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PLANT ANIMAL INTERACTIONS

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Effects of elevated atmospheric CO_2 on the nutritional ecology of C_3 and C_4 grass-feeding caterpillars

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Abstract It is plausible that the nutritional quality of C_3 plants will decline more under elevated atmospheric CO_2 than will the nutritional quality of C_4 plants, causing herbivorous insects to increase their feeding on C_3 plants relative to C_4 plants. We tested this hypothesis with a C_3 and C_4 grass and two caterpillar species with different diet breadths. *Lolium multiflorum* (C_3) and *Bouteloua curtipendula* (C_4) were grown in outdoor open top chambers at ambient (370 ppm) or elevated (740 ppm) CO_2. Bioassays compared the performance and digestive efficiencies of *Pseudaletia unipuncta* (a grass-specialist noctuid) and *Spodoptera frugiperda* (a generalist noctuid). As expected, the nutritional quality of *L. multiflorum* changed to a greater extent than did that of *B. curtipendula* when grown in elevated CO_2; levels of protein (considered growth limiting) declined in the C_3 grass, while levels of carbohydrates (sugar, starch and fructan) increased. However, neither insect species increased its feeding rate on the C_3 grass to compensate for its lower nutritional quality when grown in an elevated CO_2 atmosphere. Consumption rates of *P. unipuncta* and *S. frugiperda* were higher on the C_3 grass than the C_4 grass, the opposite of the result expected for a compensatory response to the lower nutritional quality of the C_4 grass. Although our results do not support the hypothesis that grass-specialist insects compensate for lower nutritional quality by increasing their consumption rates more than do generalist insects, the performance of the specialist was greater than that of the generalist on each grass species and at both CO_2 levels. Mechanisms other than compensatory feeding, such as increased nutrient assimilation efficiency, appear to determine the relative performance of these herbivores. Our results also provide further evidence against the hypothesis that C_4 grasses would be avoided by insect herbivores because a large fraction of their nutrients is unavailable to herbivores. Instead, our results are consistent with the hypothesis that C_4 grasses are poorer host plants primarily because of their lower nutrient levels, higher fiber levels, and greater toughness.

Keywords Lepidoptera · Poaceae · Nutrients · Global change · Herbivore

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Introduction

A large amount of work has been done to predict the effects of rising levels of atmospheric CO_2 on C_3 plants (e.g., Poorter 1993; Poorter et al. 1996, 1997). During this century a doubling of CO_2 levels is expected to increase plant biomass and nonstructural carbohydrates, but decrease protein (nitrogen) concentrations in leaves (Poorter et al. 1996; Bezemer and Jones 1998). The nutritional consequences of these changes have been examined with over 40 species of plant-feeding insects on over 40 plant species (Bezemer and Jones 1998). Leaf-chewing insects (ca. 20 species) were commonly able to maintain similar growth rates and final weights on plants grown in ambient or elevated CO_2. Insect behavioral responses varied between species, and showed either increased consumption rates or no compensatory feeding at all (Lincoln et al. 1993; Bezemer and Jones 1998). These studies focused on forb- and tree leaf-feeding caterpillars, while few studies have examined grass-feeding insects (Marks and Lincoln 1996; Goverde et al. 2002).

The impact of elevated atmospheric CO_2 levels on the nutritional ecology of grass leaf-chewing insects is a useful addition to the body of information on global

change for at least three reasons: (1) grasses cover over 40% of Earth's land surface area, a greater proportion than any other vegetation type (Williams et al. 1968); (2) insects are major herbivores in some grassland ecosystems (Hewitt and Onsager 1983; Tscharntke and Greiler 1995; Belovsky and Slade 2000); and (3) grasses are nutritionally and chemically distinct from dicots. C_3 (cool season) grasses are believed to be nutritionally superior to C_4 (warm season) grasses, both in terms of nutrient concentration and digestibility. C_4 grasses have, on average, less protein and water, but more fiber and silica, and greater toughness than C_3 grasses (Caswell et al. 1973; Boutton et al. 1978; Van Soest 1982; Landa and Rabinowitz 1983; Bernays and Hamai 1987; Barbehenn 1993; but see Scheirs et al. 2001). In addition, the bundle sheath cells of C_4 grasses contain a large fraction of foliar nutrients and are believed to render them indigestible, at least to insects that rely on tissue maceration to extract nutrients (Caswell et al. 1973; Caswell and Reed 1975, 1976; Barbehenn 1992). C_3 grasses are also more responsive to elevated CO_2 than are C_4 grasses; in C_3 grasses, aboveground biomass and nonstructural carbohydrates increased 38% and 37%, respectively, while in C_4 grasses they increased only 12% and 11% (Wand et al. 1999). Similarly, nitrogen content declined 21% in C_3 grasses, but only 6% in C_4 grasses (Wand et al. 1999). Unlike most other C_3 and C_4 plants, C_3 grasses produce fructan (a fructose polymer) as a storage carbohydrate, and it is unknown whether insects can utilize these polysaccharides. Finally, mature grasses are commonly defended by their toughness and low nutrient levels, rather than by high levels of allelochemicals (Bernays and Chapman 1976, 1977; Bernays and Barbehenn 1987; Goverde et al. 2002; but see Vicari and Bazely 1993). Endophytic fungi provide an antiherbivore defense in some species (Clay et al. 1985; Clay 1988; Saikkonen et al. 1998, 1999). Levels of this defense appear to be unaffected by elevated atmospheric CO_2 (Marks and Lincoln 1996), whereas elevated CO_2 causes marked changes in the levels of defensive chemicals in some dicots (Johnson and Lincoln 1991; Traw et al. 1996; Lindroth et al. 1995; Karowe et al. 1997; Bezemer and Jones 1998). As a result of the greater decline in C_3 plant nutritional quality, it has been predicted that insect herbivores will increase their feeding damage on C_3 plants to a greater extent than on C_4 plants (Lincoln et al. 1984, 1986; Lambers 1993). Although there is some acceptance of this idea (e.g., Coviella and Trumble 1999), to our knowledge this hypothesis has not been tested.

