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Digestive utilization of ozone-exposed forage by rabbits (*Oryctolagus cuniculus*)

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ABSTRACT

A mixture of common Southern Piedmont (USA) grassland species (*Lolium arundinacea*, *Paspalum dilatatum*, *Cynodon dactylon* and *Trifolium repens*) was exposed to O_3 [ambient (non-filtered; NF) and twice-ambient (2X) concentrations] and fed to individually caged New Zealand white rabbits (*Oryctolagus cuniculus*) in a digestibility experiment. Forages and feed refusals were analyzed for concentrations of total cell wall constituents, lignin, crude protein, and soluble and hydrolyzable phenolic fractions. Neutral detergent fiber and acid detergent fiber digestibility by rabbits were significantly lower for 2X than NF forage. Decreased digestibility could not be attributed to lignin concentrations, but was associated with increased concentrations of acid-hydrolyzable and saponifiable phenolics. Exposure of forage to elevated O_3 resulted in decreased digestible dry matter intake by rabbits. Elevated O_3 concentrations could be expected to have a negative impact on forage quality, resulting in decreased nutrient utilization by mammalian herbivores in Southern Piedmont grasslands under projected future climate scenarios.

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1. Introduction

Tropospheric (ground-level) ozone (O_3) is the most significant phytotoxic air pollutant affecting vegetation in the United States (US EPA, 2006), and it is widespread globally (Chameides et al., 1994). Ozone is a secondary air pollutant that is formed in the presence of sunlight by photochemical reactions between oxides of nitrogen and volatile organic compounds, both of which are products of natural and anthropogenic processes (National Research Council, 2004). Tropospheric O_3 is also a greenhouse gas, and models predict that concentrations will increase on a global basis by approximately 20% over the next 20 years (Vingarzan, 2004). The southeastern USA has a warm climate and dense vegetative cover, a source of organic hydrocarbons that contribute to production of tropospheric O_3 (Chameides et al., 1988; Chameides and Cowling, 1995).

Ozone-sensitive plant species are important with respect to energy and nutrient cycling between trophic levels and the atmosphere, and as forage resources for domestic and wild grazing animals (Fuhrer, 1997; Ditchkoff et al., 2009). Increasing concentrations of O_3 may detrimentally affect certain O_3-sensitive plant species in grasslands (Ashmore and Ainsworth, 1995; Mills et al., 2009). These effects potentially result in alterations in community structure and function (Barbo et al., 1998; Volk et al., 2006), thus decreasing biodiversity and altering the natural state of grassland communities. Climate change also plays a major role in this process. Increasing O_3 concentrations in combination with climate change variables such as rising temperatures and altered precipitation events may cause alterations in species composition and nutritive quality in grassland communities (Suttle et al., 2007; Gilliland, 2011).

Exposure of sensitive plant species to elevated O_3 concentrations suppresses photosynthesis, accelerates senescence, causes visible foliar injury, and decreases biomass yields (Reich, 1987; Heck et al., 1988; Chappelka et al., 2003; Krupa et al., 2004; US EPA, 2006; Booker et al., 2009). Rebbeck et al. (1988) reported that elevated O_3 concentrations suppressed ladino clover (*Trifolium repens*) regrowth and starch reserves in a clover-tall fescue (*Lolium arundinacea*) pasture mixture. In a FACE (Free-Air CO_2 and O_3 Enrichment) experiment, exposure to elevated concentrations of O_3 resulted in increased concentrations of lignin and decreased *in vitro* cell-wall digestibility in *Trifolium* spp. by mixed-batch cultures of ruminal microorganisms (Muntifering et al., 2006).

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These O_3-induced changes in foliar chemistry can be expected to result in decreased intake and digestive utilization of nutrients and energy by ruminants due to decreased quality of herbaceous vegetation (Krupa et al., 2004). More specifically, due to increased lignification and potential increases in concentrations of secondary phenolic compounds in plant cell walls due to O_3 exposure, several grassland species have been shown to experience a loss of forage nutritive quality for ruminant herbivores (Bender et al., 2006; Lewis et al., 2006; Ditchkoff et al., 2009). Loss of nutritive quality can approach the same order of magnitude as observed for biomass yield depression (Krupa et al., 2004). However, a decrease in total consumable food value (fractional reduction in yield × fractional reduction in nutritive quality) is not currently considered in economic risk-assessment models under current and future global-climate scenarios (Gonzalez-Fernandez et al., 2008).

