

Effects of CO<sub>2</sub> on  
nitrogen inputs and  
losses

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# Nitrogen inputs and losses in response to chronic CO<sub>2</sub> exposure in a sub-tropical oak woodland

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## Abstract

Rising atmospheric CO<sub>2</sub> concentrations could 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 yr of elevated atmospheric CO<sub>2</sub> concentrations. We also explored the role of herbivory, carbon limitation, and competition for light and nutrients in shaping response of N fixation to elevated CO<sub>2</sub>. Elevated CO<sub>2</sub> did not significantly alter gaseous N losses, but lower recovery and deeper distribution in the soil of a long-term <sup>15</sup>N tracer indicated that elevated CO<sub>2</sub> increased leaching losses. Elevated CO<sub>2</sub> had no effect on asymbiotic N fixation, and had a transient effect on symbiotic N fixation by the dominant legume. Elevated CO<sub>2</sub> 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 N fixation to elevated CO<sub>2</sub>. These results indicate that positive responses of N fixation to elevated CO<sub>2</sub> may be transient, and that chronic exposure to elevated CO<sub>2</sub> can increase N leaching. Models that assume increased fixation or reduced N losses with elevated CO<sub>2</sub> 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 eleven years of chronic exposure to increased CO<sub>2</sub> concentrations on N inputs and losses from a subtropical oak woodland.

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5 fixation was negligible and not significant (van Groenigen et al., 2006). Elevated CO<sub>2</sub> often increases plant growth and element accumulation (Luo et al., 2006), including in the subtropical woodland studied here (Duval et al., 2013a). Therefore, increased element uptake by non-fixing plants could restrict nutrient availability for N fixing organisms, potentially limiting their response to elevated CO<sub>2</sub>.

10 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<sub>2</sub> 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<sub>2</sub>. Herbivores often prefer the tissue of N fixing plants to that of other plants, because N fixing plants have 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.

15 With the exception of episodic losses during disturbance, N losses from terrestrial ecosystems occur primarily as gaseous products of nitrification and denitrification (NO, N<sub>2</sub>O, and N<sub>2</sub>), and through leaching of NO<sub>3</sub><sup>-</sup> and organic N. Elevated CO<sub>2</sub> 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<sub>2</sub> could also reduce ammonium availability to nitrifiers, suppressing nitrification and N losses through gaseous fluxes (Hungate et al., 1997b) and NO<sub>3</sub><sup>-</sup> leaching (Torbert et al., 1998). Across studies conducted to date, elevated CO<sub>2</sub> has been found to increase N<sub>2</sub>O efflux from terrestrial ecosystems (van Groenigen et al., 2011); effects on N leaching have not been synthesized.

25 During the first six years of experimental treatment of the subtropical woodland studied here, elevated CO<sub>2</sub> increased N<sub>2</sub> 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<sub>2</sub> fixation during the full eleven years of the CO<sub>2</sub> exper-

iment, and we also assess responses of asymbiotic N<sub>2</sub> fixation to elevated CO<sub>2</sub>. We also investigate possible mechanisms shaping the responses to CO<sub>2</sub>, testing the hypotheses that selective herbivory, light competition, and changes in nutrient availability modulate the response of N<sub>2</sub> fixation to elevated CO<sub>2</sub>. We also report new data on rates of N gas losses and tracer <sup>15</sup>N recovery in deep soil to assess effects of CO<sub>2</sub> on N leaching.

## 2 Materials and methods

### 2.1 Site description

This work was conducted at the Smithsonian Environmental Research Center's elevated CO<sub>2</sub> 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<sup>2</sup>. Eight chambers were maintained at ambient atmospheric CO<sub>2</sub> concentrations and eight chambers were maintained at approximately 350 μLL<sup>-1</sup> above ambient atmospheric CO<sub>2</sub> concentration. The soils at the site were acidic Spodosols (Arenic Haplahumods 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 (*Serenoa repens*), shiny blueberry (*Vaccinium myrsinites*), rusty Lyonia (*Lyonia ferruginea*), and tarflower (*Befaria racemosa*). A native vine, Elliott's milkpea (*Galactia elliotii*) constituted only 1 % of aboveground productivity (Hungate et al., 2004) but is important for its ability to fix nitrogen.

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## 2.2 Symbiotic N<sub>2</sub> 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; Stiling et al., 2002; Stiling et al., 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 the total non-oak litter fraction as measured in from 1999–2002 (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 %N) vs. other species (lower in %N); this mixing model yielded an estimate of 73 %.

We measured N<sub>2</sub> fixation rates using isotope dilution. A nitrogen-15 tracer was added on 19 June 1998 (0.18 g N m<sup>-2</sup> (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> 99.9 atom % <sup>15</sup>N). To the extent *G. elliottii* fixes N<sub>2</sub> via symbiotic bacterial fixation from the atmosphere, *G. elliottii* leaves will be lower in δ<sup>15</sup>N than oak leaves, whose N is derived from the soil, directly increased by the δ<sup>15</sup>N value of the added tracer. The proportion of N in *G. elliottii* that was derived from N<sub>2</sub> fixation ( $p_f$ ) was calculated as:

$$p_f = (\delta^{15}\text{N}_O - \delta^{15}\text{N}_G) / (\delta^{15}\text{N}_{G_0} - \delta^{15}\text{N}_O) \quad (1)$$

where the subscript <sub>O</sub> refers to the dominant oak (*Q. myrtifolia*), <sub>G</sub> to the N-fixer, *G. elliottii*, after adding the <sup>15</sup>N tracer, and <sub>G<sub>0</sub></sub> to *G. elliottii* before adding the <sup>15</sup>N tracer. This calculation relaxes the common assumption that the δ<sup>15</sup>N value of N obtained by fixation is equal to the δ<sup>15</sup>N of atmospheric N<sub>2</sub>, and therefore accounts for biological fractionation during N<sub>2</sub> fixation (Yoneyama et al., 1986) and provides a more precise estimate of the end member of the mixing model. The δ<sup>15</sup>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

