atmospheric CO_2 concentrations and eight chambers were maintained at approximately 350 µL L^{-1} above ambient atmospheric CO_2 concentration. The soils at the site were acidic Spodosols (Arenic Haplohumods and Spodic Quartzipsamments). The vegetation was Florida coastal scrub oak palmetto (Dijkstra et al., 2002; Johnson et al., 2003; Seiler et al., 2009), dominated by three oaks (*Quercus myrtifolia*, *Q. geminata*, and *Q. chapmanii*) and several less abundant species, including saw palmetto (*Seranoa repens*), shiny blueberry (*Vaccinium myrsinites*), rusty Lyonia (*Lyonia ferruginea*), and tarflower (*Befaria racemosa*). A native vine, Elliott's milkpea (*Galactia elliottii*) constituted only 1 % of aboveground productivity (Hungate et al., 2004) but is important for its ability to fix nitrogen.

2.2 Symbiotic N_2 fixation by *Galactia elliottii*

We estimated *G. elliottii* annual aboveground production as the annual flux of *G. elliottii* mass collected in litter traps (Stiling et al., 1999, 2002, 2009). Litter of *G. elliottii* was sorted and measured separately beginning in 1999; in 1997 and 1998, *G. elliottii* litter fall was measured together with other "non-oak" species. As described previously (Hungate et al., 2004), for 1997 and 1998, we estimated that *G. elliottii* litter constituted 68 % of the non-oak litter. This estimate is the average of the proportion of *G. elliottii* litter mass in in the total non-oak litter fraction as measured from 1999 to 2002 (63 %) and the estimated proportion of *G. elliottii* litter mass in 1997 and 1998 based on a mixing model using N concentration in *G. elliottii* litter (high in nitrogen percentage) versus other species (lower in nitrogen percentage); this mixing model yielded an estimate of 73 %.

We measured N_2 fixation rates using isotope dilution. A nitrogen-15 tracer was added on 19 June 1998 (0.18 g N m^{-2} $(NH_4)_2$ SO_4 99.9 atom % ^{15}N). To the extent that *G. elliottii* fixes N_2 via symbiotic bacterial fixation from the atmosphere, *G. elliottii* leaves will be lower in $\delta^{15}N$ than oak leaves, whose N is derived from the soil, directly increased by the $\delta^{15}N$ value of the added tracer. The proportion of N in *G. elliottii* that was derived from N_2 fixation (p_f) was calculated using the standard model for ^{15}N dilution (Shearer and Kohl 1986), where the ^{15}N signature of unlabeled *G. elliottii* is the atmospheric end member:

$$p_f = (\delta^{15}N_O - \delta^{15}N_G)/(\delta^{15}N_O - \delta^{15}N_{Go}), \quad (1)$$

where the subscript $_O$ refers to the dominant oak (*Q. myrtifolia*); $_G$ to the N-fixer, *G. elliottii*, after adding the ^{15}N tracer; and $_{Go}$ to *G. elliottii* before adding the ^{15}N tracer. This calculation relaxes the common assumption that the $\delta^{15}N$ value of N obtained by fixation is equal to the $\delta^{15}N$ of atmospheric N_2; it therefore accounts for biological fractionation during N_2 fixation (Yoneyama et al., 1986) and provides a more precise estimate of the end member of the mixing model. The $\delta^{15}N$ value of *G. elliottii* prior to label application was −2.2 ‰. A second assumption of this method is that the reference plant (*Q. myrtifolia*) obtains its N from the soil rather than from N fixation. Departures from this assumption will cause the mixing model to underestimate the proportion of N derived from fixation, unlikely to be a serious error at our site because the mixing model indicated that *G. elliottii* obtained nearly all its N from fixation. The isotope dilution method also requires that the $\delta^{15}N$ tracer is sufficiently strong for the $\delta^{15}N$ value of the reference plant to remain distinct from the atmospheric source. This was the case throughout the experiment: the average $\delta^{15}N$ in oak leaves was 131.0 ± 5.3 ‰. By the final harvest, this value declined to 84.3 ± 4.2 ‰, still clearly distinct from the N_2-fixer value (−2.2 ‰), providing sufficient resolution in the mixing model such that the standard deviation for the proportion of N derived from fixation (p_f) was 0.012. The isotope dilution method also assumes that *G. elliottii*, if it takes up N from the soil, accesses approximately the same soil N available to the reference plant. Evidence suggests that this assumption is reasonable, as both fine roots (67 ± 13 %) and *G. elliottii* nodules (74 ± 9 %) were concentrated in the top 30 cm of soil.

We measured N concentration and $\delta^{15}N$ in *G. elliottii* foliage and senesced litter on an elemental analyzer inline with an isotope-ratio mass spectrometer. Total N_2 fixation was calculated as the product of *G. elliottii* biomass production, N concentration, and the proportion of N derived from fixation. To calculate p_f using Eq. 1, we used $\delta^{15}N$ values from senesced leaves because they were gathered in litter traps and therefore captured an integrative sample of *G. elliottii* tissue at the plot scale (Stiling et al., 2009). For N concentration, we used the percentage of N in green leaves to avoid underestimating N_2 fixation due to retranslocation during senescence.

We measured nodule biomass by handpicking soil from cores taken in July 2007 (0–100 cm) (Hungate et al., 2013b; Day et al., 2013). Nodules were washed free of sand, oven dried, and weighed. For comparison, we also report here data obtained during the first year of the experiment (Hungate et al., 1999), when we measured nodule mass and number in buried columns of C-horizon sand placed in the top 15 cm of soil. The earlier assay using ingrowth cores captures new nodule growth, whereas the cores at the final harvest measure the standing crop of nodules. Though not directly comparable, both assess responses of N_2-fixing nodules to the elevated-CO_2 treatment and thus are presented together here.