Another potential source of variation in the responses of insect herbivores to grasses at elevated CO_2 is their degree of feeding specialization (Scriber and Feeny 1979). Grass-specialist insects have morphological adaptations for chewing tough leaves (Isely 1944; Bernays and Hamai 1987; Bernays 1991), and such traits might allow them to perform better than generalist insects on grasses. To the extent that compensatory feeding allows grass-feeding insects to perform well on grasses with lower nutritional quality, grass-specialists might also be better able to compensate for changes in grasses grown in elevated CO_2 than generalists. We compared the performance of a grass specialist caterpillar (*Pseudaletia unipuncta*, Noctuidae) and a generalist caterpillar (*Spodoptera frugiperda*, Noctuidae) on two grass species grown at two CO_2 levels. *P. unipuncta* feeds primarily on grasses and some sedges, and has the smooth-edged mandibles that are characteristic of graminivorous Lepidoptera (Guppy 1961; Barbehenn 1992). *S. frugiperda* feeds on grasses and dicots (Crowell 1943; Chang et al. 1987), and has mandibles with incisor teeth.

Previous studies on the effects of elevated CO_2 on plant-feeding insects have commonly measured dry mass digestive efficiencies, but usually have not examined the digestive efficiencies of specific nutrients. Thus, it is commonly assumed that nutrients are utilized in proportion to their concentrations in leaves. This assumption is especially important to verify in grass-feeding insects for two reasons: (1) some carbohydrates, such as fructan and starch, may not be utilized, or utilized in proportion to their foliar concentrations; and (2) nutrients in C_4 grasses are potentially less available to herbivores than are those in C_3 grasses because they are contained in putatively uncrushable bundle sheath cells (Caswell et al. 1973). We measured the effects of elevated CO_2 on the nutritional quality of *Lolium multiflorum* (C_3) and *Bouteloua curtipendula* (C_4) (i.e., protein, sugar, starch, fructan, water, fiber, and toughness), and measured the assimilation efficiency of protein, sugar, starch, and fructan in *P. unipuncta* and *S. frugiperda* for each of four plant species × CO_2 treatment combinations. Protein is commonly regarded as the most limiting macronutrient for herbivores (Mattson 1980), although other factors, such as water, toughness, and carbohydrates, can also be important components of plant quality for caterpillars (Slansky and Scriber 1985; Martin and Van't Hof 1988; Goverde et al. 2002; Haukioja 2003).

Our work tested four main hypotheses: (1) the nutritional quality of C_3 grasses declines more than that of C_4 grasses under elevated CO_2; (2) grass-specialist caterpillars perform better and compensate more effectively than generalist caterpillars on grasses with lower nutrient content; (3) insect performance is greater on C_3 than C_4 grasses as a result of lower C_4 grass nutritional quality; and (4) the level of herbivory on C_3 grasses will increase relative to that on C_4 grasses in an elevated CO_2 atmosphere.

Materials and methods

Grasses

Lolium multiflorum Lam. (Italian ryegrass) is a common introduced C_3 pasture grass, and *Bouteloua curtipendula* (Michx.) Torr. (sideoats grama) is a native C_4 rangeland grass. They were chosen for study based on their ecological and economic importance, the size of their leaves relative to the growth chambers and insect containers, their growth rates, and the availability of seed. *L. multiflorum* and *B. curtipendula* were grown from June to early August 2000 at the University of Michigan Biological Station,

Pellston, Michigan, USA. *L. multiflorum* seed was obtained from the Michigan Department of Agriculture (East Lansing, Mich.) and *B. curtipendula* seed was supplied by the United States Department of Agriculture (Knox City, Tex.). Grass seedlings were grown in potting soil in 48-well (23 cm^2 /well) flats in a greenhouse for approximately 1–3 weeks. Seedlings were transplanted into 10×10×36-cm pots (Hummert, Springfield, Mo., USA) and grown in an 80:20 (v/v) mixture of potting soil and sand. The long, narrow shape and large volume of these pots has been found to minimize pot-binding artifacts (Arp 1991; Coleman and Bazzaz 1992; Poorter 1993; Wilsey et al. 1997). *L. multiflorum* was grown at a density of two plants per pot, while *B. curtipendula* was grown at a density of three to four plants per pot because of its slow growth rate. Pots of *L. multiflorum* (n=6) and *B. curtipendula* (n=6) were arranged in a checkerboard array in each open top chamber, and were recessed approximately 20 cm into the ground. A sheet of plastic lined the soil pit of each chamber to prohibit potential root growth into the surrounding soil. Chambers were constructed as described by Drake et al. (1989), with the exception that cubical structures (0.5 m^3) were produced with PVC pipe. Chambers were arranged into blocks, with each block containing one ambient and one adjacent elevated chamber. Within blocks, CO_2 treatment was randomly assigned to chambers. Chambers were located in a fenced field site with unobstructed sunlight, and were maintained at 370 ppm CO_2 (n=20) or at 740 ppm CO_2 (n=20) (Karowe et al. 1997). CO_2 concentrations inside each elevated CO_2 chamber were controlled by dispensing CO_2 into the inlet port of an input blower, and monitoring CO_2 concentration with continuous sampling of chamber air. Pumps delivered air from each elevated and two ambient chambers to an adjacent control house containing a microcomputer-controlled valve manifold, which directed the gas stream to an infrared CO_2 gas analyzer. The CO_2 concentration in each chamber was monitored automatically every 20 min and the data were recorded on a personal computer. Temperatures inside chambers during the day ranged from 3% warmer than values recorded immediately adjacent to the chambers (at 2000 hours) to 23% warmer (at 0800 hours), with an overall mean increase in temperature of 16%. Decreases in photosynthetically active wavelengths of light (400–700 nm) inside the chambers were assumed to be similar to those measured previously for chambers of the same materials (i.e., decreased 10–15%) (Drake et al. 1989). Grasses were watered and checked for insect herbivores daily, and were fertilized once a week (100 ml containing 25 mg of Peters 20–20–20 fertilizer). The nitrogen concentration of this fertilizing regime was equivalent to 100 kg N/hectare/year. After a 2-month period in the chambers, leaves from each grass species were collected separately from each chamber, and preserved during bioassays with caterpillars (described below).

