AoB PLANTS
The open-access journal for plant sciences

Research Article

Effects of elevated atmospheric CO_2 concentrations, clipping regimen and differential day/night atmospheric warming on tissue nitrogen concentrations of a perennial pasture grass

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Received: 4 March 2015; **Accepted:** 6 July 2015; **Published:** 13 August 2015

Associate Editor: Ulo Niinemets

Citation: Volder A, Gifford RM, Evans JR. 2015. Effects of elevated atmospheric CO_2 concentrations, clipping regimen and differential day/night atmospheric warming on tissue nitrogen concentrations of a perennial pasture grass. *AoB PLANTS* **7**: plv094; doi:10.1093/aobpla/plv094

Abstract. Forecasting the effects of climate change on nitrogen (N) cycling in pastures requires an understanding of changes in tissue N. We examined the effects of elevated atmospheric CO_2 concentration, atmospheric warming and simulated grazing (clipping frequency) on aboveground and belowground tissue N concentrations and C : N ratios of a C3 pasture grass. *Phalaris aquatica* L. cv. 'Holdfast' was grown in the field in six transparent temperature gradient tunnels (18 × 1.5 × 1.5 m each), three at ambient atmospheric CO_2 and three at 759 p.p.m. CO_2. Within each tunnel, there were three air temperature treatments: ambient control, +2.2/+4.0 °C above ambient day/night warming and +3.0 °C continuous warming. A frequent and an infrequent clipping treatment were applied to each warming × CO_2 combination. Green leaf N concentrations were decreased by elevated CO_2 and increased by more frequent clipping. Both warming treatments increased leaf N concentrations under ambient CO_2 concentrations, but did not significantly alter leaf N concentrations under elevated CO_2 concentrations. Nitrogen resorption from leaves was decreased under elevated CO_2 conditions as well as by more frequent clipping. Fine root N concentrations decreased strongly with increasing soil depth and were further decreased at the 10–60 cm soil depths by elevated CO_2 concentrations. The interaction between the CO_2 and warming treatments showed that leaf N concentration was affected in a non-additive manner. Changes in leaf C : N ratios were driven by changes in N concentration. Overall, the effects of CO_2, warming and clipping treatments on aboveground tissue N concentrations were much greater than on belowground tissue.

Keywords: C : N ratio; climate change; defoliation; grassland; tissue quality.

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Introduction

Long-term responses of plant growth to elevated atmospheric CO_2 and warming will depend in part on the availability of mineral nutrients and the way in which they are utilized by the plant (Stitt and Krapp 1999; Duval *et al.* 2012). For example, the stimulation of biomass accumulation at elevated CO_2 is generally less under nitrogen (N) limited than well-fertilized conditions (Norby and Luo 2004; Finzi *et al.* 2006; Gill *et al.* 2006; Reich *et al.* 2006). Leaf and plant N concentrations generally decrease in response to elevated atmospheric CO_2, and such decreases occur even under conditions of high soil N availability (Cotrufo *et al.* 1998). Thus, decreased tissue N concentration in response to elevated atmospheric CO_2 is not just due to a N limitation in the growth medium (Poorter *et al.* 1997; Lee *et al.* 2011). Decreased leaf N concentrations have been attributed to either lower N demand as plants utilize rubisco protein more efficiently for photosynthesis under elevated atmospheric CO_2 concentrations (Drake *et al.* 1997; Stitt and Krapp 1999), or a N-dilution effect as a result of increased carbohydrate production and storage (Coleman *et al.* 1993; Loladze 2002), or diminished ability of plants to assimilate nitrate under high CO_2 conditions (Rachmilevitch *et al.* 2004; Bloom *et al.* 2010, 2012, 2014). Regarding the negative impact of elevated CO_2 concentrations on nitrate assimilation, in C_3 species, suppression of photorespiration by elevated atmospheric CO_2 concentrations is hypothesized to limit the availability of reductant in the glutamine synthetase and glutamine:2-oxoglutarate amidotransferase cycle. Competition for reductants slows the rate at which nitrate can be converted to glutamate (Bloom 2006), the form of N used in protein synthesis. Since elevated atmospheric CO_2 and higher air temperatures have opposite effects on photorespiration (Ziska and Bunce 1995), higher temperatures may counteract the suppressing effect of elevated atmospheric CO_2 on nitrate assimilation in C_3 plants.

As both warming and elevated atmospheric CO_2 affect N uptake, translocation and assimilation, they can both be expected to affect tissue N concentrations. Warming applied under ambient atmospheric CO_2 conditions led to decreased tissue N concentration in both an unfertilized tallgrass prairie (An *et al.* 2005) and a Mediterranean shrubland (Sardans *et al.* 2008). In an interactive $CO_2 \times$ warming experiment, Lilley *et al.* (2001) found no effect of temperature on herbage N concentration in *Phalaris aquatica*, but elevated atmospheric CO_2 conditions did lower herbage N concentration. Zhou *et al.* (2011) found that elevated atmospheric CO_2 concentrations led to lower leaf N concentration in *P. arundinacea*, yet higher temperatures led to higher leaf N concentrations overall. These findings on the effect of warming contrast with those of An *et al.* (2005) and Sardans *et al.* (2008) who found lower leaf tissue N concentrations when warming was applied under ambient CO_2 conditions. Neither Lilley *et al.* (2001) nor Zhou *et al.* (2011) found an interactive effect of warming and atmospheric CO_2 concentration on leaf N concentration. Anthropogenic global warming is likely greater for night-time minima than for daytime maxima (Karl *et al.* 1991; Vose *et al.* 2005; Gershunov *et al.* 2009), which can also affect plant C balances. For example, Lilley *et al.* (2001) found that *P. aquatica* stores very little carbon (C) as starch, but it maintains high levels of soluble carbohydrates. In this study, soluble carbohydrate concentrations were enhanced by elevated atmospheric CO_2 but decreased by warming under ambient CO_2 conditions. Diurnal patterns of warming with higher night-time temperatures, a trend that has been observed in the global temperature records (Vose *et al.* 2005), may have a different effect on plant tissue quality than continuous warming. Temperate steppe grasses responded to nocturnal warming by depleting more stored carbohydrates at night and compensated for this response by enhancing daytime photosynthesis to such a degree (+19.8 %) that the steppe ecosystem switched from being a minor C source to a C sink (Wan *et al.* 2009). Such strong C balance responses to night-time warming will also likely affect tissue N concentrations and C : N ratios.

Changes in tissue N or C concentrations, or both, will affect tissue C : N ratios, with consequences for growth and decomposition, which can then affect litter decomposition rates and C and N cycling if a change in living tissue C : N ratios translates into altered litter C : N ratios. For example, exposure to increasing atmospheric CO_2 levels along a CO_2 gradient led to increased plant tissue C : N ratios, greater aboveground N storage in plant parts and increased C : N ratios in soil organic matter in a Texas grassland (Gill *et al.* 2006). In this same experiment, N mineralization rates were decreased, while C mineralization increased under elevated CO_2 conditions. One possible explanation was that increased litter C : N ratios may have stimulated microbes to start using the more recalcitrant N-rich C fractions in the soil to meet their N demands.

Few studies have reported on the effect of elevated atmospheric CO_2 on both root C and N concentrations (Nie *et al.* 2013), and data on interactive effects of atmospheric CO_2 concentration and temperature on root C and N concentration are even rarer (Dieleman *et al.* 2012). The response of tissue nutrient concentrations to CO_2 and warming is likely to be dependent on the grazing or clipping regime. Ziter and MacDougall (2013) found that grazing enhanced leaf tissue N concentrations while not affecting root N and sugar concentrations. When heavy

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grazing was compared with moderate grazing, Biondini *et al.* (1998) found that heavy grazing reduced root N concentrations. As grazing removes substantial amounts of biomass from the system and thus interferes with nutrient cycling, pastures are often fertilized.