2.3 Herbivory

From 2000 to 2006, litter of the oaks and of *G. elliottii* was scored for damage by insect herbivores and sorted, counted, and weighed separately by species and insect damage category as described previously (Stiling et al., 1999, 2003, 2009). The rate of litterfall of herbivore-damaged leaves, L (g m^{-2} $year^{-1}$), was determined for each species as the total mass of damaged litter divided by the area of the litter traps in each chamber. Live green leaves of all species were also sampled during the experiment in order to determine the average mass of an undamaged green leaf for each species. Seven plants were randomly chosen for each of the three oak species in September 2004. Fifty leaves were sampled haphazardly over the entire canopy of each plant. For *G. elliottii*, total aboveground biomass was sampled in 1/6th of each chamber in September 1999, separated into leaves and stems, and the leaves were counted. For all four species, leaves were dried in a ventilated oven at 70 °C for 72 h and weighed, and the average leaf mass was calculated. We used these measurements to estimate the amount of tissue consumed by insect herbivores. For each species, we multiplied the number of damaged leaves collected in the litter traps (# leaves m^{-2} $year^{-1}$) by the average mass of a green leaf of that species (g $leaf^{-1}$) to determine the production of all the leaves of which insects consumed at least a part,

P, ($g\,m^{-2}\,year^{-1}$). Note that this differs from total leaf production in that P only includes leaves that were damaged by insect herbivores. We then calculated herbivore consumption (C) as the difference of $P - L$. Our use of green leaf mass in calculating C is justified because herbivore damage often causes early leaf abscission (Faeth et al., 1981; Williams and Whitham, 1986; Stiling and Simberloff, 1989, 2002, 2003). Finally, we calculated total leaf production as the total number of leaves in litterfall multiplied by average green mass and then calculated the proportion of total leaf production that was consumed by herbivores, for each species, as C divided by total leaf production.

2.4 Soil micronutrient analyses

In July 2005, October 2006, and July 2007, soil was collected from the A (0–10 cm) and E (10–30 cm) horizons. Samples were collected from five locations within each chamber. In 2007 we also analyzed samples from the E2 horizon (30–60 cm). The cores from each plot were composited, yielding one A and E sample for each plot for 2005 and 2006, and one A, E, and E2 sample in 2007. We sampled foliage of *G. elliotii*, collecting fully exposed and expanded leaves from five plants per plot in May 2003 and in July 2007. Leaves were oven dried at 60 °C. Total ecosystem element mass data for 2007 were reported previously (Duval et al., 2014); here, they are shown as separate components (plant and soil), together with data from 2003, 2005, and 2006.

Soils were air dried and passed through a 2 mm sieve. Soil-available Mo, Fe, and V concentrations were determined after ammonium oxalate extraction (Liu et al., 1996), and soil-available P was determined by extraction with NaOH (Carter, 1993). Extracts were filtered, diluted 10 times, suspended in 10 mL of 0.32 M trace metal grade HNO_3, and analyzed by Inductively coupled plasma mass spectrometry (ICP-MS) as described below.

For 2007, plant samples were dried at 60 °C and ∼ 750 mg was ashed at 600 °C. Five hundred milligrams of *G. elliottii* leaves was run through a MARS microwave digester using EPA protocol 3052x, consisting of a 30 min cycle at 200 °C with 9 mL of HNO_3, 3 mL of HCl, and 2 mL of HF. All reagents were trace-metal-grade concentrated acids. The resulting solutions were dried on a hot plate and resuspended in 10 mL of 0.32 M trace-metal-grade HNO_3 prior to ICP-MS analysis.

Element concentrations were determined on a Thermo X Series quadrupole ICP-MS at the Keck Isotope Biogeochemistry Laboratory, Arizona State University, Tempe, Arizona, and a Thermo X2 Series quadrupole ICP-MS at the Isotope Geochemistry Laboratory, Northern Arizona University, Flagstaff, Arizona. In these analyses, we used the standard references cody shale (SCo-1) for soils and peach (NIST 1547) and apple leaves (NIST 1515) for plants. In the ICP-MS analysis, we had > 90 % recovery of all elements measured.

2.5 Acetylene reduction for nonsymbiotic N_2 fixation

We measured acetylene reduction to ethylene in soil incubations to estimate N_2 fixation by free-living soil heterotrophs. Soil samples were collected in January and March 2006. At each date, five cores (4 cm diameter x 15 cm deep) were collected from each chamber, shipped overnight to Florida International University (Miami, Florida), composited for each plot, and sieved through a 2 mm sieve. Soils were composited by treatment and 3 g subsamples were filled into 20 mL glass vials ($n = 4$–5). In January 2006, the experimental design included two levels of glucose addition (0 and 324 µg C g^{-1} soil) and two levels of soil moisture (3 % and 10 % volumetric). In March 2006, the experimental design included three levels of glucose addition (0, 6.7, and 34.4 µg C g^{-1} soil) and two levels of phosphorus addition (0 and 6.5 µg P g^{-1} soil). For both experiments, treatments were crossed in a fully factorial design with $n = 4$ for January and $n = 5$ for March. Acetylene (1 mL) was added to each vial, and the vials were sealed and incubated for 6 h at room temperature. Headspace samples were analyzed via gas chromatography for ethylene production using an HP5890 gas chromatograph equipped with a flame ionization detector.

2.6 Nitrous oxide and nitrogen oxide gas fluxes

We measured soil production of nitrous oxide (N_2O) and nitric oxide (NO_x) using static chambers (Hutchinson and Livingston, 1993) during 2005–2007. For N_2O, headspace samples were collected in syringes and analyzed by gas chromatography. For NO_x, the static chamber was plumbed to a chemiluminescent detector and the computer that logged real-time [NO_x]. Chambers (1.8 L) were constructed from a 10.2 cm diameter PVC pipe closed with a PVC cap. The bottom 3 cm of each chamber was tapered to allow the chamber to slide smoothly into PVC rings of similar diameter installed in each plot in 2004. Once the chamber was in place, headspace air (15 mL) was sampled through a rubber septum (fixed to the top of each chamber) using a 20 mL nylon syringe equipped with a nylon stopcock and a 23-gauge needle. Three subsequent headspace samples were taken at 15 min intervals. Syringes were maintained under pressure using a rubber band until analysis, within 12 h of sample collection. Samples were analyzed on a gas chromatograph system (Shimadzu) with Haysep-$Q$60/80- and Porapack-$Q$60/80-packed columns and equipped with an electron capture device to determine N_2O concentrations. Field fluxes were calculated using linear regression of concentrations over time. The flux rates were expressed as µg N_2O-N $m^{-2}\,d^{-1}$.

2.7 Tracer ^{15}N distribution and recovery in deep soils

During the final harvest of the experiment in 2007, each plot was cored to the water table or to a depth of 3 m (whichever was deeper). Core depth averaged 260.6 cm (SEM 13.5),

not significantly different between the CO_2 treatments ($P = 0.90$; t test). We modeled the depth distribution of tracer ^{15}N using the function

$$Y = 1 - \beta^d,$$

where d is depth, Y is the cumulative proportion of tracer ^{15}N recovered up to depth d, and β is a fitted parameter (Gale and Grigal, 1987). We used Microsoft Excel's Solver function to find the values of β, minimizing the sum of squares of the errors in Y between measured and modeled values for each plot. We used a t test to determine if elevated CO_2 altered β and used nonlinear regression to explore relationships between the vertical distribution of recovered ^{15}N and total ^{15}N recovery at the ecosystem scale (mg $^{15}N\,m^{-2}$).

