Changes in Southern Piedmont Grassland Community Structure and Nutritive Quality Under Future Climate Predictions: Tropospheric Ozone and Altered Rainfall

by

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Abstract

Tropospheric (ground-level) ozone (O_3) is the most significant phytotoxic air pollutant in the United States. It is readily transported to rural agricultural and forested areas from urban centers. Estimating the effects of elevated ozone concentrations in combination with altered rainfall amounts is important for predicting effects of future climatic changes. The Southern Piedmont region spans 17 million hectares from central Virginia to eastern Alabama. There are numerous reports demonstrating ozone-induced injury to foliage, reduction in crop growth, and yield losses in economically important plants in this region. Only recently have there been studies to determine the effects of elevated ozone concentrations on wild plants. In addition, experiments have been conducted to explore ozone-induced alterations in nutritive quality regarding mammalian herbivores. To determine the effects of ozone on grassland forage species as well as effects on herbivore nutrition, two independent experiments were designed. In the first experiment several forage species common to the region were examined for effects of ozone exposure in combination with varying rainfall amounts: tall fescue (*Lolium arundinacea*), dallisgrass (*Paspalum dilatatum*), common bermudagrass (*Cynodon dactylon*), and ladino clover (*Trifolium repens*). These forages were exposed to two levels of ozone in a randomized split-plot experiment with three levels of precipitation. Plants were grown in open-top chambers from June – Sept 2009 under non-filtered (NF) and twice-ambient (2X) ozone concentrations. Precipitation regimes were in three blocks and represented average (30-yr average for Auburn, AL); high (+ 20% of average rainfall); and low (- 20% of average). These differing ozone concentrations and rainfall amounts were representative of future predicted climatic changes. Interestingly, primary-growth (representing cumulative ozone-exposure effects) grasses differed significantly in biomass over the growing season across precipitation and ozone treatments, with 2X treatments having greater biomass. The primary-growth grasses experienced little differences in nutritive quality over the growing season between ozone treatments. Regrowth (representing one month of exposure) grasses actually responded positively to ozone effects, with lower neutral-detergent fiber (NDF) concentrations and greater relative feed value (RFV) than NF-exposed grasses throughout the growing season. Mean nutritive quality at final harvest for regrowth grasses reflected similar effects, with RFV greater in 2X treatments. Primary-growth clover exhibited decreased nutritive quality in 2X treatments with increases in NDF, ADF, and significant increases in lignin concentrations. Regrowth clover exposed to 2X ozone treatments demonstrated decreased nutritive quality; i.e. increased concentrations of cell-wall constituents (ADF and NDF) and lower RFV in the final harvest as well as over the growing season. Total plant canopy cover also decreased more rapidly at the end of the growing season in 2X-exposed communities compared with NF communities. Decreases in nutritive quality of certain forages have implications for herbivores that rely on these species for energy and nutrient consumption. Also, if grassland communities contain forage species that are "sensitive" to greater concentrations of ozone, grassland community structure and diversity could be altered.

Regarding experiment two, we hypothesized that exposure to elevated ozone concentrations would alter the chemical composition of forages affecting nutrient utilization by lagomorphs. In a balance trial (feeding stalls in the lab), New Zealand White rabbits (*Oryctolagus cuniculus*) were fed forages grown under differing ozone levels, NF and 2X, and harvested from OTCs. The rabbit is an ideal and ecologically relevant model herbivore for these experiments because rabbits utilize similar selective foraging and digestive strategies as most wild and domestic mammalian herbivores. The feeding trial was initiated in January 2010, with rabbits receiving forages grown in different ozone-treatment chambers (NF and 2X) for a total of 10 days. Neutral-detergent fiber (NDF) and acid-detergent fiber (ADF) digestibility by rabbits were significantly lower for 2X than NF diets. The decreased digestibility was not attributed to lignin concentration, but was significantly correlated with increased saponifiable phenolic concentrations. Digestible dry matter intake (DDMI) of rabbits receiving 2X diets was also significantly decreased compared with rabbits receiving NF diets. Elevated ozone was determined to increase bound-phenolic concentrations in forages that were associated strongly to decreased digestibility of forages. Based on the above results from the two independent experiments, elevated ozone concentrations appear to have a negative impact on forage quality, resulting in decreased nutrient utilization by herbivores in Southern Piedmont grassland communities.

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I. Introduction and Literature Review

Ozone Concentrations

Ozone (O_3) present in the troposphere (ground-level) is the most significant phytotoxic air pollutant affecting vegetation in the United States (US EPA 2006). Tropospheric ozone has also become widespread worldwide (Chameides *et al.* 1994; US EPA 2006). It is formed by photochemical reactions in the presence of sunlight between oxides of nitrogen and volatile organic compounds, both of which are products of natural and anthropogenic processes (National Research Council 2004). Background levels of tropospheric ozone increased by approximately 0.5 to 2% per year in the mid-latitudes of the Northern Hemisphere between the years 1970-2000 (Vingarzan 2004), which could be causing a cumulatively greater effect regarding chronic exposures of plants (Krupa and Manning 1988). Although background concentrations of ground-level ozone decreased over the entire US in that span, in the last decade of the century ozone concentrations increased in the Southern US (US EPA 2001). The Southeastern US has a warm climate and dense vegetative cover, a source of organic hydrocarbons, which aid in the production of tropospheric ozone (Chameides *et al*. 1988; Chameides and Cowling 1995). Ozone can be transported from metropolitan areas to rural agricultural and forested areas that are important to crop and forestry production, and is one of six criteria air pollutants designated by NAAQS (National Ambient Air Quality Standards) listed by the Environmental Protection Agency (US EPA 2006).

Middleton (1956) was the first to report ozone as phytotoxic, and since ozone has been found to induce visible injury to herbaceous plant species and native forest trees, including broad-leaved species across the United States and Europe (Chappelka 1994; Innes *et al.* 2001). Ozone-sensitive plants occurring in the Southern Region of the US are at risk of being harmed by these increasing concentrations of ozone. These sensitive species are important with regards to energy cycling between trophic levels and with the atmosphere, and as forage resources for wild and domestic grazing animals (Fuhrer 1997; Krupa *et al.* 2004; Ditchkoff *et al.* 2009). Ozone, and other air pollutants, can also cause plants to become more susceptible to insect predation and other stress factors such as drought (Heck *et al.* 1988).

Climate Change

The earth's climate is constantly, albeit gradually, changing, and is projected to keep changing in the foreseeable future (Parmesan 2006; Christensen *et al.* 2007; IPPC 2007; US Climate Change Science Program 2008). Global circulation models (GCM) predict continual temperature increases for the next century due to greenhouse gas emissions into the atmosphere. The Canadian model predicts increases in the range of 1.7-5.5^{o}C by 2030 while the Hadley model predicts 1.0-2.3^{o}C by 2100 in the Southeastern US (MacCracken *et al*. 2001). These two models also predict increased total yearly rainfall amounts for the Southeastern US, although the Hadley model estimates 20% greater rainfall in the summer months while Canadian model predicts 10% less (MacCracken *et al.* 2001). Other models predict ground-level ozone concentrations to increase on a global basis by approximately 20% over the next twenty years (Thompson 1992; Vingarzan 2004) due to industrialization of rural countries (Chameides *et al*. 1994; Ashmore 2005). It is important to understand how anthropogenic impacts (global climate change) are affecting structure and nutritive quality of grasslands to ensure their survival and capacity to support wildlife and agricultural production.

Ozone Effects on Individual Plants

Numerous studies have reported ozone-induced injury to foliage, reduction in crop growth and decreases in biomass and species richness in cool-season grasses and clovers, agricultural crops, and some warm-season grasses (Blum *et al.* 1982; Fuhrer 1997; Reich 1987; Booker *et al.* 2009). Long-term (chronic) exposure to elevated ozone over an entire growing season has been found to lead to overall decreased plant growth, cover, and productivity (Barbo *et al.* 1998; Krupa *et al*. 2004). Exposure of certain sensitive plants to elevated ozone concentrations has also been reported to suppress photosynthesis and accelerate leaf senescence (Reich 1987; Krupa *et* al. 2001; Booker *et al.* 2009). Barbo *et al.* (1998) determined that ozone can cause a shift in community richness and evenness as well as visible foliar symptoms in blackberry (*Rubus cuneifolius*). Szantoi *et al.* (2007) reported ozone effects on purple coneflower (*Echinacea purpurea*) grown in open-top chambers (OTC), including: foliar injury, root and biomass reduction, and greater concentrations of cell-wall constituents that have negative impacts on digestion of forages (Van Soest 1994). In addition, Szantoi *et al.* (2009) reported elevated concentrations of ozone significantly altered foliar injury, productivity, and concentrations of cell-wall constituents in cutleaf coneflower (*Rudbeckia lacianata*) plants grown in OTC. In a study in Great Smoky Mountains National Park, Chappelka *et al.* (2003) determined ground-level ozone caused foliar injury and had other deleterious effects on cutleaf coneflower and crown-beard

(*Verbesina occidentalis*). In addition to visible injury and reductions in biomass and yield, ozone can affect plant reproduction. Chappelka (2002) found that blackberry flowering and fruit production was affected by ozone. Blackberry was reported to produce greater numbers of large and ripe fruits in carbon-filtered (CF) and ambient concentrations of ozone compared with blackberry exposed to 2X concentrations of ozone.

Ozone Effects on Plant Communities

Increasing concentrations of ozone can have detrimental effects on certain "sensitive" species in plant communities having lower thresholds for ozone tolerance (Ashmore and Ainsworth 1995), with potential to cause shifts in community structure and function (Barbo *et al.* 1998; Gonzalez-Fernandez *et al.* 2008). Plant community canopies are major sites of pollutant deposition and can cause adverse effects on terrestrial landscapes (Heck *et al.* 1988*)*. Even with knowledge of effects on individual species, ozone's influence on whole-plant communities is not well understood (Barbo *et al.* 1998). Heagle *et al.* (1989) studied a clover-fescue pasture and determined that ladino clover growth was diminished due to ozone and water stress, while tall fescue (*Lolium arundinacea*) growth increased. Gonzalez-Fernandez (2008) reported that white clover nutritive quality and cover were diminished due to ozone, while ryegrass colonized areas left by the diminished clover. Barbo *et al.* (1998) reported ozone effects on total community cover, evenness, and richness, with impacts on several grassland species including: *Rubus cuneifolius, Paspalum notatum,* and *Panicum spp*. It is important that whole-plant communities be examined and the effects of ozone-exposure be quantified to gain knowledge of interspecific differences in response to ozone (Davison 1998; Heagle *et al.* 1991). Increasing ozone concentrations in combination with climate change variables such as increasing temperatures and altered precipitation events have the potential to cause shifts in species composition in grassland communities (Suttle *et al.* 2007). Lewis *et al.* (2006) observed varying responses to ozone in two important species of warm-season grasses used in grazing pastures and as a resource for wildlife species. Big bluestem (*Andropogon gerardii*) generally was not affected by ozone, while eastern gamagrass (*Tripsacum dactyloides*) grown simultaneously in the same open-top chambers had decreased nutritive quality in one growing season. Barbo *et al*. (2002) reported varying effects of ozone on seedlings of loblolly pine (*Pinus taeda*), which is a forest species potentially affected by ozone (Chappelka *et al.* 1990; Chappelka and Samuelson 1998; Skelly 2000). Contrary to other studies, Barbo *et* al. (2002) found that pine biomass was least when grown in a competitive environment in carbon-filtered (CF) chambers that represented sub-ambient levels of ozone. Barbo *et al.* (2002) reported the low pine biomass in CF chambers was due to competition from other sensitive species that out-competed pine in the pristine air.

The exposure-response of plants in the field is complex and is impacted by biotic and abiotic factors, including micro-climatic variables such as soil moisture (Reich 1987; Ball *et al.* 2002). It is important to understand differences in timing and duration of exposures to ozone, as chronic exposures (yield losses) are important to crop production and forests (Krupa and Manning 1988). Detrimental effects on plants have the potential to cause a loss of certain species in grasslands (Ashmore and Ainsworth 1995; Barbo *et al.* 1998; Gonzalez-Fernandez *et al.* 2008) which could eventually decrease biodiversity and alter the natural state of grassland communities.