The objective of this study was to investigate the interactive effects of both atmospheric warming and elevated atmospheric CO_2 concentration on N concentration and C : N ratio of leaves, roots and litter of *P. aquatica* plantings subjected to two different clipping intensities (infrequent and frequent) to simulate grazing. We reduced the impact of feedbacks due to drought or nutrient limitation by providing ample water and nutrients. Therefore, we report on the direct impacts of both elevated atmospheric CO_2 and warming on tissue quality in a managed pasture system.

Methods

Species description

Phalaris aquatica L., previously also known as *P. tuberosa* L., is a highly productive deep-rooted perennial grass originating from the Mediterranean and Middle East and was first introduced as a pasture grass in Australia in 1877 (Oram *et al.* 2009). The common name for *P. aquatica* is 'phalaris', although sometimes the name 'Hardinggras' is also used (Oram *et al.* 2009). *Phalaris aquatica* is the most widely sown perennial grass in temperate areas of south-eastern Australia. In addition, it is also used in pastures in the USA, South America and New Zealand and to a limited extent in parts of Africa and southern Europe (Oram *et al.* 2009). The cultivar used in this experiment, 'Holdfast', is grazing tolerant, winter active and exhibits low summer dormancy. The cultivar 'Holdfast' was developed by CSIRO, Canberra, Australia, to have reduced aluminium sensitivity and tolerate acid soils (Oram *et al.* 1993; Oram and Culvenor 1994).

Phalaris aquatica is considered an environmental weed in both Australia (Stone 2009) and the Western USA, where it thrives in areas with deep soil and adequate soil moisture (>500 mm rainfall). Due to low seedling vigour, the ability to establish outside cultivation is low, unless bare patches of soil are available (Stone 2009). Once established in natural ecosystems, it can outcompete native species through its ability to form dense clumps with deep root systems that allow it to survive periods of drought (Stone 2009). In general, *P. aquatica* has a high nutrient requirement, especially for N and P, which inhibits its ability to be highly productive outside agricultural situations (Stone 2009). The California Invasive Plant Council rates *P. aquatica* as moderately invasive in California (IPC 2015).

Temperature gradient tunnels and environmental conditions

The experiment site and tunnel controls are described in Volder *et al.* (2004). Briefly, six transparent ventilated temperature gradient tunnels (TGT, 18 × 1.5 × 1.5 m each) were established on a uniform flat fallow field at Ginninderra Experimental Station (Canberra, Australian Capital Territory, Australia, lat. 35.22°S, long. 149.13°E). During the experiment, hourly averaged ambient temperatures ranged from −5.5 °C (24 August 2002) to 43.6 °C (18 January 2003) with a daily mean of 14.3 °C (Volder *et al.* 2004). Three tunnels were kept at ambient atmospheric CO_2 concentrations (i.e. no CO_2 adjustment) and three tunnels were designed to maintain elevated atmospheric CO_2 concentrations (target: 750 p.p.m.) by injecting CO_2 into the airstream at two locations. CO_2 concentrations in the airstream were measured (Model ADC-2000, Analytical Development Co. Ltd, Hoddesdon, UK) at downstream locations every 0.3 s and pulse lengths were adjusted every 1 s accordingly (Volder *et al.* 2004). The daily CO_2 concentration over the whole experiment in the three elevated atmospheric CO_2 tunnels was 759 ± 12.6 p.p.m. In the ambient CO_2 tunnels, the daily average was 403 p.p.m. During the day, the drawdown between the start and the end of the tunnels was ∼7 p.p.m. The tunnels were constructed using a thin aluminium framework with Teflon (Nowoflon ET-film 6235, Nowoflon Kunststoffprodukte GmbH and Co., Siegsdorf, Germany) panels. Radiation energy intercepted by the tunnel structure varied between 20 and 40 %, with the highest proportional interception at midday and in winter (Volder *et al.* 2004).

Within each tunnel, three plant sections (3 × 1.5 × 1.5 m each) were established where the air temperature was either kept at ambient, or a constant warming of +3.0 °C above ambient (constant warming), or +2.2 °C warming above ambient during the day and +4.0 °C warming during the night (high night-time warming). Warming was accomplished mostly by passive solar heating and variable fan speed as air moved through the tunnels during the day and by using a combination of air heaters and drawing in cool air at night (Volder *et al.* 2004). Temperatures were controlled using double-shielded, continuously aspirated thermistors, located 1 m above the surface in each plant section, connected to a Microzone II controller that controlled fan speed and activity of the air heaters. Temperatures were measured every 0.1 s, and fan speed and heater outputs were adjusted every 0.5 s based on the measured temperature differential from the target temperature. Thermistors used for control were independent from the thermistors used to log section air temperatures to

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ensure data integrity. Daily averaged air temperature warming was +2.9 °C in the high night-time warming treatment and +3.0 °C in the constant warming treatment.

The plots were maintained at non-limiting soil water levels, using overhead irrigation with 16 nozzles per section to ensure even distribution. Soil water content was measured using Theta probes (Delta-T devices Ltd., Cambridge, UK) installed at 5 cm depth in the infrequently clipped plots. In Year 1, soil water content was maintained at 0.32–0.38 $m^3\ m^{-3}$ (77–92 % of field capacity). Starting 25 September 2002, soil water content was reduced to between 0.20 and 0.25 $m^3\ m^{-3}$. Soil water levels were checked daily and when a Theta probe read below 0.20 $m^3\ m^{-3}$, water was applied to all plots until the driest probe read 0.25 $m^3\ m^{-3}$. All sections received the same amount of water. There was always a higher soil moisture content (by 0.02–0.05 $m^3\ m^{-3}$) in the elevated CO_2 treatment (Volder *et al.* 2004).

Perennial temperate pasture grass (*P. aquatica* cv. Holdfast) was winter-sown at a density of 250 plants m^{-2} using a row spacing of 8 cm on 3 May 2001. Within each temperature treatment, there were two clipping regimes, both at 7 cm above the ground in 1.5 × 1.5 m areas. One regime was clipped two times as often as the other, using visual criteria to decide the time for clipping. The frequently clipped treatment dates were 25 October and 21 November 2001; 8 January, 19 February, 4 April, 1 May, 28 August, 25 September, 6 November, 11 December 2002 and 5 February, 5 March, 8 April and 20–21 May 2003. Plots for the infrequent clipping were clipped every second date. A border zone of 30 cm width was established around each measured plot to avoid edge effects. Both border and measured plot (90 × 90 cm) zones were clipped at each harvest, but border zone material was discarded. Random subsamples (3–6 % of total dry mass) were taken from the bulk sample at each harvest during the harvesting of each plot. The subsamples were divided into standing dead (i.e. litter), green leaf lamina and remainder (sheath, stem and flowers). Sheath, stems and reproductive parts were placed together as most of the stem growth occurs in support of the reproductive parts. All fractions were dried for a week at 80 °C and weighed. Total C and N were determined using a Europa elemental analyser (Sercon, Cheshire, UK). At the final harvest, stubble remaining below the clipping height was harvested from two 20 × 20 cm quadrats placed randomly within each plot. All stubble material including crowns was cleaned free of soil and any roots were discarded. Total C and N were measured as above.

Soil cores to 30 cm depth were collected with 32-mm diameter push tubes from each CO_2 × temperature × clipping combination on 25 September 2001 (start of treatments), 20 February 2002, 26 September 2002 and 5 March 2003. At the end of the experiment, cores were collected down to 1 m using a hydraulic corer. Cores were cut into 0–10, 10–20 and 20–30 cm segments, with additional segments for the final deeper cores at 50–60 and 80–90 cm. Roots were then washed with distilled water, sieved through a 2-mm sieve, followed by a 0.5-mm sieve (Volder *et al.* 2007) and split into two size classes, fine laterals (first-order roots, less than ∼0.3 mm diameter) and lateral-bearing coarse roots (generally >0.3 mm diameter). Fine laterals were removed from the coarse roots and placed in the fine fraction. All fractions were dried for a week at 80 °C, weighed and total C and N was determined using a Europa elemental analyser (Sercon).