2.8 Statistical analyses

We used analysis of variance (ANOVA) to test for effects of elevated CO_2, using repeated measures to test for effects of time and interactions between CO_2 and time, and split-plot ANOVA to test for differences among soil depths. When necessary, data were log-transformed (N_2 fixation, soil element concentrations) to meet assumptions of ANOVA. For nodule biomass in 1996 and 1997, we used a Kruskal–Wallis test because data did not meet assumptions of ANOVA and values of zero precluded log-transformation. We used an α threshold of 0.10. We omitted one data point from the analysis of foliar Mo of *G. elliottii* in 2007 based on a Grubb's outlier test; omitting the data point did not influence the significance of any statistical comparison. We used simple and multiple linear regressions to explore relationships between N_2 fixation and possible drivers. We also used ANOVA for the acetylene reduction assay to test effects of CO_2, glucose, and water (in January 2006) or phosphorus (March 2006) on rates of N_2 fixation by free-living soil microorganisms. We note that compositing soils from plots by CO_2 treatment compromised the independence of replicates within CO_2 treatments. The absence of any significant effects of CO_2 on nonsymbiotic N_2 fixation (see below) provides some protection from the dangers of false inference caused by pseudoreplication (Hurlbert, 1984). We assessed effects of N_2 fixation as a function of time since disturbance by calculating time since disturbance as the number of years that had elapsed between the date of the measurement and the most recent disturbance, whether by fire at the beginning of the experiment or by hurricane in September 2004 (Hungate et al., 2013a). We expressed the effect of elevated CO_2 on N_2 fixation as the absolute difference between elevated and ambient CO_2 plots.

Table 1. Proportion of N derived from fixation and N concentration in *Galactia elliottii* during 11 years of exposure to elevated CO_2 and results from repeated measures ANOVAs for effects of time, elevated CO_2, and their interaction.

Year	*p*Fixation		%N	
	Ambient	Elevated	Ambient	Elevated
1996			1.37 ± 0.16	1.20 ± 0.08
1997			2.20 ± 0.16	2.33 ± 0.16
1998	0.911 ± 0.026	0.910 ± 0.026	2.13 ± 0.10	1.86 ± 0.10
1999	0.939 ± 0.008	0.918 ± 0.005	1.86 ± 0.12	1.48 ± 0.05
2000	0.932 ± 0.006	0.875 ± 0.017	1.99 ± 0.14	1.95 ± 0.08
2001	0.953 ± 0.008	0.924 ± 0.025	2.13 ± 0.10	1.83 ± 0.07
2002	0.941 ± 0.009	0.88 ± 0.012	2.26 ± 0.13	1.70 ± 0.12
2003	0.965 ± 0.006	0.958 ± 0.009	1.98 ± 0.05	1.75 ± 0.07
2004	0.964 ± 0.008	0.908 ± 0.046	1.94 ± 0.06	1.79 ± 0.09
2005	0.992 ± 0.004	0.99 ± 0.008	1.92 ± 0.04	1.75 ± 0.08
2006	0.991 ± 0.008	0.998 ± 0.001	2.05 ± 0.05	1.64 ± 0.14
2007	0.994 ± 0.002	0.987 ± 0.004	1.76 ± 0.09	2.12 ± 0.11
CO_2		0.005	0.006	
Time		0.018	< 0.001	
$CO_2 \times$ Time		0.453	0.002	

3 Results

3.1 N_2 fixation by *G. elliottii*

N_2 fixation by *Galactia elliottii* varied over time ($P < 0.001$), with high rates in 1998 and low rates in 2000 and 2005 (Fig. 1). Elevated CO_2 increased N_2 fixation by *G. elliottii* during the first six months of experimental treatment (Fig. 1a and as reported in Hungate et al., 1999). However, by the third year of exposure to elevated, CO_2, N_2 fixation was not different between the two treatments ($CO_2 \times$ time interaction, $P = 0.070$). Subsequently (years 5–7), elevated CO_2 suppressed *G. elliottii* N_2 fixation. Rates equalized again by year 9 (2005). Accumulated over the 11-year exposure period, *G. elliottii* fixed 3.92 ± 0.50 g $N\,m^{-2}\,year^{-1}$ in the ambient CO_2 treatment compared to 3.51 ± 0.71 g $N\,m^{-2}\,year^{-1}$ in the elevated-CO_2 treatment, a nonsignificant difference (repeated measures ANOVA, $P = 0.376$). The effect of elevated CO_2 dissipated with time since disturbance (Fig. 1b).

G. elliottii derived nearly all of its foliar N from fixation, increasing from 92 % in 1998 to 100 % from 2005 to 2007 (Table 1). Given the small range of variation in reliance on atmospheric N_2, temporal changes in N_2 fixation (Fig. 1) were driven by effects of time and treatment on the productivity of *G. elliottii*. Nevertheless, the proportion of N derived from the atmosphere by *G. elliottii* was sensitive to temporal variation and to the CO_2 treatment. Across all years, elevated CO_2 reduced the reliance of *G. elliottii* on N from the atmosphere, though the effect was small, from an average of 96.9 % for the ambient CO_2 treatment to 94.7 % for the elevated treatment. Foliar nitrogen percentage of *G. elliotti* was

