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Chemical composition and digestibility of *Trifolium* exposed to elevated ozone and carbon dioxide in a free-air (FACE) fumigation system

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Summary

1. Tropospheric ozone (O_3) and carbon dioxide (CO_2) are significant drivers of plant growth and chemical composition. We hypothesized that exposure to elevated concentrations of O_3 and CO_2, singly and in combination, would modify the chemical composition of *Trifolium* and thus alter its digestibility and nutritive quality for ruminant herbivores.

2. We tested our hypothesis by collecting samples of Red Clover (*Trifolium pratense*) and White Clover (*Trifolium repens*) from the understoreys of Trembling Aspen (*Populus tremuloides*)–Sugar Maple (*Acer saccharum*) communities that had been exposed since 1998 to ambient air, elevated CO_2, elevated O_3 or elevated CO_2 + O_3 at the Aspen Free-Air CO_2 and O_3 Enrichment (FACE) site located near Rhinelander, WI, USA. Foliage samples were analysed for (1) concentrations of N, total cell wall constituents, lignin and soluble phenolics; and (2) *in vitro* dry-matter digestibility (IVDMD) and *in vitro* cell-wall digestibility (IVCWD) using batch cultures of ruminal micro-organisms.

3. Significant air-treatment effects were observed for lignin concentration, IVDMD and IVCWD, and between Red and White Clover for all dependent variables. No air treatment × clover species interactions were detected.

4. Exposure to elevated O_3 resulted in increased concentration of lignin and decreased IVDMD and IVCWD compared with exposure to ambient air, and the response was similar regardless of whether plants had been coexposed to elevated CO_2. Exposure to elevated CO_2 alone did not affect chemical composition or *in vitro* digestibility, nor did it ameliorate the negative effect of elevated O_3 on these determinants of nutritive quality for ruminant herbivores.

5. In contrast to recent reports of a protective effect of elevated CO_2 against growth reduction in plants under O_3 stress, our results indicate that elevated CO_2 would not be expected to ameliorate the negative impact of elevated O_3 on nutritive quality of *Trifolium* under projected future global climate scenarios.

Key-words: nutritive quality, *Trifolium*, lignin, digestibility, ozone, carbon dioxide, FACE, climate change

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Introduction

Tropospheric ozone (O_3) is the most significant phytotoxic air pollutant affecting vegetation in industrialized regions of the world, and background levels have risen by approximately 0·5–2% per year in the mid-latitudes of the Northern Hemisphere during the 30-year period between 1970 and 2000 (Vingarzan 2004). Chronic exposure to elevated O_3 over a growing season can lead to decreased plant growth and productivity resulting from decreased photosynthetic rate, reduced photosynthetic area, reduced photosynthate translocation and/or allocation, accelerated foliar senescence and increased respiration (Runeckles & Chevone 1992; Chappelka & Samuelson 1998; Fuhrer & Booker 2003). Evidence has accumulated recently that O_3-induced changes in foliar chemistry can result in decreased

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nutritive quality of herbaceous vegetation for ruminant herbivores (Krupa, Muntifering & Chappelka 2004), which is significant because loss of consumable food value is a potentially significant effect of O_3 that is not currently considered in economic risk-assessment models.

Plant growth is nearly always stimulated by elevated carbon dioxide (CO_2), especially in C_3 plants, and a doubling of atmospheric concentrations is projected to occur in this century if current rates of CO_2 emissions are left unchecked (Bolin *et al*. 1986). Most reports indicate that elevated CO_2 increases photosynthesis, water- and nutrient-use efficiency, and economic yield. Foliar chemistry responses to elevated CO_2 have included decreased nitrogen concentration resulting from increased C/N ratio (Rogers, Runion & Krupa 1994); increased synthesis of secondary phenolic compounds, notably lignin (Gifford, Barrett & Lutze 2000); and accumulation of non-structural carbohydrates (Heagle *et al*. 2002). Picon-Cochard *et al*. (2004) observed increased water-soluble sugar concentration, decreased fibre concentration and increased *in vitro* digestibility of bulk forage from a semi-natural grassland community exposed to elevated CO_2. In contrast, other investigators have reported that forage quality was adversely affected by plant growth under elevated CO_2 as a result of increased fibre concentration (Owensby, Cochran & Auen 1996) and decreased *in vitro* digestibility (Morgan *et al*. 2004).