The seeds and leaves of each grass species were surveyed for fungal endophytes (Latch et al. 1987; White and Chambless 1991). Seeds (n=6/species) were soaked overnight in 5% sodium hydroxide, rinsed 3 times with distilled water, and soaked for 1 h in distilled water. Seeds were deglumed, squashed under a cover slip in 1–2 drops of aniline blue, and examined for intercellular hyphae (400×). Leaf sections (culms) were also stained and examined. No fungi were observed in any case.

Insects

Eggs of *P. unipuncta* and *S. frugiperda* were obtained from colonies kept at the Boyce Thompson Institute (Ithaca, N.Y., USA) and the USDA (Tifton, Ga., USA), respectively. *P. unipuncta* and *S. frugiperda* were reared to the fifth instar on *Triticum aestivum* (wheat; C_3) seedlings and *Cynodon dactylon* (bermudagrass; C_4) at 25°C and 20°C, coinciding with a 16:8 h light:dark cycle in an environmental chamber. Larvae were fed *T. aestivum* or *C. dactylon* alternately for 72-h periods in 150×25-mm petri dishes containing moistened filter paper. At the beginning of the fifth instar, test larvae of each species were split into two groups and fed either *T. aestivum* or *C. dactylon* for approximately 3 days. Larvae last feeding on *T. aestivum* were used for feeding trials with *L. multiflorum* and those last feeding on *C. dactylon* were used for feeding trials with *B. curtipendula*. This pre-treatment was designed to provide a strength-building period for insects that would feed on the tougher C_4 grass, thereby minimizing any potential period of adjustment during the experiment (Bernays and Hamai 1987).

Bioassays

After grasses had grown in chambers for 2 months, newly-molted sixth-instar *P. unipuncta* and *S. frugiperda* larvae were assigned at random to one of the grass species × CO_2 treatment combinations. Feeding trials were started over a 5-day period. Test larvae were weighed and placed individually in 100×25-mm petri dishes lined with moistened filter paper, and replaced in the environmental chamber in which the larvae were reared (at ambient CO_2). The initial dry weight of each larva was estimated from the percent dry weight of newly-molted sixth-instar larvae fed either *T. aestivum* or *C. dactylon* (n=17–30/treatment). No differences were observed between the percent dry weights of larvae as a result of feeding on *T. aestivum* or *C. dactylon* during the fifth instar. Grass leaves were provided to caterpillars by cutting the tops of tillers, including the first three leaves and a short length of the culm, and placing the culm in water in a microcentrifuge tube. Fresh weights of food samples and the number of the chamber from which they were taken were recorded. Larvae were given freshly-cut leaves after 24 h from uncut plants from the same chamber as used to feed them originally, and were allowed to feed for a total of 48 h. Thus, within each caterpillar species, each larva fed on plants from a different chamber. Representative leaves from several plants of each species within each of the 40 chambers were collected (n=20 samples/species and CO_2 treatment) during the feeding trials and stored at −80°C until they were freeze-dried. The initial dry weights of leaves were estimated from their fresh weights based on the average percent dry weight of representative leaves from each species and CO_2 treatment. All uneaten food was dried at 70°C for at least 72 h and weighed. Consumption was determined as the difference between the initial and final dry weights of the food. Frass was collected daily, frozen at −80°C and then freeze-dried.

At the end of each bioassay, larvae were starved for 2 h and frozen (−80°C). Larvae were freeze-dried to determine their final dry weights. Rehydrated larvae were found to still contain leaf material in their guts, and eight larvae per treatment were dissected to determine the weights of their gut contents. Gut content weights in the remaining larvae were estimated by calculating regressions of larval weight versus gut content weight in each species and treatment (R^2 values ca. 0.5). The weights of larvae were reduced and weights of frass increased by the mass of their gut contents. Relative consumption rate (RCR), relative growth rate (RGR) and nutritional indices were calculated on a dry weight basis, using the arithmetic mean weights of larvae for RCR and RGR (Waldbauer 1968). Assimilation efficiencies for carbohydrates and protein were calculated as (mg nutrient ingested − mg nutrient egested)/mg ingested nutrient × 100. Relative assimilation rates (RAR) for carbohydrates and protein were calculated as mg nutrient assimilated/mg average insect dry weight/day, where assimilation is defined as the amount ingested minus the amount egested. Thus, the two components of the experiments (grass growth and insect bioassays) were separate, with the chamber array designed to serve as a source of four types of homogeneous foliage.

Chemical and physical analyses

To estimate the nutritional quality of the leaves ingested by caterpillars in the bioassays, we examined grass samples selected haphazardly from a subset of the 40 chambers, equally representing the four species × CO_2 treatments (see Table 1 for sample sizes). Samples were ground to a homogeneous powder using a dental amalgamator, and stored in screw-cap centrifuge tubes at room temperature or 4°C in the dark. Frass samples were prepared in the