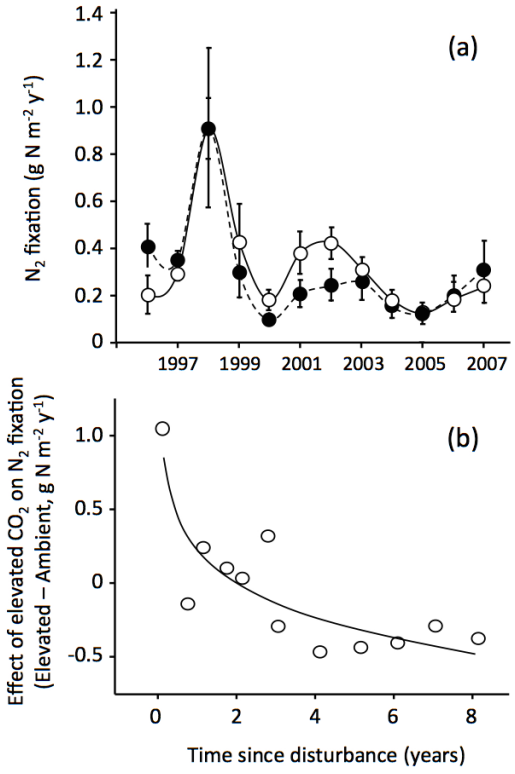

Figure 1. Rate of N_2 fixation **(a)** and effect of elevated CO_2 on N_2 fixation as a function of time since disturbance **(b)** for *Galactia elliottii* during 11 years of experimental exposure to increased carbon dioxide concentrations. For panel **(a)** Values are means ± standard error of the mean ($n = 8$) for the ambient (open circles) and elevated (filled circles) CO_2 treatments. For panel **(b)**, values are differences of means for each year of the experiment.

lower in the elevated-CO_2 treatment (Table 1), an effect that varied over time. During the first year of the experiment, elevated CO_2 stimulated nodule biomass (effect of CO_2 in 1996, $P = 0.062$), but elevated CO_2 had no effect on nodule mass at final harvest (Fig. 2, Table 2), a pattern similar to that found for N_2 fixation.

N_2 fixation by *G. elliottii* (g N m^{-2} year^{-1}) was positively correlated with *G. elliottii* foliar N concentration, negatively correlated with the total mass of the oak leaves, negatively correlated with herbivory, and unrelated to leaf area index (Table 3). Therefore, as the dominant plants grew larger, N_2 fixation declined, suggesting competition. The absence of any relationship with leaf area index argues against competition for light. N_2 fixation also declined as foliar nitrogen percentage declined, which can indicate limitation by non-N nutrients (Rogers et al., 2009). Finally, high proportional consumption of *G. elliottii* tissue was associated with lower rates of N_2 fixation, suggesting some control of N_2 fixation by herbivory (Table 3).

Table 2. Nodule mass (g m^{-2}) recovered in soil cores at the final harvest in 2007. P values from split-plot ANOVA are shown in the last three rows.

Depth	Ambient	Elevated
0–10 cm	108 ± 56	66 ± 29
10–30 cm	70 ± 62	71 ± 60
30–60 cm	122 ± 98	35 ± 35
60–100 cm	32 ± 13	10 ± 5
0–100 cm	332 ± 143	182 ± 108
CO_2		0.416
Depth		0.498
CO_2 × Depth		0.812

Table 3. Multiple regression of rates of N_2 fixation (g N m^{-2} year^{-1}) as a function of foliar N concentration in *G. elliottii* (g g^{-1} × 100 %), herbivory (% of leaf production consumed), leaf area index (m^2 m^{-2}), and total oak biomass (kg m^{-2}). The overall regression is significant ($F_{4,103} = 8.543$; $P < 0.001$; adjusted $r^2 = 0.22$).

Effect	Coefficient ± SE	P
Constant	0.0467 ± 0.1682	0.782
N concentration	0.1594 ± 0.0583	0.007
Ln(Herbivory)	−0.0549 ± 0.0147	< 0.001
Leaf area index	−0.0194 ± 0.0474	0.683
Oak Biomass	−0.0233 ± 0.0104	0.027

3.2 Soil-extractable micronutrient concentrations

Concentrations of soil-extractable Fe, Mo, and V declined from 2005 to 2007 (Fig. 3, Table 4). Elevated CO_2 significantly reduced soil-extractable concentrations of Mo and V, and tended to reduce Fe. Some assays of soil P availability collected during the experiment indicated that elevated CO_2 reduced soil P availability (ion exchangeable P in 1997, extractable P in 2001), though this effect was not apparent at the final harvest (Fig. 4). Reductions in soil element availability under elevated CO_2 corresponded with reduced foliar concentrations in *G. elliottii*: elevated CO_2 reduced foliar concentrations of Fe in 2003 and 2007 (repeated measures ANOVA (RMA), $P = 0.013$), and, for 2003, Mo (Hungate et al., 2004), although foliar P was not affected in either year (Fig. 5; RMA, $P = 0.886$). However, the rate of N_2 fixation was not related to soil-extractable nutrient concentration (data not shown, $P > 0.10$ for all regressions).

3.3 Herbivory

Herbivores in this subtropical woodland consumed a higher percentage of *G. elliottii* leaf production (24.0 ± 1.8 %) than of oak leaf production (14.1 ± 0.6 %, $P < 0.001$, Fig. 6). The percentage consumed of *G. elliottii* was not affected by the

Table 4. Effect of elevated CO_2, time, and soil depth on extractable nutrient concentrations for the last three years of the experiment (2005, 2006, and 2007). Values are P values from repeated measures, split-plot ANOVAs testing for the main effect of CO_2 treatment, repeated measures effect of the year, and split-plot effect of the depth, as well as all interactions. All data were log-transformed before analysis.

Element	CO_2	Year	Depth	CO_2 × year	CO_2 × depth	Year × depth	CO_2 × $Y \times D$
Fe	0.223	< 0.001	0.056	0.731	0.720	0.035	0.417
Mo	0.024	< 0.001	0.012	0.150	0.674	0.088	0.796
V	0.010	< 0.001	< 0.001	0.376	0.209	0.036	0.844

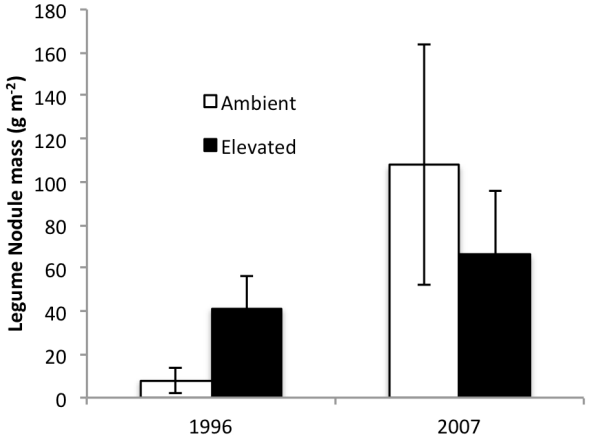

Figure 2. Mass of *G. elliottii* nodules recovered in the top 10 cm of soil in 1996 (from ingrowth soil cores) and in 2007 (from intact cores). Values are means ± standard errors.