A limited number of studies of interactive effects of elevated O_3 and CO_2 on herbaceous vegetation have produced variable results. Phytotoxic effects of elevated O_3 on plant growth and physiological processes are often ameliorated in a CO_2-enriched environment (Fuhrer 2003). Elevated CO_2 largely ablated the negative effect of elevated O_3 on soybean (*Glycine max*) residue biomass production, but plant chemistry and decomposition rates in the combined gas treatment were similar to those in the elevated CO_2 and O_3 treatments administered singly, which in turn were only marginally different from those in the ambient-air treatment (Booker *et al*. 2005). Increasing concentrations of laminar cell-wall constituents in White Clover (*Trifolium repens*) across a range of O_3 concentrations was essentially nullified under a CO_2-enriched atmosphere (Burns, Heagle & Fisher 1997).

While information from these and other controlled-environment experiments provides evidence of protection by elevated CO_2 against negative effects of O_3 on plant growth and chemistry, the interactive effect of these atmospheric contaminants on the chemical composition of herbaceous vegetation as it relates to nutritive quality has not been investigated under free-air conditions. Given the widespread occurrence and importance of *Trifolium* spp. as a food resource for ruminant herbivores, we investigated the chemical composition and nutritive quality of *Trifolium* growing naturally in forest understorey communities and exposed to elevated O_3 and CO_2, singly and in combination, in a free-air (FACE) fumigation system.

Methods

STUDY SITE

The Aspen FACE site (32 ha) is located in northern Wisconsin near Rhinelander (longitude 89·5°, latitude 45·6°), on the Harshaw Experimental Farm of the USDA Forest Service. The legal description of the site is SW80, sect. 21, T37N, R7E, Cassian Township, Oneida County, WI, USA. The site is old agricultural land that was farmed for potatoes (*Solanum tuberosum*) and small grains for more than 50 years. The Forest Service purchased the farm in 1972 for use as a short-rotation intensive culture and mixed-genetics forest research facility. Approximately 80% of the 32-ha Aspen FACE site was planted with different hybrid poplar (*Populus* spp.) clones and some larch (*Larix* spp.) from 1976 to 1990, and the remaining area reverted to 'old-field' vegetation. The study site currently comprises 12 30-m-diameter rings in which the concentrations of CO_2 and tropospheric O_3 are controlled (Dickson *et al*. 2000). All poplar and larch plantings were cleared from the site in 1996 and 1997. Stumps in the ring areas were removed, and the rings were disked and planted in rye (*Secale cereale*) overcrop in the summer of 1996. All rings were situated at least 100 m apart from each other. Each ring was divided into three forest communities: Trembling Aspen (*Populus tremuloides*) only (half ring), Aspen–Paper Birch (*Betula papyrifera*, quarter ring), and Aspen–Sugar Maple (*Acer saccharum*, quarter ring), planted at 1 × 1-m spacing in the summer of 1997.

The Aspen FACE site is level to gently rolling Pandus sandy loam (mixed, frigid, coarse loamy Alfic Haplorthod). The sandy loam topsoil (≈15 cm deep) grades into a plough layer–clay loam accumulation layer (≈30 cm deep) and then grades back into a sandy loam, stratified sand and gravel substratum. Occasional clay layers at 30–60 cm are found throughout the field, primarily in the northern 16 ha. As a basis for future comparisons, soils within each ring were analysed in 1997. Soil properties differed little among the 12 rings.

Trees inside the rings were irrigated with overhead sprinklers as needed (two to three times per growing season) in 1997 and 1998, and vegetation within 0·25 m of each tree was controlled by either hand weeding or applications of herbicide (two to three times per growing season as needed) from 1997 through 1999. With the exception of controlling Forest Tent Caterpillar (*Malacosoma disstria*) in two growing seasons, no additional management was conducted in the rings as vegetation from the seed bank was allowed to develop naturally.

Air treatments consisted of three rings (blocks) of four treatments: control; elevated carbon dioxide (CO_2); elevated ozone (O_3); or elevated $CO_2 + O_3$ using a randomized block design (12 rings total). O_3 and CO_2 were released during the daylight hours. Target levels were 1·5 × ambient and 560 p.p.m., respectively,

for the O_3 and CO_2 exposures. Ozone was not administered during periods of cold weather, or when surfaces of leaves on the trees were wet from fog, dew or precipitation. Additional Aspen FACE site information, including a complete description of the air-fumigation system design and exposure regimens, is given by Dickson *et al*. (2000) and Karnosky *et al*. (2003).