All plots were fertilized three times per year with 100 kg N ha^{-1} per occasion using a slow release fertilizer (Osmocote, Scotts Company, Marysville, OH, USA), which also included 26.7 kg P ha^{-1} and 50.6 kg K ha^{-1}. Fertilizer was applied on 28 September 2001; 20 February, 18 June and 29 September 2002 and 6 March 2003.

Statistical analyses

When necessary to improve homogeneity of variances, data were natural log transformed (Zar 1984). Effects of CO_2, temperature and clipping frequency were analysed with ANOVA (residual maximum likelihood procedure) using JMP Pro for windows version 10 (SAS Institute, Cary, NC, USA). The design was a split–split plot with CO_2 level (ambient, 750 p.p.m.) as the main plot factor, warming treatment (+0, +2.2/+4.0 and +3.0 °C) as subplots and clipping frequency (regular, frequent) as randomly assigned sub-subplots within each warming treatment.

Results

Across treatments and harvests, harvested green leaf tissue N concentrations were decreased by 15.6 % under elevated atmospheric CO_2 concentration (Fig. 1A) and increased by 32.0 % by frequent clipping (Fig. 1B). The influence of elevated CO_2 and clipping frequency on leaf N concentration varied with harvest period (Fig. 1, Table 1, **see Supporting Information—Tables S1–S3** for tissue C and N concentrations and C : N ratios on each harvest date). The decline in leaf tissue N concentration due to elevated CO_2 was stronger in spring and summer (November–May and September–March) than during the winter (Fig. 1A, $P_{\text{harvest date}\times CO_2} < 0.001$), while the effect of clipping frequency was stronger in the winter and fall (May–December, March–May) than in the spring and summer (Fig. 1B, $P_{\text{harvest date}\times\text{ clipping}} < 0.001$).

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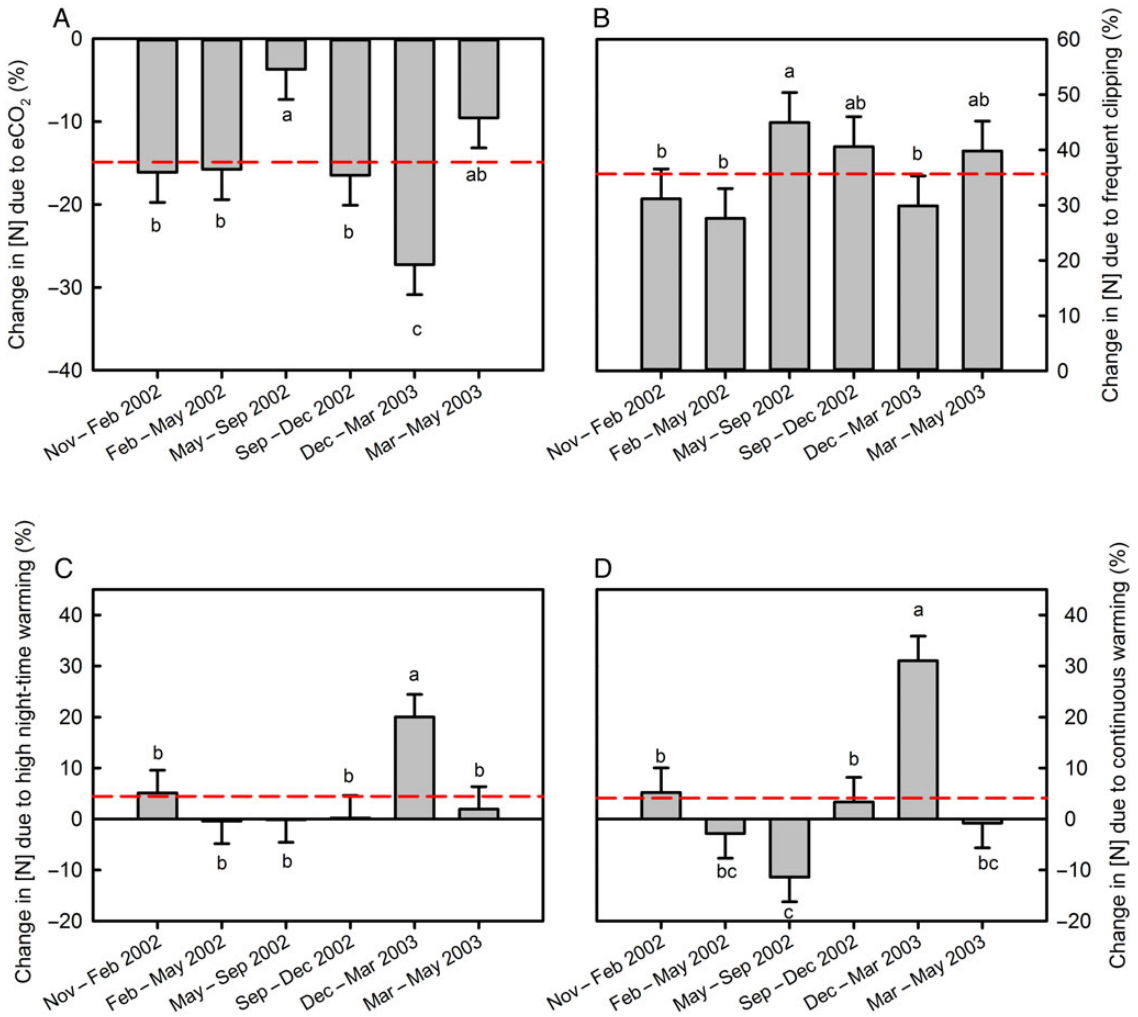


Figure 1. Change in green leaf N concentrations due to (A) increased atmospheric CO_2 concentration (756 p.p.m.) compared with ambient atmospheric CO_2 concentration (405 p.p.m.), (B) increased clipping frequency, (C) higher night-time warming compared with ambient air temperatures (+2.0/+4.4 °C above ambient, day/night) and (D) continuous warming above ambient (+3.0 °C), as affected by harvest period. The dashed lines indicate the average response to elevated atmospheric CO_2 levels (A), increased clipping frequency (B), high night-time warming (C) and continuous warming (D). Data presented are least square means and SEM based on a full model. Different letters indicate statistically significant differences between harvest periods at $P < 0.05$ using Student's *t* LSD test.

Warming was without any overall effect on green leaf N concentration when averaged across all the other treatments and harvest times (Table 1). However, there were strong interactive effects of warming with CO_2 and harvest period. Averaged across CO_2 treatments, both warming treatments greatly increased leaf N concentration in the second summer ($P_{harvest \times warm} < 0.001$, December–March 2003, Fig. 1C and D). In contrast, continuous warming decreased leaf N concentration in comparison with ambient temperatures in the winter (May–September 2002, Fig. 1C). When averaged across all dates and clipping frequencies, warming significantly increased leaf N concentrations under ambient CO_2, but under elevated CO_2, leaf N concentration was not affected by warming (Fig. 2A). The negative effect of increased atmospheric CO_2 concentration on leaf N concentration was substantially enhanced by both warming treatments, from 7.6 % decrease under ambient temperature to 17.5 and 21.4 % decrease under continuous and high night-time warming ($P_{CO_2 \times warm} = 0.036$).

Elevated CO_2 increased leaf C : N ratios, particularly under the high night-time warming scenario ('HN', $P_{CO_2 \times warming} = 0.053$, from 18.0 to 22.9, Fig. 2B). Changes in leaf C : N ratio in response to elevated CO_2 reflect changes in leaf N concentration. There was no relationship between changes in C : N ratio and changes in C concentration in response to elevated CO_2. Increased clipping frequency decreased green leaf C : N ratios by an average of 24 %, from 23.6 to 17.9 (Fig. 3A, $P < 0.001$).