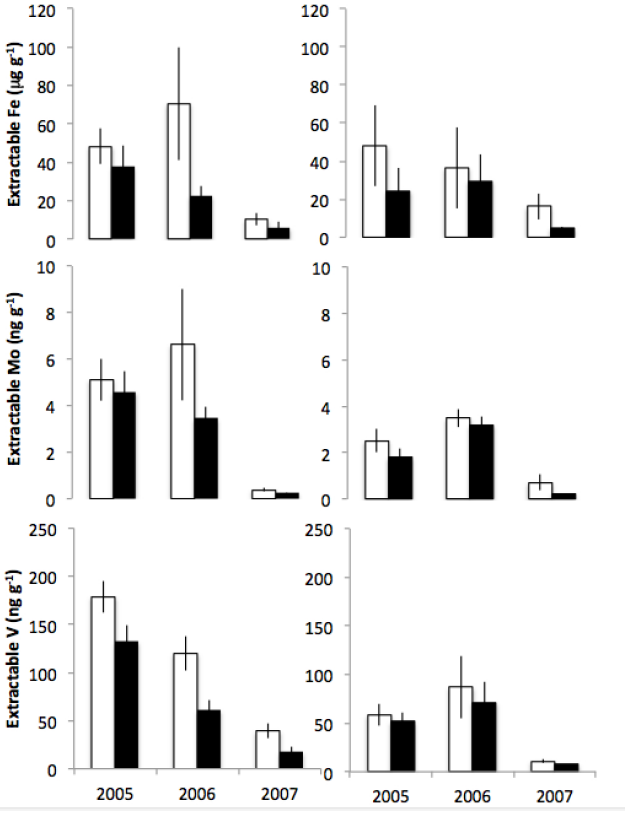

Figure 3. Soil-extractable Fe, Mo, and V concentrations for 0–10 cm (left three panels) and 10–30 cm (right three panels) soil depths over the last three years of the experiment. Values are means ± standard error of the mean. Units are µg g^{-1} soil (for Fe) and ng g^{-1} soil (for Mo and V).

elevated-CO_2 treatment ($P = 0.443$), nor did it vary significantly from year to year ($P = 0.700$; no interactions were significant in the repeated measures ANOVA; $P > 0.30$ for all).

3.4 Nonsymbiotic N_2 fixation

Elevated CO_2 had no effect on acetylene reduction in soil laboratory incubations (Tables 5 and 6). Acetylene reduction was also unresponsive to phosphorus addition or soil water content. Acetylene reduction increased with glucose addition, consistent with carbon limitation of heterotrophic nonsymbiotic N_2 fixation in these soils.

3.5 Nitrous oxide and nitric oxide fluxes

Across all measurements conducted during the CO_2 enrichment experiment, N_2O efflux averaged 0.346 mg N_2O-N m^{-2} d^{-1} for the ambient CO_2 treatment and 0.377 mg N_2O-N m^{-2} d^{-1} for the elevated-CO_2 treatment, a nonsignificant difference of 0.035 mg N_2O-N m^{-2} d^{-1} (5 and 9 % confidence limits, −0.070 and 0.132). Scaled to the entire 11-year period and assuming that N_2O constitutes 10 % of the total N losses in denitrification (with 90 % as N_2 production), our best estimate is that elevated CO_2 increased losses of N_2O-N by 1.4 g N m^{-2} , though this difference (elevated – ambient) is not significant, with 5 and 95 % confidence limits that span zero (−2.9 to 5.3). Across all measurements, NO_x losses averaged 0.075 mg N m^{-2} d^{-1} for the ambient CO_2 treatment and were somewhat lower for the elevated-CO_2 treatment at 0.027 mg N m^{-2} d^{-1}, a difference between elevated and ambient treatments of −0.047 mg N m^{-2} d^{-1} (5 and 95 % confidence limits, −0.116 to −0.002 mg N m^{-2} d^{-1}). Scaled to the entire 11-year period, the elevated-CO_2-treated plots lost 0.20 g N m^{-2} less NO_x

Table 5. Rates of acetylene reduction ($\mu mol\, C_2H_2\, g^{-1}\, h^{-1}$) as a proxy for nonsymbiotic N_2 fixation, measured in soils from the ambient- and elevated-CO_2 treatments from laboratory incubations with added glucose and either added water (January 2006) or added phosphorus (March 2006). Values are means ± standard errors of the mean ($n = 4$, January 2006; $n = 5$, March 2006).

	Glucose $\mu g\, g^{-1}$	Ambient CO_2	Elevated CO_2	Ambient CO_2	Elevated CO_2
Jan 2006		3 % H_2O		10 % H_2O	
	0	21.5 ± 3.2	23.8 ± 3.0	20.4 ± 2.3	21.8 ± 2.5
	324	27.4 ± 1.4	25.8 ± 4.7	32.4 ± 3.2	30.0 ± 4.5
Mar 2006		0 P		6.5 P	
	0	36.0 ± 1.7	37.7 ± 4.1	37.2 ± 4.2	37.5 ± 1.6
	6.7	42.0 ± 3.8	42.7 ± 4.5	34.6 ± 1.2	36.0 ± 2.7
	34.4	42.4 ± 3.1	44.9 ± 3.1	43.6 ± 3.5	41.0 ± 2.8

Table 6. P values from ANOVAs testing the effects of chronic CO_2 exposure and short-term additions of glucose, water, and phosphorus on acetylene reduction. Columns under each date show P values for main effects and interactions tested on that date, H_2O for January and phosphorus for March (note: the first P value in each column is a main effect).

Jan 2006		Mar 2006	
CO_2	0.964	CO_2	0.727
Glucose	< 0.001	Glucose	0.011
CO_2 × glucose	0.329	CO_2 × glucose	0.816
H_2O	0.351	P	0.151
H_2O × CO_2	0.773	P × CO_2	0.670
H_2O × glucose	0.071	P × glucose	0.604
H_2O × CO_2 × glucose	1.000	P × CO_2 × glucose	0.673