While a number of studies have documented the negative impact of O_3 on nutritive quality of forage crops and crop residues for ruminant herbivores as determined by *in vitro* incubation experiments and/or chemical composition using wet-chemistry methods (Powell et al., 2003; Gonzalez-Fernandez et al., 2008; Frei et al., 2010, 2011), there are no published reports on nutritive quality of O_3-exposed herbaceous vegetation that are relevant to non-ruminant herbivores, notably hindgut fermentors. In this regard, rabbits (*Oryctolagus cuniculus*) represent an ideal and ecologically relevant non-ruminant model herbivore for *in vivo* digestibility experiments, because they utilize similar selective foraging and digestive strategies as many wild and domestic mammalian herbivores, are common in most countries in the world, and readily utilize a high-roughage diet (McNitt et al., 1996). Rabbits are hindgut fermentors (Cheeke, 1987) that have a simple, non-compartmentalized stomach along with an enlarged cecum and colon inhabited by a fibrolytic microbial (primarily *Bacteroides* spp.) population (Irlbeck, 2001).

Based on the lack of published digestibility studies with live animals and nutritive quality responses to O_3 in non-ruminant herbivores, our goal was to quantify and characterize mechanistically the *in vivo* digestive utilization of O_3-exposed grass-legume forage in rabbits. Our overall hypothesis was that rabbits receiving forages exposed to elevated O_3 concentrations would have decreased intake and digestive utilization of forages compared with those receiving those grown under ambient O_3 concentrations. Specific objectives were to (1) determine dry matter consumption and utilization by rabbits fed O_3-exposed forage under controlled experimental conditions, and (2) relate these to effects of O_3 exposure on chemical composition of forages.

2. Materials and methods

2.1. Field site (forage) establishment

The Atmospheric Deposition Site of the School of Forestry and Wildlife Sciences at Auburn University, AL was utilized for field-fumigation. The site is located approximately 5 km from campus and is representative of a rural agricultural area. There are 24 large (4.8 m height × 4.5 m diameter) open-top chambers (OTC, Heagle et al., 1989) at this site, 12 of which were aerially seeded with a mixture of 3 grass and 1 legume species commonly found in the Southern Piedmont region of the USA. These species included tall fescue (*Lolium arundinacea*), dallisgrass (*Paspalum dilatatum*), common bermudagrass (*Cynodon dactylon*) and ladino (white) clover (*Trifolium repens*), which were selected to be representative of a grassland community managed for livestock and wildlife in the Southern Piedmont region of the US (Ball et al., 2002). Tall fescue, a cool season-adapted C_3 bunchgrass, and white clover, a cool season-adapted legume, were seeded in October 2007 at rates of 7.00 and 0.84 kg pure live seed (PLS)/ha, respectively. Prior to seeding, the site was prepared by mowing existing vegetation within each chamber, after which the stubble was treated with glyphosate. One week later, the soil was tilled and all dead vegetation was removed, then the ground surface was raked and seeded. Seeds were covered with approximately 1 cm soil and wheat straw. Each chamber was misted daily to facilitate germination. Dallisgrass and common bermudagrass, both warm season-adapted C_4 grasses, were seeded in March 2008 at rates of 4.20 and 2.80 kg PLS/ha, respectively. Seeding rates for all species were established on the basis of Alabama Cooperative Extension System recommendations, corrected for viability as determined by germination test, and designed to produce uniform stands of forage consisting of approximately equal proportions of each specie in late spring/early summer of the 2008 growing season. Using principal component analysis (Gomez and Gomez, 1984), 6 OTC (three chambers for each treatment) were selected for fumigation of forages with controlled concentrations of O_3 on the basis of uniformity of plant communities and soil characteristics. Non-filtered (NF) air was representative of ambient air in rural areas of the Southern Piedmont region (US EPA, 2001), and air containing 2 × ambient-O_3 concentration (2X; 2 × NF) was considered to be representative of concentrations currently found in the vicinity of large metropolitan areas in the Southeastern USA such as Atlanta and Birmingham (Chameides and Cowling, 1995). Consideration was given to chamber effects (Fuhrer, 1994), as the effective area was 0.5 m from the inside edge of the chambers.

