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C_3 grasses have higher nutritional quality than C_4 grasses under ambient and elevated atmospheric CO_2

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Abstract

Grasses with the C_3 photosynthetic pathway are commonly considered to be more nutritious host plants than C_4 grasses, but the nutritional quality of C_3 grasses is also more greatly impacted by elevated atmospheric CO_2 than is that of C_4 grasses; C_3 grasses produce greater amounts of nonstructural carbohydrates and have greater declines in their nitrogen content than do C_4 grasses under elevated CO_2. Will C_3 grasses remain nutritionally superior to C_4 grasses under elevated CO_2 levels? We addressed this question by determining whether levels of protein in C_3 grasses decline to similar levels as in C_4 grasses, and whether total carbohydrate : protein ratios become similar in C_3 and C_4 grasses under elevated CO_2. In addition, we tested the hypothesis that, among the nonstructural carbohydrates in C_3 grasses, levels of fructan respond most strongly to elevated CO_2. Five C_3 and five C_4 grass species were grown from seed in outdoor open-top chambers at ambient (370 ppm) or elevated (740 ppm) CO_2 for 2 months. As expected, a significant increase in sugars, starch and fructan in the C_3 grasses under elevated CO_2 was associated with a significant reduction in their protein levels, while protein levels in most C_4 grasses were little affected by elevated CO_2. However, this differential response of the two types of grasses was insufficient to reduce protein in C_3 grasses to the levels in C_4 grasses. Although levels of fructan in the C_3 grasses tripled under elevated CO_2, the amounts produced remained relatively low, both in absolute terms and as a fraction of the total nonstructural carbohydrates in the C_3 grasses. We conclude that C_3 grasses will generally remain more nutritious than C_4 grasses at elevated CO_2 concentrations, having higher levels of protein, nonstructural carbohydrates, and water, but lower levels of fiber and toughness, and lower total carbohydrate : protein ratios than C_4 grasses.

Keywords: carbohydrate, C_3 grasses, C_4 grasses, elevated CO_2, nutrient, Poaceae, protein

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Introduction

Grasslands cover a greater fraction of Earth's land surface than any other plant type (Williams *et al*., 1968). In northern latitudes and higher elevations, grasses (Poaceae) with the C_3 photosynthetic pathway are more abundant, while grasses with the C_4 photosynthetic pathway are more abundant in hotter climates (Teeri & Stowe, 1976). C_3 and C_4 grasses are also often separated in time, as the terms 'cool-season' (C_3) and 'warm-season' (C_4) grasses suggest. The adaptation of C_4 grasses to high-temperature and high-light environments has produced anatomical and biochemical features that impact their nutritional quality for herbivores. The leaves of C_4 grasses have narrowly spaced veins, each of which is composed of a vascular bundle surrounded by concentric layers of bundle sheath cells and mesophyll cells (Laetsch, 1974). As a result of their greater photosynthetic efficiency, C_4 grasses frequently have lower levels of photosynthetic enzymes and lower protein levels overall compared with C_3 grasses. Caswell *et al*. (1973) hypothesized that C_4 grasses are nutritionally inferior to C_3 grasses based

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on (1) differences in nutrient levels between the two grass types and (2) the belief that the thick-walled bundle sheath cells of C_4 grasses are indigestible. Most studies comparing the nutritional quality of C_3 and C_4 grasses at ambient CO_2 levels have supported the first component of the above hypothesis: C_3 grasses commonly contain higher levels of nonstructural carbohydrates, protein, and water, and lower levels of fiber, silica and toughness than C_4 grasses (Wilson *et al*., 1983; Bernays & Hamai, 1987; Barbehenn & Bernays, 1992; Barbehenn, 1993; Van Soest, 1994). However, a recent comparison found no significant differences between protein or water levels in C_3 and C_4 grasses (Scheirs *et al*., 2001). Given that there is substantial variation between grasses within each photosynthetic pathway, one of the aims of our research was to expand the comparison of C_3 and C_4 grass nutritional quality to include a larger number of species.

CO_2 levels in Earth's atmosphere are widely expected to double during this century (e.g., Crane, 1985; Post *et al*., 1990), potentially changing the relative nutritional quality of C_3 and C_4 grasses. C_3 grasses commonly are more strongly affected by elevated CO_2 than are C_4 grasses. In previous studies on C_3 grasses, total nitrogen (N) in leaves decreased 21% and nonstructural carbohydrates increased 37% under elevated CO_2, while in C_4 grasses total N decreased only 6% and nonstructural carbohydrates increased only 11% (Poorter, 1993; Poorter *et al*., 1996; Wand *et al*., 1999). However, little work has been done on the effects of elevated CO_2 on specific foliar carbohydrates, fiber, water or toughness in grasses. Such changes in nutritional quality have potential importance for the many species of vertebrates and insects that feed on grasses as their main source of food. Nutritional qualities of grasses that affect the fitness of ruminants and small mammalian grass feeders include levels of protein and indigestible fiber (primarily lignin) (Van Soest, 1994; Young Owl & Batzli, 1998). Insect fitness is also affected by these factors, as well as by water, toughness and nonstructural carbohydrates (e.g., Scriber, 1977; Mattson, 1980; Bernays & Barbehenn, 1987; Goverde *et al*., 2002).

An unusual trait of C_3 grasses, not shared by C_4 grasses, is their synthesis of fructan (fructose polymers) as a storage carbohydrate (Meier & Reid, 1982). Fructan storage is increased by factors that reduce carbon sink strength (e.g., cold temperatures) and also by factors that increase carbon fixation (e.g., high light levels) (Pollock & Cairns, 1991). For example, fructan levels of over 30% dry weight are not uncommon in C_3 grasses grown at low temperatures (Chatterton *et al*., 1989). Fructan is efficiently digested by ruminants (Van Soest, 1994), and recent studies have found that grass fructan is also efficiently assimilated by leaf-chewing insects (Barbehenn *et al*., 2004a, b). However, little is known about the effects of elevated CO_2 levels on fructan synthesis, and one of the aims of this study was to examine whether fructan levels increase in C_3 grasses under elevated CO_2 to the extent that they do in cold temperatures.