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Table 1. *P*-values of the effect of atmospheric CO_2 concentration (CO_2), warming treatment (*W*), clipping frequency (*C*) and harvest period (*H*) on green leaf N concentration, C concentration and C : N ratio. [1]Numerator, denominator. [2]Data were ln transformed prior to analysis. [3]Missing data prevented analysis of the four-way interaction term. *P* values <0.10 but >0.05 are given in italics, while *P* values <0.05 are given in bold.

	df[1]	Green leaf						Litter					
		N		C		C : N ratio		N[2]		C		C : N ratio	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
CO_2	1,4	130	**<0.001**	0.99	0.375	98.1	**<0.001**	0.90	0.385	6.39	*0.064*	0.22	0.651
Warming (*W*)	2,8	0.83	0.470	5.88	**0.027**	0.38	0.694	4.25	**0.047**	8.61	**0.010**	0.23	0.798
$CO_2 \times W$	2,8	5.17	**0.036**	0.48	0.633	3.48	*0.082*	2.44	0.140	3.37	*0.087*	1.80	0.219
Clipping frequency (*C*)	1,12	196	**<0.001**	0.07	0.799	160	**<0.001**	55.0	**<0.001**	2.10	0.171	29.6	**<0.001**
$C \times CO_2$	1,12	0.16	0.691	0.07	0.797	2.32	0.153	0.00	0.947	0.21	0.652	0.48	0.498
$C \times W$	2,12	0.49	0.615	2.45	0.128	1.24	0.325	0.24	0.788	1.72	0.217	0.18	0.838
$C \times W \times CO_2$	2,12	0.55	0.584	0.41	0.674	0.70	0.518	0.42	0.668	0.68	0.523	0.52	0.607
Harvest (*H*)	5,120	79.5	**<0.001**	61.1	**<0.001**	39.8	**<0.001**	6.25	**<0.001**	15.3	**<0.001**	0.98	0.435
$H \times CO_2$	5,120	6.39	**<0.001**	1.35	0.249	5.00	**<0.001**	1.05	0.392	1.38	0.236	0.33	0.893
$H \times W$	10,120	4.43	**<0.001**	2.89	**0.003**	4.05	**<0.001**	1.15	0.334	1.02	0.434	0.66	0.761
$H \times CO_2 \times W$	10,120	0.75	0.662	1.22	0.286	0.91	0.528	1.17	0.317	1.20	0.296	0.67	0.752
$H \times$ Clip	5,120	5.17	**<0.001**	14.2	**<0.001**	2.02	*0.080*	16.7	**<0.001**	11.9	**<0.001**	13.4	**<0.001**
$H \times$ Clip $\times CO_2$	5,120	*2.05*	*0.075*	0.19	0.964	0.85	0.515	0.47	0.796	1.46	0.207	0.41	0.839
$H \times$ Clip $\times W$	10,120	0.78	0.652	1.48	0.156	0.61	0.806	1.51	0.143	1.47	0.157	1.07	0.392
$H \times$ Clip $\times CO_2 \times W$	10,120	0.42	0.936	1.07	0.393	0.408	0.941		NA[3]		NA[3]		NA[3]
Model r^2			0.898		0.779		0.874		0.649		0.620		0.621
Model *P*			<0.001		<0.001		<0.001		<0.001		<0.001		<0.001

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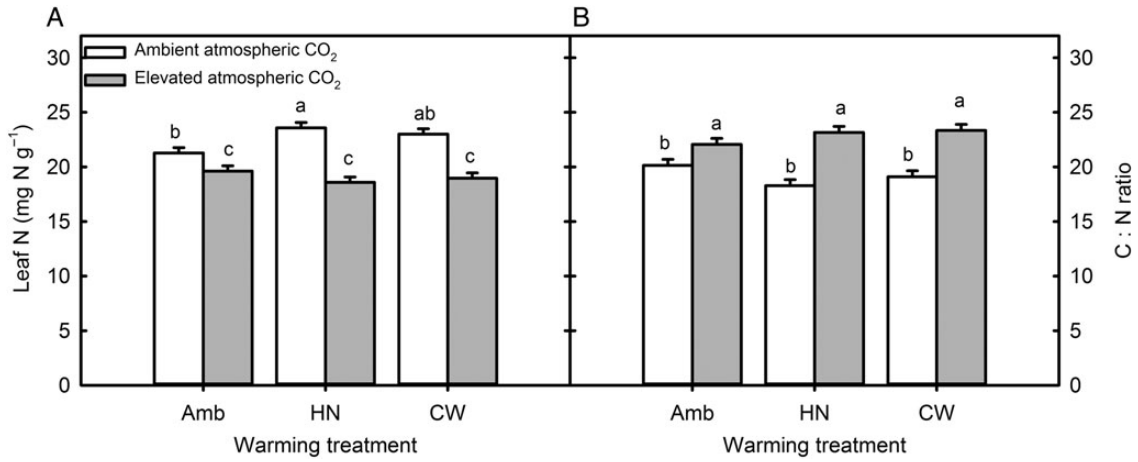


Figure 2. Effect of warming treatment, Amb = ambient temperature, HN = +2.2/+4.0 °C (day/night warming), CW = +3.0 °C continuous warming and atmospheric CO_2 concentration (ambient, 405 p.p.m., and elevated, 756 p.p.m.) on (A) green leaf N concentration and (B) green leaf C : N ratio. Data presented are least square means and SEM averaged across harvests and clipping frequencies. Different letters indicate statistically significant differences at $P < 0.05$ using Student's *t* LSD test.

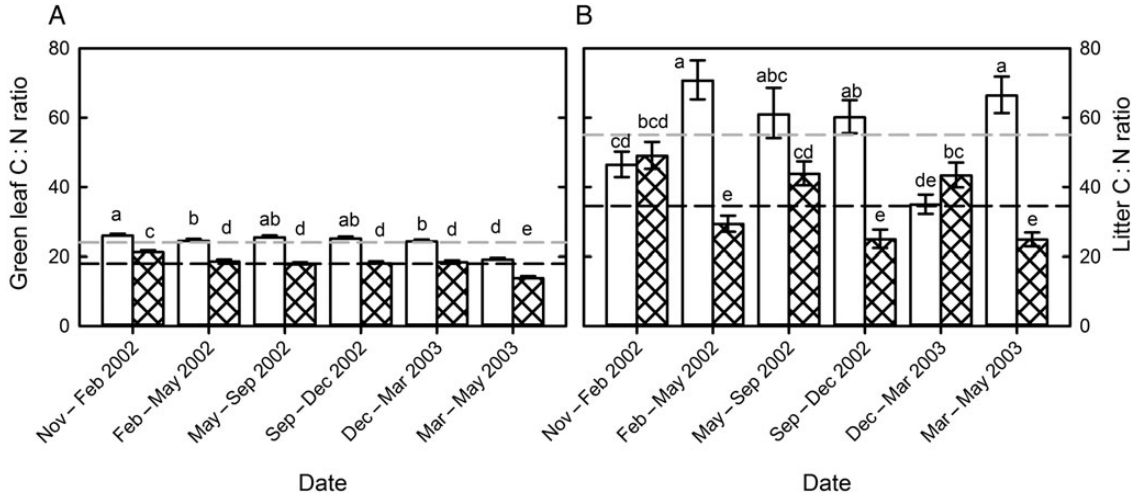


Figure 3. Effect of clipping frequency (open bar = infrequent, hashed bar = frequent) on C : N ratios of (A) green leaf material and (B) litter, through time averaged across CO_2 and warming treatments. Dashed line indicates overall mean across dates, CO_2 and warming treatment for each clipping frequency, infrequent (grey) and frequent (black). Different letters indicate statistically significant differences between C : N ratios at $P < 0.05$ using Student's *t* LSD test.