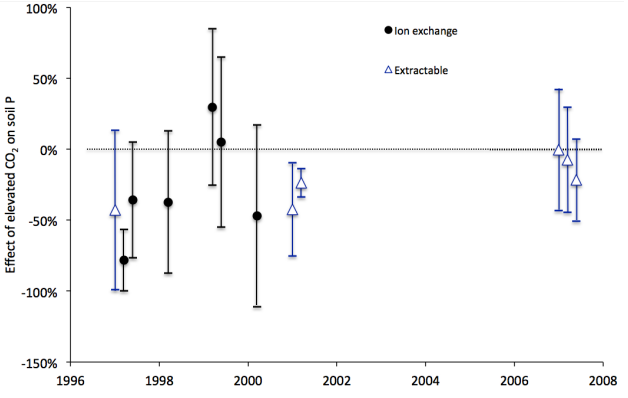

Figure 4. Effect of elevated-CO_2 concentration on available P in soils over time using two methods, extractable soil P (open triangles) and ion-exchange resins (filled circles). Values are the effect size of elevated CO_2, expressed as a percentage: $(E - A)/A \times 100$ %. Bars denote 5 and 95 % confidence limits. (Data from 1997 to 2001 were calculated from raw data reported in Johnson et al., 2001, 2003.).

compared to the controls (5 % and 95 % confidence limits, 0.01 to 0.47). If these rates are typical for N_2O and NO_x losses over the experiment, the elevated-CO_2-treated plots lost 1.2 g N m^{-2} more N in gaseous fluxes compared to the ambient CO_2 treatment, but this difference was not significant (−3.3 to 5.3; 5 and 95 % confidence limits).

3.6 ^{15}N tracer recovery and distribution as an indicator of N leaching

Nitrogen movement from the O and A horizons into deeper soil (> 15 cm) was measured directly during the first 3 years of the experiment using resin lysimeters (Johnson et al., 2001), with a tendency for CO_2 to reduce vertical N movement. Yet whole-system recovery and distribution of the ^{15}N tracer were consistent with increased ^{15}N losses via leaching in the elevated-CO_2 treatment. Elevated CO_2 reduced total ^{15}N recovery (Hungate et al., 2013), and of ^{15}N that remained in mineral soil, more was found deeper in the profile in the elevated-CO_2 treatment as indicated by a higher β value ($\beta = 0.81 \pm 0.01$ for ambient CO_2 and 0.89 ± 0.03 for elevated CO_2, $P = 0.03$). These findings were associated such that reduced ^{15}N recovery occurred in plots where the recovered ^{15}N was distributed deeper in the soil profile (Fig. 7). Thus, elevated CO_2 reduced ^{15}N recovery and promoted ^{15}N movement down the soil profile, consistent with increased N leaching losses.

4 Discussion

4.1 Effects of CO_2 on processes regulating ecosystem N accumulation

Findings reported here explain the absence of any stimulation of N accumulation in response to 11 years of exposure of this subtropical oak woodland to elevated carbon dioxide concentration (Hungate et al., 2013b). Elevated atmospheric CO_2 either elicited no response in processes favoring N accumulation (nonsymbiotic N_2 fixation) or caused only a transient and quantitatively negligible response (symbiotic

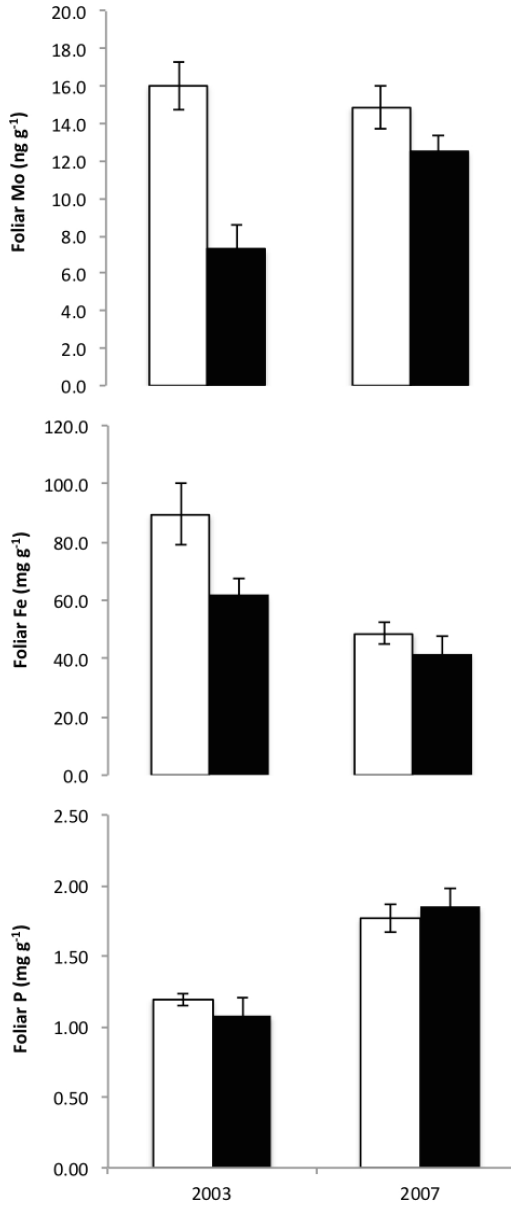

Figure 5. Effects of elevated CO_2 concentration on Mo, Fe, and P concentrations in leaves of *G. elliottii* sampled in 2003 and 2007 (values for Mo and Fe from 2003 are from Hungate et al., 2004).

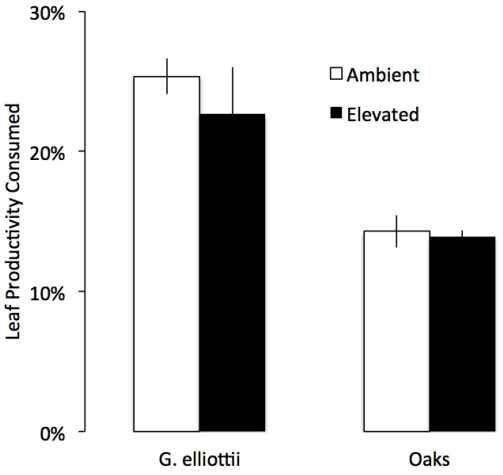

Figure 6. Percent of leaf productivity consumed by herbivores for the N_2-fixing vine, *G. elliottii*, and for the three co-dominant oak species in the ambient- and elevated-CO_2-treated plots.