Because of the greater direct and indirect effects of elevated CO_2 on C_3 grass protein levels (Drake & Gonzalez-Meler, 1996; Wand *et al*., 1999), and because protein is a limiting nutrient for herbivores (Mattson, 1980), we tested the hypothesis that the reduction in protein levels in C_3 grasses will eliminate a major difference in nutritional quality between C_3 and C_4 grasses under elevated CO_2. Although total N has commonly been measured in lieu of protein in elevated CO_2 studies, changes in total N can be potentially difficult to relate to changes in protein. Nonprotein nitrogenous compounds, such as chlorophyll and nitrate, comprise between 20% and 40% of total N, and can also change under elevated CO_2 (Cave *et al*., 1981; Van Soest, 1994; Poorter *et al*., 1997), and factors for converting N to protein are typically unknown and vary substantially between plant species (Milton & Dintzis, 1981). Therefore, protein was measured as total amino acids in whole-leaf hydrolysates. To compare the overall nutritional quality of C_3 and C_4 grasses under ambient and elevated atmospheric CO_2, foliar sugars, starch, fructan, water, fiber and toughness were measured, in addition to protein.

The grasses examined in this study represent a taxonomically diverse group of common native and introduced species (Table 1). Endophyte-free plants of each species were grown at ambient (370 ppm) or elevated (740 ppm) CO_2 concentrations in outdoor open-top chambers.

Materials and methods

Grasses

Grass species were selected for study based on their size (i.e., compatibility with the space available in open-top chambers), growth rates, ecological and economic importance and the availability of seed. *Buchloe dactyloides* (Nutt) Engelm., *Bouteloua curtipendula* (Michx.) Torr. and *Panicum virgatum* L. are important native rangeland and prairie grasses in the western and midwestern United States. *Lolium multiflorum* Lam., *Dactylis glomerata* L. and *Festuca arundicacea* Schreb. are widely planted pasture grasses from Europe. *Agropyron desertorum* (Fisch ex. Link) Schultes (Eurasian) and *Elymus canadensis* L. (native) are also important rangeland grasses in western North America. *Paspalum*

Table 1 Foliar carbohydrate and protein levels in C_3 and C_4 grasses grown at ambient (370 ppm) or elevated (740 ppm) atmospheric CO_2 concentrations†

		Carbohydrate and protein (% dry weight)					
Grass species	$[CO_2]$ (ppm)	Hexose‡	Sucrose	Starch	Fructan	TNC‡	Protein
C_3 grasses							
2000							
Agropyron desertorum	370	2.1 ± 0.3	5.5 ± 0.6	3.1 ± 0.4	2.2 ± 1.0	12.6 ± 1.9	25.4 ± 1.7
	740	2.8 ± 0.3	7.0 ± 1.0	5.2 ± 0.7*	6.6 ± 1.5*	21.1 ± 3.2*	23.5 ± 1.6
Festuca arundinacea	370	0.9 ± 0.1	5.5 ± 1.0	1.5 ± 0.2	0.2 ± 0.1	7.9 ± 1.2	26.6 ± 0.6
	740	1.7 ± 0.3*	9.3 ± 1.6	3.0 ± 0.6	1.9 ± 0.7*	15.7 ± 2.7*	24.6 ± 1.4
Lolium multiflorum	370	1.9 ± 0.2	8.3 ± 0.9	2.8 ± 0.5	3.1 ± 0.8	16.3 ± 1.7	25.8 ± 1.4
	740	2.2 ± 0.2	9.4 ± 1.1	5.0 ± 0.8*	6.2 ± 1.4*	22.0 ± 1.9*	20.5 ± 1.2*
2001							
Dactylis glomerata	370	2.5 ± 0.2	2.5 ± 0.2	3.0 ± 0.5	0.4 ± 0.1	8.5 ± 0.6	23.7 ± 1.1
	740	4.0 ± 0.4*	4.3 ± 0.4*	4.9 ± 0.5*	1.3 ± 0.2*	14.6 ± 0.9*	22.0 ± 1.3
Elymus canadensis	370	2.6 ± 0.2	2.8 ± 0.4	2.4 ± 0.3	0.4 ± 0.1	8.2 ± 0.8	29.0 ± 1.4
	740	3.8 ± 0.4*	5.3 ± 0.5*	3.2 ± 0.3	1.0 ± 0.1*	12.9 ± 0.6*	25.6 ± 1.0*
L. multiflorum	370	5.3 ± 0.5	6.1 ± 0.6	2.3 ± 0.4	1.0 ± 0.2	15.0 ± 1.2	27.9 ± 1.6
	740	7.2 ± 0.6*	9.4 ± 0.8*	4.1 ± 0.5*	1.9 ± 0.3*	23.1 ± 1.4*	22.8 ± 1.5*
C_4 grasses							
2000							
Bouteloua curtipendula	370	0.8 ± 0.1	2.3 ± 0.2	8.1 ± 1.4	nd	11.1 ± 1.6	18.2 ± 0.8
	740	0.7 ± 0.1	2.1 ± 0.3	9.6 ± 1.9	nd	11.4 ± 1.9	18.1 ± 1.4
Panicum virgatum	370	0.9 ± 0.2	2.8 ± 0.3	5.6 ± 1.3	nd	9.3 ± 1.5	23.7 ± 1.0
	740	1.6 ± 0.2*	3.5 ± 0.3	7.7 ± 1.2	nd	12.8 ± 1.5	23.7 ± 0.9
Paspalum notatum	370	0.6 ± 0.1	0.8 ± 0.2	5.0 ± 0.9	nd	5.4 ± 1.1	24.2 ± 0.9
	740	0.9 ± 0.1	0.8 ± 0.1	4.7 ± 0.6	nd	5.7 ± 0.7	22.3 ± 0.8
2001							
B. curtipendula	370	1.6 ± 0.2	3.2 ± 0.3	7.5 ± 0.9	nd	11.4 ± 1.3	20.2 ± 1.4
	740	2.1 ± 0.2	3.1 ± 0.2	8.0 ± 0.9	nd	13.0 ± 1.1	19.5 ± 0.9
Buchloe dactyloides	370	1.8 ± 0.2	1.6 ± 0.2	4.2 ± 0.6	nd	7.1 ± 0.8	20.1 ± 1.3
	740	2.1 ± 0.2	1.6 ± 0.2	4.5 ± 0.7	nd	7.2 ± 0.6	18.9 ± 1.1
Digitaria sanguinalis	370	2.7 ± 0.2	2.9 ± 0.3	8.6 ± 1.0	nd	12.8 ± 1.6	20.1 ± 1.2
	740	2.1 ± 0.3	2.8 ± 0.4	13.4 ± 1.1*	nd	18.3 ± 1.4*	16.7 ± 1.0*