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N derived from fixation, unlikely to be a serious error at our site because the mixing model indicated that *G. elliotii* obtained nearly all its N from fixation. The isotope dilution method also requires that the  $\delta^{15}\text{N}$  tracer is sufficiently strong that the  $\delta^{15}\text{N}$  value of the reference plant remains distinct from the atmospheric source. This was the case throughout the experiment: the average  $\delta^{15}\text{N}$  in oak leaves was  $131.0 \pm 5.3\text{‰}$ . By the final harvest, this value declined to  $84.3 \pm 4.2\text{‰}$ , still clearly distinct from the N<sub>2</sub>-fixer value ( $-2.2\text{‰}$ ), 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. elliotii*, if it takes up N from the soil, accesses approximately the same soil N available to the reference plant. Evidence suggests that this first assumption is reasonable, as both fine roots ( $67 \pm 13\%$ ) and *G. elliotii* nodules ( $74 \pm 9\%$ ) were concentrated in the top 30 cm of soil.

We measured N concentration and  $\delta^{15}\text{N}$  in *G. elliotii* foliage and senesced litter on an elemental analyzer inline with an isotope-ratio mass spectrometer. Total N<sub>2</sub> fixation was calculated as the product of *G. elliotii* biomass production, N concentration, and the proportion of N derived from fixation. To calculate  $p_f$  using Eq. (1), we used  $\delta^{15}\text{N}$  values from senesced leaves because they were gathered in litter traps and therefore captured an integrative sample of *G. elliotii* tissue at the plot scale (Stiling et al., 2009). For N concentration, we used %N in green leaves, to avoid underestimating N<sub>2</sub> 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.

### 2.3 Herbivory

From 2000–2006, litter of the oaks and of *G. elliotii* was scored for damage by insect herbivores and sorted, counted and weighed separately by species and insect damage

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category as described previously (Stiling et al., 1999, 2003, 2009). The rate of litterfall of herbivore-damaged leaves,  $L$  ( $\text{g m}^{-2} \text{yr}^{-1}$ ), 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 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. elliotii*, 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, weighed, and average leaf mass 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 ( $\# \text{ leaves m}^{-2} \text{ yr}^{-1}$ ) by the average mass of a green leaf of that species ( $\text{g leaf}^{-1}$ ) to determine the production of all the leaves of which insects consumed at least a part,  $P$ , ( $\text{g m}^{-2} \text{ yr}^{-1}$ ). Note, 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; Stiling et al., 2002, 2003). Finally, we calculated total leaf production as the total number of leaves in litterfall times average green mass, and then 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 5 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., 2013a); 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; Duval et al., 2013b), 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<sub>3</sub>, and analyzed by ICP-MS as described below.

For 2007, plant samples were dried at 60 °C and ~ 750 mg was ashed at 600 °C. Five hundred mg of *G. elliotii* 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<sub>3</sub>, 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 re-suspended in 10 mL of 0.32 M trace metal grade HNO<sub>3</sub> 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, 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 asymbiotic N<sub>2</sub> fixation

We measured acetylene reduction to ethylene in soil incubations to estimate N<sub>2</sub> fixation by free-living soil heterotrophs. Soil samples were collected in January and March 2006. At each date, five cores (4 cm diameter × 15 cm deep) were collected from each chamber, shipped overnight to Florida International University, (Miami, Florida),

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composited for each plot, and sieved through a 2 mm sieve. Soils were composited by treatment, and 3 g subsamples weighed into 20 mL glass vials ( $n = 4-5$ ). In January 2006, the experimental design included two levels of glucose addition (0 and  $324 \mu\text{gCg}^{-1}$  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  $34.4 \mu\text{gCg}^{-1}$  soil), and two levels of phosphorus addition (0 and  $6.5 \mu\text{gPg}^{-1}$  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 GC equipped with a flame ionization detector.

### 2.6 Nitrous oxide and nitrogen oxide gas fluxes

We measured soil production of nitrous oxide (N<sub>2</sub>O) and nitric oxide (NO<sub>x</sub>) using static chambers (Hutchinson and Livingston, 1993) during 2005–2007. For N<sub>2</sub>O, headspace samples were collected in syringes and analysed by gas chromatography. For NO<sub>x</sub>, the static chamber was plumbed to a chemoluminescent detector and computer that logged real time [NO<sub>x</sub>]. Chambers (1.8 L) were constructed from 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<sub>2</sub>O concentrations. Field fluxes were calculated using linear regression of concentrations over time. The flux rates were expressed as  $\mu\text{gN}_2\text{O-Nm}^{-2}\text{d}^{-1}$ .

## 2.7 Tracer $^{15}\text{N}$ 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  $\text{CO}_2$  treatments ( $P = 0.90$ ,  $t$  test).

5 We modeled the depth distribution of tracer  $^{15}\text{N}$  using the function

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

where  $d$  is depth and  $Y$  is the cumulative proportion of tracer  $^{15}\text{N}$  recovered up to depth  $d$ , and  $\beta$  is a fitted parameter (Gale and Grigal, 1987). We used Microsoft Excel's Solver function to find the values of  $\beta$ , 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  $\text{CO}_2$  altered  $\beta$ , and used non-linear regression to explore relationships between the vertical distribution of recovered  $^{15}\text{N}$  and total  $^{15}\text{N}$  recovery at the ecosystem scale ( $\text{mg } ^{15}\text{N m}^{-2}$ ).