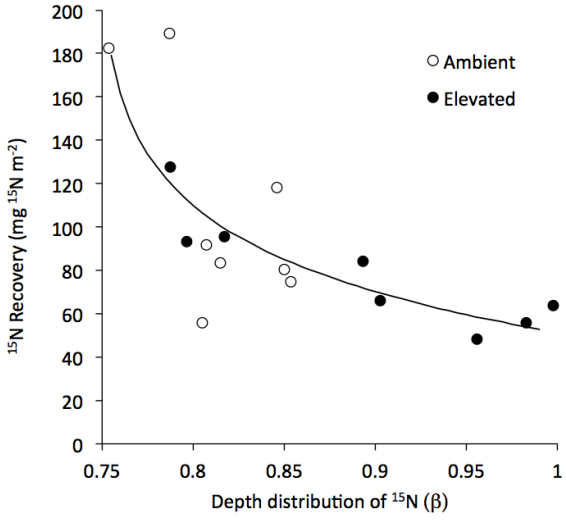

Figure 7. Recovery of added tracer ^{15}N as a function of its depth distribution. Higher β values indicate relatively more ^{15}N in deeper soil layers, whereas low β values indicate concentration of ^{15}N at the soil surface.

N_2 fixation). Reduced gaseous N losses from the elevated-CO_2-treated plots were observed for NO_x, but NO_x was a minor component of the ecosystem N budget such that the changes observed were insufficient to promote N accumulation. Moreover, the reduced ^{15}N recovery and pattern of greater ^{15}N recovery deep in the soil indicates that elevated CO_2 enhanced leaching losses of N. Our results indicate that processes promoting N loss were more responsive to elevated CO_2 than were processes promoting N accumulation.

4.2 Effects of CO_2 on N_2 fixation

N_2 fixation by the leguminous vine, *G. elliottii*, was only temporarily responsive to the CO_2 treatment (Fig. 1). Growth and N_2 fixation in N_2-fixing plants can respond positively to elevated CO_2 (Cernusak et al., 2011), but in many cases these responses are absent, e.g., N-fixers in temperate grasslands (Garten et al., 2008; Zhang et al., 2011), *Alnus* (Temperton et al., 2003; Millett et al., 2012), and ocean cyanobacteria (Czerny et al., 2009; Law et al., 2012). Chronic CO_2 exposure was found to reduce cyanobacterial abundance in desert crusts (Steven, 2012). Our finding that nonsymbiotic

N_2 fixation in soil was also insensitive to the CO_2 treatment is consistent with observations in other forest ecosystems (Hofmockel and Schlesinger, 2007). In general, responses appear to be more muted under field conditions in long-term experiments than in short-term (van Groenigen et al., 2006; Leuzinger et al., 2011), so our finding that elevated CO_2 did not increase N_2 fixation is not unusual. Nitrogen availability, light limitation, herbivory, and non-N nutrient limitation are plausible explanations for the absence of a significant response of N_2 fixation after the first year of the experiment.

4.3 Nitrogen availability

Increased N availability is well known to reduce N_2 fixation (Streeter, 1988), as uptake of N from the soil is energetically favorable to fixation. In the scrub oak ecosystem studied here, elevated CO_2 increased plant N uptake from soil, in part by enhanced turnover of soil organic matter (Carney et al., 2007; Langley et al., 2009; Hungate et al., 2013b). The slight reduction in the reliance of *G. elliottii* on atmospheric N in response to elevated CO_2 (Fig. 1, Table 1) is consistent with a CO_2-stimulation of soil N availability. N_2 fixation can decline with shading during canopy development, yet in this system leaf area index was not a significant predictor of N_2 fixation (Table 3), possibly because the N_2-fixers are vines, capable of climbing rapidly to the top of the short canopy to alleviate light limitation (Kurina and Vitousek, 1999). On the other hand, the negative relationship between N_2 fixation and total oak biomass (Table 3) suggests competition for other resources between the oaks and the N_2-fixing vine, possibly competition for nutrients.

4.4 Non-N nutrients

When supplied with sufficient non-N nutrients, N_2-fixing plants will increase nodule biomass and rates of N_2 fixation (van Groenigen et al., 2006; Rogers et al., 2009), while at the same time maintaining foliar N concentration in response to elevated CO_2. Under nutrient-limiting conditions, foliar N declines, nodule growth diminishes, and rates of N_2 fixation are depressed (Rogers et al., 2009), a pattern consistent with the positive correlation between foliar N concentration and N_2 fixation shown here (Table 3).

The responses of N_2 fixation and growth of N-fixing plants to elevated CO_2 may depend on availability of non-N nutrients. In our system, there was some evidence that the availability of soil P declined, particularly during the first 6 years of the experiment (Fig. 4, and see Johnson et al., 2001, 2003, a response also observed in a rice–wheat rotation, see Ma et al., 2007). However, reduced P availability is not a universal response to increased CO_2 concentration (Dijkstra et al., 2012; Khan et al., 2008, 2010). Other elements critical for N_2 fixation such as Mo, Fe, and V either declined significantly in soil and in foliage or showed a tendency to decline (Figs. 3, 5), suggesting their role in modulating the response of N_2 fixation to elevated CO_2. Natali et al., 2009 found that total soil metal content increased in the surface soils of a loblolly pine and sweetgum plantations, though total metal concentration may be a poor indicator of extractable metal concentration and metal availability to plants. Foliar concentrations of most metal elements were substantially lower at this scrub oak site compared to the loblolly pine and sweetgum plantations (Natali et al., 2009) (Duval et al., 2014). Sandy texture and low pH are two soil properties typically associated with low metal availability because sandy soils have low ion exchange capacity and because metal availability is very sensitive to pH (Vlek and Lindsay, 1977; Sposito, 1984; Goldberg et al., 1996; Kabata-Pendias, 2001). Thus, compared to other ecosystems, this subtropical woodland may be more likely to exhibit micronutrient limitation of ecosystem processes, such as N_2 fixation.

Several experiments have demonstrated the importance of P availability for the responses of N_2 fixation to elevated CO_2: the stimulation of growth and N_2 fixation by elevated CO_2 was higher with P addition for clover (Edwards et al., 2006), *Azolla* (Cheng et al., 2010), soybean (Lam et al., 2012), chickpea, and field pea (Jin et al., 2012). The CO_2-stimulation of N-fixer growth and N_2 fixation in several grasslands experiments was higher with P addition compared to controls without supplemental P (Niklaus et al., 1998; Grunzweig and Korner, 2003). In general, with supplemental nutrients added, elevated CO_2 often increases N_2 fixation (Lee et al., 2003; Otera et al., 2011), but in experiments without exogenous nutrient addition, N_2 fixation is generally not responsive to elevated CO_2 (van Groenigen et al., 2006).