*Significant differences ($P < 0.05$) between treatment means within each grass species.

†Data are presented as mean ± SE ($N = 10$–20/treatment). nd, not determined (not present in C_4 grasses).

‡Hexose is the sum of glucose and fructose levels. Total nonstructural carbohydrates (TNC) is the sum of hexose, sucrose, starch and fructan levels.

notatum Flügge is native to the southeastern US, and is commonly used as livestock forage and as a turfgrass. Finally, *Digitaria sanguinalis* (L.) Scop. is a weedy native species that is widely distributed in the US. All C_3 grasses are in the subfamily Pooideae (tribes Triticeae and Poeae). *B. curtipendula* (PCK subtype) and *B. dactyloides* (NAD-ME subtype) are in the Chloridoideae, while *D. sanguinalis* (NADP-ME subtype), *P. virgatum* (PCK subtype) and *P. notatum* (NAD-ME subtype) are in the Panicoideae. Seeds of *P. virgatum* (var. Dacotah), *A. desertorum* (var. Nordan) and *E. canadensis* (var. Mandan) were obtained from the USDA-ARS (Mandan, SD, USA). *L. multiflorum*, *F. arundinacea* (var. Chieftan II) and *D. glomerata* were obtained from the Michigan Department of Agriculture (East Lansing, MI, USA). *P. notatum* (var. Pensacola) and *B. curtipendula* were obtained from the USDA-NRCS (Americus, GA, USA and Knox City, TX, USA, respectively). *B. dactyloides* (var. Sharpshooter) was purchased from Sharp Brothers (Healy, KS, USA). *D. sanguinalis* (native var.) was purchased from Elstell Farm and Seeds (Ardmore, OK, USA).

Grasses were grown from seed from the beginning of June to early August 2000 and 2001, at the University of Michigan Biological Station (Pellston, MI, USA). This period of growth avoided potential decreases in plant nutritional quality due to post-flowering senescence, especially in C_3 grasses. Seeds and/or seedlings were confirmed to be endophyte free by staining them with aniline blue and examining them for hyphae under a

microscope (Latch *et al.*, 1987). Seeds were germinated in a greenhouse at ambient CO_2 and were transplanted to 3 L pots in outdoor open-top chambers, as described previously (Barbehenn *et al.*, 2004b). *L. multiflorum* was grown at a density of two plants per pot because of its rapid growth rate, while the other species were grown at a density of three to four plants per pot. Grasses were grown in a mixture of commercial topsoil and sand (80 : 20). Plants were well watered as needed, and fertilized weekly with 100 mL of Peters 20–20–20 fertilizer (0.25 mg mL^{-1}) (W. R. Grace, Fogelsville, PA, USA). Open-top chambers (0.5 m^3) were constructed of PVC tubing, covered on four sides and a frustrum with clear PVC film (Livingstone Coating Corp., Charlotte, NC, USA) (Drake *et al.*, 1989). Air containing either ambient (370 ppm) or elevated (740 ppm) CO_2 was blown into the base of each chamber, and was continuously monitored with an infrared gas analyzer (Li-cor 6200, Li-cor, Lincoln, NE, USA) and adjusted as necessary (Karowe *et al.*, 1997). Forty chambers were arranged into 20 blocks, with each block containing one ambient CO_2 and one adjacent elevated CO_2 chamber. Within blocks, CO_2 treatment was randomly assigned to chambers. Chambers were located in a fenced field site with unobstructed sunlight. Each chamber housed 16 randomly arranged pots, with at least one pot of each of six grass species in each chamber. In 2000, we grew *A. desertorum*, *F. arundinacea* and *L. multiflorum* (C_3 grasses) and *B. curtipendula*, *P. virgatum* and *P. notatum* (C_4 grasses). In 2001, we grew *D. glomerata*, *E. canadensis* and *L. multiflorum* (C_3 grasses) and *B. curtipendula*, *B. dactyloides* and *D. sanguinalis* (C_4 grasses). The growth of *L. multiflorum* and *B. curtipendula* in both years provided a measure of year-to-year variation in biomass, water, fiber and toughness, and possibly in the composition of the other nutrients measured.

Daytime temperatures inside chambers ranged from an average minimum of 16 °C to a maximum of 39 °C, with an overall average of 25 °C throughout the 2-month experiment (in 2001). These temperatures averaged 3% warmer than values recorded immediately adjacent to the chambers (at 20:00 hours) to 23% warmer (at 08:00 hours), with an overall mean increase in temperature of 16% during the day. Decreases in photosynthetically active wavelengths of light (400–700 nm) inside the chambers were assumed to be similar to those measured for the PVC film by the manufacturer (i.e., decreased 9–14%) (Drake *et al.*, 1989).