Litter (i.e. standing dead leaf) N concentrations were unaffected by atmospheric CO_2 concentration (Table 1 and **see Supporting Information—Table S1**). There was a marginally significant warming effect (Table 1, $P_{warming} = 0.047$) where litter N concentration averaged across harvests was higher under a continuous warming scenario (9.1 mg g^{-1}) than under ambient warming (7.6 mg g^{-1}). The average of the two warming treatments increased litter N concentration by 25.3 % (Fig. 4C and D). Litter N concentration was most affected by clipping frequency; increased clipping frequency increased litter N concentrations by an average of 95.9 % (Fig. 4B). Consequently, frequent clipping reduced litter C : N ratio from 55.0 to 34.5 averaged across harvests and CO_2 and warming treatments (Fig. 3B and **see Supporting Information—Table S3**). Nitrogen resorption efficiency, calculated as [100 × (green leaf N concentration − litter N concentration)/green leaf N concentration], was decreased from 56.7 % at ambient atmospheric CO_2 concentration to 50.9 % under elevated CO_2 when averaged across warming and clipping treatments (Fig. 5A). Increased clipping frequency decreased N resorption efficiency from 58.9 % in infrequently clipped plots to 48.7 % in frequently clipped plots when averaged across warming and CO_2 treatments (Fig. 5A). Averaged across clipping and CO_2 treatment, both warming treatments reduced

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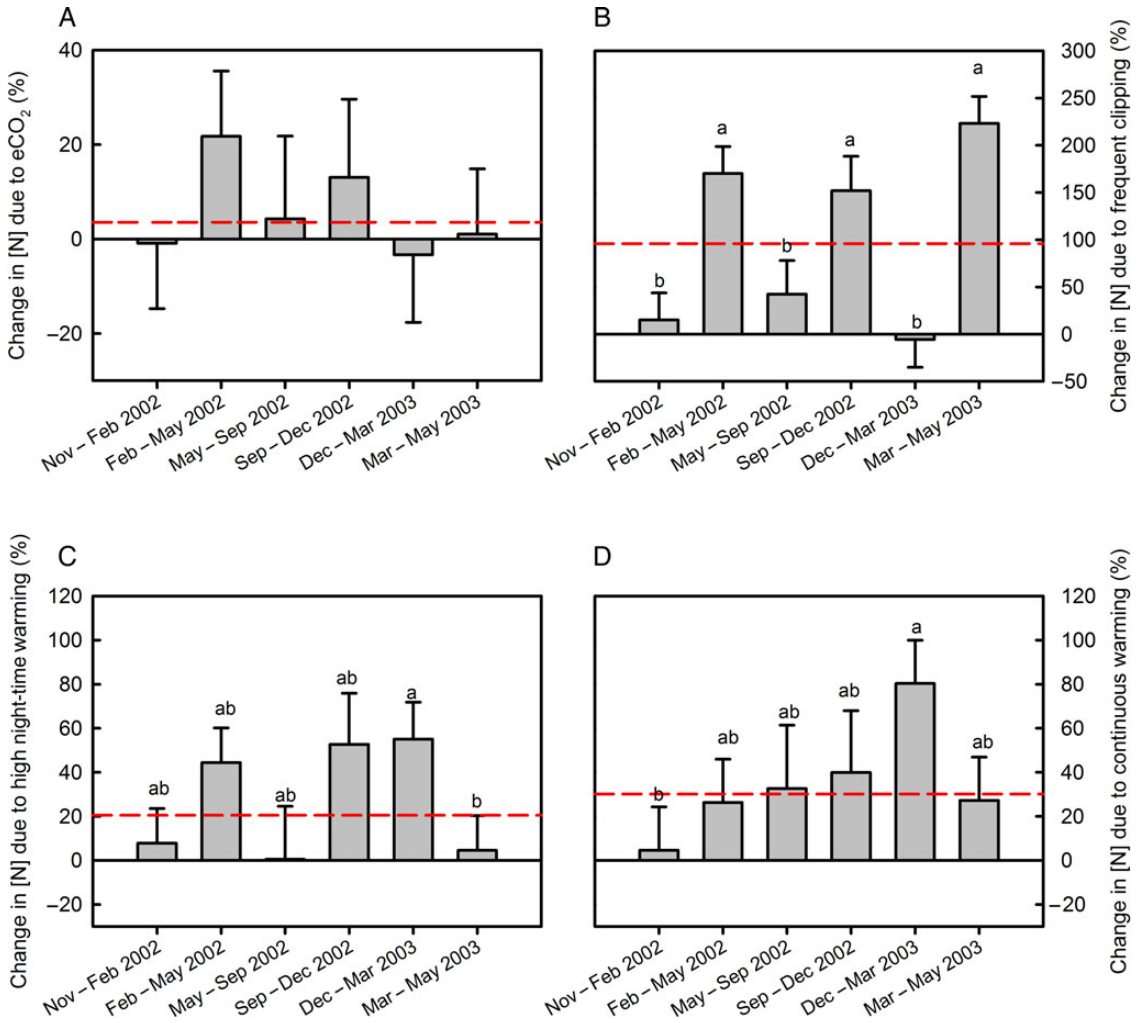


Figure 4. Percent change in litter N concentrations due to (A) increased atmospheric CO_2 concentration (756 p.p.m.) compared with ambient atmospheric CO_2 concentration (405 p.p.m.), (B) increased clipping frequency, (C) higher night-time warming compared with ambient air temperatures (+2.0/+4.4 °C above ambient, day/night) and (D) continuous warming above ambient (+3.0 °C), as affected by harvest period. The dashed lines indicate the average response to elevated atmospheric CO_2 levels (A), increased clipping frequency (B), high night-time warming (C) and continuous warming (D). Data presented are least square means and SEM based on a full model. Different letters indicate statistically significant differences between harvest periods at $P < 0.05$ using Student's *t* LSD test.

N resorption efficiency from 59.1 % in the unwarmed treatment to 52.8 and 49.5 % in the high night-time and continuously warmed treatments, respectively.

Fine root N concentrations decreased strongly with depth in the soil on all three harvest dates (Fig. 6, Table 2). Elevated atmospheric CO_2 had no systematic effect on fine root N concentration, although there was a small significant decline under high CO_2 in March 2003, in the 20–30 cm soil depth (Fig. 6A). Increased clipping frequency had no overall effect on fine root N concentration but did increase fine root N concentration in just the 0–10 cm soil layer in March 2003 (Fig. 6B). Overall, averaged across CO_2, clipping frequency and harvests, warming was without effect on root N concentration. However, small effects of warming varied with depth and date and were limited to the shallower (0–10 and 10–20 cm) soil depths (Fig. 6C). In February 2002, high night-time warming led to slightly increased fine root N concentration compared with continuous warming in the 0–10 and 10–20 cm soil depths, while in March 2003, continuous warming led to higher fine root N concentrations in the continuous warming treatment compared with ambient warming in the 0–10 cm soil depth (Fig. 6C). At the final harvest (March 2003), where we also collected deeper soil cores, fine root N concentration decreased further with soil depth below 50 cm. There was a strong atmospheric CO_2 concentration × depth interaction effect ($P_{CO_2 \times depth} = 0.009$, Table 2) where elevated atmospheric CO_2 levels reduced fine root N concentrations at the 10–20, 20–30 and 50–60 cm core depths,

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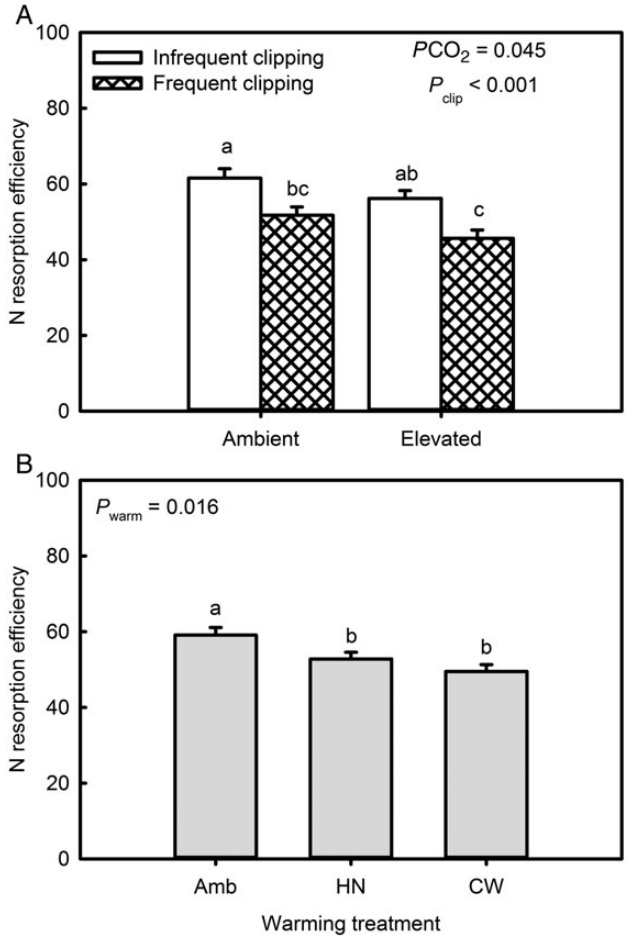


Figure 5. (A) Effect of clipping frequency and atmospheric CO_2 concentration on N resorption efficiency. (B) Effect of warming treatment on N resorption efficiency, Amb = ambient, HN = +2.2 °C/+4.0 °C day/night warming, CW = +3.0 °C continuous warming. Different letters indicate statistically significant differences at $P < 0.05$ using Student's *t* LSD test.

but not in the 0–10 and 80–90 cm core depths (Fig. 7). There were no additional effects of clipping frequency or air warming on fine root N of roots growing below 50 cm soil depth (Fig. 7B and C).