Ozone Effects on Ruminant Nutrition

Recently, studies have been conducted to explore ozone-induced alterations in nutritive quality for ruminant animals. There is evidence that ozone-induced changes in foliar chemistry can result in decreased utilization of nutrients and intake of energy by ruminant herbivores due to decreases in the quality of herbaceous vegetation (Krupa *et al.* 2004). Muntifering *et al.* (2000) reported that ozone had deleterious effects on nutritive quality of bahiagrass (*Paspalum notatum*) planted early (May) during the growing season and these effects were sufficient enough as to possibly cause nutritional and/or economical losses in ruminant animal production due to decreases in dry matter digestibility and intake (Linn and Martin 1989; Van Soest 1991). Bender *et al.* (2006) grew Kentucky bluegrass (*Poa pratensis*) in pots (mesocosms) alone and in competitive mixed cultures and found that it experienced early season (April and May) foliar injury and decreased relative food value (RFV; Rohweder *et al.* 1978) due to ozone exposure. In a FACE (Free-Air CO_2 and O_3 Enrichment) experiment, Muntifering *et al*. (2006) determined exposure to elevated concentrations of ozone resulted in increased concentrations of lignin and decreased cell-wall digestibility of *Trifolium* spp. in Rhinelander, WI. Powell *et al.* (2003) observed that elevated ozone decreased the nutritive quality of two important agricultural and wildlife warm-season forages: *Sericea lespedeza* and little bluestem (*Schizachyrium scoparium*). Gonzalez-Fernandez *et al*. (2008) determined white clover (*Trifolium repens*) nutritive quality was decreased by exposure to ozone due to increases in concentration of cell wall constituents. More specifically, due to increased lignification and possibly increases in concentrations of secondary phenolic compounds of plant cell walls, many grassland species have been shown to experience decreased nutritive quality for mammalian herbivores (Bender et al. 2006; Lewis et al. 2006; Booker 2009). Ditchkoff *et al.* (2009) reported ozone effects on highbush blackberry that resulted in decreased relative food value of the plant. These changes were sufficient enough that it could potentially affect feeding behaviors of mammalian herbivores in the wild. Current economic risk-assessment models do not consider the potentially significant effect of elevated ozone concentrations in the troposphere on the consumable food value of herbaceous vegetation for these animals (Gonzalez-Fernandez *et al.* 2008). Still, little is known about the effect that elevated ozone concentrations is having on wildlife species (Lewis *et al*. 2006; Ditchkoff *et al*. 2009). Furthermore, previous experiments with ozone report digestibility as IVDMD (*in-vitro* dry matter digestibility) or IVNDFD (*in-vitro* neutral detergent fiber digestibility), (Powell *et al.* 2003; Muntifering *et al.* 2006) which are mathematical indices of forage, not actual digestibility coefficients as calculated after a feeding trial with live animals.

Overall Goal

To our knowledge, there is no study that has considered physiological and community responses to increasing ozone concentrations in concert with differing rainfall amounts as suggested by several global climate models (MacCracken *et al*. 2001). This study will advance our knowledge of the effects of rainfall amounts in combination with different levels of ozone-exposure on local plant species and eventually on plant/animal interactions as well. We also hoped to gain further knowledge about the exposure-response of certain common grassland species in the Southeastern United States: tall fescue (*Lolium arundinacea*), dallisgrass (*Paspalum dilatatum*), common bermudagrass (*Cynodon dactylon*), and ladino clover (*Trifolium repens*). Studies suggest tall fescue is relatively insensitive to ozone (Richards *et al.* 1980; Flagler and Youngner 1985; Rebbeck *et al.* 1988) and it can even thrive when grown in combination with an ozone-sensitive species such as white clover (Rebbeck *et al.* 1988; Heagle *et al.* 1989). Bermudagrass is also thought to be insensitive from a study that found little effects of ozone on it compared with cool-season grasses (Richards *et al*. 1980). The relative sensitivity of dallisgrass is unknown, although Muntifering *et al.* (2000) reported that bahiagrass (*Paspalum notatum*), same genus as dallisgrass, grown in pots under controlled conditions, experienced declines in dry matter yield and quality of regrowth.

Due to the absence of nutritional studies with live animals and responses to ozone-exposed forages, our goal was to relate nutritional results of *in vitro* digestibility studies (Muntifering *et al.* 2000; Powell *et al*. 2003; Lewis *et al*. 2006; Gonzalez-Fernandez *et al*. 2008; Ditchkoff *et al.* 2009) by conducting a feeding trial with lagomorphs. Rabbits (lagomorphs) are relatively inexpensive and arguably an ideal and ecologically relevant model herbivore for a feeding trial to study the effects of ozone on forage digestibility because they utilize similar selective foraging and digestive strategies as most wild and domestic mammalian herbivores (Cheek 1987; Irlbeck 2001). Domestic rabbits (*Oryctolagus cuniculus*) are also found in most countries in the world and can utilize a low-grain and high-roughage diet with a fast transit time (19 hours) (McNitt *et al.* 1996). Rabbits are considered concentrate selectors (Cheeke 1987) and have a simple, non-compartmentalized stomach along with an enlarged cecum and colon inhabited by a microbial population (*Bacteroides*) (Irlbeck 2001). Rabbits receiving forages exposed to elevated ozone concentrations were predicted to have different intake, digestive utilization, and metabolism of energy when compared with rabbits receiving forages grown under ambient ozone concentrations.

To accomplish the above goals two separate experiments, one field and the other laboratory based, were developed. The overall objective of the field fumigation experiment was to examine the effects of elevated ozone alone and in combination with altered rainfall amounts on several species of plants found in Southern Piedmont grassland communities. The forages in this study were grown in OTC and exposed to two levels of ozone with three levels of precipitation in competitive growth environments over an entire growing season (May to September). Nutritive quality was calculated monthly, based on foliar concentrations of neutral-detergent fiber (NDF), acid-detergent fiber (ADF), acid-detergent lignin (ADL), and crude protein (CP).

The main objective of the laboratory based (feeding trial) experiment was to quantify ozone effects on forage nutritional value for mammalian herbivores. Nutritive quality characteristics of forages (diets and orts) [i.e., cell-wall constituents and phenolics] and digestibility coefficients by rabbits were determined after completion of the feeding trial. The information from both experiments will provide valuable knowledge in regards to setting a secondary NAAQS (national ambient air quality standard) based on ecological impact of tropospheric ozone in the Southeastern United States.

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II. Changes in Southern Piedmont Grassland Community Structure and Function Under Future Climate Predictions: Tropospheric Ozone and Altered Rainfall

Summary

Several species common to the Southern Piedmont region [tall fescue (*Lolium arundinacea),* dallisgrass (*Paspalum dilatatum),* common bermudagrass (*Cynodon dactylon*), and ladino clover (*Trifolium repens*)] were investigated for effects of ozone exposure in combination with varying rainfall amounts. Plants were seeded in twelve open-top chambers (OTC) in March 2007 and exposed to two levels of ozone in a randomized split-plot experiment with three levels of precipitation from June – Sept 2009. Ozone treatments included non-filtered (NF) and twice-ambient (2X) ozone concentrations. Precipitation regimes were in three blocks and represented average (30-yr average for Auburn, AL), high (+ 20% of average rainfall), and low (- 20% of average) for the area. Primary-growth (representing cumulative ozone-exposure effects) and regrowth forages (plots representing one month of regeneration) were harvested from each chamber starting one month (June 30) after fumigation began. Grasses were combined in each chamber to obtain effects based on type of forage (grasses vs. legumes), until the final harvest where an additional harvest separated grasses by species. Dry matter (DM) yield did not differ over the growing season in forage types with exception of primary-growth grasses for which DM yield was greater in 2X than NF treatments. In the final harvest, DM yield was greater in regrowth grasses and less in regrowth clover for 2X than NF treatments. Regrowth grasses actually responded

positively to 2X exposure, with greater relative food value (RFV) and percent crude protein (CP) than NF-exposed regrowth grasses over the growing season. Final harvest nutritive quality means for regrowth grasses reflected similar effects with greater RFV in 2X treatments. Primary-growth clover, however, decreased in nutritive quality in 2X treatments with increases in neutral detergent fiber (NDF), acid detergent fiber (ADF), and significant increases in lignin (ADL) concentrations over the entire growing season. Regrowth clover exposed to 2X treatments exhibited decreased nutritive quality; i.e., increased concentrations of cell-wall constituents (ADF and NDF) and lower RFV, in the final harvest as well as over the growing season. Regrowth clover in general experienced the greatest decrease in nutritive quality, while the grasses were not adversely affected by 2X ozone. Precipitation did not have a significant effect over the growing season on primary or regrowth forages except in primary-growth grasses for which DM yield was greater in chambers with high precipitation. Total canopy cover was significantly greater over the growing season in chambers receiving above-average (+ 20%) rainfall amounts. Canopy cover increased early in the growing season and decreased in September. Decreases in nutritive quality and canopy cover in combination with fluctuations in DM yield over the growing season of certain forages have implications to herbivores that rely on these species for energy and nutrient consumption. Plant community structure and function can also be altered with shifts in energy allocation in species producing more cell-wall constituents as the result of ozone-exposure and differing rainfall amounts.

Introduction

Tropospheric (ground-level) ozone is a phytotoxic air pollutant that is globally widespread and can be transported from metropolitan areas to rural agricultural lands

(Chameides *et al.* 1994; Ashmore 2005). Ground-level ozone is formed by photochemical reactions between oxides of nitrogen from natural and anthropogenic sources and volatile organic compounds in the presence of sunlight (National Research Council 2004). Middleton (1956) was the first to report ozone as phytotoxic, and since then the US EPA (2006) has recognized ozone as the most significant phytotoxic air pollutant affecting vegetation in the United States. The Southeastern US has a warm climate and dense vegetative cover, which is a source of organic hydrocarbons, both of which facilitate in the production of tropospheric ozone (Chameides *et al*. 1988). Numerous studies have shown ozone-induced injury to foliage, and decreases in biomass and species richness on cool-season grasses and clovers as well as some warm-season grasses in the Southeastern US as well as Europe (Blum *et al.* 1982; Fuhrer 1997; Reich 1987; Booker *et al.* 2009). Innes *et al.* (2001) reported visible foliage damage due to ozone in several broadleaved species exposed to ozone in OTC as well as field conditions including: *Acer*, *Prunus*, and *Salix* spp. Ozone and other abiotic air pollutants can also cause plants to become more susceptible to insect predation and other stress factors such as drought (Heck *et al.* 1988).

The earth's climate has changed dramatically over the last century and is projected to keep changing (Christensen *et al*. 2007; US Climate Change Science Program 2008). Some models predict that tropospheric ozone concentrations will increase on a global basis by approximately 20% (0.3 – 2.0% per year) over the next twenty years (Thompson 1992; Vingarzan 2004). Also, summer rainfall may either decrease or increase based on model predictions for the Southeastern United States (MacCracken *et al*. 2001). The Canadian and Hadley global circulation models (GCM) predict varying increases in temperatures as well as total yearly rainfall amounts for the Southeastern US, although the Hadley model estimates 20% greater rainfall in the summer months while the Canadian model predicts 10% less (MacCracken *et al.* 2001). Increasing ozone concentrations in combination with climate change aspects such as increasing temperatures and altered precipitation events have potential to cause alterations in species composition and function in grassland communities (Ashmore and Ainsworth 1995; Suttle *et al.* 2007).

Plant community canopies are major sites of pollutant deposition, which has implications of adverse affects on terrestrial landscapes (Heck *et al.* 1988*).* It is important that whole-plant communities be studied and the effects of ozone exposure be quantified. Indirect effects, or community level responses, of ozone on cool-season grasses have been reported in Europe (Ashmore *et al*. 1995; Power and Ashmore 2002). Long-term exposure to elevated ozone over an entire growing season can lead to decreased plant growth and productivity and have adverse effects such as accelerated leaf senescence (Reich 1987; Barbo *et al.* 1998; Krupa *et al*. 2004; Muntifering *et al.* 2006). In addition, there is evidence that ozone-induced changes in foliar chemistry can result in decreased utilization of nutrients by ruminant herbivores because of decreases in the quality of herbaceous vegetation (Krupa *et al.* 2004).