Grasses were harvested haphazardly within chambers (one plant/pot), alternating between ambient and elevated CO_2 chambers within blocks. The first fully expanded (collared) leaves from one or more plants (ca. 400 mg fresh weight) from each species in each chamber were collected for chemical analysis ($N = 20$/treatment). Leaf samples were quickly frozen (−80 °C) and freeze-dried (2000) or placed directly in a forced-draft oven at 70 °C (2001) in loosely arranged open envelopes to promote rapid drying. Following the removal of a leaf for nutrient analysis, the remaining aboveground biomass of a single sampled plant per species in each chamber was weighed fresh and oven-dried (70 °C). Biomass was measured as a general indicator of the overall response of the plants to growth under elevated CO_2.

Freeze drying and oven drying have both been found to preserve total nonstructural carbohydrates (TNC) in grass samples well (Smith, 1973). No significant decreases in TNC in *L. multiflorum* or *B. curtipendula* were observed from oven drying (assuming actual levels were similar across years), suggesting that oven drying prevented losses from respiration (Wolf & Carson, 1973). The potential interconversion of carbohydrates from invertase, amylase and/or fructanase activity during oven drying would have lead to lower levels of sucrose, starch and fructan, respectively. However, no significant decreases between drying methods (years) were observed for sucrose (which increased in *B. curtipendula*) or starch were observed (Table 1). We calculated that the decrease in fructan in *L. multiflorum* in 2001 would not have produced enough fructose to account for the increase in hexose (fructose) observed under elevated CO_2 in 2001. In addition, it seems unlikely that fructanase, but not invertase and amylase, would have been activated by drying. No decreases in protein levels in *L. multiflorum* and *B. curtipendula* resulted from oven drying, as might be expected if Maillard products were formed (Van Soest, 1994) (Table 1). Potential proteolytic activity during drying, as occurs during senescence, would be expected to increase amino acid levels (Thomas, 1978), but this would not change the total amino acid (protein) content measured with the ninhydrin method. Therefore, our results suggest that nutrient levels were preserved as well by drying leaf samples at 70 °C as they were by freeze drying.

Chemical and physical analysis

A subgroup of dried leaf samples from each species × CO_2 treatment combination was selected haphazardly for chemical analysis. Each of the samples within species was from a different chamber. Samples were ground to a homogeneous powder using a dental amalgamator (Foremost Dental MFG, Englewood, NJ, USA), and stored in screw-cap microcentrifuge tubes in the dark at room temperature, or 4°C for long-term storage. No loss of protein or carbohydrates was found in dried samples that were reanalyzed after a year

(unpublished data). Hexose sugars (glucose and fructose), sucrose and fructan were extracted in a sequence of 80%, 50% and 20% (v/v) ethanol, and extracts were pooled for each sample ($N = 10$–20/treatment) (Barbehenn *et al.*, 2004b). Extracted carbohydrates were measured by sequentially converting each to glucose enzymatically and measuring changes in total glucose with a glucose test kit (Sigma Chemical Co, St. Louis, MO, USA) (Hendrix, 1993). Fructan was measured separately in C_3 grasses after it was hydrolyzed with 1.0 M HCl, as described previously (Barbehenn *et al.*, 2004b). The difference in the amount of sugars (measured with the above procedure) between matched pairs of hydrolyzed and unhydrolyzed samples was defined as fructan. Starch was hydrolyzed with α-amylase and amyloglucosidase in the residue remaining after ethanol extraction, and was measured as glucose with a glucose test kit (Hendrix, 1993). All reaction mixtures were scaled to fit in 96-well microtiter plates (200 μL), using a single aliquot (100 μL) of glucose color reagent to measure all sugars in each sample. Absorbance measurements were made with a Bio-Rad Benchmark (Bio-Rad, Hercules, CA, USA) microplate reader at 490 nm. Protein was measured as total amino acids in 6 M HCl hydrolysates with ninhydrin ($N = 10$–20/treatment) (Barbehenn, 1995). No correction was made for the small overestimation of protein with this method. Neutral detergent fiber (cellulose, hemicellulose and lignin) was measured gravimetrically after solubilizing and removing non-fiber compounds ($N = 10$/treatment) (Van Soest *et al.*, 1991). TNC was defined as the sum of sugars, fructan and starch, while total carbohydrate also included structural carbohydrates (fiber).

Leaf toughness was measured on fresh leaves at the time of plant harvest. Toughness was measured with a custom-made penetrometer (Barbehenn *et al.*, 2004b), and was expressed as the mass (g) necessary to punch a 2 mm diameter hole through a leaf ($N = 20$/treatment). The middle of the first fully expanded leaf was tested, while avoiding thickened midribs. Leaves of *B. dactyloides* were too narrow to measure with our penetrometer.

Statistical analysis

Measures of the nutritional quality of five C_3 vs. five C_4 grasses were compared by split-plot nested ANOVA, with photosynthetic pathway, grass species (nested within photosynthetic pathway) and CO_2 concentration as main effects, and block and $CO_2 \times$ block as random effects (PROC MIXED; SAS, 2000). The full model included the photosynthetic pathway $\times$ CO_2 concentration interaction (to compare the effect of CO_2 on C_3 vs. C_4 grasses) and the CO_2 concentration $\times$ species interaction (to compare the effect of elevated CO_2 on individual species within each photosynthetic pathway). Small, but significant, differences in some measures were observed between years for *L. multiflorum* and *B. curtipendula* (e.g., hexose in both species, sucrose in *B. curtipendula*, and fructan in *L. multiflorum*) (Table 1), precluding the pooling of data across years. Therefore, data from 2001 were used for these two species in the overall comparison because of the larger sample sizes available during this year. The normality of residuals was tested with PROC UNIVARIATE (SAS, 2000). Where necessary (hexose, toughness and biomass), log transformations were used to normalize the residuals. If residuals could not be normalized (sucrose, fructan and water), the significance of main effects was determined with Kruskal–Wallis tests (Wilkinson, 2000). Kruskal–Wallis tests were also used to compare the ratios of total carbohydrates : protein among the four photosynthetic pathway $\times$ CO_2 concentration groups. Pairwise differences within grass species between CO_2 treatments were examined by differences of least-squares means (SAS, 2000), or by Kruskal–Wallis tests when data could not be transformed. Toughness in C_3 and C_4 grasses was compared using ANCOVA, with fiber as the covariate, to compare the slopes of the regression lines (Fig. 3) (SAS, 2000).