Discussion

Across the three tissues examined, green leaf, litter and fine root, green leaves had the highest tissue N concentration and were the most responsive to elevated atmospheric CO_2 concentration and warming. Elevated atmospheric CO_2 concentration generally reduced tissue N concentrations while the effect of warming depended on the CO_2 concentration. Of the three treatments, frequent clipping had the greatest overall impact on leaf and litter N concentrations, with increased clipping frequency strongly increasing tissue N concentrations. Because treatment impacts varied by tissue type, we will discuss treatment effects by tissue type.

Green leaf

More frequent clipping strongly increased green leaf N concentration by an average of 33 % across the other treatments and harvest dates (Fig. 1B). This was expected because the frequently clipped plants have shoot tissue that is about half the age of shoot tissue of infrequently clipped plants. Younger tissue generally has higher N concentrations (Field 1983). Just why the magnitude of this clipping-frequency effect was greater in the slower growing winter months (May–September) is not clear.

Despite being well fertilized, elevated CO_2 concentration decreased green leaf N concentrations in these field grown C_3 grasses. That result is consistent with the literature (Cotrufo *et al.* 1998). The mean decrease in leaf N concentration in response to elevated CO_2 was 15 % (Fig. 1A), which is similar to the 17 % decrease in N concentration for non-woody C_3 species reported by Cotrufo *et al.* (1998). The magnitude of lower leaf N concentration in response to elevated atmospheric CO_2 varied seasonally with a much smaller response to elevated CO_2 in the slower growing winter season. The biomass production response to elevated atmospheric CO_2 in this same experiment was also smaller during the cold months (Volder *et al.* 2004), consistent with the notion (Coleman *et al.* 1993; Loladze 2002) that dilution of leaf N concentration by enhanced growth in response to elevated CO_2 plays a role at least in part. However, the strong relationship between changes in leaf N concentration and changes in leaf C : N ratio, as well as the lack of a relationship between changes in leaf C concentration and changes in leaf C : N ratio, supports the idea that effects of elevated CO_2 on green leaf tissue C : N ratio are mostly driven by the effects of CO_2 on leaf N concentrations rather than C accumulation in response to elevated CO_2 (Gifford *et al.* 2000).

Atmospheric warming treatments, when averaged across all harvests, increased green leaf N concentrations of plants grown under ambient atmospheric CO_2 concentrations by 11.4 and 8.6 % for the higher night and continuous warming treatments, respectively (Fig. 2A, open bars). However, warming had no significant effect on leaf N concentrations of the swards grown under elevated CO_2 (Fig. 2A, closed bars). The effects of CO_2 concentration and warming on leaf N concentrations were not simply additive; warming of ambient CO_2 plants enhanced green leaf N concentrations by ~10 %, elevated CO_2 decreased leaf N concentrations by 7.6 %, while combined warming and elevated CO_2 decreased green leaf N concentrations by ~11 % (Fig. 2A). Non-additivity of warming and CO_2 responses, with the response to CO_2 dominating, is also consistent with a meta-analysis of leaf tissue N in six temperature × CO_2 manipulation

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Table 2. *P*-values of the effect of atmospheric CO_2 concentration (CO_2), warming treatment (*W*), clipping frequency (*C*) and soil depth (*D*) on fine root N and C concentrations, and C : N ratio through time. [1]Depth classes also included 50–60 and 80–90 cm. [2]Numerator, denominator. [3]Data were ln transformed prior to analysis.

Treatment	February 2002 (summer)							September 2002 (early spring)							March 2003 (early fall)[1]						
	df[2]	N		C		C : N[3]		df	N		C		C : N[3]		df	N		C		C : N[3]	
		F ratio	*P*	*F* ratio	*P*	*F* ratio	*P*		*F* ratio	*P*	*F* ratio	df	*F* ratio	*P*		*F* ratio	*P*	*F* ratio	*P*	*F* ratio	*P*
CO_2	1,4	0.01	0.942	1.01	0.370	0.64	0.466	1,4	0.03	0.883	3.63	0.127	0.69	0.453	1,4	6.93	*0.059*	0.62	0.475	7.42	*0.054*
Warming (*W*)	2,8	*3.22*	*0.096*	0.76	0.500	2.45	0.166	2,8	0.10	0.906	0.02	0.976	0.96	0.959	2,8	4.46	**0.049**	1.19	0.352	3.20	*0.095*
$CO_2 \times W$	2,8	1.08	0.386	0.01	0.987	0.58	0.589	2,8	0.70	0.527	0.47	0.640	0.60	0.602	2,8	0.30	0.746	0.32	0.737	0.12	0.886
Clipping (*C*)	1,12	1.31	0.275	*3.84*	*0.076*	0.03	0.866	1,12	0.11	0.742	0.45	0.515	0.91	0.915	1,12	2.37	0.150	8.54	**0.013**	22.5	**<0.001**
$CO_2 \times$ Clip	1,12	2.39	0.148	0.05	0.825	1.89	0.197	1,12	0.04	0.852	0.21	0.656	0.73	0.734	1,12	1.63	0.226	0.13	0.730	4.50	*0.055*
$W \times$ Clip	2,12	0.53	0.602	0.40	0.681	0.10	0.910	2,12	0.41	0.670	0.23	0.799	0.45	0.448	2,12	0.41	0.673	1.96	0.182	2.73	0.105
$CO_2 \times W \times$ Clip	2,12	1.24	0.324	2.08	0.171	0.10	0.910	2,12	0.15	0.859	0.28	0.762	0.48	0.477	2,12	2.27	0.146	0.06	0.940	3.01	*0.087*
Depth (*D*)	2,44	78.1	**<0.001**	3.82	**0.029**	69.5	**<0.001**	2,46	135	**<0.001**	28.9	**<0.001**	47.8	**<0.001**	2,95	514	**<0.001**	16.5	**<0.001**	427	**<0.001**
$D \times CO_2$	2,44	1.07	0.351	0.28	0.758	1.00	0.377	2,46	1.00	0.375	0.37	0.695	1.12	0.335	2,95	3.59	**0.009**	3.62	**0.009**	3.52	**0.010**
$D \times W$	4,44	1.40	0.251	1.40	0.249	0.33	0.858	4,46	0.84	0.507	1.64	0.179	0.660	0.665	4,95	1.23	0.291	1.95	*0.062*	1.03	0.420
$D \times CO_2 \times W$	4,44	0.75	0.566	2.04	0.105	0.66	0.627	4,46	0.79	0.537	1.07	0.381	0.48	0.752	4,95	0.75	0.645	1.41	0.202	1.35	0.228
$D \times C$	2,44	0.39	0.676	0.70	0.504	0.38	0.683	2,46	0.56	0.576	0.86	0.432	1.38	0.262	2,95	1.74	0.147	2.36	*0.059*	1.31	0.273
$D \times CO_2 \times C$	2,44	0.39	0.681	3.53	**0.038**	1.83	0.175	2,46	0.79	0.459	2.74	*0.075*	1.94	0.156	2,95	0.98	0.422	0.73	0.574	0.71	0.584
$D \times W \times C$	4,44	0.95	0.445	0.70	0.599	0.61	0.656	4,46	0.40	0.807	4.84	**0.002**	2.32	*0.071*	4,95	0.54	0.822	0.60	0.774	1.00	0.442
$D \times CO_2 \times W \times C$	4,44	0.21	0.932	0.56	0.691	0.84	0.506	4,46	0.64	0.640	1.32	0.277	0.06	0.994	4,95	1.55	0.152	0.63	0.754	1.02	0.425
Model r^2			0.721		0.448		0.741			0.854		0.681		0.716			0.955		0.566		0.948
Model *P*			<0.001		<0.001		<0.001			<0.001		<0.001		<0.001			<0.001		<0.001		<0.001