Barbo *et al.* (1998) determined that ozone can cause a shift in community richness and evenness, as well as cause visible foliar injury in blackberry (*Rubus cuneifolius*). Plant communities exposed to CF (carbon-filtered; pristine air) had greater species richness and diversity as well as a plant community that was more vertically dense compared with treatments of ambient and twice-ambient ozone concentrations.

Increasing concentrations of ozone have detrimental effects on certain "sensitive" plant species in grasslands that have lower thresholds for ozone tolerance (Ashmore and Ainsworth 1995) and can cause a shift in community structure and function (Barbo *et al.* 1998; Gonzalez-Fernandez *et al.* 2008). In a study to determine ozone's effects on cool-season clover-fescue pastures, Heagle *et al.* (1989) reported that white clover (*Trifolium repens*) growth was diminished due to ozone and water stress while tall fescue (*Lolium arundinacea*) growth increased. Gonzalez-Fernandez (2008) reported white clover nutritive quality and cover were diminished due to ozone, while ryegrass growth occupied areas left by the diminished clover. In a study of warm-season grasses Lewis *et al.* (2006) reported big bluestem (*Andropogon gerardii*) was generally not affected by ozone while eastern gamagrass (*Tripsacum dactyloides*), grown simultaneously in the same open-top chambers, exhibited decreased nutritive quality over one growing season. It is important that whole-plant communities be investigated and the effects of ozone-exposure be quantified to gain knowledge of inter-specific differences in response to ozone and competition (Chappelka 1990; Heagle *et al.* 1989; Heagle *et al.* 1991; Barbo *et al.* 1998; Davison 1998).

The overall hypothesis of this study was that elevated tropospheric ozone concentrations alone, or in combination with elevated/decreased rainfall amounts would adversely affect resource allocation and canopy structure of dominant forage species found in the Southern Piedmont region. Specific objectives included: 1) determining vegetative growth types that are more sensitive to elevated concentrations of ozone, i.e. clover, and if those species are more adversely affected than non-sensitive species (grasses). 2) It was also important to ascertain if ozone effects interact with differing rainfall amounts and if species composition and productivity of the communities are impacted by the interactions. Estimations of ozone, rainfall, and harvest effects on biomass, species abundance and diversity, relative food value, and crude protein percentages, would ultimately be used for estimation of threshold ozone concentrations which elicit adverse effects on certain species of plants.

Materials and Methods

Study Site, Ozone Exposures, and Rainfall Amounts

The study site was located approximately 5 km from Auburn University and was representative of managed grasslands located throughout the Southern Piedmont region (USDA 1981). The ozone-exposure system consisted of twelve large open-top chambers (OTC), 4.8m height X 4.5m in diameter (Heagle *et al.* 1989). Each OTC had a rain hood at the top to control for precipitation, but which also allowed for ambient air circulation (Manning and Krupa 1992). Using principal component analysis (Gomez and Gomez 1984), chambers were selected for fumigation of forages with controlled concentrations of ozone on the basis of uniformity of initial plant communities and soil characteristics. Each chamber was seeded in March 2007 with a combination of three common grass and one legume species found in the Southern Piedmont region: tall fescue (*Lolium arundinacea*, C_3, cool-season grass), dallisgrass (*Paspalum dilatatum*, C_4, warm-season grass), ladino clover (*Trifolium repens*, C_3, cool-season legume), and common bermudagrass (*Cynodon dactylon*, C_4, warm-season grass) (Franzluebbers and Stuedemann 2006).

Water lines were placed underground and came up to the center of each chamber, approximately ½ m from ground-level, for irrigation purposes. Individual radial sprinklers were attached to each water line that were operated by automatic timers and manipulated in zones of four chambers each (one zone per rain treatment). Limitations in the irrigation system allowed for precipitation to be grouped into three zones which were then used in the split-split plot design with two ozone treatments per precipitation zone. An individual water meter was attached to each sprinkler to measure the amount of water dispensed per rain event. Water was applied three times per day with the amount dispensed recorded weekly and converted to cm of rainfall to ensure chambers were receiving target rainfall amounts. Styrofoam cups were also randomly placed in each chamber to ensure uniform water distribution throughout the chambers. The three different precipitation blocks were used to simulate future predicted rainfall amounts: zone 1: average (30-yr average monthly rainfall for Auburn, AL), zone 2: average monthly rainfall + 20% (Hadley GCM), zone 3: 20% less than average (representative of Canadian GCM) (MacCracken *et al.* 2001). These rainfall amounts were selected based on the uncertainty of future rainfall predictions for the Southeastern US (Burkett *et al.* 2001; MacCracken *et al.* 2001; Christensen *et al.* 2007) and on average monthly rainfall in the Auburn, AL area from 1971-2000 (Alabama Weather Information System).

Ozone fumigation in the chambers was initiated on June 1, 2009 and continued daily until September 30, 2009. Ozone was generated by passing pure O_2 through a high-intensity electrical discharge source (Griffin Inc., Lodi, NJ) and added to the chambers 12 hr/day (0900–2100 hr), 7 days/wk. Fans were turned off from 2300–0500 hr to permit natural dew formation within the chambers. Instruments were calibrated according to US EPA quality assurance guidelines. The two ozone treatments applied in this study consisted of: non-filtered air, representative of ambient air found in rural areas in the

Piedmont region (US EPA 2006), and air enriched to twice-ambient ozone concentrations, representative of future ozone predictions for rural areas in the Southern Piedmont region (Lefohn *et al.* 1992; Thompson 1992; Vingarzan 2004). There were a total of six replications (chambers) of both the non-filtered (NF) and twice-ambient (2X) ozone levels.

Plant Measurements

In each chamber, two randomly selected permanent plots of 0.5 m diameter were established for harvest each month by placing circular metal rings in the ground. These permanent regrowth plots were harvested at the end of each month to determine regrowth capacity of each forage type (clover & grasses). Along with the two permanent plots from each chamber (regrowth), two random plots (0.5 meter in diameter) were harvested (for primary-growth) from each chamber every month. Plots within chambers were assigned random numbers and designated for selection by a random numbers plot in Microsoft Excel. The random primary-growth plots were marked so as to be sampled only once throughout the growing season; these plots represented cumulative exposure to ozone (Barbo *et al.* 1998; Ditchkoff *et al.* 2009). Primary and regrowth plots were established because ozone adversely affects certain species when there is a co-occurrence of elevated concentrations of ozone with sensitive growth stages of the plant, such as younger plants (Muntifering *et al.* 2000; Bender and Weigel 2003). Since seasonal distribution of forage growth varies among species (Ball *et al*. 2002), such harvests throughout the growing season could be expected to contain an optimal representation of cool-season C_3 grasses, warm-season C_4 grasses, and clover. Also, based on previous research with similar species (Muntifering *et al*. 2000; Muntifering *et al*. 2006; González-

Fernández *et al*. 2008), cumulative ozone-exposure by mid-summer is sufficient such that the 2X forages could be expected to have decreased nutritive quality as predicted by laboratory analysis. Consideration was given to edge effects associated with OTC (Fuhrer 1994) and accounted for by having the effective area inside chambers 0.5 m from the inside rim of the chambers.

A point-frame canopy cover count was conducted before each harvest using a method modified from two previous studies (Bonham 1989; Barbo *et al*. 1998). This point-frame device was circular in design and covered an area of 0.2 m^2, the approximate area of each plot. The device contained 140 holes in which 25 were selected randomly for pin placement covering approximately 18% of the area inside each plot, similar to that of Barbo *et al.* (1998). Total percent canopy cover as well as individual species cover was evaluated monthly throughout the four harvests of this study. The total effective area in each chamber was 9.5 m^2 and with each plot having an area of 0.2 m^2, we sampled approximately 10% of the total effective area each month.

Laboratory Analysis

Samples from each primary and regrowth plot were cut to ground-level with scissors and placed into paper sacks during each harvest. The samples were then separated manually into grasses and clover, with all grass species from each plot combined for one "grass" sample (one regrowth, one primary-growth) from each chamber. This procedure was implemented to compare different forage types, grass vs. legume, because white clover is known for its sensitivity to ozone (Heagle *et al.* 1989; Davison 1998; Gonzalez-Fernandez *et al.* 2008). After separation of forage types, harvested material was dried in a conventional forced-air oven at 55-60° C to constant weight, air-equilibrated, and weighed for DM yield (biomass). Samples were then ground in a Thomas Wiley mill to pass through a 1-mm screen. Representative samples were sequentially fractionated according to Van Soest *et al*. (1991) for calculation of cell-wall constituents (hemicellulose, cellulose, and lignin): neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL). An ANKOM fiber analyzer (ANKOM Technology Corporation, Fairport, NY) was used for fractionation of samples. DM percentages and concentrations of crude protein (CP; Kjeldahl N x 6.25) in samples were calculated according to the Association of Analytical Chemists (1995).

Cell-wall constituents are partially and variably digestible and have a considerable impact on forage nutritive quality and value to ruminant herbivores (Van Soest 1994; Powell *et al.* 2003; Krupa *et al.* 2004; Gonzalez-Fernandez *et al*. 2008). According to Van Soest (1994) neutral detergent fiber is inversely related to free-range voluntary forage intake; as forage concentration of the NDF fraction increases, forage intake decreases. Acid detergent fiber is inversely related to forage digestibility; as ADF concentration increases forage digestibility decreases (Van Soest 1994). Relative food values (RFV) were then calculated for each sample throughout the season. Relative food value is a mathematical estimation of forage quality (Rohweder *et al.* 1978) and uses concentrations of NDF and ADF in prediction equations for its calculation (Linn and Martin 1989). Relative food value is important in agriculture as well as to wildlife because RFVs allow for comparisons of different forage types; i.e., grass vs. legume, with a standard forage RFV (100) containing 53% NDF and 41% ADF (Linn and Martin 1989).

Individual Species Harvest

A total of five monthly harvests (primary and regrowth harvests of grasses & clover) were conducted with an additional harvest of individual species on the day of the final harvest (September 30, 2009). During this additional harvest, all species were collected and bagged individually from each of the twelve chambers. Species were collected from the remaining non-harvested primary-growth plants throughout the chambers. These species included the four original species: white clover, dallisgrass, tall fescue, and bermudagrass with an additional species, foxtail grass (*Setaria glauca*) collected. Foxtail was sampled and analyzed in the final harvest due to its abundance in the chambers during the last month of the season. There were a total of 60 samples collected in the individual species final harvest (12 chambers with five species from each chamber). The separation by species level was conducted to calculate species-specific individual nutritive values (RFV, lignin, crude protein, ADF, NDF). We also wanted to determine interspecific differences in the grass component.

In addition to sequential fiber fractionation (Van Soest *et al.* 1991), *in vitro* dry matter digestibility (IVDMD) and *in vitro* neutral detergent fiber digestibility (IVNDFD) were calculated for the 60 individual species samples. This process involved determining the digestibility of the samples with cattle ruminal fluid collected from a mature, rumen-fistulated dairy cow (*Bos taurus*) housed at the Auburn University Veterinary Clinic. The 60 samples were packed into individual filter bags, placed into jars with the ruminal fluid, and into a Daisy Ankom II digestion incubator for 48 hr at 39^{o}C for the digestion trial. Samples then underwent NDF extraction, after which they were dried overnight at 100^{o}C and weighed (Tilley and Terry 1963; Goering and Van Soest 1970). The resulting digestion estimations from *in vitro* digestion analysis were referenced with the RFV calculations, from fiber analysis (Van Soest *et al.* 1991) of the individual species, to ascertain inter-specific differences in nutritional value.

Experimental Design and Statistics

There were a total of six replications (chambers) of both the non-filtered (NF) and twice-ambient (2X) ozone levels. The twelve chambers had three zones of varying precipitation amounts among them (four chambers for each precipitation amount). Each water treatment "zone" contained two chambers receiving NF air and two chambers receiving 2X air.

The experimental design for this field fumigation study was a randomized split-plot design with two ozone treatments replicated within three zones of precipitation, and sample regrowth and primary-growth plots within each treatment. The experiment unit was the chamber; with plant communities in each chamber the individual populations. Main precipitation effects could have been altered by experimental design of blocked rain treatments, no true replication of precipitation blocks; for which we had no choice. ANOVA procedures were used to analyze the data: NDF, ADF, RFV, lignin, biomass, crude protein, and IVDMD estimations. Simple *t*-tests were used to test significance within treatments for ozone. Data were entered into a linear model with dependent variables the function of ozone treatment and precipitation. Repeated measures were utilized to test differences over harvests in the data. Analyses were conducted using the R package for statistical analysis (R Development Core Team 2010).