SAMPLE COLLECTION

Foliage of unimproved Red Clover (*T. pratense*) and White Clover (*T. repens*) growing naturally within the rings was collected on 9 and 10 August 2004 from the Aspen–Sugar Maple communities. These species are preferred forages for ruminant animals and common to all treatments. The Aspen-Sugar Maple community in each ring was used because it contained the greatest abundance of understorey vegetation, which ensured that sufficient mass of sample material was collected for the laboratory analysis (≈10–20 g DW). Representative samples of foliage were collected from multiple sectors of the Aspen–Sugar Maple community in each ring and bulked into a single composite sample (target of 40 g DW) for each species from each ring ($N = 24$).

Foliage was collected by clipping samples at approximately 2–5 cm above the ground line. Samples were then placed in a paper bag and dried at 40–50 °C on site until a constant weight was achieved (≈3 days). The dried, air-equilibrated material was immediately shipped to Auburn University. All flowers and main stems were removed, then remaining leaves and petioles were ground in a Wiley mill to pass a 1-mm screen prior to laboratory analysis for nutritive quality assessment.

LABORATORY ANALYSES

Forage samples were analysed for dry matter and N using procedures of the Association of Official Analytical Chemists (1995). Concentrations of neutral-detergent fibre (NDF) and acid-detergent lignin were determined sequentially according to Van Soest, Robertson & Lewis (1991). Soluble, non-lignin phenolics were extracted from forage samples in 70% aqueous acetone solution using sonication followed by centrifugation as described by Hagerman (1988), and concentrations were determined by reference to a gallic acid standard using the Prussian Blue assay (Price & Butler 1977) as modified by Graham (1992) for improved chromophore stabilization.

In vitro dry-matter digestibility (IVDMD) of whole-plant samples was determined by the Goering & Van Soest (1970) modification of the two-stage Tilley & Terry (1963) procedure, in which neutral-detergent extraction of fermentation residues was substituted for acid-pepsin digestion. *In vitro* cell-wall digestibility (IVCWD) was calculated as $[(NDF_i - NDF_r)/NDF_i] \times 100\%$, where NDF_i represents the amount of NDF in the original sample and NDF_r represents the mass of the final NDF residue following 48 h fermentation and extraction with neutral-detergent solution. Ruminal fluid used for *in vitro* incubations was obtained 3 h postprandial from a ruminally fistulated dairy cow (*Bos taurus*) fed Bermudagrass hay (*Cynodon dactylon*) *ad libitum* and limited quantity of a grain-concentrate mix containing rolled and cracked corn, cottonseed hulls, hominy feed, soybean meal, cottonseed meal, distillers' dried grains, soybean hulls, citrus pulp and minerals. All surgical procedures, post-surgical care, experimental protocols and maintenance of the cow were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The overall design was a split plot, replicated three times (three blocks of four treatment rings each). Whole-plot treatments were the treatment rings (O_3, CO_2, $CO_2 + O_3$ and controls), and subplots were the two clover species (Red Clover and White Clover).

Data were analysed using ANOVA and regression techniques (JMP, JMP IN 5·1, SAS Institute, Cary, NC, USA). Whole-plot treatments were partitioned into the following three orthogonal contrasts: (1) (control + O_3) *vs* [CO_2 + (CO_2 + O_3)]; (2) (control + CO_2) *vs* [O_3 + (CO_2 + O_3)]; and (3) [control + (CO_2 + O_3)] *vs* (O_3 + CO_2). These single-df contrasts provided independent tests for effects of CO_2, O_3 and $CO_2 + O_3$, respectively.

Results

Rings with elevated CO_2 had average concentrations of 516, 518 and 533 μl l^{-1} CO_2 during daylight hours in late May and the entire months of June and July, respectively, prior to sample collection. Mean background ambient CO_2 concentrations during daylight hours were 378, 376 and 368 μl l^{-1}, respectively, during these periods. Rings with elevated O_3 had average concentrations of 36·2, 47·5 and 43 ηl l^{-1} O_3 (≈1·2 × ambient), respectively, during daylight hours in these periods. Mean background ambient O_3 concentrations during daylight hours in the same periods were 35·8, 34 and 34·7 ηl l^{-1}, respectively. In addition to gas concentrations monitored in each ring, meteorological conditions at 2 m above ground surface were observed in four of the 12 rings, one ring per gas treatment. Across these rings, average temperature was 10·6, 15·6 and 18·0 °C, and average maximum photosynthetically active radiation (PAR) was 1·45, 1·49 and 1·39 mmol m^{-1} s^{-1} in May, June and July, respectively. Total precipitation from May to July was 205 mm.