Results

C_3 grasses contained higher levels of hexose sugars (glucose and fructose) and sucrose at ambient CO_2 than did C_4 grasses (Table 1, Fig. 1a, b). By contrast, C_4 grasses contained higher levels of starch at ambient CO_2 than did C_3 grasses. Thus, the levels of TNC (sugars, starch and fructan) were not significantly different between the C_3 and C_4 grasses at ambient CO_2 (Table 1, Fig. 1e).

Levels of each of the nonstructural carbohydrates in the C_3 grasses increased significantly under elevated CO_2 (Tables 1 and 3, Fig. 1a–d). Hexose, sucrose, starch and fructan increased 48%, 63%, 63% and 202%, respectively. Storage carbohydrates (starch and fructan) increased most consistently in the C_3 grasses, although in two species in which starch did not increase significantly (*F. arundinacea* and *E. canadensis*) sugars increased instead. Although levels of fructan tripled on average in C_3 grasses under elevated CO_2, their absolute levels remained relatively low, constituting no more than 7% dry weight (Table 1, Fig. 1c).

Nonstructural carbohydrates in the C_4 grasses increased to a smaller extent under elevated CO_2 than they did in the C_3 grasses, producing significant photosynthetic pathway $\times$ CO_2 concentration interactions

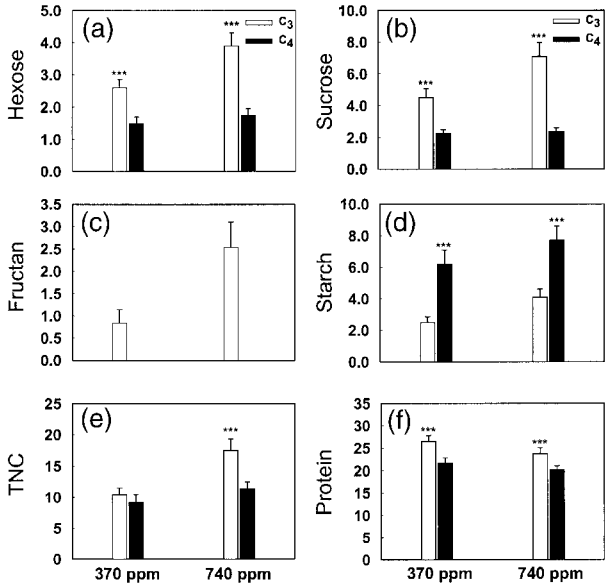

Fig. 1 Percent dry weights of nonstructural carbohydrates and protein in five C_3 and five C_4 grasses grown at ambient (370 ppm) or elevated (740 ppm) atmospheric CO_2 concentrations. Overall means (± SE) are plotted. Significant differences between C_3 and C_4 grasses at each CO_2 level are indicated by asterisks (***$P<0.001$). TNC, total nonstructural carbohydrates. Analyses used *Lolium multiflorum* and *Bouteloua curtipendula* data from 2001.

(Table 3). Under elevated CO_2, only starch increased significantly ($P = 0.004$) in the C_4 grasses, although increases in hexose levels were nearly significant ($P = 0.052$). Together, these changes resulted in a 22% increase in TNC in the C_4 grasses ($P = 0.037$). However, most of this increase occurred in *D. sanguinalis*, in which TNC increased 43%. Overall, under elevated CO_2, TNC levels became 53% higher in C_3 than in C_4 grasses (Table 1, Fig. 1e).

C_3 grasses contained 22% more protein at ambient CO_2 than did C_4 grasses (Table 1, Fig. 1f). Under elevated CO_2, protein levels in the C_3 species decreased by an average of 12% ($P<0.001$) (Table 1, Fig. 1f). By comparison, elevated CO_2 caused only a 6% decrease in protein in the C_4 species ($P = 0.114$), although the ANOVA did not reveal a significant photosynthetic pathway × CO_2 concentration interaction (Table 3). Between-species variation in CO_2 treatment effects were most notable in the C_4 grass *D. sanguinalis*, in which protein levels decreased 17%. Overall, under elevated CO_2, C_3 grasses remained significantly more protein rich than C_4 grasses, containing 19% more protein (Table 1, Fig. 1f). When protein levels were compared on a TNC-free basis, no significant differences were found between C_3 grasses at ambient or elevated CO_2, or between C_4 grasses at ambient or elevated CO_2. However, C_3 grasses still contained significantly higher levels of protein than C_4 grasses on a TNC-free basis at both CO_2 concentrations ($P<0.001$ in each case).

C_3 grasses contained on average 6% more water than C_4 grasses at ambient CO_2, but C_3 species were also more strongly affected by elevated CO_2 (Table 2, Fig. 2a). The water content of C_3 grasses declined significantly under elevated CO_2, but did not change significantly in C_4 grasses. Although non-normality precluded the use of ANOVA, these results suggest that there was a significant photosynthetic pathway × CO_2 concentration interaction. Nevertheless, under elevated CO_2, C_3 grasses still contained 3% more water than did C_4 grasses.

As expected, fiber levels were 36% higher in C_4 than C_3 grasses at ambient CO_2 (Table 2, Fig. 2b). Although there was no overall CO_2 effect on fiber levels, fiber content increased significantly under elevated CO_2 in two C_3 species (*D. glomerata* and *L. multiflorum*) and one C_4 species (*D. sanguinalis*) (Table 2). Under elevated CO_2, fiber remained at significantly higher levels in C_4 than in C_3 grasses (Table 2, Fig. 2b).