## 2.8 Statistical analyses

15 We used analysis of variance (ANOVA) to test for effects of elevated  $\text{CO}_2$ , using repeated measures to test for effects of time and interactions between  $\text{CO}_2$  and time, and split-plot ANOVA to test for differences among soil depths. When necessary, data were log-transformed ( $\text{N}_2$  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  $\alpha$  threshold of 0.10. We omitted one data point from the analysis of foliar Mo of *G. elliotii* 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  $\text{N}_2$  fixation and possible drivers. We also used ANOVA for the acetylene reduction assay

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to test effects of CO<sub>2</sub>, glucose, and (January 2006) or glucose and phosphorus additions (March 2006) on rates of N<sub>2</sub> fixation by free-living soil microorganisms. We note that compositing soils from plots by CO<sub>2</sub> treatment compromised the independence of replicates within CO<sub>2</sub> treatments. The absence of any significant effects of CO<sub>2</sub> on asymbiotic N<sub>2</sub> fixation (see below) provides some protection from the dangers of false inference caused by pseudoreplication (Hurlbert, 1984). We assessed effects of N<sub>2</sub> fixation as a function of time since disturbance calculating time since disturbance as the number of years 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<sub>2</sub> on N<sub>2</sub> fixation as the absolute difference between elevated and ambient CO<sub>2</sub> plots.

### 3 Results

#### 3.1 N<sub>2</sub> fixation by *G. elliotii*

N<sub>2</sub> fixation by *Galactia elliotii* varied over time ( $P < 0.001$ ), with high rates in 1998 and low rates in 2000 and 2005 (Fig. 1). Elevated CO<sub>2</sub> increased N<sub>2</sub> fixation by *G. elliotii* 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<sub>2</sub>, N<sub>2</sub> fixation was not different between the two treatments (CO<sub>2</sub> × time interaction,  $P = 0.070$ ). Subsequently (years 5–7), elevated CO<sub>2</sub> suppressed *G. elliotii* N<sub>2</sub> fixation. Rates equalized again by year 9 (2005). Accumulated over the 11 yr exposure period, *G. elliotii* fixed  $3.92 \pm 0.50 \text{ gNm}^{-2}\text{yr}^{-1}$  in the ambient CO<sub>2</sub> treatment compared to  $3.51 \pm 0.71 \text{ gNm}^{-2}\text{yr}^{-1}$  in the elevated CO<sub>2</sub> treatment, a non-significant difference (repeated measures ANOVA,  $P = 0.376$ ). The effect of elevated CO<sub>2</sub> dissipated with time since disturbance (Fig. 1b).

*G. elliotii* derived nearly all of its foliar N from fixation, increasing from 92 % in 1998 to 100 % from 2005 to 2007 (Table 1). Across all years, elevated CO<sub>2</sub> reduced the

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reliance of *G. elliotii* on N from the atmosphere, though the effect was small, from an average of 96.9 % for the ambient CO<sub>2</sub> treatment to 94.7 % for the elevated. Foliar %N of *G. elliotii* was lower in the elevated CO<sub>2</sub> treatment (Table 1), an effect that varied over time. During the first year of the experiment, elevated CO<sub>2</sub> stimulated nodule biomass (effect of CO<sub>2</sub> in 1996, *P* = 0.062), but elevated CO<sub>2</sub> had no effect on nodule mass at final harvest (Fig. 2, Table 2), a pattern similar to that found for N<sub>2</sub> fixation.

N<sub>2</sub> fixation by *G. elliotii* (gNm<sup>-2</sup>yr<sup>-1</sup>) was positively correlated with *G. elliotii* 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<sub>2</sub> fixation declined, suggesting competition. The absence of any relationship with leaf area index argues against competition for light. N<sub>2</sub> fixation also declined as foliar %N declined, which can indicate limitation by non-N nutrients (Rogers et al., 2009). Finally, high proportional consumption of *G. elliotii* tissue was associated with lower rates of N<sub>2</sub> fixation, suggesting some control of N<sub>2</sub> fixation by herbivory (Table 3).

### 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<sub>2</sub> 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<sub>2</sub> reduced soil P availability (ion exchange in 1997, extractable in 2001), though this effect was not apparent at final harvest (Fig. 4). Reductions in soil element availability under elevated CO<sub>2</sub> corresponded with reduced foliar concentrations in *G. elliotii*: elevated CO<sub>2</sub> 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<sub>2</sub> fixation was not related to soil extractable nutrient concentration (data not shown, *P* > 0.10 for all regressions).

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### 3.3 Herbivory

Herbivores in this subtropical woodland consumed a higher percentage of *G. elliotii* leaf production ( $24.0 \pm 1.8\%$ ) than of oak leaf production ( $14.1 \pm 0.6\%$ ,  $P < 0.001$ , Fig. 6). The percentage consumed of *G. elliotii* was not affected by the elevated  $\text{CO}_2$  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 Asymbiotic $\text{N}_2$ fixation

Elevated  $\text{CO}_2$  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 asymbiotic  $\text{N}_2$  fixation in these soils.