4.5 Herbivory

Herbivory has been postulated to reduce N_2 fixation in ecosystems because of preferential feeding on the more nutritious tissue of N_2-fixers compared to other plants (Vitousek and Howarth, 1991). This hypothesis is consistent with our finding that herbivores in this subtropical woodland consumed a larger proportion of *G. elliottii* leaf production than leaf production of oaks (Fig. 6). Herbivory could also limit the response of N_2 fixation to environmental change, if grazing on the tissue of N_2-fixers increases. Increasing herbivore pressure on the dominant N_2-fixing vine in our experiment was associated with reduced rates of N_2 fixation (Table 3), which supports this idea. Elevated CO_2 decreased the proportion of leaves with herbivore damage in this scrub oak woodland (Stiling and Cornelissen, 2007), as has been found in other systems (Lindroth, 2010; Robinson et al., 2012). Insect herbivores often respond to reduced leaf nitrogen concentration by consuming more leaf tissue (Stiling et al., 2003). In the scrub oak woodland studied here, this response resulted in no effect of elevated CO_2 on the proportion of N-fixer leaf production consumed by herbivory (Fig. 6).

4.6 C limitation of nonsymbiotic N_2 fixation

Our finding that acetylene reduction increased with glucose addition suggests C limitation of heterotrophic N_2 fixation in soil (Tables 5 and 6), as has been found previously (Billings et al., 2003). Neither P addition nor altered soil water content influenced rates of acetylene reduction, indicating that these factors were not limiting to soil N_2 fixation.

4.7 Temporal dynamics

Evidence for a single, dominating mechanism underlying the response of N_2 fixation to elevated CO_2 was weak in our experiment, possibly because the process is limited by multiple factors whose influences shift over time. The experimental site was struck by a hurricane in 2004 (Li et al., 2007a, b), a disturbance that preceded the high soil nutrient concentrations observed in 2005, which subsequently declined in 2006 and 2007 (Fig. 3). This disturbance also preceded a large positive response of oak production to elevated CO_2 found both above- and belowground (Day et al., 2013; Hungate et al., 2013a), as well as the disappearance of the CO_2 suppression of *G. elliottii* growth and N_2 fixation (Fig. 1). Consistent with this, the positive correlation between foliar Mo concentration and N_2 fixation rates found in the two years before the hurricane (Hungate et al., 2004) was no longer apparent by the final harvest (regression between foliar [Mo] and N_2 fixation for 2007, $r^2 = 0.138$; $P = 0.638$). Yet, rates of N_2 fixation were low after the hurricane disturbance in both treatments (Fig. 1), and there was no association between soil-extractable micronutrient availability and rates of N_2 fixation during the period 2005–2007; therefore, the availability of micronutrients, if they played a role, were not the only factor limiting N_2 fixation at this time.

The temporal variation in the response of N_2 fixation, in particular the finding that initially strong positive responses dissipate with time (Leuzinger et al., 2011), may be a general feature of global change experiments. While elevated CO_2 can stimulate N_2 fixation in some species and under some conditions, responses in many field studies are far more muted (van Groenigen et al., 2006) consistent with findings reported here. Thus, increased N_2 fixation is not a certain outcome of rising atmospheric CO_2 concentrations.

4.8 N Losses

N fixation is the major biological process mediating N inputs to terrestrial ecosystems, but N losses through gaseous and leaching pathways exert an equally important influence on total N pool size. Our finding that elevated CO_2 had no significant effect on N_2O production in this subtropical woodland is consistent with several past studies finding no effect of CO_2 on N_2O production (Mosier et al., 2002;Phillips et al., 2001), though increased N_2O production has been detected in others (Hagedorn et al., 2000; Ineson et al., 1998; Kammann et al., 2008; Lam et al., 2010; Smith et al., 2010). On average, elevated CO_2 tends to increase soil N_2O emissions by around 20 % (van Groenigen et al., 2011), a larger stimulation than the nonsignificant increase of 8 % we observed in this subtropical woodland. The trend for elevated CO_2 to reduce NO_x losses from this subtropical woodland indicates that NO_x losses were likely not the major pathway of increased N loss from this system in response to elevated CO_2. By contrast, the concurrence of reduced tracer ^{15}N recovery (Hungate et al., 2013b) and deeper distribution of tracer ^{15}N throughout the soil profile (Fig. 7) supports the notion that elevated CO_2 stimulated N leaching in this experiment. Increased leaching with elevated CO_2 has been observed (Korner and Arnone, 1992) and may be caused by a combination of increased plant water-use efficiency resulting in greater downward water flux through the soil profile (Jackson et al., 1998), along with increased turnover of soil organic matter in response to rising CO_2 (Drake et al., 2011; Hungate et al., 2013b). Some experiments have documented reduced N leaching with elevated CO_2 (Johnson et al., 2004), so our finding of increased leaching is likely not universal. Furthermore, during the first three years of this experiment, we found no effect of elevated CO_2 on vertical movement of N from the surface to deeper soil layers (Johnson et al., 2001). The use of the ^{15}N tracer to integrate the cumulative effects of elevated CO_2 on nitrogen losses in this experiment may be both more temporally integrative and sensitive to changes in the ecosystem-scale distribution and retention of N in response to elevated CO_2.

4.9 Summary

Eleven years of chronic exposure to increased CO_2 concentrations elicited disequilibrium in the N cycle, with increased rates of internal N transformations, no change in N inputs, and increased N losses. Elevated CO_2 accelerated the rate of soil N mineralization (Langley et al., 2009; McKinley et al., 2009), which likely contributed to increased N uptake by plants (Hungate et al., 2013). Nitrogen losses also increased, with increased turnover of N through plant tissues, as evidenced by the increased ^{15}N dilution in plants (Hungate et al., 2013) along with no change in net plant N capital (Hungate et al., 2013). Elevated CO_2 also appeared to enhance N losses at the scale of the soil profile: the pattern of lower ^{15}N recovery in plots exhibiting greater downward movement of ^{15}N in the soil profile suggests increased leaching. Thus, processes that make nutrients available to plants can also promote nutrient losses. Finally, we found no evidence that elevated CO_2 enhanced N inputs via N_2 fixation. Together, these results describe an ecosystem in which more rapid cycling of N with elevated CO_2 is unlikely to be sustained. These empirical findings contrast with model projections in which elevated CO_2 enhances N_2 fixation and reduces leaching (Thornley and Cannell, 2000). Given the strong influence of N cycling and N accumulation on the C cycle, the changes

in N cycling reported here, if general, would tend to dampen the biosphere's capacity to assimilate and store C in the face of rising atmospheric CO_2 concentrations (Thornton et al., 2007; Churkina et al., 2009; Arneth et al., 2010; Zaehle and Dalmonech, 2011).

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