same manner. Protein was measured as total amino acids in 6 M HCl hydrolysates of grass and frass samples (5–10 mg) using ninhydrin (Sigma) (Barbehenn 1995). Sugar and fructan were measured in ethanol extracts, as modified from Hendrix (1993). Briefly, ground samples (5–15 mg) were extracted in 80% ethanol (600 µl, 45°C, 20 min) in a shaker. Supernatant solutions were removed after centrifugation (10,000 rpm, 3 min). The extraction procedure was repeated using 50 and 20% ethanol, and the three ethanol extracts were combined for each sample, producing a final ethanol concentration of 50%. Aliquots (10 or 15 µl) of the ethanol extracts and glucose, fructose and sucrose standards were dried in a microtiter plate, and resolubilized in 40 µl of double-distilled water. Sugars were converted to glucose in stages enzymatically (Hendrix 1993), and measured as the change in glucose concentration with a glucose test kit (Sigma 115A). Thus, following the measurement of the initial glucose concentration, fructose was measured as the increase in absorbance (490 nm) after the addition of phosphoglucose isomerase (ten enzyme units/20 µl, Roche Molecular Biochemicals), and incubation for 15 min (37°C). Sucrose was then measured as the increase in absorbance following the addition of invertase (300 EU/20 µl, Sigma), and incubation for 30 min (37°C). A single aliquot (100 µl) of glucose color reagent was used for measuring all sugars in each sample. Fructan was measured separately in *L. multiflorum* extracts (10 or 15 µl) after they were dried, hydrolyzed in 20 µl of 1.0 M HCl (15 min, 37°C), and neutralized with 20 µl of 1.0 M NaOH. The difference in the amount of sugar (measured with the above procedure) between matched pairs of hydrolyzed and unhydrolyzed samples was defined as fructan. Starch was measured in the pellet remaining after sugar and fructan were extracted. Pellets were boiled in distilled water (1.0 ml) for 1 h in capped centrifuge tubes. Starch was hydrolyzed with α-amylase (Roche) by adding 360 EU in 200 µl of pH 5.5 sodium acetate buffer (50 mM) per sample for 75 min at 45°C, followed by amyloglucosidase (Sigma) (122 EU/200 µl of 50 mM sodium acetate buffer, pH 4.5) at 45°C overnight (Hendrix 1993). Amylopectin, amylose and soluble potato starch were used as positive controls. Hydrolysis of these starches was complete, with glucose recovery averaging 106±6% (n=8). All reaction mixtures were scaled to fit in 96-well microtiter plates (200 µl), and absorbance measurements were made with a Bio-Rad Benchmark microplate reader. Standard curves were constructed using glucose, fructose and sucrose to convert absorbance to µg sugar. The data were expressed as total sugar for simplicity, since the sugars are each readily utilized by insects. Average nutrient levels across the 5-day bioassay period are presented in Table 1. These values were also used to calculate nutrient assimilation efficiencies and rates.

Nitrogenous waste products (allantoin, allantoic acid and uric acid) were measured in frass from five larvae of *P. unipuncta* and *S. frugiperda* from each grass species × CO_2 treatment combination. Samples (10 mg) were extracted in 0.6% Li_2CO_3 (500 µl, pH 11.5). Allantoin and allantoic acid were measured according to Van Zyl et al. (1998), and uric acid was measured with uricase (Martin and Van't Hof 1988). The allantoin + allantoic acid and uric acid concentrations were multiplied by 2.0 and 2.4, respectively, to account for their color factors in the ninhydrin assay. Contrary to the methods described previously (Barbehenn 1995), the nitrogenous waste products are interfering substances once they have been subjected to acid hydrolysis. Mean nitrogenous waste concentrations were used to correct fecal protein measurements. The use of corrected fecal protein values increased protein assimilation efficiencies by approximately 10 percentage points.

Leaf toughness was measured with a penetrometer (Feeny 1970), using a punch constructed from a flat-ended metal rod (2 mm diameter) to mimic the cutting edge of an insect mandible (Bernays and Hamai 1987). The first fully-expanded leaf was punctured midway between the base and tip, usually at a position that avoided the midrib. Toughness was expressed as the mass (g) necessary to puncture the leaf. To examine a factor that contributes to toughness, neutral detergent fiber (cellulose, hemicellulose and lignin) was measured (Van Soest et al. 1991).

Statistical analysis

Measures of C_3 and C_4 grass nutritional quality and specialist and generalist insect performance were analyzed with a split-plot, Type III ANOVA (PROC MIXED) (SAS 2000). Models for analyzing grass quality included grass species and CO_2 level as main effects, and CO_2 block as random effects, and also included the CO_2 × grass species interaction. Models for analyzing insect performance included insect species, grass species and CO_2 level as main effects and CO_2 block as random effects, and also included all two-way and three-way interactions among insect species, grass species and CO_2 levels. The normality of residuals was tested with PROC UNIVARIATE (SAS 2000). Where necessary, log or square root transformations were used to normalize residuals. If residuals could not be normalized, the significance of main effects was determined by Kruskal–Wallis tests (Wilkinson 2000). Pairwise differences among grass and insect treatment groups were examined by differences of least-squares means (SAS 2000). These multiple comparisons tested a priori hypotheses, and consequently differences significant at P=0.05 are indicated in the tables by non-overlapping letters.

Initial dry weights were higher for *S. frugiperda* than *P. unipuncta* larvae (P=0.004), and higher for larvae that fed exclusively on *T. aestivum* prior to the experiment (P=0.011 for insect × plant species interaction). Analysis of growth and consumption rates by ANCOVA using initial dry weight as a covariate has been recommended for such data (Raubenheimer and Simpson 1992), but was not possible due to a significant larval weight x plant species interaction. Two other approaches for calculating consump-

Table 1 Effect of atmospheric CO_2 concentration on levels of nutrients, fiber and toughness in *L. multiflorum* (C_3) and *B. curtipendula* (C_4). Data are presented as mean ± SE. *ND* Not determined C_4 grasses do not produce fructan). Non-overlapping letters designate statistically significant differences between means within columns (P<0.05). P-values <0.10 are listed, and *NS* indicates P>0.10. The significance of sugar effects were determined by Kruskal–Wallis tests, where possible