### 3.5 Nitrous oxide and nitric oxide fluxes

Across all measurements conducted during the  $\text{CO}_2$  enrichment experiment,  $\text{N}_2\text{O}$  efflux averaged  $0.346 \text{ mg N}_2\text{O-N m}^{-2} \text{ d}^{-1}$  for the ambient  $\text{CO}_2$  treatment and  $0.377 \text{ mg N}_2\text{O-N m}^{-2} \text{ d}^{-1}$  for the elevated  $\text{CO}_2$  treatment, a non-significant difference of  $0.035 \text{ mg N}_2\text{O-N m}^{-2} \text{ d}^{-1}$  (5% and 95% confidence limits,  $-0.070$  and  $0.132$ ). Scaled to the entire 11 yr period, and assuming that  $\text{N}_2\text{O}$  constitutes 10% of the total N losses in denitrification (with 90% as  $\text{N}_2$  production), our best estimate of the effect of elevated  $\text{CO}_2$  on  $\text{N}_2\text{O}$  losses over the 11 yr period is an increase of  $1.4 \text{ g N m}^{-2}$  (with 5% and 95% confidence limits,  $-2.9$  to  $5.3$ ). Across all measurements,  $\text{NO}_x$  losses averaged  $0.075 \text{ mg N m}^{-2} \text{ d}^{-1}$  for the ambient  $\text{CO}_2$  treatment, and were somewhat lower for the elevated  $\text{CO}_2$  treatment at  $0.027 \text{ mg N m}^{-2} \text{ d}^{-1}$ , a difference between elevated and ambient treatments of  $-0.047 \text{ mg N m}^{-2} \text{ d}^{-1}$  (5% and 95% confidence limits,  $-0.116$  to  $-0.002 \text{ mg N m}^{-2} \text{ d}^{-1}$ ). Scaled to the entire 11 yr period, the elevated  $\text{CO}_2$ -treated plots lost  $0.20 \text{ g N m}^{-2}$  less  $\text{NO}_x$  compared to the controls, (5% and 95% confidence limits,

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tion), or caused only a transient and quantitatively negligible response (symbiotic N<sub>2</sub> fixation). Reduced gaseous N losses from the elevated-CO<sub>2</sub> treated plots were observed for NO<sub>x</sub>, but NO<sub>x</sub> was a minor component of the ecosystem N budget, such that the changes observed were insufficient to promote N accumulation. Moreover, the reduced <sup>15</sup>N recovery and pattern of greater <sup>15</sup>N recovery deep in the soil indicates that elevated CO<sub>2</sub> enhanced leaching losses of N. Our results indicate that processes promoting N loss were more responsive to elevated CO<sub>2</sub> than were processes promoting N accumulation.

## 4.2 Effects of CO<sub>2</sub> on N<sub>2</sub> fixation

N<sub>2</sub> fixation by the leguminous vine, *G. elliotii*, was only temporarily responsive to the CO<sub>2</sub> treatment (Fig. 1). Growth and N<sub>2</sub> fixation in N<sub>2</sub>-fixing plants can respond positively to elevated CO<sub>2</sub> (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<sub>2</sub> exposure was found to reduce cyanobacterial abundance in desert crusts (Steven et al., 2012). Our finding that asymbiotic N<sub>2</sub> fixation in soil was also insensitive to the CO<sub>2</sub> 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 experiments in the lab or greenhouse (van Groenigen et al., 2006; Leuzinger et al., 2011), so our finding that elevated CO<sub>2</sub> did not increase N<sub>2</sub> 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<sub>2</sub> fixation after the first year of the experiment.

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### 4.3 Nitrogen availability

Increased N availability is well known to reduce N<sub>2</sub> fixation (Streeter, 1988), as uptake of N from the soil is energetically less costly than fixation. In the scrub-oak ecosystem studied here, elevated CO<sub>2</sub> 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. elliotii* on atmospheric N in response to elevated CO<sub>2</sub> (Fig. 1, Table 1) is consistent with a CO<sub>2</sub>-stimulation of soil N availability. N<sub>2</sub> fixation can decline with shading during canopy development, yet in this system leaf area index was not a significant predictor of N<sub>2</sub> fixation (Table 3), possibly because the N<sub>2</sub> 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<sub>2</sub> fixation and total oak biomass (Table 3) suggests competition for other resources between the oaks and the N<sub>2</sub> fixing vine, possibly competition for nutrients.

### 4.4 Non-N nutrients

When supplied with sufficient non-N nutrients, N<sub>2</sub>-fixing plants will increase nodule biomass and rates of N<sub>2</sub> fixation (van Groenigen et al., 2006; Rogers et al., 2009), while at the same time maintaining foliar N concentration in response to elevated CO<sub>2</sub>. Under nutrient limiting conditions, foliar N declines, nodule growth diminishes, and rates of N<sub>2</sub> fixation are depressed (Rogers et al., 2009), a pattern consistent with the positive correlation between foliar N concentration and N<sub>2</sub> fixation shown here (Table 3).

The responses of N<sub>2</sub> fixation and growth of N fixing plants to elevated CO<sub>2</sub> 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 yr of the experiment (Fig. 4, Johnson et al., 2001, 2003), a response also observed in a rice-wheat rotation (Ma et al., 2007). However, reduced P availability is not a universal response to increased CO<sub>2</sub> concentration (Dijkstra et al., 2012; Khan et al., 2008, 2010). Other elements crit-

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ical for N<sub>2</sub> fixation such as Mo, Fe, and V either declined significantly in soil and in foliage or showed a tendency to decline (Figs. 3 and 5), suggesting their role in modulating the response of N<sub>2</sub> fixation to elevated CO<sub>2</sub>. 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., 2013a). 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 micro-nutrient limitation of ecosystem processes, such as N<sub>2</sub> fixation.