Significant differences were observed between Red and White Clover for all dependent variables, and significant O_3 effects were observed for lignin concentration, IVDMD and IVCWD (Table 1). Foliar

Table 1. *P* values for selected nutritive quality indices in Red and White Clover at Aspen FACE site for data collected in August 2004

Source	df	*N*	NDF	Lignin	Soluble phenolics	IVDMD	IVCWD
Block	2	0·3290	0·1835	0·0048	0·9895	0·8214	0·4548
Treatment	3	0·4123	0·3238	0·0002	0·4776	0·0458	0·1677
O_3	1	0·1365	0·3475	0·0001	0·5043	0·0101	0·0367
CO_2	1	0·5467	0·3373	0·3916	0·2353	0·5514	0·9656
O_3CO_2	1	0·9845	0·1917	0·2380	0·4649	0·3073	0·9015
Species	1	0·0002	<0·0001	<0·0001	<0·0001	<0·0001	0·0113
Species × treatment	3	0·3159	0·3453	0·0702	0·3153	0·1778	0·5476

Treatment effect separated into single-df orthogonal contrasts. Block and treatment effects tested by block × treatment (6 df); species and species × treatment tested by subplot error (8 df).
NDF, neutral-detergent fibre; IVDMD, *in vitro* dry-matter digestibility; IVCWD *in vitro* cell-wall digestibility.

Table 2. Means of selected nutritive quality indices by clover species for data collected at Aspen FACE site in August 2004

Clover species	*N* (g kg^{-1})	NDF (g kg^{-1})	Lignin (g kg^{-1})	Soluble phenolics (g kg^{-1})	IVDMD (g kg^{-1})	IVCWD (g kg^{-1})
Red	37·7	560·4	175·1	3·4	564·5	223·0
White	33·7	491·6	143·7	4·8	637·8	266·8

Statistical analyses and significant responses as reported in Table 1.
NDF, neutral-detergent fibre; IVDMD, *in vitro* dry-matter digestibility; IVCWD *in vitro* cell-wall digestibility.

Table 3. Means of selected nutritive quality indices in Red and White Clover by treatment for data collected at Aspen FACE site in August 2004

Treatment	*N* (g kg^{-1})	NDF (g kg^{-1})	Lignin (g kg^{-1})	Soluble phenolics (g kg^{-1})	IVDMD (g kg^{-1})	IVDMD (g kg^{-1})
Control	34·1	532·8	144·2	4·4	611·5	271·1
CO_2	35·0	509·8	143·2	3·4	623·8	276·8
O_3	36·4	528·7	172·0	4·3	585·9	218·5
CO_2O_3	37·2	532·6	178·2	4·0	581·1	215·8

Statistical analyses and significant responses as reported in Table 1.
NDF, neutral-detergent fibre; IVDMD, *in vitro* dry-matter digestibility; IVCWD *in vitro* cell-wall digestibility.

concentrations of N, NDF and lignin were higher, whereas the concentration of soluble phenolics was lower in Red than in White Clover (Table 2). There was no effect of CO_2 on the foliar concentration of any chemical constituent, but lignin concentrations were higher in the O_3 and $CO_2 + O_3$ treatments than in the control and CO_2 treatments (Table 3). No significant air treatment × clover species interactions were detected, although lignin concentration in Red Clover that had been exposed to elevated CO_2 (160 mg g^{-1} DM) tended ($P < 0·07$) to be higher than in the control treatment (152 mg g^{-1} DM), whereas the opposite pattern was observed in White Clover that had been exposed to elevated CO_2 (126 mg g^{-1} DM) compared with the control treatment (136 mg g^{-1} DM). Across clover species and air treatments, there was a positive relationship ($r^2 = 0·57$, $N = 24$, $P < 0·001$) between concentrations of lignin and NDF.

White Clover had higher IVDMD and IVCWD than did Red Clover (Table 2), and the O_3 and $CO_2 + O_3$ treatments had lower IVDMD and IVCWD than did the control and CO_2 treatments (Table 3). Across clover species and air treatments, negative relationships were observed between IVDMD and NDF concentration ($r^2 = 0·66$, $N = 23$, $P < 0·0001$); IVDMD and lignin concentration ($r^2 = 0·71$, $N = 23$, $P < 0·0001$); and IVCWD and lignin concentration ($r^2 = 0·38$, $N = 23$, $P < 0·002$). Concentration of NDF averaged 526 mg g^{-1} DM across clover species and air treatments, and variability in IVDMD was largely attributable ($r^2 = 0·60$, $N = 23$, $P < 0·0001$) to IVCWD.