Results

Climatic Data and Ozone Exposures

Monthly air temperatures were similar to the 30-yr averages throughout the growing season with the exception of September, which was five degrees warmer than average (Table 1). Precipitation in the Auburn, AL area for August and September 2009 was much greater than average for those months, with 3.5 and 5 cm more than the average, respectively (Table 1). Although ambient rainfall did not directly fall into the chambers, above-average rainfall in the area may have affected ozone concentrations due to cloud cover and low sunlight. The rain exclosures on the chambers prevented this precipitation from reaching the forages except through soil percolation. Average simulated monthly rainfall amounts for the three rainfall regimes [12, 15, and 9.5 cm for average, +20% (high), and -20% (low), respectively] were close to target amounts of average monthly rainfall (approximately 11 cm for average, 13 cm high, 9 cm low) for each precipitation zone each month (Table 2).

Mean 12-h daytime (0900-2100 hr) ozone concentrations over the four-month experiment were 31 and 56 nl l^{-1} (ppb) respectively for NF and 2X treatments (Table 3). Average peaks in ozone concentrations were 39 and 77 nl l^{-1} for NF and 2X treatments. Peak 1-hr concentrations were 73 and 155 nl l^{-1} on average for NF and 2X treatments respectively. The AOT 40s for the two treatments throughout the 2009 growing season were low compared to some previous studies in the Auburn, AL area (Muntifering *et al.* 2000; Powell *et al*. 2003).

Plant Measurements

Primary Growth

Primary-growth grasses, on average, experienced increased growth in 2X treatments over the growing season, with an 18% increase in DM yield in 2X treatments compared with NF-exposed grasses (Table 4). Grasses also exhibited a trend towards greater nutritive quality in 2X treatments compared with NF treatment grasses. Clover exhibited decreased nutritive quality in 2X treatments with significant (P = 0.05) increases in lignin (Table 4). Clover in the 2X treatments was determined to contain greater amounts of cell wall constituents (ADF, NDF, and ADL), throughout the growing season and in the final harvest, which resulted in lower RFV than NF-exposed clover. In the final harvest, CP concentration was 6% greater in 2X clover compared with NF clover; CP concentrations were also greater in 2X treatments throughout the majority of the growing season (Table 4 & 6).

Precipitation did not have a significant effect over the growing season on primary-growth forages (grasses or clover) except for grass DM yield, which was 46% greater in chambers with high (+20% of average) precipitation compared with average and low precipitation treatments (Table 4). A precipitation × ozone interaction was observed for grass DM yield over the growing season as well. Low (-20% of average) and high precipitation/2X ozone treatments' DM yield of grasses was greatly increased when compared with average precipitation/NF treatments (Table 6). Harvest (month) effects were significant in grasses over the growing season for DM yield and nutritive quality, with both declining in the later harvests (Table 4).

Regrowth

Regrowth grasses exposed to 2X concentrations of ozone had 65% greater DM yield in the final harvest than NF grasses (Table 7). Grasses also had 5% greater RFV in the final harvest for 2X compared with NF regrowth grasses. Grasses exposed to 2X ozone concentrations on average, had 1.06% lower concentrations of NDF throughout the season than did the NF grasses; non- significant, but indicated a trend of insensitivity to ozone (Table 5). Grasses also had 2.2% greater RFV and approximately 1% greater concentrations of CP when compared with NF exposed grasses throughout the season. Clover exposed to 2X treatments exhibited significant increases in concentrations of NDF (P = 0.10) and ADL (P < 0.03) over the growing season than NF-exposed clover. These greater concentrations of NDF and ADL caused a 4% decrease in RFV in 2X clover compared with NF clover over the season (Table 5). During the final harvest, 2X clover experienced a 60% decrease in biomass production compared with NF clover. Nutritive quality of final harvest was also significantly decreased in 2X clover, which experienced an 8% decrease in RFV compared with that of NF clover.

Precipitation alone did not have significant effects on regrowth forages (grasses or clover) over the growing season or in the final harvest, although high precipitation amounts generally resulted in greater DM yield (Table 5 & 7). There were, however, significant interactions with precipitation and ozone in grasses (Table 5). The ozone by precipitation interaction resulted in greater biomass in grasses exposed to 2X treatments in combination with high and low precipitation amounts compared with NF/average treatments.

Canopy Cover

Total canopy cover did not significantly differ between ozone treatments over the season but was significantly decreased in the final harvest (Table 8 & 9; Figure 1 & 2). Grasses, in general, did not differ among ozone treatments but did decrease slightly in the final months. Clover also did not experience differences due to ozone treatment or harvest.

Total percent canopy cover differed significantly between precipitation treatments during the growing season (Table 8 & 9; Figure 1), and was 6% greater in the above-average rainfall treatments compared with average rainfall. Clover represented approximately 79% less cover in the average precipitation chambers compared with above-average and below-average precipitation chambers (Table 9). Biomass of clover in the average precipitation chambers (1-4) was insufficient to enable fiber analysis and consequently was omitted, leaving only comparisons of above and below-average precipitation treatments in clover forage analysis.

Individual Species

The additional final harvest of individual grass species (September) did not indicate any differences across ozone treatments except in bermudagrass and tall fescue. These two species had increased IVDMD and IVNDFD in 2X treatments compared with those collected from NF treatments (Table 10).

Discussion

To our knowledge, there is no study that has considered physiological and grassland community responses to increasing ozone concentrations in concert with differing rainfall amounts as suggested by several global climate models (MacCracken *et al*. 2001). The ozone concentrations experienced in June – September, 2009, were relatively low compared to those from similar studies in the Auburn, AL area (Powell *et al*. 2003; Szantoi *et al.* 2007; Szantoi *et al.* 2009), but still had detrimental effects on clover. Ambient rainfall (occurring outside of the chambers) was above average for the Auburn, AL area in 2009. Although we tried to isolate the chambers from ambient rain with rain hoods on the chambers, precipitation effects could have been affected by water infiltration through the soil. Soil moisture measurements were taken starting in June to ensure soil moisture was accurate with rainfall treatments, but measurements were inconsistent and therefore discontinued.

Even with the relatively low ozone concentrations, especially in the final two months, differences were still observed in some aspects of nutritive quality, DM yield, and canopy cover of clover. It is interesting to note that ozone did not interact with precipitation in having differing effects on nutritive quality of forages. Consequently, clover could experience adverse effects due to ozone in various rainfall amounts. Primary-growth clover, on average, experienced declines in nutritive quality in 2X treatments compared with NF treatments over the harvest months (June – September), with no differences in DM yield. Species-specific responses to various ozone concentrations have been reported in other studies (Reich 1987; Heagle *et al.* 1989; Heagle *et al.* 1991; Davison 1998; Bender *et al.* 2006; Lewis *et al*. 2006) with similar responses to ozone experienced among sensitive and insensitive species in communities. Results may have been influenced by the relatively low ozone concentrations and very high rainfall patterns experienced in 2009.

Regrowth clover exposed to 2X ozone treatments experienced declines in nutritive quality over the entire season, but no differences in DM yield were detected. However, regrowth clover exposed to 2X treatments did have significantly decreased DM yield in the final harvest compared with NF treatments. Our data are similar to other studies (Heagle *et al.* 1989; Blum *et al*. 1982; Blum *et al.* 1983) where DM yield of both primary and regrowth clover decreased in response to ozone. Nutritive quality of clover (especially regrowth clover) was negatively affected by increasing concentrations of ozone, while grasses in general were less sensitive. These data are consistent with other findings (Rebbeck *et al.* 1988; Muntifering *et al.* 2006; Gonzalez-Fernandez *et al.* 2008). Grasses as a whole did not experience detrimental ozone effects, and even experienced some increases in biomass (DM yield) over the season. Regrowth grasses exposed to 2X treatments had greater DM yield in 2X treatments over the growing season with no discernable change in canopy cover. This is explained by the forage type (bunchgrass) of dallisgrass and tall fescue.

Our results indicate the overall sensitivity of clover as evidenced by decreases in nutritive quality in response to increasing concentrations of ozone and potential impacts that timing of harvests can have on species and communities. Type of vegetation in communities may alter biomass and overall community structure such that community level responses are not realized by simple harvest biomass or canopy cover numbers. Decreases in nutritive quality due to ozone in clover and other sensitive species such as highbush blackberry could be having detrimental effects on wildlife that forage on these species (Krupa *et al.* 2004; Ditchkoff *et al.* 2009).

Nutritive quality decline due to increased ozone concentrations with no significant effects on biomass production were reported in other studies with warm-season forages (Powell *et al.* 2003; Lewis *et al.* 2006). The lack of canopy cover changes due to ozone is important because grasses did not exhibit significant changes in canopy cover but did have greater nutritive quality in 2X treatments compared with NF treatments. Clover canopy cover also did not differ among ozone treatments, but clover experienced decreased nutritive quality in 2X treatments compared with NF treatments. This is interpreted to mean that communities could appear unchanged by simple canopy appearance, but losses in nutritive quality or biomass could be occurring. Also, while there were no visible foliar injuries to any plants inside the chambers (Chappelka personal comm.), nutritive quality was still decreased.

Community structure and function is an important but indirect effect of ozone, with the direct effects of differing ozone and precipitation amounts experienced by individuals in the communities. Regarding individual species effects, studies suggest that tall fescue is relatively insensitive to ozone (Richards *et al*. 1980; Flagler and Youngner 1985) and it can even thrive when grown in combination with an ozone-sensitive species such as white clover (Rebbeck *et al*. 1988; Heagle *et al*. 1989). Tall fescue in this study was not adversely affected by ozone concentrations during the growing season. Fescue exposed to 2X treatments in the final harvest had greater digestibility coefficients (IVDMD and IVNDF) compared with NF-exposed fescue. Bermudagrass is also thought to be insensitive to ozone due to a study which found little effects of ozone on it compared with cool-season grasses (Richards *et al*. 1980). Bermudagrass in the final harvest also experienced increased digestibility in 2X treatments compared with forages grown in NF chambers. Muntifering *et al.* (2000) reported that bahiagrass (*Paspalum notatum*), which is in the same genus as dallisgrass, grown under controlled conditions experienced declines in dry matter yield and quality of re-growth. Dallisgrass experienced a general decline in nutritive quality in 2X chambers in the final harvest, as well as declines in canopy cover. The relative insensitivity of grasses to ozone in this study suggests that the pooling of grasses into one collective "grass" sample throughout the season was appropriate. The comparison of grasses (insensitive) to clover (sensitive) is important in determining species or growth-specific responses to different ozone and precipitation treatments (climate change).

Conclusions

It is important to determine the effects that altered rainfall amounts in combination with differing levels of ozone exposures may have on local plant species in the Southern Piedmont region, and eventually the plant/animal interactions in those communities as well. The Southern Piedmont region spans 17 million hectares from central Virginia into east-central Alabama (USDA 1981) and is mainly managed grasslands and pastures which include C_3 and C_4 grasses and clovers such as the species examined in this study. The differences in ozone sensitivities of the species in the Southern Piedmont region leads to the hypothesis of community-level responses as well as species-specific responses to the combination of altered rainfall amounts and increasing ozone concentrations (Krupa *et al*. 2004). These results have management implications in that ruminant herbivores may be forced to alter feeding strategies to account for declines in nutritive quality of forages. Natural declines in forage quality could be further compounded by detrimental effects of increasing concentrations of ozone as rural and forested areas become more urbanized in the future.

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Table 1

Mean monthly air temperatures, rainfall amounts, and 30-yr means from June 1 – Sept 30, 2009 for the Auburn, AL, USA area.

	Air Temperature (^{o}C)		Precipitation (cm)	
Month	2009	30-yr avg.	2009	30-yr avg.
June	25.9	24.9	10.2	10.3
July	24.9	26.2	10.1	14.9
Aug	25.1	26.1	12.7	9.2
Sept	28.2	23.3	14.6	9.6

Data obtained from National Climatic Data Center (ncdc.noaa.gov), for Auburn, AL 2009. 30-yr averages obtained from Alabama Weather Information System (AWIS), Auburn, AL.