Several experiments have demonstrated the importance of P availability for the responses of N<sub>2</sub> fixation to elevated CO<sub>2</sub>: the stimulation of growth and N<sub>2</sub> fixation by elevated CO<sub>2</sub> 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<sub>2</sub>-stimulation of N-fixer growth and N<sub>2</sub> fixation in several grasslands experiments was higher with P addition compared to controls without supplemental P (Niklaus et al., 1998; Grunzweig and Körner, 2003). In general, with supplemental nutrients added, elevated CO<sub>2</sub> often increases N<sub>2</sub> fixation (Lee et al., 2003; Otera et al., 2011), but in experiments without exogenous nutrient addition, N<sub>2</sub> fixation is generally not responsive to elevated CO<sub>2</sub> (van Groenigen et al., 2006).

## 4.5 Herbivory

Herbivory has been postulated to reduce N<sub>2</sub> fixation in ecosystems because of preferential feeding on the more nutritious tissue of N<sub>2</sub>-fixers compared to other plants (Vitousek and Howarth, 1991). This hypothesis is consistent with our finding that her-

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bivores in this subtropical woodland consumed a larger proportion of *G. elliotii* leaf production than of oaks (Fig. 6). Herbivory could also limit the response of N<sub>2</sub> fixation to environmental change, if grazing on the tissue of N<sub>2</sub> fixers increases. Increasing herbivore pressure on the dominant N<sub>2</sub> fixing vine in our experiment was associated with reduced rates of N<sub>2</sub> fixation (Table 3), in support of this idea. Elevated CO<sub>2</sub> 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<sub>2</sub> on the proportion of N-fixer leaf production consumed by herbivory (Fig. 6).

### 4.6 C limitation of asymbiotic N<sub>2</sub> fixation

Our finding that acetylene reduction increased with glucose addition suggests C limitation of heterotrophic N<sub>2</sub> 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<sub>2</sub> fixation.

### 4.7 Temporal dynamics

Evidence for a single, dominating mechanism underlying the response of N<sub>2</sub> fixation to elevated CO<sub>2</sub> 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<sub>2</sub> found both above- and below-ground (Day et al., 2013; Hungate et al., 2013a), as well as the disappearance of the CO<sub>2</sub> suppression of *G. elliotii* growth and N<sub>2</sub> fixation (Fig. 1). Consistent with this, the positive correlation between foliar Mo con-



the concurrence of reduced tracer  $^{15}\text{N}$  recovery (Hungate et al., 2013b) and deeper distribution of tracer  $^{15}\text{N}$  throughout the soil profile (Fig. 7) supports the notion that elevated  $\text{CO}_2$  stimulated N leaching in this experiment. Increased leaching with elevated  $\text{CO}_2$  has been observed (Körner and Arnone, 1992), and may be caused by increased turnover of soil organic matter in response to rising  $\text{CO}_2$  (Drake et al., 2011; Hungate et al., 2013b). Some experiments have documented reduced N leaching with elevated  $\text{CO}_2$  (Johnson et al., 2004), so our findings may not be universal. Furthermore, during the first three years of this experiment, we found no effect of elevated  $\text{CO}_2$  on vertical movement of N from the surface to deeper soil layers (Johnson et al., 2001). The use of the  $^{15}\text{N}$  tracer to integrate the cumulative effects of elevated  $\text{CO}_2$  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  $\text{CO}_2$ .

## 4.9 Summary

Overall, our findings of no sustained effect on  $\text{N}_2$  fixation and increased N losses via leaching provide an empirical point of contrast with model projections in which elevated  $\text{CO}_2$  enhances  $\text{N}_2$  fixation and reduces leaching (Thornley and Cannell, 2000). This is an important conclusion for modeling responses of future C uptake by the biosphere, given the strong influence of N cycling and N accumulation on the biosphere's capacity to assimilate and store C (Thornton et al., 2007; Churkina et al., 2009; Arneeth et al., 2010; Zaehle and Dalmonech, 2011).

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**Table 1.** Proportion of N derived from fixation and N concentration in *Galactia elliotii* during 11 yr of exposure to elevated CO<sub>2</sub>, and results from repeated measures ANOVAs for effects of time, elevated CO<sub>2</sub>, and their interaction.

Year	pFixation		%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 <sub>2</sub>	0.005		0.006
	Time	0.018		< 0.001
	CO <sub>2</sub> × Time	0.453		0.002

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**Table 2.** Nodule mass ( $\text{gm}^{-2}$ ) 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 <sub>2</sub>		0.416
Depth		0.498
CO <sub>2</sub> × Depth		0.812

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

Effect	Coefficient ± SE	<i>P</i>
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

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**Table 4.** Effect of elevated CO<sub>2</sub>, 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 main effect of CO<sub>2</sub> treatment, repeated measures effect of Year, and split-plot effect of Depth, as well as all interactions. All data were log-transformed before analysis.

Element	CO <sub>2</sub>	Year	Depth	CO <sub>2</sub> × year	CO <sub>2</sub> × depth	Year × depth	CO <sub>2</sub> × Y × 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

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**Table 5.** Rates of acetylene reduction ( $\mu\text{mol C}_2\text{H}_2\text{g}^{-1}\text{h}^{-1}$ ) as a proxy for asymbiotic N<sub>2</sub> fixation, measured in soils from the ambient and elevated CO<sub>2</sub> treatments from laboratory incubations with added glucose and either added water (January 2006), or added phosphorus (March 2006). Values are means  $\pm$  standard errors of the mean ( $n = 4$ , January 2006;  $n = 5$ , March 2006).