Discussion

The Aspen FACE site is unique in that it enables investigation of long-term effects of CO_2 and O_3, singly and in combination, on ecosystem processes and attributes from seedling establishment onward (Karnosky *et al.* 2005). Because the communities are not enclosed, there is no significant modification of the ambient environment other than from elevation of these trace atmospheric gases. Also, the communities are sufficiently

large to enable investigation of effects that are often difficult to detect in small chambers (e.g. competition, nutrient fluxes, pest epidemiology), and the state-of-the-art fumigation system is devoid of artefacts that often occur in small-chamber systems such as low winds, above-ambient temperatures, altered hydrology, increased humidity and reduced light levels (Karnosky *et al*. 2005).

Meteorological conditions in understoreys of the Aspen–Sugar Maple communities were characteristic of a cool, moist northern-latitude environment to which Red and White Clover are well adapted. While there were no significant air treatment × clover species interactions detected for any of the nutritive quality variables measured, Red Clover would be expected to have a 22% lower mean food value (capacity to support intake of digestible dry matter) than White Clover across all air treatments, based on observed values for IVDMD and predicted DM intake calculated from concentrations of NDF (Rohweder, Barnes & Jorgensen 1978). Nutritive quality is affected in large measure by plant maturity, and the decline in forage quality with age results primarily from increasing stem : leaf ratio and foliar concentrations of cell-wall constituents (Van Soest 1994). Across all air treatments, chemical composition and *in vitro* digestibility of Red and White Clover were characteristic of these species at mid- to full bloom; therefore nutritive quality was lower than would be expected for less-mature forage earlier in the growing season. While N concentration was greater for Red than White Clover, it was sufficiently high in both forages so as not to be limiting to fibrolytic activity of ruminal micro-organisms or to host-animal physiological processes (Van Soest 1994). Also, while there is quite extensive evidence for detrimental effects of low molecular-weight, soluble phenolics on fibrolytic digestion and animal physiological processes (Fahey & Jung 1989), the small difference in concentrations of soluble phenolics between Red and White Clover would not be expected to be biologically significant.

A diversity of findings has been reported with respect to CO_2 and O_3 effects on forage nutritive quality, and a lack of consensus has yet to emerge from the few studies published thus far. Investigators have utilized different methods and criteria for assessing forage quality, which explains differences in results to some degree. To illustrate, forage quality was not affected by CO_2 (Schenk, Jäger & Weigel 1997) or O_3 (Fuhrer *et al*. 1994; Pleijel *et al*. 1996) in experiments that employed the Weende crude-fibre methodology, which results in incomplete recovery of lignin and hemicellulose and thus nullifies the statistical association that exists between forage concentration of total cell wall constituents and its nutritive quality for ruminants (Van Soest 1994). In other studies, some investigators have included non-structural carbohydrates (NSC) in their assessment of forage-quality response to CO_2 and/or O_3, with variable relationships observed with respect to NDF concentration and *in vitro* digestibility. While forage concentration of NSC may be positively correlated with digestibility when there is a concomitant negative correlation of each of these with NDF concentration (Picon-Cochard *et al*. 2004), NSC are generally much less important quantitatively than cell-wall constituents as a source of energy from forages for ruminants. Coupled with the fact that NSC are rapidly and completely fermented by ruminal micro-organisms, concentration of NSC is not a good statistical predictor of nutritive quality in terms of intake of digestible DM (Van Soest 1994). These concepts are illustrated by the findings of Lilley *et al*. (2001), who reported that concentration of NSC in *T. subterraneum* was increased by an average of 28% in response to elevated CO_2, but *in vitro* digestibility was unaffected. Likewise, Heagle *et al*. (2002) reported that CO_2 enrichment caused a linear increase in NSC concentration in *T. repens*, but there was no effect on NDF concentration or *in vitro* digestibility.