2.2. O_3 fumigation system

Ozone fumigation was initiated on May 13, 2008 and continued through July 2, 2008. Ozone was generated by passing pure oxygen through a high-intensity electrical discharge source (Griffin Inc., Lodi, NJ), and was applied proportionally above ambient to the 2X chambers daily (0900–2100 h, 7 days/wk). Concentrations of O_3 were continuously monitored in each OTC, and US EPA quality assurance guidelines were used for calibration of the instruments. All six chambers were exposed to ambient concentrations of precipitation, as the chambers were open at the top to allow rainfall to reach the forages.

2.3. Sample (forage) collection and processing

Forages from each OTC were clipped to a height of 5 cm aboveground on June 9 and July 2, 2008, dried to constant weight in a forced-air oven at 55–60 °C, air-equilibrated and weighed. Seasonal distribution of forage growth varies among the species (Ball et al., 2002) such that a harvest at these times was expected to contain an optimal representation of cool-season C_3 grass, cool-season legume, and warm-season C_4 grasses. Also, based on previous research with the same or very similar species (Muntifering et al., 2000, 2006; Gonzalez-Fernandez et al., 2008), cumulative O_3 exposure by mid-summer is usually sufficient to expect that 2X forages would likely exhibit decreased nutritive quality in the Auburn, AL area as predicted by laboratory analysis. Forages from each of the 6 OTC (3 chambers per treatment) were bulked by treatment from each harvest and compressed into 50-g blocks using a hydraulic press.

2.4. Rabbit feeding trial

We procured (Myrtle's Rabbitry, Thompsons Station, TN) 16 freshly weaned, 8-week-old, half-sib female New Zealand white rabbits (*Oryctolagus cuniculus*) in early December 2009 and housed them at the Biological Research Facility, Division of Laboratory Animal Health, College of Veterinary Medicine at Auburn University. Purpose-bred, specific-pathogen-free laboratory rabbits were utilized in the feeding trial in order to reduce between-animal variation and achieve compliance with institutional standards of animal welfare and biosecurity. Rabbits were maintained individually in slatted-floor, stainless steel cages (61 × 76 × 46 cm), each with its own feeding trough. During the month of December 2009, rabbits were transitioned from a high-concentrate weanling diet to commercial pellets (Rabbit Chow® Complete Blend, Purina Mills LLC, Gray Summit, MO) fed ad libitum, and subsequently beginning in early January 2010 to NF forage grown in 2008. Following complete transition to NF forage in late January, each rabbit received four 50-g forage blocks daily, and daily voluntary consumption of NF forage by rabbits was monitored for consistency over a 2-week adaptation period. Ten rabbits exhibiting the most consistent forage intake and fecal output (dynamic steady-state) during this adaptation period were then stratified into five outcome groups based on body weight; within groups, one rabbit was randomly assigned to receive forage blocks grown under 2X-ambient O_3 conditions, and one to receive forage blocks grown under ambient-O_3 conditions (five rabbits/forage treatment). Beginning in early February 2010, rabbits received two 50-g blocks of experimental forage from the June 9, 2008 harvest each day at 1400 h; this daily forage allocation was established to maintain near-constant intake and steady-state conditions based on voluntary forage intake recorded during the previous 2-week adaptation period, and to minimize feed refusals (orts). Rabbits were offered forage blocks for 5 days, and water was available for *ad libitum* consumption. Rabbits were subsequently offered blocks of forage from the July 2, 2008 harvest in a 6-day digestibility experiment that consisted of quantitative collection of fecal output for each rabbit. Orts for each rabbit were collected each day at 1330 h and pooled for chemical analysis, and feces were separated from urine and recovered daily from steel trays located under the rabbits' cages.