	Glucose $\mu\text{g g}^{-1}$	Ambient CO <sub>2</sub>	Elevated CO <sub>2</sub>	Ambient CO <sub>2</sub>	Elevated CO <sub>2</sub>
Jan 2006		3% H <sub>2</sub> O		10% H <sub>2</sub> O	
	0	21.5 $\pm$ 3.2	23.8 $\pm$ 3.0	20.4 $\pm$ 2.3	21.8 $\pm$ 2.5
	324	27.4 $\pm$ 1.4	25.8 $\pm$ 4.7	32.4 $\pm$ 3.2	30.0 $\pm$ 4.5
Mar 2006		0 P		6.5 P	
	0	36.0 $\pm$ 1.7	37.7 $\pm$ 4.1	37.2 $\pm$ 4.2	37.5 $\pm$ 1.6
	6.7	42.0 $\pm$ 3.8	42.7 $\pm$ 4.5	34.6 $\pm$ 1.2	36.0 $\pm$ 2.7
	34.4	42.4 $\pm$ 3.1	44.9 $\pm$ 3.1	43.6 $\pm$ 3.5	41.0 $\pm$ 2.8

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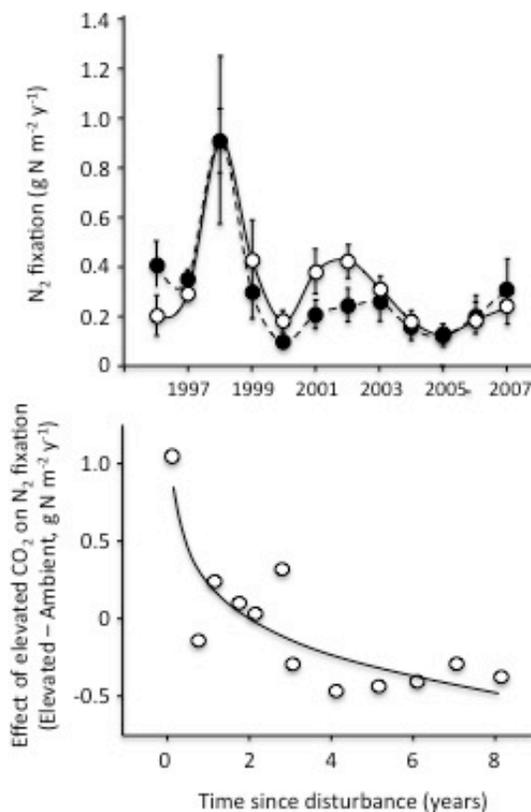


**Table 6.** *P* values from ANOVAs testing the effects of chronic CO<sub>2</sub> exposure and short-term additions of glucose, water, and phosphorus on acetylene reduction. Columns under each date show *P* values for main effects and interactions tested on that date, H<sub>2</sub>O for January and phosphorus for March (note: the first *P* value in each column is a main effect).

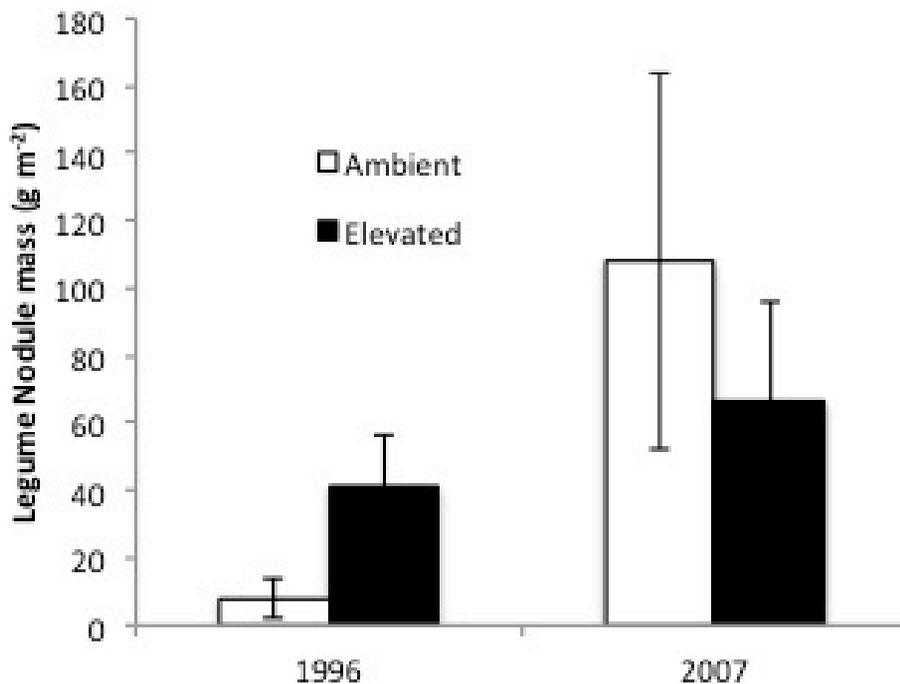
	Jan 2006		Mar 2006
CO <sub>2</sub>	0.964	CO <sub>2</sub>	0.727
Glucose	< 0.001	Glucose	0.011
CO <sub>2</sub> × glucose	0.329	CO <sub>2</sub> × glucose	0.816
H <sub>2</sub> O	0.351	P	0.151
H <sub>2</sub> O × CO <sub>2</sub>	0.773	P × CO <sub>2</sub>	0.670
H <sub>2</sub> O × glucose	0.071	P × glucose	0.604
H <sub>2</sub> O × CO <sub>2</sub> × glucose	1.000	P × CO <sub>2</sub> × glucose	0.673

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**Fig. 1.** Rate of N<sub>2</sub> fixation **(a)** and effect of elevated CO<sub>2</sub> on N<sub>2</sub> fixation as a function of time since disturbance **(b)** for *Galactia elliotii* during 11 yr of experimental exposure to increased carbon dioxide concentrations. Values are means ± standard error of the mean ( $n = 8$ ), for the ambient (open circles) and elevated (filled squares) CO<sub>2</sub> treatments.