Leaf toughness, like fiber, was significantly greater in C_4 than in C_3 grasses at ambient CO_2 (Table 2, Fig. 2c). Under elevated CO_2, C_3 grasses became 11% tougher than at ambient CO_2 ($P<0.001$), while the toughness of C_4 grasses was not significantly affected. However, this differential response to elevated CO_2 by C_3 and C_4 grasses was not sufficiently large to produce a significant photosynthetic pathway × CO_2 interaction (Table 3). Overall, under elevated CO_2, C_4 species remained 16% tougher than C_3 species. Leaf toughness was significantly correlated with fiber content for both C_3 ($P = 0.007$) and C_4 grasses ($P<0.001$) (Fig. 3). The steeper slope of the regression line for C_4 grasses ($P = 0.043$) indicates that a given increase in fiber produces a greater increase in toughness in C_4 grasses than in C_3 grasses.

Aboveground biomass was significantly greater for C_4 than for C_3 grasses under ambient CO_2 (Table 2, Fig. 2d). The aboveground biomass of C_3 grasses increased by 47% under elevated CO_2 ($P<0.001$), but that of C_4 grasses was not significantly affected, producing a significant photosynthetic pathway × CO_2 interaction (Table 3). Because of this differential response to elevated CO_2 by the two types of grasses, biomass did not differ significantly between the C_3 and C_4 grasses under elevated CO_2, though it varied considerably among species.

C_4 grasses had significantly higher total carbohydrate (TNC + fiber) : protein ratios than did C_3 grasses at ambient CO_2 (Fig. 4). Under elevated CO_2 the carbohydrate : protein ratio increased by 31% in C_3 grasses

Table 2 Foliar nutritional quality and aboveground biomass of C_3 and C_4 grasses grown at ambient (370 ppm) or elevated (740 ppm) atmospheric CO_2 concentrations†

		Nutritional quality and biomass			
Grass species	[CO_2] (ppm)	Water (%FW)	Fiber (%DW)	Toughness (g)	Biomass (g)
C_3 grasses					
2000					
Agropyron desertorum	370	74.5 ± 1.2	36.8 ± 1.1	460 ± 24	0.62 ± 0.11
	740	71.2 ± 1.3*	34.7 ± 1.1	471 ± 19	0.84 ± 0.09*
Festuca arundinacea	370	81.7 ± 0.4	38.4 ± 0.7	428 ± 16	0.63 ± 0.09
	740	80.5 ± 0.5	35.6 ± 1.2	476 ± 15*	0.88 ± 0.11*
Lolium multiflorum	370	81.0 ± 0.5	34.6 ± 1.3	399 ± 18	1.57 ± 0.15
	740	78.7 ± 0.8*	34.2 ± 1.9	464 ± 17*	1.74 ± 0.17
2001					
Dactylis glomerata	370	81.0 ± 0.6	37.0 ± 0.6	416 ± 30	0.38 ± 0.04
	740	80.1 ± 0.5	42.1 ± 1.1*	489 ± 28*	0.51 ± 0.05
Elymus canadensis	370	76.5 ± 0.4	38.1 ± 0.4	428 ± 22	0.29 ± 0.04
	740	75.0 ± 0.5*	39.9 ± 0.6	460 ± 22	0.47 ± 0.05*
L. multiflorum	370	81.7 ± 0.9	29.1 ± 0.7	306 ± 21	1.10 ± 0.14
	740	78.5 ± 1.2*	32.1 ± 1.4*	369 ± 28*	1.63 ± 0.16*
C_4 grasses					
2000					
Bouteloua curtipendula	370	67.0 ± 0.2	55.5 ± 1.0	669 ± 38	0.64 ± 0.08
	740	66.4 ± 1.1	55.8 ± 0.8	751 ± 56	0.72 ± 0.08
Panicum virgatum	370	78.4 ± 0.4	47.6 ± 1.2	575 ± 22	0.97 ± 0.14
	740	78.9 ± 0.4	44.5 ± 1.5	615 ± 21	0.83 ± 0.10
Paspalum notatum	370	82.3 ± 0.3	44.6 ± 0.8	387 ± 18	0.33 ± 0.06
	740	82.8 ± 0.8	45.2 ± 1.0	401 ± 15	0.40 ± 0.08
2001					
B. curtipendula	370	70.4 ± 0.8	55.3 ± 1.3	706 ± 33	0.47 ± 0.04
	740	69.9 ± 1.0	57.0 ± 1.8	718 ± 28	0.53 ± 0.04
Buchloe dactyloides	370	69.9 ± 0.6	58.0 ± 0.9	nd	0.40 ± 0.05
	740	70.9 ± 0.9	57.9 ± 1.1	nd	0.43 ± 0.07
Digitaria sanguinalis	370	73.9 ± 2.2	38.4 ± 1.0	309 ± 17	2.58 ± 0.39
	740	76.2 ± 0.7	42.3 ± 1.4*	355 ± 19	2.35 ± 0.19

†Data are presented as mean ± SE (N = 10–20/treatment). nd, not determined due to narrow leaf blades.
*Significant differences ($P < 0.05$) between treatment means within each grass species.

Table 3 P-values from split-plot nested ANOVA testing differences between five C_3 and five C_4 species grown at ambient (370 ppm) or elevated (740 ppm) atmospheric CO_2 concentrations†

Effect	Hexose‡	Sucrose	Starch	Fructan	TNC‡	Protein	Water	Fiber	Toughness	Biomass
Photosynthetic path	<0.001	<0.001	<0.001	nd	<0.001	<0.001	<0.001	<0.001	<0.001	ns
Species (path)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
CO_2 level	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	ns	ns	0.002	<0.001
Path × [CO_2]	0.009	nd	ns	nd	<0.001	ns	nd	ns	ns	0.005
Species × [CO_2]	ns	nd	0.014	nd	ns	ns	nd	<0.001	ns	ns

†Grasses were grown at ambient (370 ppm) and elevated (740 ppm) atmospheric CO_2 levels. nd, not determined; ns, not significant (P-values < 0.1 are reported). Sucrose, fructan and water were analyzed with Kruskal–Wallis tests. Analyses used *Lolium multiflorum* and *Bouteloua curtipendula* data from 2001.
‡Hexose is the sum of glucose and fructose levels. Total nonstructural carbohydrates (TNC) is the sum of hexose, sucrose, starch and fructan levels.