Table 2

Monthly average target precipitations and realized precipitations (cm) over the growing season for chambers by month and precipitation regimes

	Average		High (+20%)		Low (-20%)	
Month	Target	Achieved	Target	Achieved	Target	Achieved
June	11.43	11.80	13.72	15.68	9.14	9.70
July	11.43	12.22	13.72	16.01	9.14	9.59
Aug	11.43	11.23	13.72	15.28	9.14	9.45
Sept	11.43	12.22	13.72	15.01	9.14	9.81
Total	45.72	47.47	54.88	61.98	36.56	38.55
Season monthly average	11.43	11.87	13.72	15.50	9.14	9.66

Average target= average precipitation in chambers 1-4, (30-yr avg. from 1971-2000 for Auburn area); High= chambers 5-8, 20% greater than average; Low= chambers 9-12, 20% less than average (Canadian GCM).

Table 3

Mean daytime (0900 – 2100 h) 12-hr ozone concentrations (ppb: *n*l l^{-1}), average ozone peaks, 1-hr peaks, and AOT 40_12hr (ppb-hr) concentrations from June 1 – Sept 30, 2009.

	12-hr ozone concentrations		Avg. ozone peak conc.		Peak 1-hr concentrations		AOT 40 (ppb-hr)	
Month	NF	2X	NF	2X	NF	2X	NF	2X
June	35	68	44	91	59	126	742	10620
July	35	61	43	79	73	155	713	8642
Aug	26	47	34	66	56	113	169	4981
Sept	26	50	35	73	55	136	188	5630
Season	31	56	39	77	73	155	1811	29874

NF = non-filtered ambient, 2X = enriched to twice ozone concentrations.

Table 4

Significant levels for ANOVA of biomass and nutritive quality for primary-growth grasses and clover exposed to differing ozone and precipitation amounts in 2009 (*P*-values).

	df	biomass (g)	NDF (%)	ADF (%)	ADL (%)	RFV	CP (%)
<u>Primary grasses</u>							
precip	2	0.1^{+}	0.75	0.38	0.63	0.62	0.35
O_3	1	0.02*	0.62	0.81	0.89	0.70	0.17
harvest	3	0.001**	0.001**	0.01**	0.11	0.001**	0.001**
O_3 x precip	2	0.02*	0.12	0.38	0.61	0.17	0.48
precip x harvest	6	0.05*	0.02*	0.02*	0.42	0.02*	0.21
O_3 x harvest	3	0.65	0.16	0.54	0.08^{+}	0.34	0.25
O_3 x precip x harvest	6	0.73	0.03*	0.27	0.76	0.05*	0.67
<u>Primary clover</u>							
precip	1	0.69	0.47	0.79	0.62	0.53	0.46
O_3	1	0.57	0.12	0.12	0.05*	0.13	0.06^{+}
harvest	3	0.004**	0.43	0.01**	0.001**	0.50	0.001**
O_3 x precip	1	0.95	0.55	0.23	0.74	0.41	0.91
Precip x harvest	3	0.39	0.11	0.04*	0.15	0.13	0.86
O_3 x harvest	3	0.83	0.17	0.11	0.30	0.18	0.89
O_3 x precip x harvest	3	0.95	0.14	0.12	0.37	0.14	0.99

Significance at the $^{**}P < 0.01$, $^{*}P < 0.05$, and $^{+}P < 0.10$ level. Biomass (g); NDF, % neutral detergent fiber; ADF, % acid detergent fiber; ADL, acid detergent lignin; RFV, relative feed value; CP, % crude protein. Due to lack of clover in average precipitation treatments, chambers with average precipitation were excluded from analysis of clover.

Table 5

Significant levels for ANOVA of biomass and nutritive quality for regrowth grasses and clover exposed to ozone and differing precipitation amounts in 2009 (*P*-values).

	df	biomass (g)	NDF (%)	ADF (%)	ADL (%)	RFV	CP (%)
<u>Regrowth grasses</u>							
precip	2	0.12	0.38	0.40	0.84	0.33	0.25
O_3	1	0.19	0.02*	0.23	0.77	0.03*	0.001**
harvest	3	0.001**	0.001**	0.001**	0.001**	0.001**	0.06^+
O_3 x precip	2	0.001**	0.69	0.92	0.04*	0.55	0.83
precip x harvest	6	0.77	0.69	0.96	0.14	0.82	0.38
O_3 x harvest	3	0.68	0.85	0.73	0.39	0.58	0.92
O_3 x precip x harvest	6	0.84	0.34	0.88	0.34	0.96	0.92
<u>Regrowth clover</u>							
precip	1	0.65	0.79	0.65	0.62	0.93	0.32
O_3	1	0.64	0.10^+	0.14	0.03*	0.06^+	0.93
harvest	3	0.002**	0.02*	0.001**	0.001**	0.001**	0.02*
O_3 x precip	1	0.90	0.25	0.53	0.29	0.33	0.31
precip x harvest	3	0.82	0.50	0.20	0.39	0.35	0.64
O_3 x harvest	3	0.74	0.18	0.25	0.02*	0.12	0.35
O_3 x precip x harvest	3	0.84	0.75	0.75	0.59	0.67	0.74

Significance at the $^{**}P < 0.01$, $^{*}P < 0.05$, and $^{+}P < 0.10$ level. Biomass (g); NDF, % neutral detergent fiber; ADF, % acid detergent fiber; ADL, acid detergent lignin; RFV, relative feed value; CP, % crude protein. Due to lack of clover in average precipitation treatments chambers with average precipitation were excluded from analysis of clover.

Table 6

Nutritive quality and biomass significance among ozone and precipitation treatments, for final harvest data, of primary-growth grasses and clover.

	biomass (g)	NDF (%)	ADF (%)	ADL (%)	RFV	CP (%)
Ozone conc.						
Grasses						
NF	78.22	69.33	32.91	2.18	85.18	6.79
2X	96.84	69.80	33.36	2.02	83.86	7.13
p-value	0.25	0.73	0.63	0.24	0.63	0.35
Clover						
NF	3.15	31.44	18.59	3.38	220.55	19.22
2X	1.37	34.18	20.67	3.80	199.88	20.49
p-value	0.45	0.35	0.15	0.23	0.31	0.10^{+}
Precipitation						
Grasses						
Avg	75.80	70.90	33.56	1.96	82.37	5.89
High	119.9	70.42	34.33	2.33	82.16	7.04
Low	66.95	67.36	31.53	2.01	89.02	7.97
p-value	0.17	0.21	0.21	0.52	0.20	0.20
Clover						
High	1.59	34.21	19.70	3.65	201.81	20.12
Low	6.47	31.40	19.95	3.60	217.98	19.92
p-value	0.23	0.43	0.88	0.91	0.50	0.93

Significance at the $^{**}P < 0.01$, $^{*}P < 0.05$, and $^{+}P < 0.10$ level. Biomass (g); NDF, % neutral detergent fiber; ADF, % acid detergent fiber; ADL, acid detergent lignin; RFV, relative feed value; CP, % crude protein. Due to lack of clover in average precipitation treatments, chambers with average precipitation were excluded from analysis of clover.

Table 7

Nutritive quality and biomass significance among ozone and precipitation treatments, for final harvest data, of regrowth grasses and clover.

	biomass (g)	NDF (%)	ADF (%)	ADL (%)	RFV	CP (%)
Ozone conc.						
Grasses						
NF	11.41	68.90	34.76	2.27	83.50	9.39
2X	18.86	67.47	33.82	2.19	88.04	10.14
p-value	0.09$^+$	0.15	0.19	0.44	0.03*	0.13
Clover						
NF	2.58	29.04	17.55	3.55	241.27	20.32
2X	1.03	31.24	18.56	3.62	221.88	19.17
p-value	0.06$^+$	0.05*	0.14	0.78	0.03*	0.29
Precipitation						
Grasses						
Avg	9.83	68.10	34.75	2.00	84.59	9.86
High	17.09	67.58	33.94	2.46	86.06	10.02
Low	18.51	68.88	34.14	2.21	86.65	9.42
p-value	0.33	0.66	0.67	0.29	0.79	0.53
Clover						
High	1.52	29.73	17.35	3.69	236.30	20.00
Low	2.08	30.54	18.76	3.48	226.84	19.49
p-value	0.62	0.59	0.28	0.56	0.48	0.70

Significance at the $**P < 0.01$, $*P < 0.05$, and $^+P < 0.10$ level. Biomass (g); NDF, % neutral detergent fiber; ADF, % acid detergent fiber; ADL, acid detergent lignin; RFV, relative feed value; CP, % crude protein. Due to lack of clover in average precipitation treatments, chambers with average precipitation were excluded from analysis of clover.

Table 8

Significances (*P*-values) from repeated measures analysis for total canopy cover and canopy cover of selected species exposed to ozone and altered rainfall amounts over four harvests (June – Sept) 2009 in Auburn, AL, USA

Source of variation	df	Canopy cover	Clover	Dallisgrass	Bermuda grass	Tall fescue
Ozone	1	0.54	0.19	0.17	0.86	0.48
Precip	2	0.001**	0.001**	0.64	0.66	0.13
Harvest	3	0.004**	0.38	0.05*	0.001**	0.001**
Ozone x Precip	2	0.89	0.99	0.10^{+}	0.30	0.88
Ozone x Harvest	3	0.41	0.74	0.23	0.99	0.82
Precip x Harvest	6	0.05*	0.61	0.35	0.84	0.97
Ozone x Precip x Harvest	6	0.99	0.79	0.87	0.88	0.70

$^{**}P < 0.01$, $^{*}P < 0.05$, $^{+}P < 0.1$. Harvest effects are for harvests from June, July, August, and September 2009. Ozone effects represent variation between 2X and NF treatment over the harvests. Precip represents significance among different rain treatments (avg, high, low) over the four harvests.

Table 9

Means and statistics for univariate analysis of total canopy cover (%) and canopy cover (%) during the four harvests (average of June, July, August, and September) for selected species exposed to different ozone concentrations and rainfall amounts during 2009, in Auburn, AL, USA.

Treatment	Total canopy cover	Clover	Dallisgrass	Bermuda grass	Tall fescue
Ozone					
2X	96.12 a	32.33 a	22.09 a	75.75 a	6.75 a
NF	95.70 a	24.25 a	18.17 a	75.00 a	8.25 a
p-value	0.69	0.24	0.19	0.88	0.54
Precip					
Avg	93.00 a	8.130 a	18.25 a	75.38 a	8.25 ab
High	98.75 b	35.50 b	21.00 a	77.76 a	9.75 a
Low	96.13 b	41.25 b	21.13 a	73.01 a	4.50 b
p-value	0.001	0.001	0.68	0.72	0.19

Values with the same letter are not significantly different from each other using pair-wise t-tests at $P < 0.5$ level. Species were counted as part of canopy cover if contacted by rods during point-frame analysis. This method allows for multiple species to account for a single are of canopy cover.

Table 10

Nutritive quality and digestibility of individual species at final harvest (Sept. 30, 2009) between ozone treatments.

Species	Treatment	CP (%)	NDF (%)	ADF (%)	ADL (%)	RFV	IVDMD (%)	IVNDFD (%)
clover	NF	20.32	27.01	17.85	3.12	259.37	88.66	58.07
	2X	21.23	27.77	17.59	3.21	252.07	88.33	58.00
	p-value	0.44	0.40	0.61	0.69	0.43	0.64	0.97
dallisgrass	NF	8.16	67.40	36.12	2.84	85.06	63.31	46.51
	2X	8.03	66.75	35.15	2.56	86.97	62.69	44.80
	p-value	0.94	0.87	0.72	0.51	0.82	0.92	0.77
bermudagrass	NF	7.00	70.66	34.76	1.87	81.61	61.56	45.54
	2X	7.70	70.13	33.30	1.71	83.63	64.33	49.15
	p-value	0.19	0.75	0.20	0.18	0.51	0.05*	0.05*
foxtail	NF	5.42	66.46	38.12	2.72	83.18	65.26	47.68
	2X	5.95	67.24	37.34	2.56	82.94	63.74	46.17
	p-value	0.41	0.65	0.63	0.45	0.95	0.50	0.58
fescue	NF	5.22	76.03	42.70	4.97	68.16	44.98	27.65
	2X	5.39	76.27	42.25	4.70	68.51	49.17	33.29
	p-value	0.76	0.88	0.80	0.66	0.91	0.08^{+}	0.06^{+}

Significant at the *$P < 0.05$, $^{+}P < 0.10$ level. NDF, % neutral detergent fiber; ADF, % acid detergent fiber; ADL, % acid detergent lignin; RFV, relative food value; CP, % crude protein; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fiber digestibility. Due to lack of clover in average precipitation treatments, chambers with average precipitation were excluded from analysis of clover.