Grass species	CO_2 (ppm)	Protein (%DW) n=10	Sugar (%DW) n=15	Starch (%DW) n=15	Fructan (%DW) n=15	TNC (%DW) n=15	Water (%FW) n=20	Fiber (%DW) n=10	Toughness (g) n=20
L. multiflorum	370	25.8±1.4^b	10.2±0.9^b	2.8±0.5^a	3.1±0.8^a	15.8±1.5^a	81.0±0.5^c	34.6±1.3^a	399±18^a
L. multiflorum	740	20.5±1.2^a	11.6±1.2^b	5.0±0.8^a	6.2±1.4^b	22.5±1.7^c	78.7±0.8^b	34.2±1.9^a	464±17^a
B. curtipendula	370	18.2±0.8^a	3.0±0.2^a	8.1±1.4^b	ND	11.9±1.6^b	67.0±0.2^a	55.5+1.0^b	669±38^b
B. curtipendula	740	18.1±1.4^a	2.8±0.3^a	9.6±1.9^b	ND	12.6±2.1^b	66.4±1.1^a	55.8±0.8^b	751±56^b
Significance of effects									
Grass species		<0.001	<0.001	<0.001	ND	<0.001	<0.001	<0.001	<0.001
CO_2		0.055	NS	NS	0.036	0.054	NS	NS	0.055
CO_2× grass species		0.050	ND	NS	ND	0.022	NS	NS	NS

tion and growth rates include using initial dry weights (Farrar et al. 1989) or "exponential" mean weights (Gordon 1968) instead of the arithmetic mean weight. The use of each of these alternative approaches did not change the patterns observed or the conclusions presented, compared with the use of the arithmetic mean weight for RGR and RCR. Therefore, the arithmetic mean weight was used in calculations presented here, e.g.,

RGR
= dry weight gained/[(initial dry weight + final dry weight)/2]/day.

Results

Grasses

Overall, protein levels were significantly higher in the C_3 species *L. multiflorum* than in the C_4 species *B. curtipendula* (Table 1). Growth under elevated CO_2 resulted in an overall decrease in protein that was nearly significant. However, there was a significant CO_2 × grass species interaction, with protein content decreasing 20% in *L. multiflorum*, but only 1% in *B. curtipendula* when the grasses were grown under elevated CO_2. Thus, *L. multiflorum* contained 42% more protein than *B. curtipendula* at ambient CO_2, but did not differ significantly from the C_4 grass under elevated CO_2.

Sugar levels were 206% higher in *L. multiflorum* than in *B. curtipendula*, and were not significantly affected by elevated CO_2 (Table 1). Since residuals could not be normalized, ANOVA could not be used to test for a CO_2× grass species interaction. However, pairwise Kruskal–Wallis comparisons within each grass species indicated that there was no significant change in sugar content for either species under elevated CO_2. Overall, the starch content in *B. curtipendula* was 130% higher than in *L. multiflorum*, and was not significantly affected by elevated CO_2 in either species. Fructan (not produced by C_4 grasses) increased significantly (by 100%) in *L. multiflorum* under elevated CO_2 (Table 1). Total nonstructural carbohydrates (TNC; sugar, starch and fructan) were significantly higher (by 56%) in *L. multiflorum* than in *B. curtipendula*, and were nearly significantly higher under elevated CO_2 than under ambient CO_2. There was a significant grass species × CO_2 interaction, indicating that TNC increased to a greater extent under elevated CO_2 in *L. multiflorum* than in *B. curtipendula*. TNC were 33% higher in *L. multiflorum* than in *B. curtipendula* under ambient CO_2, and 79% higher in *L. multiflorum* under elevated CO_2 (Table 1).

Overall, the water content in *L. multiflorum* was 19% higher than in *B. curtipendula* (Table 1), but fiber and toughness were significantly greater in *B. curtipendula* than in *L. multiflorum* (by 62 and 65%, respectively). However, none of these measures of nutritional quality was affected by growth under elevated CO_2, nor did any exhibit a significant CO_2× grass species interaction.

Insects

Both the specialist, *P. unipuncta*, and the generalist, *S. frugiperda*, had significantly higher RGR on *L. multiflorum* than on *B. curtipendula* (Table 2). However, *P. unipuncta* grew faster than *S. frugiperda* on both grass

Table 2 Growth, consumption, and digestion efficiencies of *P. unipuncta* (specialist) and *S. frugiperda* (generalist) caterpillars on *L. multiflorum* (C_3) and *B. curtipendula* (C_4) grown at ambient or elevated CO_2 levels. Data are presented as mean ± SE. Non-overlapping letters designate statistically significant differences between means within columns ($P<0.05$). *RGR* Relative growth rate, *RCR* relative consumption rate, *AD* approximate digestibility, *ECI* efficiency of conversion of ingested mass, *ECD* efficiency of conversion of digested mass. $P<0.10$ are listed, and *NS* indicates $P>0.10$