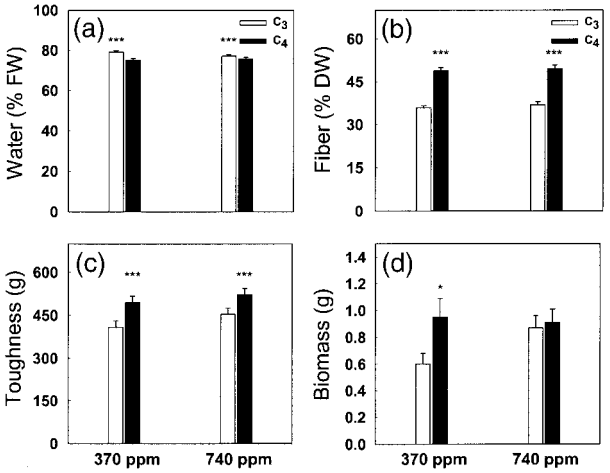

Fig. 2 Foliar nutritional quality and aboveground biomass of five C_3 and five C_4 grasses grown at ambient (370 ppm) or elevated (740 ppm) atmospheric CO_2 concentrations. Overall means (± SE) are plotted. Significant differences between C_3 and C_4 grasses are indicated by asterisks (***$P<0.001$, *$P<0.05$). Analyses used *Lolium multiflorum* and *Bouteloua curtipendula* data from 2001.

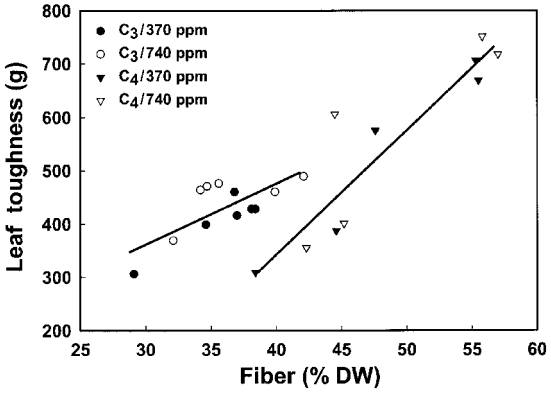

Fig. 3 The association between C_3 and C_4 grass fiber content and leaf toughness. Ambient and elevated CO_2 data are plotted for each species except *Buchloe dactyloides*. $R^2 = 0.537$ for C_3 species, $R^2 = 0.840$ for C_4 species.

($P<0.001$), but only by 14% in C_4 grasses ($P = 0.060$). There was substantial variation among the C_4 grasses, with total carbohydrate : protein ratios in species such as *B. curtipendula* and *P. notatum* relatively unaffected by elevated CO_2, while in *D. sanguinalis* the changes were as large as those in C_3 grasses. Overall, under elevated CO_2 total carbohydrate : protein ratios remained 33% higher in C_4 than in C_3 grasses.

Discussion

The results of this study support the hypothesis that C_3 grasses are nutritionally superior to C_4 grasses; levels of

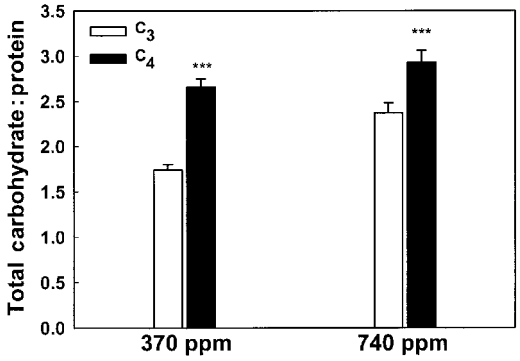

Fig. 4 Ratios of total carbohydrates (TNC + fiber) to protein in C_3 and C_4 grasses grown at ambient or elevated atmospheric CO_2 concentrations. Overall means (± SE) are plotted. Significant differences between C_3 and C_4 grasses are indicated by asterisks (***$P<0.001$). Analyses used *Lolium multiflorum* and *Bouteloua curtipendula* data from 2001.

nutrients (e.g., sugars, protein and water) are higher in C_3 grasses than in C_4 grasses on average at ambient CO_2. As expected, elevated CO_2 (740 ppm) significantly reduces protein levels in C_3 species, consistent with their dilution by nonstructural carbohydrates, but has little effect on most C_4 grasses. Despite greater decreases in protein levels in C_3 than in C_4 grasses under elevated CO_2, protein levels remain significantly higher in C_3 grasses at elevated CO_2. An additional indicator of plant quality, the ratio of total carbohydrates to protein also remains lower (better) in C_3 grasses at elevated CO_2. Therefore, the hypothesis that C_3 and C_4 grasses will become nutritionally equivalent with respect to protein under elevated CO_2 is not supported.