Figure 1. Seasonal patterns of total (all species) percentage canopy cover by precipitation treatments in 2009: avg: average rainfall in Auburn, AL based on 30-yr average of 137 cm/yr; high: +20% of average; low: -20% of average.

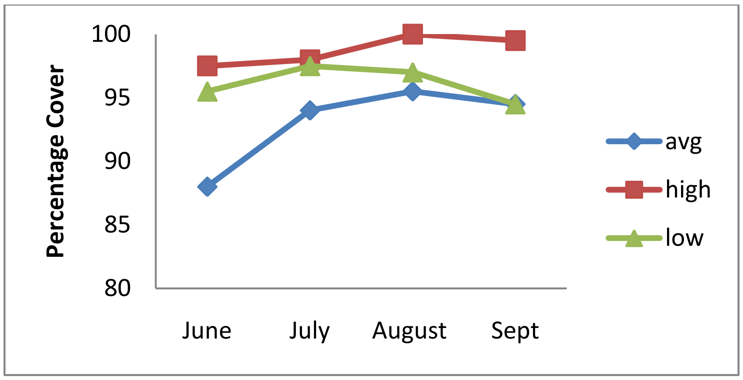
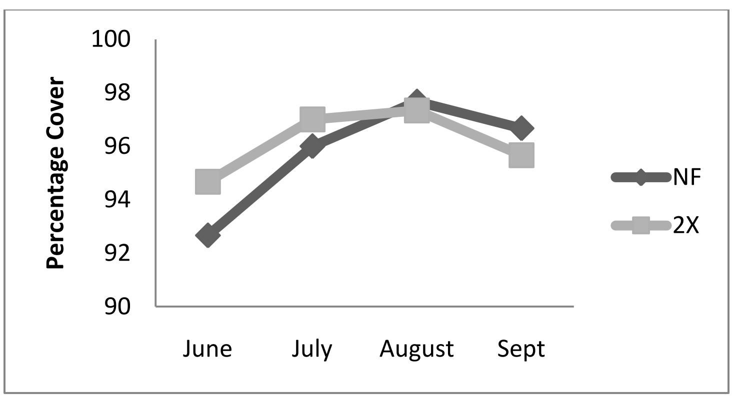


Figure 2. Seasonal patterns of total (all species) percentage canopy cover by O_3 treatments in 2009: NF (non-filtered chambers); 2X (twice-ambient chambers).



III. Nutrient Utilization by a Mammalian Herbivore (*Oryctolagus cuniculus*) Receiving Ozone-Exposed Forage

Summary

1. Tropospheric ozone (O_3) can play a significant role in the growth and nutritive quality of plant species. We hypothesized that exposure to elevated concentrations of O_3 would alter the chemical composition of forages such that nutrient utilization by a mammalian herbivore would be affected.
2. We tested our hypothesis by feeding common a mixture of Southern Piedmont grassland species: tall fescue (*Lolium arundinacea),* dallisgrass (*Paspalum dilatatum),* common bermudagrass (*Cynodon dactylon),* and ladino clover (*Trifolium repens)* exposed to differing levels of O_3 [ambient (non-filtered; NF) and twice-ambient (2X) concentrations] to rabbits (*Oryctolagus cuniculus*). Foliage samples (diets and orts) were analyzed for concentrations of total cell wall constituents, lignin, crude protein, and phenolics.
3. Neutral-detergent fiber (NDF) and acid-detergent fiber (ADF) digestibility by rabbits were significantly lower for 2X diets than NF diets. The decreased digestibility was not attributed to lignin content, as lignin concentrations did not differ between the two treatment diets, but was significantly associated with increased concentrations of saponifiable (i.e., alkali-labile) phenolics.

4. Exposure to elevated O_3 resulted in decreased digestible dry matter intake (DDMI) and digestibility by rabbits receiving 2X diets compared with rabbits receiving NF diets.
5. In contrast to reports of increased cell wall lignification affecting forage digestibility by mammalian herbivores, our results indicate that elevated O_3 increases forage concentrations of cell wall-bound phenolics that are predictably associated with decreased digestibility of forages in a hindgut-fermenter. Elevated O_3 concentrations would be expected to have a negative impact on forage quality resulting in decreased nutrient (dry matter) utilization by both ruminant and non-ruminant mammalian herbivores in Southern Piedmont grasslands under projected future climate scenarios.

Keywords: Ozone; Air pollution; *Oryctolagus cuniculus*; forage quality; herbivore nutrition; phenolics

Introduction

Tropospheric (ground-level) ozone (O_3) is the most significant phytotoxic air pollutant affecting vegetation in the United States (US EPA 2006) and is widespread globally (Chameides *et al.* 1994). It is formed by photochemical reactions in the presence of sunlight between oxides of nitrogen and volatile organic compounds, both of which are products of natural and anthropogenic processes (National Research Council 2004). Models predict tropospheric O_3 concentrations will increase on a global basis by approximately 20% over the next twenty years (Vingarzan 2004). The Southeastern US has a warm climate and dense vegetative cover, a source of organic hydrocarbons, which aid in the production of tropospheric O_3 (Chameides *et al*. 1988).

O_3-sensitive plant species are important with regards to energy and nutrient cycling between trophic levels and with the atmosphere, and as forage resources for wild and domestic grazing animals (Ditchkoff *et al.* 2009; Fuhrer 1997). Increasing concentrations of O_3 can have detrimental effects on certain "sensitive" plant species in grasslands (Ashmore and Ainsworth 1995), with potential to cause a shift in community structure and function (Barbo *et al.* 1998). This in turn could lower biodiversity and alter 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 aspects such as increasing temperatures and altered precipitation events can cause changes in species composition and nutritive quality in grassland communities (Suttle *et al.* 2007; Gilliland *et al.* unpublished).

Exposure of sensitive plants to elevated O_3 concentrations has been reported to suppress photosynthesis, accelerate senescence, cause visible foliar injury, and decrease yields (Reich 1987; Heck *et al.* 1988; Krupa *et* al. 2004; US EPA 2006; Booker *et al.* 2009). Szantoi *et al.* (2009) reported that O_3 caused foliar injury and altered productivity and concentrations of cell-wall constituents of cutleaf coneflower (*Rudbeckia lacianata*) plants under controlled conditions. Recently there have been studies to determine the effects of elevated O_3 concentrations on wild plants. Chappelka *et al.* (2003) reported ground-level O_3 in Great Smoky Mountains National Park caused foliar injury and had other deleterious effects on cutleaf coneflower and crown-beard (*Verbesina occidentalis*).

Recently studies have been conducted to investigate O_3-induced alterations in nutritive quality for ruminant animals. Muntifering *et* al. (2006) determined that exposure to elevated concentrations of O_3 in a FACE (Free-Air CO_2 and O_3 Enrichment) experiment resulted in increased concentrations of lignin and decreased cell-wall digestibility of *Trifolium* spp. in Rhinelander, WI. Nutritive quality losses in mammalian herbivore systems have been documented in other studies as well (Muntifering *et al.* 2000; Powell *et al.* 2003; Gonzalez-Fernandez *et al*. 2008). There is evidence that O_3-induced changes in foliar chemistry can result in decreased utilization of nutrients and intake of energy by ruminant herbivores due to decreased quality of herbaceous vegetation (Krupa *et al.* 2004). More specifically, because of increased lignification and possibly increases in concentrations of secondary phenolic compounds in plant cell-walls due to exposure to O_3, many grassland species have been shown to experience a loss of forage nutritive quality for mammalian herbivores (Bender et al. 2006; Lewis et al. 2006; Ditchkoff *et al.* 2009). Current economic risk-assessment models do not consider the potentially significant effect of elevated O_3 concentrations in the troposphere on the consumable food value for these animals (Gonzalez-Fernandez *et al.* 2008). Still, relatively little is known about the effect elevated O_3 concentrations are having on wildlife species. Furthermore, previous experiments with O_3 and nutritive quality of forages refer to digestibility as IVDMD (*in-vitro* dry matter digestibility) or IVNDFD (*in-vitro* neutral detergent fiber digestibility), (Powell *et al.* 2003; Gonzalez-Fernandez *et al.* 2008), not actual digestibility coefficients as determined by a feeding trial with live animals.

Rabbits in general, are relatively inexpensive and represent an ideal and ecologically relevant model herbivore for these experiments because lagomorphs utilize similar selective foraging and digestive strategies as most wild and domestic mammalian herbivores. Domestic rabbits (*Oryctolagus cuniculus*) are common in most countries in the world and readily utilize a low-grain, high-roughage diet with consistent (19 h) transit time (McNitt *et al.* 1996). Rabbits are considered concentrate selectors (Cheeke 1987) and have a simple, non-compartmentalized stomach along with an enlarged cecum and colon inhabited by a microbial population (*Bacteroides*) (Irlbeck 2001).

Based on the absence of nutritional studies with live animals and nutritional responses to O_3, our goal was to verify results from previous *in vitro* studies (Muntifering *et al.* 2000; Powell *et al*. 2003; Bender *et al*. 2006; Lewis *et al*. 2006; Gonzalez-Fernandez *et al*. 2008) in a mammalian *in vivo* model. Our overall hypothesis was: rabbits receiving forages exposed to elevated O_3 concentrations would have decreased intake and digestive utilization of forages when compared with rabbits receiving forages grown under ambient O_3 concentrations. Based on these factors, our specific objectives were to: 1. determine O_3 effects on dry matter consumption and utilization of ruminant herbivores; and, 2. relate these effects to intake and digestion of forage cell-wall constituents and crude protein.

Methods

Field Site (Forage) Establishment

The Atmospheric Deposition Site of the School of Forestry and Wildlife Sciences at Auburn University, AL was the location used for field-fumigation of the forages. The site was located approximately 5 km from Auburn University and was a good representative microcosm of a rural agricultural area (Ball, personal comm.). There were 24 large (4.8 meter height, 4.5 m diameter) open-top chambers (OTC, Heagle *et al.* 1989) at this site, 12 of which were uniformly aerially seeded in 2007 (six ultimately used for forage collection in this experiment) with a combination of three common grass and one legume species found in the Southern Piedmont region. These species included tall fescue (*Lolium arundinacea*), dallisgrass (*Paspalum dilatatum*), common bermudagrass (*Cynodon dactylon),* and ladino clover (*Trifolium repens).* Forage species were chosen to establish a grassland community representative of the southern Piedmont region (Ball *et al.* 2002). Using principal component analysis (Gomez and Gomez 1984), six OTC (three chambers for each treatment) were selected for fumigation of forages with controlled concentrations of O_3 on the basis of uniformity of initial plant communities and soil characteristics. Non-filtered (NF) air was used as representative of ambient air in rural areas of the Southern Piedmont region in general (US EPA 2001) and air containing 2 × ambient-O_3 concentration (2 × NF) was used as a representative of concentrations currently found in the vicinity of large metropolitan areas in the Southeastern US such as Atlanta and Birmingham (Chameides and Cowling 1995). Chambers exposed to non-filtered (ambient) O_3 concentrations ultimately contained an average biomass composition of 50% clover and 50% grass with a range of 65-35%. Chambers exposed to 2X ambient ozone concentrations contained a mixture of 62% grass and 38% clover with a range of 91-9% (Gilliland, unpublished data). Consideration was given to chamber effects (Fuhrer 1994) as the effective area inside the chambers was 0.5 m from the inside edge of the chambers.

O_3 Fumigation System

O_3 fumigation began on May 13, 2008 and continued through July 2, 2008. Of the six chambers, three were designated for elevated O_3 exposure (2X, two-times ambient O_3 concentrations) and three chambers were exposed to non-filtered ambient O_3 concentrations (NF). Ozone was generated by passing pure oxygen through a high-intensity electrical discharge source (Griffin Inc., Lodi, NJ) and was applied to chambers daily (0900-2100 hrs, 7 days/wk). O_3 concentrations were continuously monitored in each OTC, and US EPA quality assurance guidelines were used for calibration of the O_3 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.