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2.5. Laboratory analysis

Orts and fecal pellets were dried at 55–60 °C in a forced-air oven and weighed. Forages offered, orts, and feces were ground through a 1-mm screen in a laboratory mill (Thomas Wiley Co., Philadelphia, PA) and analyzed for concentrations of dry matter (DM) and crude protein (CP = $N \times 6.25$) according to procedures of AOAC (1995). Cell-wall constituents were analyzed by sequential detergent fractionation according to Van Soest et al. (1991). This method first removes soluble cell components such as non-structural carbohydrates, lipids, pectin, and soluble protein while isolating insoluble structural cell-wall components [neutral detergent fiber (NDF)] consisting primarily of β-linked carbohydrate polymers (cellulose and hemicellulose) and lignin. After NDF is obtained, the acid detergent fiber (ADF) fraction consisting primarily of cellulose and lignin is isolated by solubilizing hemicellulose and cell wall-bound protein, allowing subsequent isolation of lignin and other recalcitrant materials [acid detergent lignin (ADL); Van Soest et al., 1991]. Quantities and concentrations of chemical fractions (DM, CP, NDF and ADF) in forages fed, orts and feces were used in calculation of coefficients of apparent digestibility as follows: $[(\text{fraction}_{intake} - \text{fraction}_{excreted}) \div \text{fraction}_{intake}] \times 100\%$ where fraction_{intake} represents the quantity of chemical fraction consumed (quantity fed − quantity in orts) and $\text{fraction}_{excreted}$ represents the quantity of chemical fraction excreted in feces over the 6-day quantitative collection period (Van Soest, 1994). Digestible DM intake (DDMI) was calculated as DM intake (g/day) × coefficient of apparent DM digestibility (%).

Soluble phenolics in experimental forages and orts were assayed using a modified version of the Folin-Ciocalteu (FC) assay (Blum, 1997; Booker and Miller, 1998). Soluble phenolics were expressed as *p*-coumaric acid equivalents based on a standard curve obtained with *p*-coumaric acid using the FC method. Soluble phenolic fractions were extracted from sample residues (50 mg) with 1 ml of 250 mM citrate buffer containing 0.04% sodium bisulfite (3×), centrifuged at 15,000 × *g*, and supernatants pooled. Residues were washed with 1 ml of water (1×), 95% ethanol (3×), dried in a rotary evaporator at 45 °C, and weighed. Extractive-free residues were then incubated in either 1 ml of 2 N HCl at 80 °C for 1 h (acid hydrolysis) or 1 ml of 1*N* NaOH overnight (alkaline hydrolysis) at room temperature. Afterward, samples were centrifuged, supernatants collected, residues washed with 1 ml of water (2×), centrifuged, and supernatants pooled by sample. Aliquots (250 µl) of alkaline-hydrolysate samples were acidified with 25 µl of 6*N* HCl to precipitate acid-insoluble phenolics, centrifuged and supernatants collected. Each sample (50–100 µl) was assayed for total phenolics using the FC assay. All extractions and assays were conducted in duplicate. A protein precipitation assay of the samples for tannins (Waterman and Mole, 1994) was negative.

2.6. Experimental design and statistical analysis

The experimental design was completely randomized, with treatments assigned randomly to body weight-stratified outcome groups. Analyses were conducted using the R package for statistical analysis (R Development Core Team, 2010). Analysis of variance (ANOVA) techniques were used to analyze the data as well as paired *t*-tests. Linear regression analyses were used to examine relationships of digestibility coefficients with specific phenolic fractions in the experimental forages.

3. Results

3.1. Climatic and O_3 exposure data

Mean monthly air temperatures for May through July 2008 were, on average, 1.3 °C above the 30-yr (1971–2000) average for Auburn, AL (Table 1), and monthly precipitation values for May–July were 6.4 cm below the 30-yr average. Mean monthly 12-h NF and 2X O_3 concentrations ranged from 21–32 and 37–56 ppb (nl l^{-1}), respectively, over the 8-wk exposure period; slightly less compared with other similar studies in the Auburn, AL area (Ditchkoff et al., 2009; Powell et al., 2003). Mean peak 1-h O_3 concentrations were greater in May and June than July for both treatments (Table 2), and average peak concentrations across all months were 49 ppb and 102 ppb for the NF and 2X O_3 treatments, respectively (Table 2). Mean AOT40 values for the period May 13–June 9 were 1645 and 16,340 ppb h, and for the period June 10–July 2 were 1894 and 14,113 ppb•h for NF and 2X treatments, respectively (data not shown).

Table 1
Mean monthly air temperatures, rainfall amounts, and 30-yr (1971–2000) means from May 13–July 2, 2008; Auburn, AL, USA.[a]

Month	Air temperature (°C)		Precipitation (cm)	
	2008	30-yr avg.	2008	30-yr avg.
May	22.1	21.2	8.3	9.7
June	26.8	24.9	3.1	10.3
July	27.2	26.2	4.2	14.9

[a] Data obtained from Alabama Weather Information System (AWIS) Inc., Auburn, AL.