Insect species	Grass species	CO_2 (ppm)	RGR	RCR	AD	ECI	ECD	Final weight (mg)	*n*
P. unipuncta	*L. multiflorum*	370	0.55 ± 0.02^{d}	2.39 ± 0.12^{ab}	57.7 ± 2.7^{d}	23.6 ± 1.2^{d}	43.1 ± 3.7^{a}	51.7 ± 3.0^{b}	14
P. unipuncta	*L. multiflorum*	740	0.55 ± 0.01^{d}	2.41 ± 0.12^{ab}	59.3 ± 2.5^{d}	23.8 ± 1.1^{d}	41.9 ± 3.5^{a}	51.5 ± 2.4^{b}	14
P. unipuncta	*B. curtipendula*	370	0.36 ± 0.02^{b}	2.14 ± 0.09^{a}	42.1 ± 2.0^{b}	16.7 ± 0.8^{b}	41.7 ± 3.4^{a}	33.8 ± 1.8^{a}	15
P. unipuncta	*B. curtipendula*	740	0.38 ± 0.02^{b}	2.18 ± 0.08^{a}	39.7 ± 3.1^{b}	16.6 ± 1.1^{b}	47.8 ± 4.8^{a}	34.6 ± 1.8^{a}	13
S. frugiperda	*L. multiflorum*	370	0.46 ± 0.01^{c}	2.42 ± 0.11^{ab}	50.8 ± 2.4^{d}	19.8 ± 0.8^{c}	41.5 ± 3.2^{a}	53.4 ± 1.9^{b}	20
S. frugiperda	*L. multiflorum*	740	0.49 ± 0.01^{c}	2.66 ± 0.12^{b}	47.1 ± 2.3^{c}	19.0 ± 0.7^{c}	42.6 ± 3.3^{a}	52.6 ± 2.8^{b}	17
S. frugiperda	*B. curtipendula*	370	0.28 ± 0.01^{a}	2.13 ± 0.07^{a}	39.0 ± 2.4^{b}	13.4 ± 0.6^{a}	37.5 ± 3.4^{a}	32.6 ± 1.5^{a}	18
S. frugiperda	*B. curtipendula*	740	0.27 ± 0.03^{a}	2.15 ± 0.17^{a}	32.6 ± 2.4^{a}	12.5 ± 1.1^{a}	42.0 ± 5.7^{a}	30.3 ± 1.8^{a}	12
Significance of effects									
Grass species			<0.001	<0.001	<0.001	NS	<0.001	<0.001	
Insect species			<0.001	NS	<0.001	NS	<0.001	NS	
CO_2			NS	NS	NS	NS	NS	NS	
CO_2 × grass species			NS	NS	NS	NS	NS	NS	
CO_2 × insect species			NS	NS	NS	NS	NS	NS	
Grass species × insect species			NS	NS	NS	NS	NS	NS	
CO_2 × grass species × insect species			NS	NS	NS	NS	NS	NS	

species. Despite significant changes in the nutritional quality of *L. multiflorum* under elevated CO_2, no effect on the RGR of either caterpillar species on either grass species resulted.

The RCR of both caterpillar species were significantly higher on *L. multiflorum* than on *B. curtipendula*, but did not differ between insect species or CO_2 levels (Table 2). The approximate digestibility (AD) of *L. multiflorum* was significantly higher than that of *B. curtipendula* for both *P. unipuncta* and *S. frugiperda*. *P. unipuncta* digested both grass species significantly more efficiently than did *S. frugiperda*. Similarly, the efficiency of conversion of ingested mass (ECI) to body mass was significantly higher for both insect species on *L. multiflorum* than on *B. curtipendula*, and was significantly higher for *P. unipuncta* than for *S. frugiperda* on both grass species. The efficiency of conversion of digested mass (ECD) to body mass did not differ between insect species or grass species, and none of the nutritional indices showed a significant effect of CO_2 or a CO_2 × insect species interaction. As would be expected, the final weights of caterpillars were higher on *L. multiflorum* than on *B. curtipendula* for both insect species, but did not differ between insect species or CO_2 levels (Table 2).

Protein assimilation efficiency was significantly higher for *P. unipunctata* than for *S. frugiperda*, and higher on *L. multiflorum* than on *B. curtipendula* (Table 3). In addition, there was a significant CO_2× insect species × grass species interaction, reflecting the fact that protein assimilation efficiency increased under elevated CO_2 only for *S. frugiperda* feeding on *B. curtipendula*.

Sugar assimilation efficiency was significantly higher for insects feeding on *L. multiflorum* than for those feeding on *B. curtipendula*, albeit by a small amount (Table 3). Sugar assimilation efficiencies in *P. unipunctata* were less affected by the grass species eaten than were those in *S. frugiperda*, resulting in a significant grass species × insect species interaction. Insects also assimilated sugar more efficiently from plants grown at ambient CO_2 than from plants grown at elevated CO_2.

Starch assimilation efficiencies were substantially higher from the C_3 than the C_4 grass, a pattern that did not differ between insect species or CO_2 levels (Table 3). Fructan was assimilated from *L. multiflorum* more efficiently by *P. unipunctata* than by *S. frugiperda*. While both species assimilated fructan more efficiently from *L. multiflorum* grown under ambient than elevated CO_2, giving rise to a significant CO_2 effect, the difference was significant only for *S. frugiperda*.

The combined effects of foliar nutrient content, leaf consumption rate and nutrient assimilation efficiency were expressed as RAR for protein and carbohydrates (Table 4). Protein RAR was 63% higher in insects feeding on *L. multiflorum* than in those feeding on *B. curtipendula*. Protein RAR exhibited a significant CO_2 × grass species interaction, resulting from decreased protein RAR under elevated CO_2 in both species of insect on *L. multiflorum*, but not in those that fed on *B. curtipendula*. Similarly, carbohydrate RAR was significantly higher in insects that fed on *L. multiflorum* than in those on *B. curtipendula*, primarily from the consumption of larger amounts of nonstructural carbohydrates in the C_3 grass. For both insect species, carbohydrate RAR increased significantly under elevated CO_2 only on *L. multiflorum*, resulting in both a significant CO_2 effect and a significant CO_2 × grass species interaction.

Table 3 Assimilation efficiencies for protein and carbohydrates from *L. multiflorum* (C_3) and *B. curtipendula* (C_4) by *P. unipuncta* (specialist) and *S. frugiperda* (generalist) caterpillars at ambient or elevated CO_2 levels. Data are presented as mean ± SE. *ND* Not determined C_4 grasses do not produce fructan). Non-overlapping letters designate statistically significant differences between means within columns ($P<0.05$). $P<0.10$ are listed, and *NS* indicates $P>0.10$