Sample (Forage) Collection and Processing

Forages from each OTC were clipped to a height of 10 cm aboveground in June and July 2008, bulk collected for each chamber, dried to constant weight in a forced-air oven at 55-60° C, air-equilibrated and weighed. Seasonal distribution of forage growth varies among species (Ball *et al*. 2002) such that a harvest at this time could be expected to contain an optimal representation of cool-season C_3 grasses, warm-season C_4 grasses, and clover. Also, based on previous research with similar species (Muntifering *et al*. 2000; Muntifering *et al*. 2006; González-Fernández *et al*. 2008), cumulative O_3-exposure by mid-summer is sufficient enough to expect that 2X forages could exhibit decreased nutritive quality as predicted by laboratory analysis. Forages from each of the six OTCs (3 chambers per treatment) were bulked by month from each chamber and compressed into 50-g forage blocks using a large hydraulic press.

Rabbit Feeding Trial

We obtained 16 weaned, 8-week old, female New Zealand White rabbits (*Oryctolagus cuniculus*) in December 2009 and housed them at the Biological Research Facility, Division of Laboratory Animal Health, Auburn University. Rabbits were maintained in slatted-floor, stainless steel cages [61 width x 76 length x 46 ht (cm)], with individual feeding troughs. Over the first few weeks in December, rabbits were transitioned from *ad libitum* commercial rabbit chow pellets to 200 g/day of NF diet created from the forages grown in 2008. Each rabbits received four 50-g forage blocks daily during transitioning. Rabbits were monitored for total and consistent consumption of NF diet over the transition days (two weeks). Rabbits demonstrating the most consistent foraging activity, based on constancy of forage intake and fecal output (steady-state), were used in the feeding trial. The ten rabbits selected were then stratified into live-weight outcome groups based on mass; within groups, each rabbit was randomly assigned treatment forages, with one group receiving forage blocks grown under 2X ambient O_3 conditions and the second group receiving forage blocks grown under ambient-ozone conditions.

Beginning in January 2010, rabbits received two 50-g forage blocks each day at 1400 h. The daily forage allocation was established based on the initial feeding results. Rabbits were provided with these forage blocks for ten days; water was available *ad libitum*. Orts for each rabbit were collected each day at 1330 h and pooled for future chemical analysis for all 10 days. The final six days consisted of fecal and urinary output collection for each rabbit. Orts, feces, and urine were collected from steel trays located under the rabbits' cages.

Laboratory Analysis

Orts and fecal pellets were collected all ten days, pooled, dried at 55-60^{o} C in a forced-air oven and weighed. Daily urine output for each rabbit was collected, stored in glass containers under refrigeration, and pooled into individual rabbit composites for N analysis by the Kjeldahl method (AOAC 1995). Urine was analyzed for crude protein (CP) content (N × 6.25) and used in CP digestibility calculations. Forages offered (diets), orts, and feces were ground through a 1-mm screen in a Thomas Wiley mill and analyzed for concentrations of dry matter (DM) and crude protein (N × 6.25) as previously described (AOAC 1995). Cell-wall constituents (cellulose, hemicellulose, and lignin) were analyzed by sequential detergent fractionation according to Van Soest *et al*. (1991). The Van Soest *et al.* (1991) method of sequential extraction first removes soluble cell components such as non-structural carbohydrates, lipids, pectin, and soluble protein while isolating partially-soluble, total cell-wall components [neutral detergent fiber (NDF)]. The partially-soluble cell-wall components (structural carbohydrates) consist mainly of the β-linked polymers cellulose and hemicellulose. After NDF is obtained, the acid detergent fiber (ADF) fraction is isolated by solubilizing the hemicellulose in the cell wall. Hemicellulose and protein bound to cell walls is removed from the cells, which allows for isolation of lignin and other recalcitrant materials [acid detergent lignin (ADL)] (Van Soest *et al.* 1991). These fractions are used to calculate relative food value (RFV) of forages according to Rohweder *et al.* (1978). RFV is a mathematical estimation of the quality of particular forages and is calculated by reference to digestible dry matter intake (DDMI), and was adopted to standardize forages with RFV of 100, containing 53% NDF and 41% ADF (Rohweder *et al.* 1978; Linn and Martin 1989).

Total phenolics were assayed using a modified version of the Folin-Ciocalteu (FC) assay (Blum 1997; Booker and Miller 1998). Total 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 (3x), centrifuged at 15,000 x *g* and supernatants were pooled. Residues were then washed with 1 ml of water

(1 X), 95% ethanol (3 X), dried in a rotary evaporator at 45^{o}C, and weighed. Extractive-free residues were then incubated in either 1 ml of 2N HCl at 80^{o}C for 1 h (acid hydrolysis) or 1 ml of 1N NaOH overnight at room temperature (alkaline hydrolysis). Afterward, samples were centrifuged, supernatants collected, residues were washed with 1 ml of water (2 X), centrifuged, and supernatants were pooled by sample. Aliquots (250 *u*l) of alkaline hydrolysate samples were acidified with 25 *u*l of 6N HCl to precipitate acid-insoluble lignin, centrifuged and supernatants collected. Each sample (50 – 100 *u*l) was assayed for total phenolics using the FC assay. Total phenolics were expressed as *p*-coumaric acid equivalents based on a standard curve obtained with *p*-coumaric acid using the FC method. All extractions and assays were conducted in duplicate. A protein precipitation assay for tannins (Waterman and Mole 1994) was performed with no indication of a significant amount of tannins in the samples.

Concentrations of phenolics in orts and diets were used in calculation of actual phenolics consumed. Phenolics consumed were calculated: [(phenolic concentrations in diet mg g^{-1} × g total DM fed) minus (phenolic concentrations in orts mg g^{-1} × g total DM orts)] / g total DM consumed. Similar calculations were used to determine cell wall constituents consumed and digestibility coefficients.

Experimental Design and Statistical Analysis

The experimental design was completely randomized, with treatments assigned randomly as 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 differences and phenolic compositions in the diets between and across treatments.

Results

Climatic and O_3 Exposure Data

Mean monthly air temperatures for May through July 2008 were, on average, 1.3 ^{o}C above the 30-yr (1971-2000) average for Auburn, AL (Table 1). 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 in June (66 and 126 ppb) were the greatest for the NF and 2X treatments (Table 2). Average monthly peak concentrations were 49 ppb and 102 ppb for the NF and 2X O_3 concentrations, respectively (Table 2).

Forage Nutritional Characteristics

Rabbits receiving O_3-exposed forages were fed a diet containing approximately 4.5% greater concentration of NDF than those receiving NF diets. The percent of ADF in the two diets was approximately equal (Table 3). NF diets on average contained 3% more crude CP than the 2X diets, probably due to the presence of more clover in the NF forages (Gilliland *et al*., unpublished). Acid detergent lignin in the two treatment diets constituted approximately the same percentage of the forages (Table 3). The NF diet contained 1.15 times the concentration of soluble phenolics as the 2X diet. The NF diet contained 18.5% lower concentration of acid-hydrolyzed phenolics than the 2X diet. Alkaline hydrolysable phenolics in the NF diet were 9.5% less than in the 2X diets, while the acid-soluble fraction of the alkaline hydrolysates was 20% less in the NF diet compared with the 2X diet.

Consumed Diet Nutritional Characteristics

Based on analysis of ort samples, average diets consumed for each treatment group differed significantly in nutritive quality. Rabbits receiving 2X diets consumed a diet containing significantly lower (1.2%) concentrations of ADF than rabbits receiving NF diets (Table 3). Rabbits receiving 2X forages consumed, on average, diets consisting of significantly greater percentages of NDF (54.1%) than rabbits receiving NF forages (50.31%) ($P < 0.001$). Mean percentages of lignin (ADL) in consumed diets were not significantly different between the two treatments (Table 3). Crude protein constituted 3.5% more of consumed diets for rabbits receiving NF forages and differed significantly from CP concentrations for 2X rabbit consumed diets.

Acid detergent fiber was less digestible in the 2X diet than the NF diet; 20.08% digestible for the 2X diet and 32.16% for the NF diet; $P < 0.03$ (Table 5). Neutral detergent fiber was also less digestible in the 2X diet at 31.7% digestibility compared with 39.6% digestibility in NF diets ($P < 0.10$). Overall DM digestibility for the 2X diets was on average approximately 6% less digestible than NF diets, although not statistically significant (P=0.14) (Table 4). Digestible dry matter intake was 5.5 g per day greater for rabbits receiving NF diets than rabbits receiving 2X forages ($P < 0.05$). Treatment groups consumed, on average, approximately the same percentage of body weight (2%) in dry matter for the experiment on an individual-rabbit basis (Table 4). Crude protein digestibility was 4.5% less for 2X than NF diets, but not statistically different between the two treatments (Table 5).

Rabbits receiving NF diets ingested (10 day total) 1.15 times the concentration of soluble phenolics as those fed 2X forages (Table 6). These consumed diets were significantly different in amount of soluble phenolics ingested with ($P < 0.001$) and were strongly associated ($r^2 = 0.86$). Regression of soluble phenolics with DM digestibility revealed a marginally significant positive relationship between soluble phenolics ingested and DM digestibility ($P < 0.075$, r^2=0.34) (Table 7). Regression of soluble phenolics ingested with NDF and ADF digestibility also revealed a positive relationship between soluble phenolics ingested ($P < 0.05$) and cell wall digestibility ($P < 0.05$) (Table 7). Decreased soluble phenolic concentrations in forages may simply be a reflection of decreased bound phenolics.

Acid hydrolysis releases ester-bound and ether-bound phenolic acids, mainly ferulic and *p*-coumaric acids, from cell walls. Rabbits receiving NF diets ingested 21% lower forage concentration of acid hydrolysable phenolics (10 day total) than 2X-fed rabbits (Table 6). Regression of DM digestibility and ADF digestibility with acid hydrolysable phenolics indicated a negative relationship, with ADF digestibility significantly decreasing as ingestion of acid hydrolysable phenolics increased ($P < 0.05$, r^2=0.41) (Table 7).

Alkaline hydrolysis releases ester-bound phenolic acids and alkaline-soluble lignin in a ligno-carbohydrate complex (LCC). The LCC is thought to decrease digestibility by bonding with cellulose in plant cell walls; forming strong bonds which are not as readily digested by micro-organisms in the digestive tract (Morrison 1979; Pigden and Heaney 1969). Rabbits receiving NF diets ingested a collective 10-day average of 48.84 mg g^{-1} of alkaline hydrolyzed phenolics while rabbits fed the 2X diet consumed an average of 52.89 mg g^{-1}; 1.08 times greater concentration ($P < 0.001$, r^2=0.84) (Table 6). Regression of alkaline–hydrolyzed "alkali-labile" phenolics with DM, NDF, and ADF digestibility indicated a negative effect of alkaline hydrolyzed phenolics on digestibility of the diets. Regression of alkaline hydrolysate with DM digestibility indicated a negative trend of ingested alkaline hydrolysate on DM digestibility ($P < 0.11$, r^2=0.28) (Table 7). The concentration of alkaline hydrolysate ingested was determined to have a marginally significant negative relationship with NDF and a significant negative relationship with ADF digestibility ($P < 0.10$, r^2=0.30 and $P <$ 0.02, r^2=0.49, respectively) (Table 7).

Acid-soluble, alkaline-hydrolyzed phenolics were also assayed. This is the alkaline-hydrolysate fraction from which the ligno-carbohydrate complex has been precipitated. Rabbits receiving the NF diet consumed a collective ten-day average of 16% lower concentration of acid-soluble alkaline hydrolysate than the 2X rabbits ($P <$ 0.001 level, r^2: 0.95) (Table 6). Regression of DM digestibility and NDF digestibility with acid-soluble alkaline hydrolysate ingested indicated a significant negative correlation of these phenolics on DM and NDF digestibility (Table 7). ADF digestibility also was negatively correlated with acid-soluble alkaline hydrolysate ingested (P-value = 0.02, r^2=0.54) (Table 7).

