# C$_3$ grasses have higher nutritional quality than C$_4$ grasses under ambient and elevated atmospheric CO$_2$

RAYMOND V. BARBEHENN*, ZHONG CHEN†, DAVID N. KAROWE‡ and ANGELA SPICKARD§

*Departments of Molecular, Cellular and Developmental Biology and Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA, †School of Forestry, Northern Arizona University, Flagstaff, AZ 86011-5018, USA, ‡Western Michigan University, Department of Biological Sciences, Kalamazoo, MI 49008-5410, USA, §Environmental Protection Agency, Ann Arbor, MI 48105, USA*

### 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

Correspondence: Raymond V. Barbehenn, fax + 1 734 647 0884, e-mail: rvb@umich.edu

'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

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[†]

| Grass species | [CO$_2$] (ppm) | Carbohydrate and protein (% dry weight) | | | | | Protein |
| | | Hexose[‡] | Sucrose | Starch | Fructan | TNC[‡] | |
|---|---|---|---|---|---|---|---|
| **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 ($N = 20$/treat-

ment). 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$_2$ × block as random effects (PROC MIXED; SAS, 2000). The full model included the photosynthetic pathway × CO$_2$ concentration interaction (to compare the effect of CO$_2$ on C$_3$ vs.

C$_4$ grasses) and the CO$_2$ concentration × 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 × 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 × CO$_2$ concentration interactions
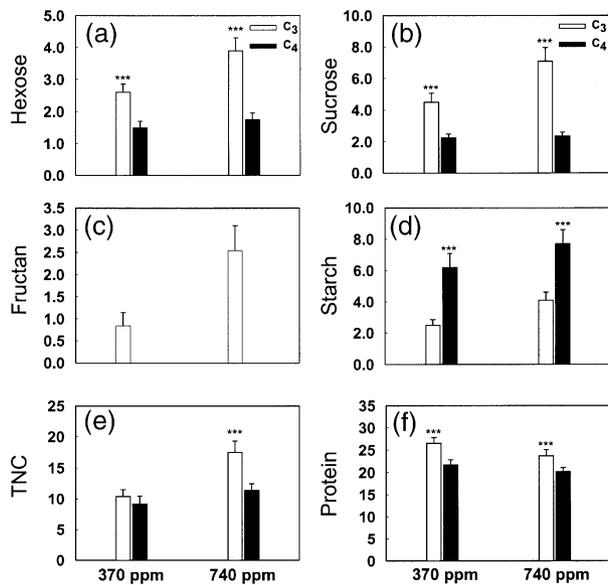
**Fig. 1** Percent dry weights of nonstructural carbohydrates and protein in five $C_3$ and five $C_4$ grasses grown at ambient (370 ppm) or elevated (740 ppm) atmospheric $CO_2$ concentrations. Overall means ( $\pm$ SE) are plotted. Significant differences between $C_3$ and $C_4$ grasses at each $CO_2$ 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 $\times CO_2$ 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 $\times CO_2$ 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 $\times CO_2$ 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 $\times CO_2$ 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[†]

| Grass species | [CO$_2$] (ppm) | Nutritional quality and biomass | | | |
| --- | --- | --- | --- | --- | --- |
| | | 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.
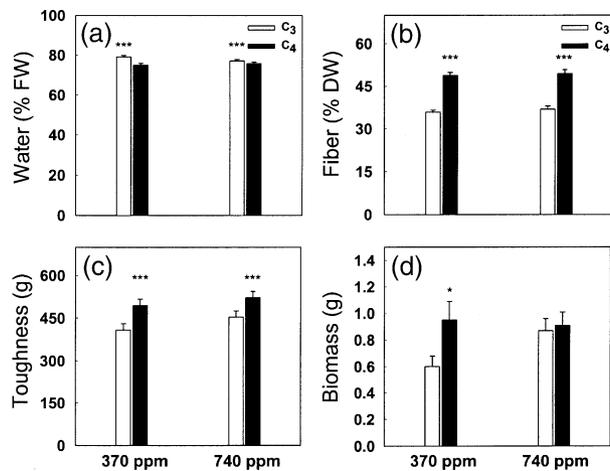
**Fig. 2** Foliar nutritional quality and aboveground biomass of five $C_3$ and five $C_4$ grasses grown at ambient (370 ppm) or elevated (740 ppm) atmospheric $CO_2$ concentrations. Overall means ( $\pm$ SE) are plotted. Significant differences between $C_3$ and $C_4$ grasses are indicated by asterisks (***$P < 0.001$, *$P < 0.05$). Analyses used *Lolium multiflorum* and *Bouteloua curtipendula* data from 2001.
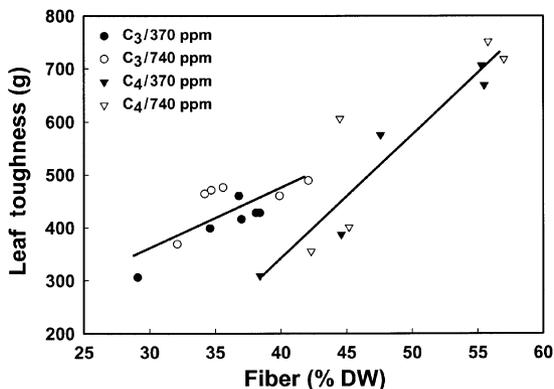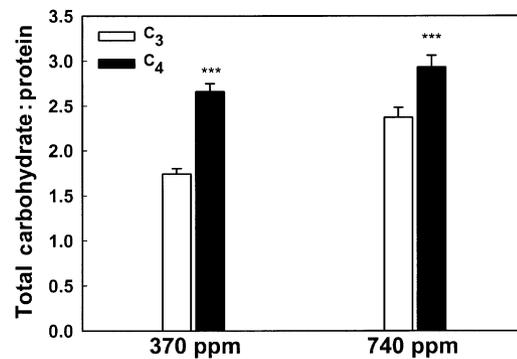


**Fig. 3** The association between $C_3$ and $C_4$ grass fiber content and leaf toughness. Ambient and elevated $CO_2$ data are plotted for each species except *Buchloe dactyloides*. $R^2 = 0.537$ for $C_3$ species, $R^2 = 0.840$ for $C_4$ species.



**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 ( $\pm$ 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.

($P < 0.001$), 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 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 sub-types, 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).

## Acknowledgements

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