**Fig. 2.** Mass of *G. elliotii* nodules recovered in the top 10 cm of soil in 1996 (from ingrowth soil cores) and in 2007 (from intact cores). Values are means  $\pm$  standard errors.

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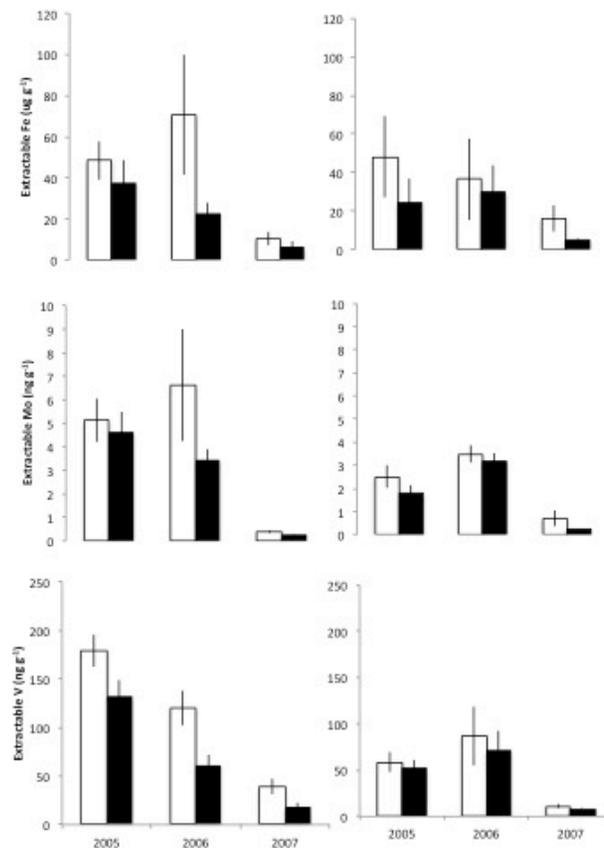
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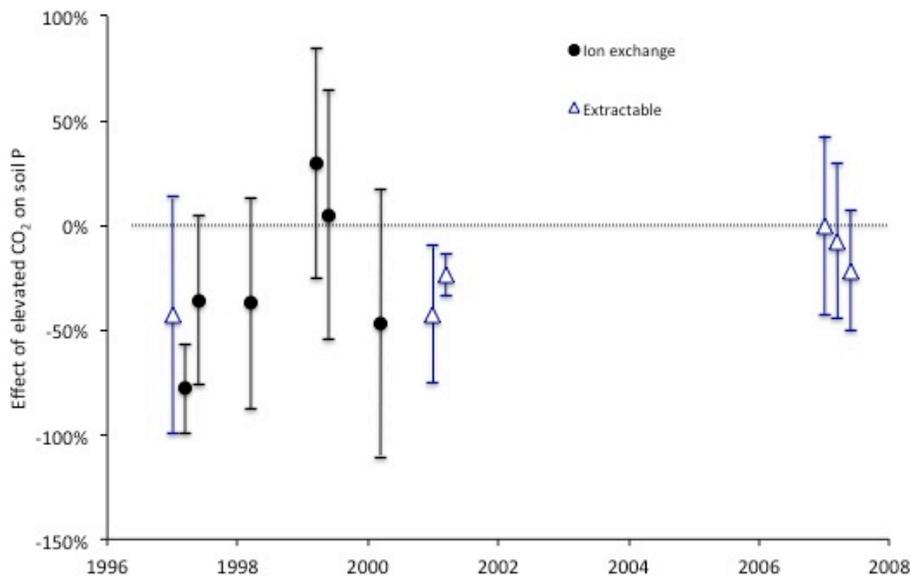
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**Fig. 3.** Soil extractable Fe, Mo, and V concentrations for 0–10 cm (left 3 panels) and 10–30 cm (right 3 panels) soil depths over the last three years of experiment. Values are means  $\pm$  standard error of the mean. Units are  $\mu\text{g g}^{-1}$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

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**Fig. 4.** Effect of elevated CO<sub>2</sub> 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<sub>2</sub>, expressed as a percentage:  $(E - A)/A \times 100\%$ . Bars denote 5% and 95% confidence limits. (Data from 1997–2001 were calculated from raw data reported in Johnson et al., 2001, 2003.)