Our initial conclusion differs from a recent study testing the 'C_3–C_4 hypothesis' (Scheirs *et al.*, 2001), in which no difference in protein levels was found between C_3 and C_4 grasses. There is little overlap in the grass species examined in the two studies, and the large variation between some species within each photosynthetic pathway might explain the differing conclusions. However, there are methodological differences between the studies that also could produce different conclusions regarding protein levels in C_3 and C_4 grasses. Only a small fraction of the total protein in grass foliage is extracted in a pH 7.6 buffer (Scheirs *et al.*, 2001). For example, in the two species examined in both studies (*D. glomerata* and *D. sanguinalis*) the sums of protein plus free amino acids were only 1.7% and 3.2% dry weight in each species when measured in buffered extracts, but were 23.7% and 20.1% dry weight in this study. We suggest that differences between our conclusions and those of some previous studies might be due to the use of a method of protein analysis that

measures total protein in leaf samples, and increased sample sizes in this study (Barbehenn & Bernays, 1992; Scheirs *et al*., 2001). In addition, we note that a large fraction of the total protein in grasses is utilized by leaf-chewing herbivores; *Pseudaletia unipuncta*, a grass-specialist caterpillar, assimilates 59% of the total protein from *D. glomerata* (C_3) and 62% from *D. sanguinalis* (C_4) (unpublished data).

Our work focused on nutrient levels in C_3 and C_4 grasses and did not address the putative differences in nutrient digestibility between these two types of grasses (Caswell *et al*., 1973). Although recent evidence shows that certain ruminants digest N-containing compounds more efficiently from C_3 than C_4 grasses (Sponheimer *et al*., 2003), among grass-chewing insects the available evidence shows that the bundle sheath cell anatomy of C_4 grasses either does not impede the digestion of protein or reduces digestion by a relatively small amount (Barbehenn & Bernays, 1992; Barbehenn *et al*., 2004a, b).

Contrary to our expectation, fructan is a minor component of the nonstructural carbohydrates that are increased by elevated CO_2 in C_3 grasses. On average, at elevated CO_2 fructan comprises 14% of the nonstructural carbohydrates measured in the C_3 grasses in this study (Fig. 1). Previous studies have also shown moderate increases in fructan in *Triticum aestivum* (Nie *et al*., 1995) at elevated CO_2 levels. Therefore, unless other environmental factors, such as cold temperatures, are coupled with elevated CO_2 levels, C_3 grasses would not be expected to produce large quantities of fructan (e.g., 30% dry weight) (Chatterton *et al*., 1989).

Additional changes in leaf composition under elevated CO_2 that could reduce the nutritional quality of C_3 grasses include an increase in toughness and a decrease in water content (Fig. 2). Such changes are generally associated with decreased performance by herbivorous insects (Scriber, 1977). Our results show a significant positive relationship between levels of fiber and toughness, consistent with previous work (Choong *et al*., 1992; Wright & Illius, 1995). Although fiber increased in some grasses under elevated CO_2, it is clear that increased toughness cannot be explained simply by increased fiber in several of the species that we examined. Another potentially important factor, specific leaf mass (SLM; $g\,m^{-2}$) also commonly increases in C_3 grasses under elevated CO_2 (Wand *et al*., 1999). Any of a number of factors that can increase SLM could potentially increase leaf toughness, e.g., leaf thickness (Lincoln *et al*., 1993), fiber deposition in secondary cell walls (MacAdam, 2002), and possibly nonstructural carbohydrates (Volk *et al*., 2000). The few previous examinations of changes in fiber and cell wall structure under elevated CO_2 have shown mixed results (Akin *et al*., 1994, 1995; Owensby *et al*., 1996; Hartley *et al*., 2000; Watling *et al*., 2000). The greater rate of increase in toughness as fiber increases in C_4 grasses (Fig. 3) suggests that there may be differences in the fiber compositions of the two grass types, such as a greater fraction of lignin in C_4 grasses. Further work is needed to determine what group of factors, including leaf thickness and chemical composition, are primarily responsible for changes in leaf toughness under elevated CO_2.

It is noteworthy that the C_3 and C_4 grasses with the strongest responses to elevated CO_2 in terms of carbohydrate composition, *L. multiflorum* and *D. sanguinalis*, are the only annuals in our study. Previous studies have suggested that annual plants may be more responsive to elevated CO_2 levels than perennial plants (Zangerl & Bazzaz, 1984; Smith *et al*., 1987). NADP-ME C_4 grasses, which include *D. sanguinalis*, can have stronger responses to elevated CO_2 than other subtypes, but results have been mixed in the small number of species that have been examined (LeCain & Morgan, 1998; Kellog *et al*., 1999). A larger comparison of the effects of elevated CO_2 on the carbohydrate composition of C_4 annual and perennial NADP-ME and NAD-ME species is needed to examine these patterns further.

Some caution is needed in generalizing from our results to long-term effects in the field. For example, further consideration of the potential effects of endophytic and mycorrhizal fungi on changes in foliar nutritional quality is warranted (e.g., Monz *et al*., 1994, Marks & Lincoln, 1996). We are aware of few studies on the effects of these symbionts on grass nutritional quality and none regarding whether they may have differential effects on foliar nutrients in C_3 and C_4 grasses under elevated CO_2 (Marks & Lincoln, 1996; Goverde *et al*., 2000). In addition, the general effects of elevated CO_2 on total carbohydrate : protein ratios observed in our study have been observed widely in field-grown and potted plant experiments (as C : N ratios) (Poorter, 1993; Poorter *et al*., 1996; Wand *et al*., 1999), suggesting that fungal associations do not alter the effects of elevated CO_2 on grass nutritional quality substantially.

In previous studies with a single pair of C_3 and C_4 grasses, it was concluded that the higher performance of caterpillars and grasshoppers on a C_3 grass was primarily due to higher nutritional quality in *L. multiflorum* (C_3) than in *B. curtipendula* (C_4) at ambient and elevated CO_2 levels (Barbehenn *et al*., 2004a, b). Based on the results of this study on a larger number of C_3 and C_4 grasses under ambient and elevated CO_2 conditions, we conclude that, on average, other C_3 grasses will also continue to be more nutritious than C_4 grasses in future

atmospheric conditions. This conclusion is consistent with the common belief that plants with higher protein levels and lower C : N ratios are more nutritious for both insect (Lincoln *et al.*, 1993; Lindroth, 1996) and vertebrate herbivores (Ehleringer *et al.*, 2002).

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