Table 2
Mean 12-h (0900–2100 h) ozone concentrations for the study duration May 13–July 2, 2008; Auburn, AL, USA.

Month[a]	O_3 concentration (ppb)					
	NF			2X		
	Mo. mean	Avg. peaks	1 h peaks	Mo. mean	Avg. peaks	1 h peaks
May	32	51	66	56	104	125
June	30	49	66	55	104	126
July	22	28	51	38	52	99
Season	30	49	67	55	102	126

[a] Duration = May 13, 2008–July 2, 2008; NF = non-filtered ambient air chambers; 2X = twice-ambient O_3 concentrations.

3.2. Forage chemical composition

Forage exposed to NF O_3 contained approximately 8% less concentration of NDF than 2X forage, but concentrations of ADF and ADL in the two forages were approximately equal (Table 3). The NF forage contained 22.1% greater concentration of CP, 15% greater concentration of soluble phenolics, and 18.5% lower concentration of acid-hydrolyzable phenolics than 2X forage. Concentration of alkaline-hydrolyzable phenolics in NF forage was 9.5% less than in 2X forage, while concentration of the acid-soluble fraction of the alkaline hydrolysates was 20% less in NF than 2X forage.

3.3. Digestive utilization of forage DM, cell-wall fractions and CP

Intake and apparent digestibility of forage DM were not significantly different between treatments, although DDMI was 5.5 g per day greater ($P < 0.05$) for rabbits receiving NF than 2X forage (Table 4). Apparent digestibility of CP was not significantly different between treatments. Acid detergent fiber was less ($P < 0.03$) digestible in 2X (20.08%) than NF (32.16%) forages (Table 5). Similarly, NDF was less ($P < 0.10$) digestible in 2X (31.7%) than NF (39.6%) forage.

Forage concentrations of soluble phenolics accounted for one- to two-thirds of the variability in digestibility of forage DM and cell-wall fractions. Regression of forage DM and ADF digestibility coefficients on forage concentrations of acid hydrolyzable phenolics indicated a negative relationship, with ADF digestibility significantly decreasing ($P < 0.05$, $r^2 = 0.41$) as concentration of acid

Table 3
Chemical composition of forages fed to New Zealand white rabbits (mg g^{-1} forage DM).

Chemical fraction[a]	NF	2X
NDF	510.3	556.6
ADF	306.8	308.2
ADL	53.1	51.0
CP	166.6	136.4
SP	58.7	50.9
ACHP	20.4	12.7
ALHP	49.0	53.7
ASALH	38.8	46.5

Phenolics are expressed as mg *p*-coumaric acid equivalents g^{-1} forage DM.
[a] NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin, CP = crude protein, SP = soluble phenolics, ACHP = acid-hydrolyzable phenolics, ALHP = alkaline-hydrolyzable phenolics, ASALH = acid-soluble alkaline hydrolysate.

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Table 4
Dry matter utilization by rabbits receiving NF and 2X forages.

Treatment[a]	DM intake (g/day)	DM intake (% of body wt)	DM apparent digestibility (%)	DDMI (g/day)
NF	62.78	2.05	52.17	32.58
2X	59.03	1.97	45.92	27.10
P-value	0.30	0.67	0.14	0.05*

[a] *Significance at $P < 0.05$; DM = dry matter, DDMI = digestible dry matter intake.

hydrolyzable phenolics increased (Table 6). Regression of DM ($P < 0.11$, $r^2 = 0.28$), NDF ($P < 0.10$, $r^2 = 0.30$) and ADF ($P < 0.02$, $r^2 = 0.49$) digestibility coefficients on forage concentrations of alkaline-hydrolyzable phenolics indicated negative statistical relationships. Similarly, regression of DM ($P < 0.09$, $r^2 = 0.32$), NDF ($P < 0.07$, $r^2 = 0.35$) and ADF ($P < 0.02$, $r^2 = 0.54$) digestibility coefficients on forage concentrations of acid-soluble, alkaline-hydrolyzable phenolics revealed negative statistical relationships.

4. Discussion

The objective of this study was to assess the effects of elevated O_3 concentrations on forage chemical composition, intake and digestibility by a mammalian (non-ruminant, hindgut fermentor) herbivore. This study is believed to be the first of its kind involving live-animal bioassay of digestive utilization of O_3-exposed forages.