Insect species	Grass species	CO_2 (ppm)	Assimilation efficiency				*n*
			Protein	Sugar	Starch	Fructan	
P. unipuncta	*L. multiflorum*	370	88.3±0.7^{d}	94.7±0.8^{c}	77.2±2.1^{c}	85.7±5.9^{b}	
P. unipuncta	*L. multiflorum*	740	91.1±0.9^{d}	90.9±1.2^{b}	73.9±3.9^{bc}	73.6±5.5^{b}	14
P. unipuncta	*B. curtipendula*	370	81.1±1.6^{bc}	90.9±0.8^{b}	43.3±5.1^{a}	ND	15
P. unipuncta	*B. curtipendula*	740	82.2±1.6^{bc}	88.7±1.2^{ab}	44.7±4.6^{a}	ND	13
S. frugiperda	*L. multiflorum*	370	83.7±1.4^{c}	95.4±0.5^{c}	71.2±2.4^{bc}	70.3±6.5^{b}	20
S. frugiperda	*L. multiflorum*	740	82.2±2.1^{bc}	93.1±0.6^{c}	63.7±3.6^{b}	51.3±7.1^{a}	17
S. frugiperda	*B. curtipendula*	370	72.4±2.1^{a}	89.7±0.8^{b}	47.5±5.2^{a}	ND	18
S. frugiperda	*B. curtipendula*	740	77.3±1.5^{b}	87.4±1.0^{a}	38.2±3.3^{a}	ND	12
Significance of effects							
Grass species			<0.001	<0.001	<0.001	ND	
Insect species			<0.001	NS	NS	0.003	
CO_2			0.094	<0.001	NS	0.041	
CO_2× grass species			NS	NS	NS	ND	
CO_2× insect species			NS	NS	NS	NS	
Grass species × insect species			NS	0.012	NS	ND	
CO_2× grass species × insect species			0.042	NS	NS	ND	

Table 4 Protein and carbohydrate relative assimilation rates (RAR) in *P. unipuncta* (specialist) and *S. frugiperda* (generalist) caterpillars fed *L. multiflorum* (C_3) or *B. curtipendula* (C_4) grown at ambient or elevated CO_2 levels. *RAR* mg nutrient assimilated/mg larva/day. Data presented as mean ± SE. Non-overlapping letters designate statistically significant differences between means within columns ($P<0.05$). $P<0.10$ are listed, and *NS* indicates $P>0.10$

Insect species	Grass species	CO_2 (ppm)	Protein RAR	Carbohydrate RAR	*n*
P. unipuncta	*L. multiflorum*	370	0.54+0.03^{c}	0.31±0.02^{b}	14
P. unipuncta	*L. multiflorum*	740	0.46±0.03^{b}	0.45±0.04^{c}	14
P. unipuncta	*B. curtipendula*	370	0.31±0.02^{a}	0.14±0.01^{a}	15
P. unipuncta	*B. curtipendula*	740	0.31±0.01^{a}	0.15±0.01^{a}	13
S. frugiperda	*L. multiflorum*	370	0.52±0.03^{bc}	0.30±0.02^{b}	20
S. frugiperda	*L. multiflorum*	740	0.45±0.04^{b}	0.46±0.04^{c}	17
S. frugiperda	*B. curtipendula*	370	0.28±0.01^{a}	0.15±0.02^{a}	18
S. frugiperda	*B. curtipendula*	740	0.31±0.03^{a}	0.14±0.02^{a}	10
Significance of effects					
Grass species			<0.001	<0.001	
Insect species			NS	NS	
CO_2			NS	0.009	
CO_2× grass species			0.025	0.012	
CO_2× insect species			NS	NS	
Grass species × insect species			NS	NS	
CO_2 × grass species × insect species			NS	NS	

Discussion

As expected, this study demonstrated that the C_3 grass *L. multiflorum* has higher nutrient levels than the C_4 grass *B. curtipendula*. In addition, the results are consistent with the hypothesis that the nutritional quality of C_3 grasses changes more than that of C_4 grasses when they are grown under an elevated CO_2 atmosphere. Under elevated CO_2, the concentration of protein declined in the C_3 grass, presumably as a result of lower concentrations of photosynthetic proteins and increased concentrations of nonstructural carbohydrates (Drake et al. 1997). To the extent that protein is the most limiting of the macronutrients examined, these changes represent a decline in the nutritional quality of the C_3 grass. Although protein levels in *L. multiflorum* grown in elevated CO_2 declined to levels as low as those found in *B. curtipendula*, the overall nutritional quality of the C_3 grass remained superior at elevated CO_2; *L. multiflorum* had higher water content, lower toughness and higher carbohydrate concentrations than *B. curtipendula* at elevated CO_2. Consistent with these differences in foliar nutritional quality, the performance of both caterpillar species examined in this study remained greater on *L. multiflorum* than on *B. curtipendula* grown under elevated CO_2, supporting the hypothesis that insect performance is greater on C_3 than C_4 grasses as a result of lower C_4 grass nutritional quality.

The results of this study are also consistent with the hypothesis that grass specialist caterpillars are more efficient than generalist species at utilizing C_3 and C_4 grasses. *P. unipuncta* had higher AD, ECI and RGR than did *S. frugiperda* on both grass species and CO_2 levels. Thus, the greater efficiency of the specialist, and its ability to maintain high growth rates on leaves with relatively low nutrient levels, are due to a combination of higher efficiencies of nutrient assimilation (Simpson and Simpson 1990; Wheeler and Slansky 1991; Hättenschwiller and Schafellner 1999) and possibly more efficient food handling, but not due to higher consumption rates. For example, food handling costs might be lower for grass specialists if their scissor-like mandibles allow tough grass leaves to be ingested with less energy per bite (Vincent 1982, 1991; Barbehenn 1992; Choong et al. 1992).

It has been hypothesized that a large fraction of the nutrients in C_4 plants are unavailable to insect herbivores as a result of the containment of these nutrients in uncrushable bundle sheath cells (Caswell et al. 1973; Caswell and Reed 1975, 1976). The results of this study show that starch and protein were less digestible from the C_4 grass than the C_3 grass, but do not support the belief that nutrients in the bundle sheath cells are unavailable. For example, the digestibility of protein from *B. curtipendula* (C_4) by *P. unipuncta* was only 13% lower than from *L. multiflorum* (C_3), and 18% lower for *S. frugiperda*. It can be calculated that if protein in bundle sheath cells was unavailable, protein digestibility from *B. curtipendula* would have been approximately 45% and 41% by *S. frugiperda* and *P. unipuncta*, respectively (Barbehenn and Bernays 1992), compared to actual protein digestive efficiencies of 75% and 82% in these caterpillars. We conclude that both generalist and specialist caterpillars are able to extract a substantial fraction of the nutrients contained in the bundle sheath cells of C_4 grasses.