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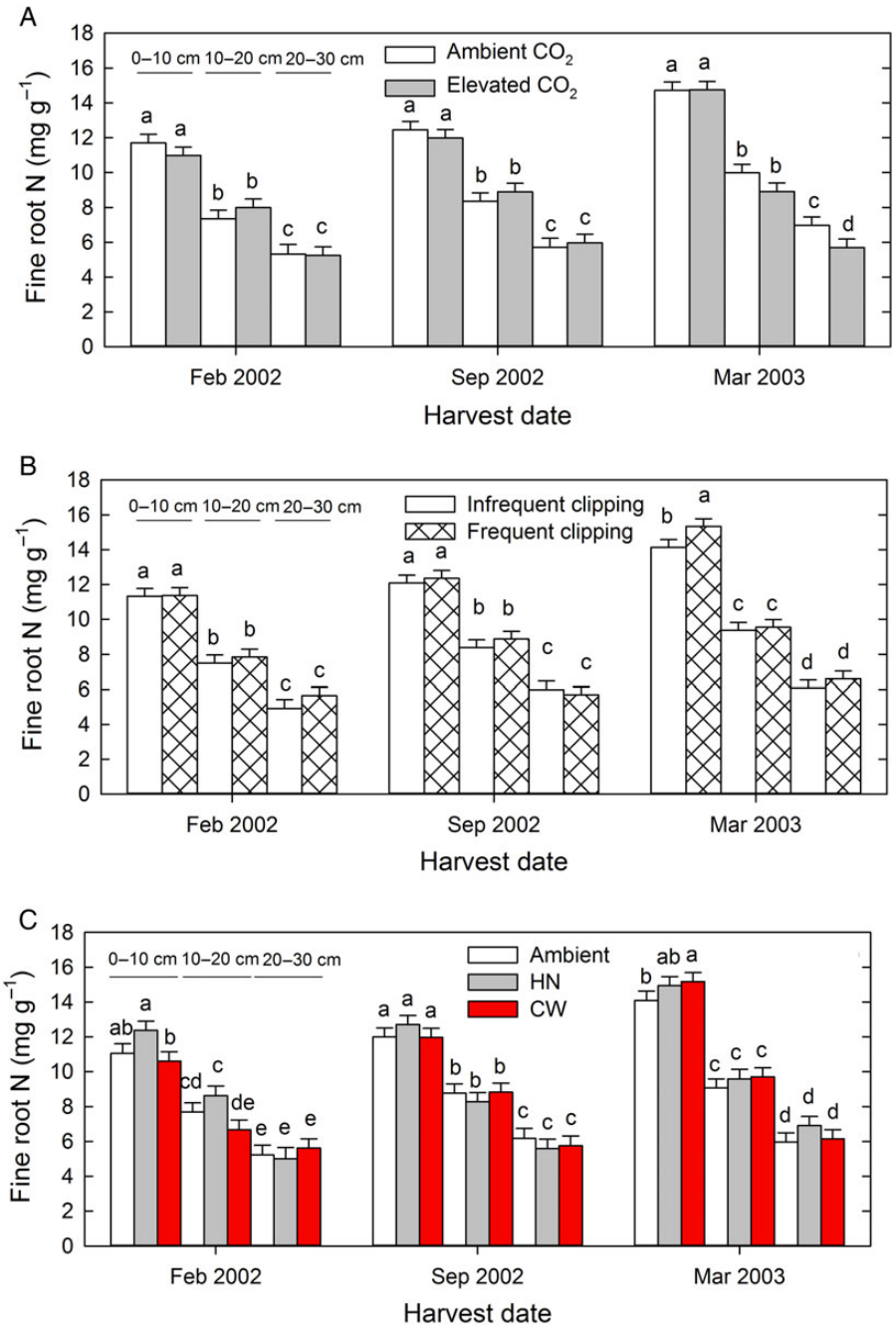


Figure 6. Effect of atmospheric CO_2 concentration (A), clipping frequency (B) and warming treatment (C) on fine root N concentrations at three harvest dates and three depths. HN = +2.2/+4.0 °C day/night, CW = +3.0 °C continuous warming. Different letters indicate statistically significant differences within a harvest date at $P < 0.05$ using Student's *t* LSD test.

studies by Dieleman *et al.* (2012). The increase in tissue N in response to warming at ambient atmospheric CO_2 levels is consistent with the idea that shoot nitrate assimilation depends on photorespiration (Rachmilevitch *et al.* 2004; Bloom *et al.* 2010, 2012). Under conditions where photorespiration is enhanced (i.e. ambient CO_2 and warming), nitrate assimilation is stimulated, while under conditions where photorespiration is repressed (i.e. high atmospheric CO_2 conditions), increasing temperatures will not affect tissue N concentrations. This suggests that the impact of future climate warming on tissue quality and N cycling cannot be predicted based on warming experiments alone.

Our finding that green leaf tissue N concentration increased in response to warming under ambient atmospheric CO_2 concentrations differs from An *et al.* (2005),

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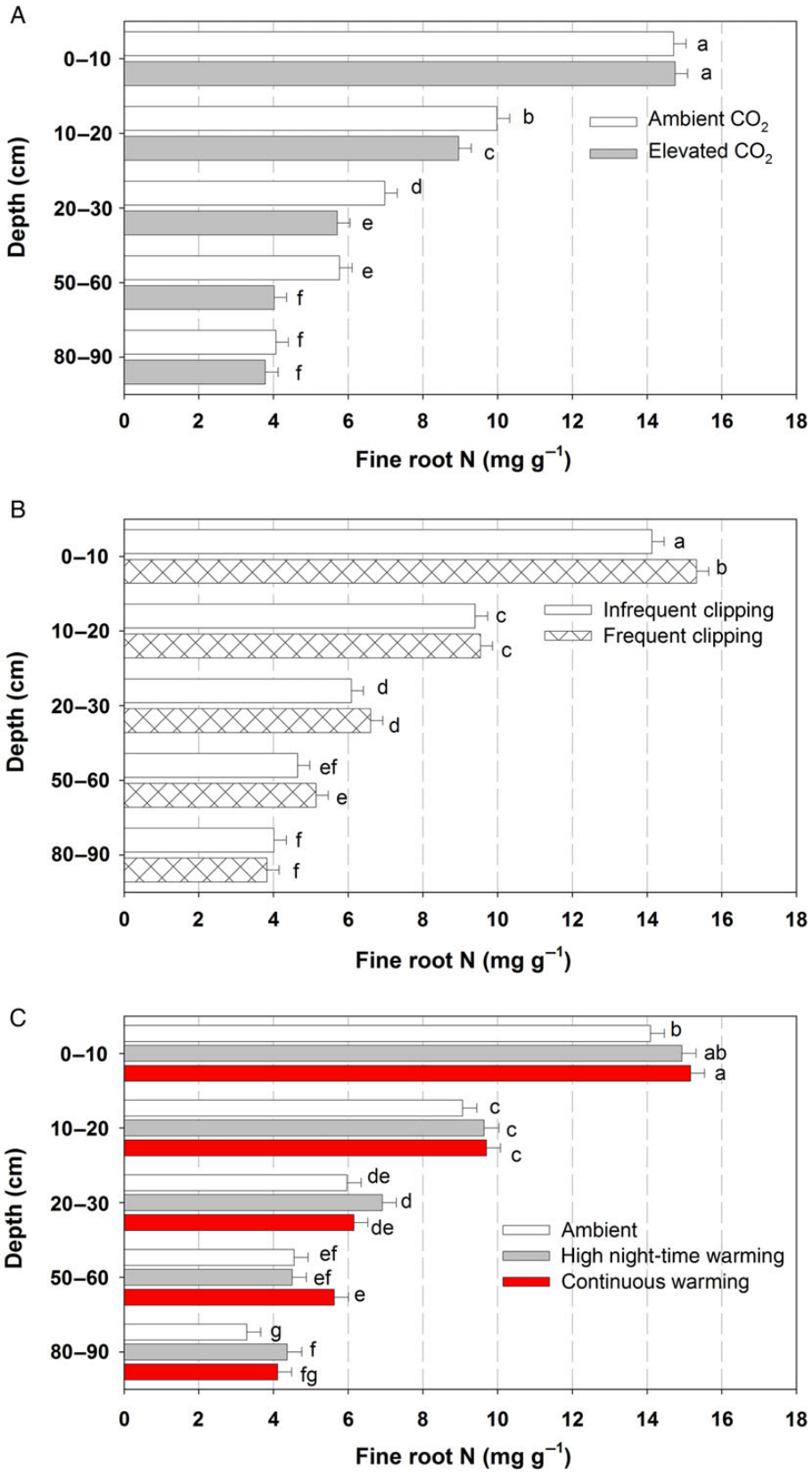