Digestibility coefficients for DM, NDF and ADF in the present study compare very favorably with those reported elsewhere for fibrous substrates typically fed to rabbits (deBlas et al., 1986; Garcia et al., 1999). Based on concentrations of cell-wall constituents in forages offered and orts refused, NF and 2X forages in the present study would not be expected to differ materially in their relative feed value (RFV) predicted from concentrations of NDF and ADF. RFV is a mathematical index of the nutritive quality of forages that is reported by reference to a standard forage with an RFV of 100 that contains 53% NDF and 41% ADF (Rohweder et al., 1978; Linn and Martin, 1989). The NDF fraction of forage consists of partially and non-uniformly digestible cell-wall constituents (primarily cellulose and hemicellulose) that are inversely related to voluntary DM intake, whereas the ADF fraction includes the less digestible and indigestible (e.g., lignin) cell-wall constituents that are inversely related to DM digestibility (Van Soest, 1994). Thus, the lower the concentrations of NDF and ADF in forage, the greater is its predicted DDMI and nutritive quality, expressed as RFV. Calculated RFV was 118 and 109 for NF and 2X forage, respectively, which is reflective of a medium-quality grass/legume forage (Linn and Martin, 1989), and sustained daily consumption at a rate of 2% of body weight as observed in the present study would be sufficient for maintaining body condition and modest growth in rabbits (Irlbeck, 2001). Although treatment groups did not consume significantly different quantities (g) of DM/day, DDMI was 5.5 g/day greater for rabbits receiving NF than 2X forage because intake and digestibility of the former tended to be greater. DDMI integrates DM intake and DM digestibility, and represents the amount of digestible energy (DE) that is available post-absorptive for metabolism. Thus, rabbits receiving NF forage consumed more DE and absorbed more food energy for metabolism than those fed 2X forage. Coefficients of apparent digestibility of CP were not different between treatments, but the lower concentration of CP in 2X than NF forage could be expected to compound the negative effect of decreased DE intake on rabbit growth because of decreased intake of digestible CP (National Research Council, 1977).

Table 5
Coefficients of apparent digestibility (%) of cell wall constituents and crude protein in NF and 2X forages.

Treatment[a]	Neutral detergent fiber (NDF)	Acid detergent fiber (ADF)	Crude protein (CP)
NF	39.59	32.16	53.60
2X	31.66	20.08	51.21
P-value	0.10*	0.03**	0.67

[a] Significance = *$P < 0.1$; ** significance at $P < 0.05$.

Forages exposed to elevated O_3 concentrations have been reported to contain greater concentrations of lignin in some studies (Muntifering et al., 2000; Powell et al., 2003; Muntifering et al., 2006; Szantoi et al., 2007), but not in others (Booker and Miller, 1998), and lignin has a negative impact on forage digestibility by mammalian herbivores (Van Soest et al., 1991; Van Soest, 1994; Muntifering et al., 2000; Krupa et al., 2004; Frei et al., 2010). This negative relationship between lignin concentration and digestibility of total plant DM is not reflected explicitly in calculation of RFV, but it is implied from the negative relationship that exists between lignin concentration and digestibility of cell-wall constituents, of which lignin is a structural and analytical subset (Van Soest, 1994). Interestingly, in the present study there were significant differences in digestibility of NDF and ADF and in DDMI, but there was no difference in concentration of lignin between NF and 2X forages. Similarly, Powell et al. (2003) reported that little bluestem (*Schizachyrium scoparium*) exposed to elevated O_3 had decreased *in vitro* digestibility of both DM and cell-wall fractions that could not be explained on the basis of lignin concentration, suggesting involvement of other inhibitory factors (e.g., phenolics) that are not recovered in the cell-wall fractions isolated by the Van Soest detergent-fractionation scheme (Van Soest et al., 1991).