Plant nutritional quality is often compared in terms of C:N ratios. It is noteworthy, therefore, that differences between ratios of nonstructural carbohydrate:protein (digestible C:N) did not explain the observed variation in caterpillar performance between C_3 and C_4 grasses at two CO_2 levels. Carbohydrate:protein ratios are strikingly

similar between *L. multiflorum* and *B. curtipendula* at ambient CO_2 levels (ca. 40:60), but increase to 52:48 in *L. multiflorum* at elevated CO_2 levels. However, the results of our study show consistent differences in insect performance between the C_3 and C_4 grasses, but no significant differences in insect performance between CO_2 levels. By contrast, concentrations of protein and nonstructural carbohydrates were each 30% less in *B. curtipendula* than in *L. multiflorum* at ambient CO_2 levels. Therefore, our results are consistent with the hypothesis that the lower nutritional quality of C_4 grasses is a result of their lower nutrient concentrations relative to C_3 grasses, and that nutrient concentration is more limiting than nutrient availability for C_4 grass-feeding caterpillars. Previous work comparing the nutritional quality of a larger variety of C_3 and C_4 grasses for a grass-specialist hesperiid caterpillar reached the same conclusion (Barbehenn and Bernays 1992). Although this study did not include replication of grass species within each photosynthetic type, we note that our results should provide a robust test of the relative ability of caterpillars to utilize C_3 and C_4 grasses, since *L. multiflorum* and *B. curtipendula* share the distinct anatomical features of other C_3 and C_4 grasses, i.e., the presence or absence of bundle sheath cells.

Fructan can reach levels ranging from 5% to 45% dry weight in C_3 grasses exposed to cold temperatures (Volenec and Nelson 1984; Chatterton et al. 1989; Pollock and Cairns 1991). Previous work on the effects of elevated CO_2 on fructan synthesis in wheat (*T. aestivum*) found that fructan increased to 9.0% dry weight (a 20% increase at 550 ppm CO_2) (Nie et al. 1995). In this study, elevated CO_2 caused a doubling of the fructan concentration in *L. multiflorum*, but the absolute levels remained relatively low compared with those produced by cold temperatures. In conditions that greatly elevate levels of fructan, C_3 grasses represent a potentially rich energy source for insects that are able to digest them. Further work is needed to examine the effects of elevated CO_2 on fructan concentrations in a wider variety of C_3 grasses.

Increased concentrations of carbohydrates have been viewed either as diluents of essential nutrients (negative) or as energy sources that can benefit insect fitness (positive) (Lincoln et al. 1993; Lindroth et al. 1995; Lindroth 1996; Goverde et al. 2002). It is commonly assumed that nonstructural carbohydrates are readily utilized. However, this is not necessarily true for some carbohydrates, such as starch and fructan. The ability to digest starch varies widely among caterpillars (Waldbauer 1968; Harvey 1975), and one fructan (inulin) was not digested by several caterpillar species (Harvey 1975; Dadd 1977). The results of this study show that both starch and grass fructan are digested and assimilated by *P. unipuncta* and *S. frugiperda*, albeit to a more limited extent than are sugar and protein. Different results on fructan digestion may represent variation between insect species or chemical differences between fructans. For example, it is possible that the β (2→1) glycosidic linkages in inulin are not hydrolyzed as readily by caterpillars as are the β (2→6) linkages primarily found in grass fructan. Variation in the ability of herbivores to digest certain types of carbohydrates could help explain the variable effects of elevated CO_2 on these insects, i.e., whether additional carbohydrates act as indigestible diluents or energy sources.

Contrary to our expectations, neither caterpillar species significantly increased its consumption rate to compensate for the lower concentration of protein in a C_3 grass grown in an elevated CO_2 atmosphere. This result does not support the hypothesis that C_3 plants will be subject to greater rates of herbivory relative to C_4 plants in future atmospheric conditions (Lincoln et al. 1984). In addition, neither insect species compensated for the lower nutritional quality of *B. curtipendula* by increasing its rate of consumption of this plant relative to the C_3 grass. Instead, *P. unipuncta* and *S. frugiperda* fed at a higher rate on the C_3 grass than the C_4 grass, the opposite of the pattern expected. There are potential consequences to insect fitness and population dynamics from the lack of compensatory feeding on the C_4 grass, since the final weights of the insects were lower on the C_4 grass; smaller adults are known to produce fewer eggs, have decreased longevity, and have lower energy reserves for flight and mate finding (Goverde et al. 2002, and references therein).

As in any two-species comparison, caution is required in generalizing from single examples within each category (Garland and Adolph 1994). More studies are required involving different insect species and different grass species before it will be possible to identify patterns of behavioral responses and fitness consequences in grass-feeding insects on grasses grown in elevated CO_2 levels. However, it is evident already that there will not be a single pattern that characterizes all grass feeders. While the consumption and growth rates of *P. unipuncta* and *S. frugiperda* caterpillars were unaffected by elevated CO_2 levels in this study, when *S. frugiperda* fed on *Festuca arundinacea* (C_3) grown in elevated CO_2 it exhibited both increased consumption and growth rates (Marks and Lincoln 1996). By contrast, elevated CO_2 levels reduced pupal weight and developmental rate when a grass-specialist satyrid caterpillar fed on several C_3 grasses (Goverde and Erhardt 2003). Although there are many examples of compensatory feeding among C_3 leaf-feeding insects on plants grown under elevated CO_2, our results demonstrate that among the many species that do not exhibit compensatory feeding responses, post-ingestive mechanisms could provide a sufficient means of compensation for the lower nutritional quality of C_3 plants grown under elevated CO_2.

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