Figure 7. Effect of (A) atmospheric CO_2 concentration, (B) clipping frequency and (C) warming treatment on fine root N concentration at five depths at the end of the experiment (March 2003). Treatments started in September 2001. Data are the least square means + SEM across treatments. Different letters indicate statistically significant differences at $P < 0.05$ using Student's *t* LSD test.

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who found that warming decreased tissue N concentrations in five grassland species. An *et al.* (2005) observed the effects of warming after 1 year of treatment, whereas in our study, warming did not have a statistically significant effect on green leaf N concentrations until well into the second season of treatments. Thus, the effect of warming may not be as immediate and is more subtle than the effects of elevated atmospheric CO_2 and clipping frequency.

In contrast to the effects of combined warming and atmospheric CO_2 on leaf N concentration, there was no interaction between clipping frequency and CO_2 concentration or between clipping frequency and warming treatment. This suggests that the effects of clipping management are additive to the effects of the climate change drivers. Our earlier findings on aboveground biomass production (Volder *et al.* 2004) and new root production (Volder *et al.* 2007) showed non-additivity, where the effect of elevated CO_2 was dependent on clipping frequency. Thus, as expected, whether effects of climate change drivers are additive or not depends on the response variable measured and the management regimen of the ecosystem (Dieleman *et al.* 2012; Xu *et al.* 2013).

Standing dead and leaf litter

The decreases in leaf N concentration due to elevated atmospheric CO_2 concentrations (Figs 1A and 2A) did not translate into reduced standing dead leaf litter N concentration or increased litter C : N ratios (Fig. 4A). Neither litter N concentration nor litter C : N ratio were significantly affected by elevated CO_2 (Table 1). Increased clipping frequency strongly decreased C : N ratios of both leaf (from 24.0 to 18.0) and litter (from 55.0 to 34.5, Fig. 3). Frequent clipping increased green leaf N concentration and also reduced N resorption efficiency from 59.2 to 53.6 %, thus leaving a greater amount of N per gram litter biomass. As more N is left in senescing tissues of frequently clipped vegetation and litter C : N ratios are reduced, it is possible that frequent clipping would speed up the rate of mineralization of litter N from a given amount of litter added to the soil (Booth *et al.* 2005), if soil moisture and soil temperature remain similar. However, whether that would lead to actual increased N availability in soils of frequently cut swards also depends on the total amount of litter returned to the soil. Previous published results (Volder *et al.* 2004) showed that frequent clipping strongly decreased biomass production, and thus the total amount of N returned to the soil from litter may be reduced, even if mineralization rates are increased because of decreased litter C : N ratios.

Roots

Root N concentrations were very strongly affected by soil depth; the concentrations in fine roots at 80–90 cm depth were less than a third of those in the top 10 cm (Fig. 7). Effects of warming, CO_2 and clipping frequency were minor in comparison with the depth effect. These minor effects of elevated CO_2 concentration and warming at depth were most evident at the final harvest. Although most CO_2 responses occurred in roots below 10 cm soil depth in our experiment, others have found negative impacts of elevated CO_2 concentrations on root N concentration in shallow ($<$10 cm) soil layers in grassland systems (Kitchen *et al.* 2009). The lack of a clipping effect on root N concentration was surprising given that increased clipping frequency increased root turnover rate (Volder *et al.* 2007), which would lead to a younger root system on average. Younger roots have been shown to have higher rates of nitrate uptake and higher tissue N concentrations (Volder *et al.* 2005); however, the proportional change in average root age may not have been large enough to affect the average tissue N concentration in our bulk samples.

Often root research is limited to the top soil layer because that is the zone where generally $>$50 % of root length occurs (Schenk and Jackson 2002). However, our data suggest that when evaluating the impact of climate on root parameters, some major changes in tissue N concentrations may be occurring deeper in the soil profile. It is important to note that our experiment involved a managed pasture grass system where water and nutrients were supplied at high levels—soil water content was kept at 20 % or higher (Volder *et al.* 2004), and the plots were fertilized three times per year at a rate of 100 kg N ha^{-1} per occasion. Thus, plant responses in our system were mostly decoupled from soil system feedbacks (Type I system, Körner 2006) when compared with other climate change experiments in grasslands, which generally take place in systems where water and/or nutrients are limited (Morgan *et al.* 2004).

Conclusions

While increasing atmospheric CO_2 concentration decreased green leaf N concentrations considerably, this was not propagated to leaf litter, as leaf litter N concentration was unaffected by elevated CO_2. Atmospheric warming increased green leaf N under ambient CO_2 but did not significantly affect leaf N concentration at elevated CO_2 concentration. The increase in leaf N concentration under warming at ambient CO_2 was reflected in increased litter N concentration. For fine roots, elevated CO_2 tended to decrease N concentration ($P = 0.059$) and increase C : N ratio by the end of the experiment

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with the magnitude of the effect increasing deeper in the soil. The effects of continuous uniform warming were similar to differential day/night warming.

In general, the non-additivity of CO_2, warming and management treatment effects with unexplained time variability of specific interactive effects that are exhibited in this data set presents considerable problems for predicting long-term climate change impacts on pasture ecophysiology by either rules of thumb or simulation modelling.

Sources of Funding

This work was funded by the Cooperative Research Centre for Greenhouse Accounting and internal funding from CSIRO Division of Plant Industry, Australia.

Contributions by the Authors

A.V. collected and processed samples, performed data analysis and wrote the manuscript. R.M.G. and J.R.E. collected samples and co-wrote the manuscript.

Conflict of Interest Statement

None declared.

Acknowledgements

We thank Bruce Robertson, Philip Measham, Michael Halliday, staff of the CSIRO Plant Industry Workshop and Ginninderra Experiment Station and Richard Blyton for invaluable advice and help with this project.

Supporting Information

The following additional information is available in the online version of this article –

Table S1. Tissue N concentrations (mg N g^{-1}) through time as affected by atmospheric CO_2 concentration, warming treatment, ambient, higher night-time warming and continuous warming, and clipping frequency.

Table S2. Tissue C concentrations (g C g^{-1}) through time as affected by atmospheric CO_2 concentration, warming treatment, ambient, higher night-time warming and continuous warming, and clipping frequency.

Table S3. Tissue C : N ratios through time as affected by atmospheric CO_2 concentration, warming treatment, ambient, higher night-time warming and continuous warming, and clipping frequency.

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