Phenolic compounds are the most widely distributed group of allelochemicals encountered by herbivores, and their accumulation represents a general response to a variety of biotic and abiotic stresses in many plant species (Kangasjärvi et al., 1994). Studies indicate that exposure to elevated O_3 can cause forages to accumulate increased concentrations of plant secondary metabolites in response to oxidative stress (Booker and Miller, 1998; Chen and Gallie, 2005; Booker et al., 2009; Frei et al., 2010). Phenolic compounds are also produced in response to herbivory (Waterman and Mole, 1994), and they can have deleterious effects on digestibility of forages (Fahey and Jung, 1989). Concentration of soluble phenolics was lower in 2X than NF forage, in agreement with a number of studies with the same or very similar forage species as those utilized in the current study (Muntifering et al., 2000, 2006; Powell et al., 2003), but in contrast with other reports of increased phenolics in O_3-exposed forage (Saviranta et al., 2010) and agricultural-crop residues (Frei et al., 2010, 2011). Decreased concentration of phenolics has been observed in species containing certain phenolic compounds that are degraded under environmental stress (Fletcher et al., 2005). An alternative mechanism that could explain a reduction in soluble phenolics in 2X forage is their conversion to insoluble phenolics. We have previously observed a negative relationship between concentrations of lignin and soluble phenolics in O_3-exposed forages (Muntifering et al., 2006) that we and others have theorized may result from up-regulation of polyphenol oxidases and polymerization of soluble phenolics to form insoluble core lignin (Kangasjärvi et al., 1994; Booker and Miller, 1998). Even though forage concentration of soluble phenolics accounted for one- to two-thirds of the variability in digestibility of forage DM and cell-wall fractions, their positive correlation is counterintuitive with nutritional theory, and for this reason fails to provide a satisfactory mechanistic explanation for decreased digestibility of 2X forage by rabbits in the present study.

In contrast to pre-gastric fermentation and particle size reduction characteristic of the ruminant digestive process, the relatively indigestible fibrous components of forages are separated out in the large intestine (colon) of the rabbit and voided in feces (Irlbeck,

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Table 6
Regression of DM, NDF, and ADF digestibility with forage concentrations of phenolic fractions.[a]

Phenolic fraction	DM digestibility				NDF digestibility				ADF digestibility			
	Intercept	Slope	R^2	P-value	Intercept	Slope	R^2	P-value	Intercept	Slope	R^2	P-value
Soluble	−0.011	0.009	0.34	0.08*	−0.301	0.011	0.44	0.04**	−0.669	0.016	0.62	0.01***
Acid hydrolyzable	0.707	−0.018	0.18	0.22	0.633	−0.023	0.22	0.17	0.711	−0.038	0.41	0.05**
Alkaline hydrolyzable	1.270	−0.015	0.28	0.11	1.275	−0.018	0.30	0.10*	1.668	−0.028	0.49	0.02**
Acid-soluble alkaline hydrolysate	0.884	−0.100	0.32	0.09*	0.830	−0.012	0.35	0.07*	0.964	−0.017	0.54	0.02**

[a] Significance = $^{*}P < 0.1$, $^{**}P < 0.05$, $^{***}P < 0.01$. DM = dry matter, NDF = neutral detergent fiber, ADF = acid detergent fiber.

2001). The sieving and selective rejection of fibrous feed from entry into the cecum may be accompanied by release of loosely bound, low-molecular-weight phenolic compounds from lignin by the action of microbial enzymes. Evidence of the inhibitory effects on fibrolytic ruminal microorganisms by acid-hydrolyzable phenolics that are loosely bound to non-core lignin via ester and ethereal linkages is quite extensive (Fahey and Jung, 1989). Alkaline hydrolysis also releases ester-bound phenolic acids and alkaline-soluble lignin in a ligno-carbohydrate complex that decreases digestibility by binding with cellulose in plant cell walls, forming strong bonds that are not as readily digested by microorganisms in the ruminant fore-stomach (Morrison, 1979; Pigden and Heaney, 1969). Given the absence of an O_3 effect on cell wall lignification in the present study, the significant negative relationships between hydrolyzable phenolics and NDF, ADF and DM digestibility in the present study indicate that inhibitory action of these non-lignin, cell wall-bound phenolic fractions might explain mechanistically the lower digestibility of O_3-exposed forages in a non-ruminant hindgut fermentor such as the rabbit.

Implications of lower digestibility and utilization of forages to nutrient and energy cycling in herbivore-dominated grasslands is important for survival of both plant and animal species (Ashmore, 2005; Booker et al., 2009). Greater concentrations of ground-level O_3 may be causing lesser amounts of nutrients and energy to be utilized by herbivores where these greater concentrations of O_3 are found. In the Southern US, nutrients (N, P, K) are thought to be limiting factors in the environment. If even less energy is utilized by herbivores due to intake of O_3-exposed forages, this could prove to be detrimental to the plant and animal species found in these grasslands.

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