We hypothesized that clover growing under elevated CO_2 would have decreased concentration of N and/or increased concentrations of secondary compounds such as lignin and low molecular-weight soluble phenolics (Rogers *et al*. 1994; Booker *et al*. 2005), resulting in decreased digestibility and nutritive quality. Conceivably, elevated CO_2 concentrations in the present study (≈1·4 × ambient) may have been too low, and/or the experimental exposure period too short, to elicit a foliar chemistry response of sufficient magnitude to be reflected in altered nutritive quality. Some experiments have shown that elevated concentrations of CO_2 caused very little change in digestibility (Fritschi *et al*. 1999; Lilley *et al*. 2001), while others (Owensby *et al*. 1996; Morgan *et al*. 2004) have shown a pronounced decrease in digestibility only when foliage was exposed to higher CO_2 concentrations (e.g. 2 × ambient, > 700 μl l^{-1}) and over longer periods (e.g. an entire growing season) than those utilized in the present study. In contrast, elevated O_3 concentrations in the present study were within a range of concentrations (40–60 ηl l^{-1}) reported by Fuhrer (1997) consistently to cause yield reductions of ≤ 10% in managed grass–clover pasture compared with lower reference exposures (20–30 ηl l^{-1}). Chronic exposure to relatively low concentrations of O_3 with periodic, random occurrences of peak concentrations or episodes can also cause accelerated foliar senescence, reflected by increased concentrations of cell-wall constituents measured as NDF (Muntifering *et al*. 2000). Furthermore, O_3 exposure can increase activities of plant metabolic pathways leading to synthesis of lignin and related phenolic compounds that are negatively associated with food value for herbivores (Krupa *et al*. 2004).

The NDF fraction consists of partially, non-uniformly digestible cell-wall constituents, primarily the β-linked polymers cellulose and hemicellulose (Van Soest 1994). Because these structural carbohydrates resist hydrolytic digestion by mammalian enzymes, the NDF fraction is digested slowly by microbial

fermentation. Also, the plant cell-wall fraction requires extensive mastication and rumination in order to reduce particle size for passage through the gastrointestinal tract, thereby increasing mean retention time of food residues compared with less-fibrous substrates. While NDF is more closely associated with intake than with digestibility (Van Soest 1994), concentration of NDF did account for two-thirds of the variability in IVDMD across clover species and air treatments. However, to the extent that there was no effect of O_3 on concentrations of NDF, the statistical association between NDF and IVDMD does not provide as satisfactory an explanation of the negative effect of O_3 on IVDMD as it does for the lower IVDMD of Red than White Clover. Clearly, the O_3 effect on IVDMD is more readily explained on the basis of increased lignin concentration and the effect of increased cell-wall lignification on IVCWD. Similarly, Powell *et al*. (2003) reported that Little Bluestem (*Schizachyrium scoparium*) exposed to elevated O_3 had decreased IVDMD and IVCWD that could not be explained on the basis of NDF concentrations alone. Based on observed values for IVDMD and predicted DM intake calculated from concentrations of NDF (Rohweder *et al*. 1978), clover exposed to elevated O_3 and $CO_2 + O_3$ would be expected to have a 7% lower mean food value compared with clover receiving the control and elevated CO_2 treatments.

Due to their role in the nutritional ecology of ruminant herbivores as negative modifiers of intake and digestibility, we were interested in ascertaining whether soluble phenolic compounds accumulated in clover exposed to elevated O_3 and/or CO_2, as has been reported for numerous plant species (Heath 1994; Rogers *et al*. 1994). There was no effect of elevated O_3 or CO_2 on concentration of soluble phenolics, but there was a trend ($r^2 = 0{\cdot}15$, $N = 21$, $P < 0{\cdot}08$) of a negative relationship between concentrations of lignin and soluble phenolics. Powell *et al*. (2003) observed a similar pattern in Sericea Lespedeza (*Lespedeza cuneata*) exposed to elevated O_3, which the authors hypothesized was due to action of polyphenol oxidases on soluble phenolics to initiate accumulation of lignin in the plant cell wall, thereby decreasing digestibility of NDF and plant total DM.

In the present study, exposure to elevated O_3 under free-air conditions caused reductions in the digestibility and nutritive quality of clover comparable with those (average 5–15%) observed in controlled-environment (e.g. chamber, glasshouse) studies with other plant species (Krupa *et al*. 2004), but these were not ameliorated by exposure to elevated CO_2 as has been observed for physiological processes reflected in plant growth and biomass production (Fuhrer 2003). Reductions in digestibility and nutritive quality of clover were of sufficient magnitude to predict nutritional and possibly economic consequences of its utilization by ruminant herbivores under existing and projected climate scenarios. Given that elevated O_3 concentrations in the present study lay within the range of present-day ambient concentrations typically found in the Northern Hemisphere, where clover is a significant food resource for ruminants, environmentally relevant concentrations of CO_2 projected for the first half of the current century would not be expected to impart a protective effect against nutritive quality reductions caused by elevated O_3.

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