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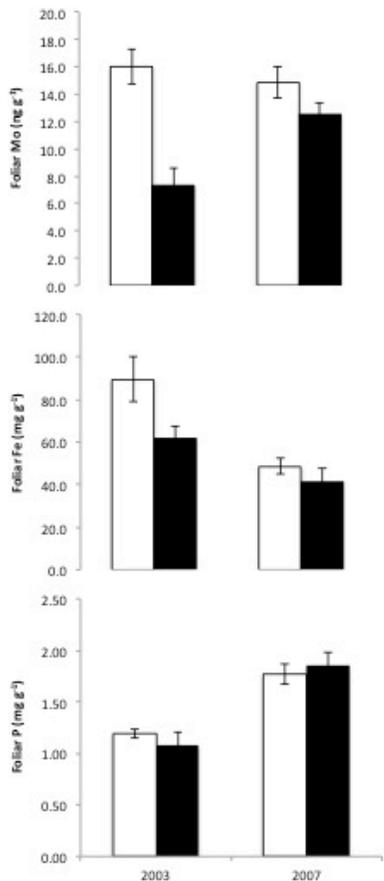
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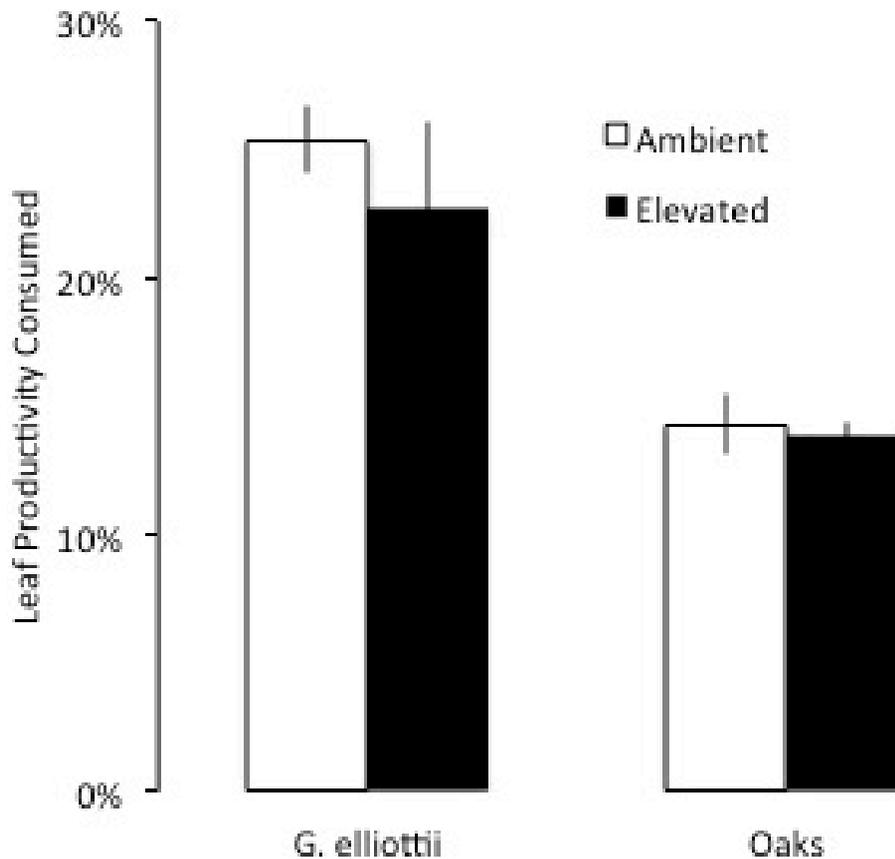
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**Fig. 5.** Effects of elevated CO<sub>2</sub> concentration on Mo, Fe, and P concentrations in leaves of *G. elliotii* sampled in 2003 and 2007 (values for Mo and Fe from 2003 are from Hungate et al., 2004).



**Fig. 6.** Percent of leaf productivity consumed by herbivores for the N<sub>2</sub>-fixing vine, *G. elliotii*, and for the three co-dominant oak species in the ambient and elevated CO<sub>2</sub> treated plots.

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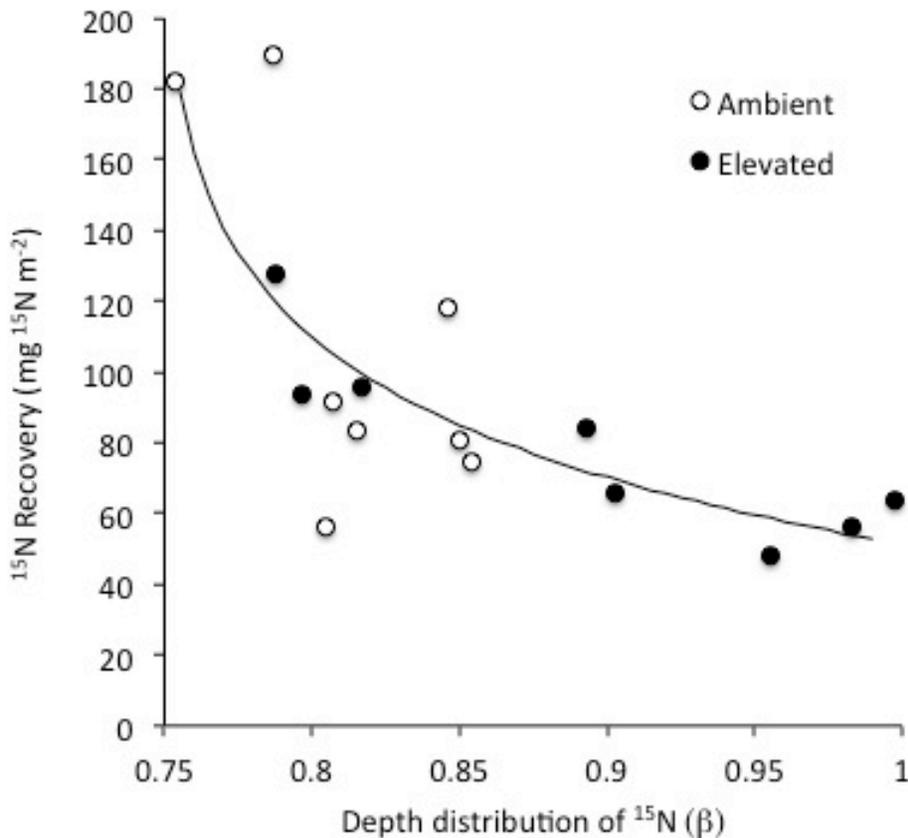
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**Fig. 7.** Recovery of added tracer  $^{15}\text{N}$  as a function of its depth distribution. Higher  $\beta$  values indicate relatively more  $^{15}\text{N}$  in deeper soil layers, whereas low  $\beta$  values indicate concentration of  $^{15}\text{N}$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