Discussion

The main objective of this study was to assess the effects of elevated ground-level O_3 concentrations on forage energy consumption and utilization by a non-ruminant, hindgut fermentor in grassland communities. Along with this objective, forage nutritive quality characteristics [neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and crude protein (CP)] were determined. The NDF and ADF fractions were used to calculate the relative feed value (RFV) of forages. RFV is a mathematical estimation of the quality of particular forages (Rohweder *et al.* 1978; Linn and Martin 1989). These calculations are important in determining the potential losses in digestibility of forages exposed to above-average concentrations of O_3 in the Southern Piedmont region (US EPA 2006). Also forages may be nutritionally available to herbivores for digestion and energy metabolism due to increased bound phenolic concentrations. This study is believed to be the first known experiment investigating O_3-exposed forage nutrient utilization by a live animal.

Although the two treatment groups did not consume statistically different amounts of dry matter per day (DM intake), Digestible dry matter intake was greater for rabbits receiving NF diets than rabbits receiving 2X diets. Digestible dry matter intake is a function of DM intake and DM digestibility and constitutes how much feed was actually available for energy metabolism. This difference indicates differing amounts of energy consumption between the two groups of rabbits, with rabbits consuming NF diets having more available energy for metabolism per day compared with rabbits consuming the 2X diet. NDF digestibility for the NF treatment was approximately 8% greater than in 2X diets. This difference is nutritionally important to herbivores because NDF digestibility is a reflection of total cell-wall digestibility and affects the amount of the forages an animal can ingest (Van Soest 1994). ADF is a portion of the plant call wall (cellulose and lignin) and affects digestion of the forage (Van Soest 1994). ADF digestibility suggested lower overall digestibility of the 2X forages. Crude protein concentrations in the two diets differed as well with NF diets consisting of approximately 3% more CP than 2X diets. The differences in fiber digestibility and crude protein concentrations can be partly attributed to differing amounts of clover to grass ratios between the two diets, with NF diets containing 10% more clover than 2X diets (Gilliland *et al*., unpublished data).

Forages exposed to elevated O_3 concentrations have been reported to contain greater lignin concentrations (Powell *et al.* 2003; Szantoi *et al.* 2007; Szantoi *et al.* 2009), and increased lignin concentration has a negative impact on forage digestibility by ruminant herbivores (Van Soest *et al.* 1991; Van Soest 1994; Muntifering *et al.* 2000; Krupa *et al.* 2004). Interestingly, in the present study there were significant differences in cell-wall digestibility and energy intake (DDMI) between the two treatments, with little difference in concentration of lignin in the diets offered or consumed. NF diets were actually found to contain slightly more lignin than 2X diets. The differences in lignin and crude protein could be attributed to the NF diets containing more clover than the 2X diets (Gilliland, unpublished). In the absence of a difference in lignin concentration of the diets or crude protein digestibility between the two treatments, cell wall digestibility differences are presumably attributable to other biochemical characteristics of the forages.

Studies indicate that exposure to elevated O_3 can cause forages to have increased concentrations of plant secondary metabolites (PSM) in response to oxidation stress (Chen and Gallie 2005; Booker 2009). Phenolic compounds are secondary plant metabolites that are produced in response to herbivory or other stressors such as pollution (Waterman and Mole 1994), and they can have deleterious effects on digestibility of forages (Fahey and Jung 1989). We observed a quantitative change in concentrations of specific phenolic fractions within forages exposed to 2X ambient O_3 concentrations. The significant negative relationships between cell wall-bound phenolics and NDF, ADF, and DM digestibility indicate that bound phenolics could account for lower digestibility of plant forages in a prototypical, non-ruminant, hindgut fermentor such as our study animals. This observation is consistent with Jung and Fahey (1981) who reported that phenolic compound removal increased digestibility of alfalfa (*Medicago sativa*). Specific phenolic fractions (saponifiable phenolics which contain LCC) were determined to have a strong correlation with digestibility depression in non-ruminant herbivore digestion of forages in this feeding trial. This digestibility depression contributed to a lower ingestion and utilization of energy in rabbits receiving 2X-exposed forages. Implications of lower digestibility and utilization of forages in herbivores is important for survival of both plant and animal species (Fraga *et al.* 1991; Ashmore 2005; Booker 2009). Ozone-sensitive forages such as white clover could become less nutritionally valuable due to increased phenolics. If herbivores preferentially select forages that are more readily digested due to community level changes such as this, it could have implications on future grassland community structure and function. If grassland species such as clover become increasingly less digestible due to increases in bound phenolics, overall grassland communities' nutritional value to herbivores could be lessened.

These are potentially significant implications for nutrient and energy cycling in grasslands. 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 areas such as the Southern US, nutrients such as nitrogen 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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Table 1. Mean monthly air temperatures, rainfall amounts, and 30-yr (1971 – 2000) means from May 13 – July 2, 2008; Auburn, AL, USA.

	Air Temperature (^oC)		Precipitation (cm)	
Month	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

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.

	O_3 concentration (ppb)					
	NF			2X		
Month	Mo. Mean	Avg. peaks	1 hr. peaks	Mo. Mean	Avg. peaks	1 hr. 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

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

Table 3

Cell wall constituents in diets offered and diet consumed [mean percentages (%) of diet for treatment groups].

Treatment	NDF	ADF	ADL	CP
	Diet Fed			
NF	51.03	30.68	5.31	16.66
2X	55.66	30.82	5.10	13.64
	Diet Consumed			
NF	50.31	29.15	4.77	15.7
2X	54.1	27.94	4.66	12.19
P-value	<0.001***	0.06*	0.52	<0.001***

Statistical significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin, CP = crude protein.

Table 4

Dry matter utilization by rabbits receiving NF and 2X diets.

Treatment	DM intake (g/day)	DM intake (% of body wt)	DM 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*

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

Table 5

Digestibility of cell wall constituents. Mean digestibility percentages (%) of cell wall constituents and crude protein on dry matter basis.

Treatment	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

*Significance at $P < 0.05$ level; $^{+}$ significance at $P < 0.1$ level.

Table 6

Phenolics consumed: concentration of ingested phenolics in diets (mg *p*-coumaric acid equivalents g^{-1}).

Phenolic fraction of cell wall	NF	2X	p-value
soluble phenolics	62.01	54.09	<0.001***
acid hydrolysable phenolics	10.53	13.39	<0.001***
alkaline hydrolysable phenolics	48.84	52.89	<0.002***
acid-soluble alkaline hydrolysate	37.29	44.55	<0.001***

***significance at $P < 0.001$ level

Table 7

DM, NDF, and ADF digestibility regression with consumed forage concentrations of phenolic fractions.

Phenolic fraction	DM Digestibility		NDF Digestibility		ADF Digestibility	
	r^2	p-value	r^2	p-value	r^2	p-value
Soluble	0.34	0.08$^+$	0.44	0.04*	0.62	0.01**
Acid hydrolyzed	0.18	0.22	0.22	0.17	0.41	0.05*
Alkaline hydrolyzed	0.28	0.11	0.30	0.10$^+$	0.49	0.02*
Acid-soluble alkaline hydrolysate	0.32	0.09$^+$	0.35	0.07$^+$	0.54	0.02*

Statistical significance (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, $^+$*P* < 0.1). DM: dry matter, NDF: neutral detergent fiber, ADF: acid detergent fiber.

IV. Overall Summary

The Southern Piedmont region spans 17 million hectares from central Virginia to eastern Alabama and consists of diverse managed grasslands (USDA 1981). Tropospheric ozone is the most significant phytotoxic air pollutant in this region, and the United States, and it is readily transported to agricultural and forested areas from urbanized centers. Estimating the effects of future climate change predictions such as increasing temperatures and differing rainfall amounts (MacCracken *et al.* 2001) as well as increasing ozone concentrations (Vingarzan 2004) is important for the Southern Piedmont region. To our knowledge, this study was the first to experiment with elevated ozone concentrations in combination with simulated rainfall amounts as predicted by future climate change models. The feeding trial with lagomorphs was also the first live-animal bioassay of herbivore response to ozone-exposed forages.

Plant Community Effects

In June – September 2009, several grassland species common to the Southern Piedmont region were examined for effects of ozone-exposure in combination with varying rainfall amounts: tall fescue (*Lolium arundinacea*), dallisgrass (*Paspalum dilatatum*), common bermudagrass (*Cynodon dactylon*), and ladino clover (*Trifolium repens*). The major findings were as follows:

- Dry matter (DM) yield of grasses was greater in 2X ozone treatments over the growing season as well as the final harvest than grasses grown in NF ozone treatments.
- Primary-growth clover (cumulative exposure over the season) decreased in nutritive quality in 2X treatments with increases in neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin (ADL).
- Regrowth grasses (one month of growth) responded positively to ozone with greater relative food value (RFV) and increased concentrations of crude protein (CP) than NF-exposed regrowth grasses.
- Regrowth clover exposed to 2X treatments exhibited decreased nutritive quality, [i.e. increased concentrations of cell-wall constituents (ADF and NDF) and lower RFV], in the final harvest as well as across the growing season.
- Precipitation alone did not have a significant effect on nutritive quality of forages or biomass production except in primary grasses for which DM yield was greater in chambers with high precipitation. Total canopy cover was significantly greater in chambers receiving above average (+ 20%) rainfall amounts.

Effects on Herbivore Nutrition (Rabbits)

In a laboratory nutrient balance experiment, caged-New Zealand White rabbits (*Oryctolagus cuniculus*) were fed forages that had been exposed to either ambient (NF) or 2-times ambient (2X) ozone concentrations in field OTCs. The nutrient-balance portion of the study (feeding trial) began in January 2010, with each treatment group (five rabbits each) receiving either NF or 2X-exposed forages consisting of dallisgrass, tall fescue, bermudagrass, and white clover. Major findings included:

- Neutral-detergent fiber and acid-detergent fiber digestibility by rabbits were significantly less for 2X than NF forages.
- Decreased digestibility of forages was significantly associated with increased saponifiable (i.e., alkali-labile) phenolic concentrations (plant secondary metabolites) in plant cell walls.
- Digestible dry matter intake (DDMI; energy consumption) of rabbits consuming 2X diets was also significantly less compared with rabbits receiving NF diets.
- Elevated ozone was determined to increase bound-phenolic concentrations in forages, especially in 2X forages, which was associated strongly with decreased digestibility of forages.

Overall Conclusions

Decreases in nutritive quality due to increased ozone concentrations, in combination with fluctuations in DM yield over the growing season, have implications for herbivores which rely on these plants for energy consumption (Barbo *et al.* 1998; Muntifering *et al.* 2000; Powell *et al.* 2003; Lewis *et al.* 2006; Szantoi *et al.* 2009; Ditchkoff *et al.* 2009; Booker *et al.* 2009). Ozone has age and species-specific effects on plants (Reich 1987; Heagle *et al.* 1989; Davison 1998; Muntifering *et al.* 2000; Bender *et al.* 2006; Gonzalez-Fernandez *et al.* 2008) resulting in increased cell wall constituents and decreased nutritive quality of ozone-sensitive species such as *Trifolium* spp., highbush blackberry, cutleaf coneflower, and Kentucky bluegrass that are important nutritional resources for ruminant and non-ruminant herbivores (Chappelka 2002; Muntifering *et al.* 2006; Bender *et al.* 2006; Gonzalez-Fernandez *et al.* 2008; Ditchkoff *et al.* 2009; Szantoi *et al.* 2009). Clover (RFV > 200) is a preferred food source of browsing herbivores. If clover nutritive quality and biomass are decreased due to ozone, while relatively insensitive species such as grasses (RFV < 100 generally) increased in biomass, these concomitant effects could have future negative impacts on herbivore nutrition. In addition to decreases in nutritive quality of plants due to increased cell wall constituents, ozone may also cause increases in concentrations of plant secondary metabolites such as saponifiable phenolics. Insoluble phenolics were negatively associated with decreases in digestibility of forages in rabbits. This could cause decreased nutritional value of plants that may not be exhibiting other ozone effects, such as visible injury or reductions in biomass.

Ozone concentrations are predicted to increase in the future, along with fluctuating rainfall patterns, and have potential to cause shifts in grassland species diversity and overall nutritive quality. Southern Piedmont grasslands are diverse communities with a mixture of warm and cool-season plant species that differ in sensitivity to ozone concentrations. Herbivores in these systems may alter their feeding strategies to compensate for declines in the more nutritionally valuable plant species that are adversely affected by ozone. Along with potential impacts on herbivore nutrition, is the long-term potential of decreased plant community biodiversity as influenced by sensitivity to ozone as well as herbivore pressure and selection.

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