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Understanding the Links Between Human Health and Climate Change: Agricultural Productivity and Allergenic Pollen Production of Timothy Grass(*Phleum pratense* L.) Under Future Predicted Levels of Carbon Dioxide and Ozone

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**UNDERSTANDING THE LINKS BETWEEN HUMAN HEALTH AND CLIMATE
CHANGE: AGRICULTURAL PRODUCTIVITY AND ALLERGENIC POLLEN
PRODUCTION OF TIMOTHY GRASS (*Phleum pratense* L.) UNDER FUTURE
PREDICTED LEVELS OF CARBON DIOXIDE AND OZONE**

A Dissertation Presented

by

JENNIFER M. ALBERTINE

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Plant and Soil Science

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ABSTRACT

UNDERSTANDING THE LINKS BETWEEN HUMAN HEALTH AND CLIMATE CHANGE: AGRICULTURAL PRODUCTIVITY AND ALLERGENIC POLLEN PRODUCTION OF TIMOTHY GRASS (*Phleum pratense* L.) UNDER FUTURE PREDICTED LEVELS OF CARBON DIOXIDE AND OZONE

SEPTEMBER 2013

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The prevalence of allergic disease is expected to increase with climate change. Grasses, which have highly allergenic pollen, are widely distributed across the globe. Changes in production and allergen content of grass pollen have not been specifically investigated. We tested the effects of elevated carbon dioxide and ozone on growth, pollen and allergen production of Timothy grass (*Phleum pratense* L.). Timothy is also used as an agricultural forage crop so changes in plant productivity can also affect humans indirectly. Plants were fumigated in eight chambers at two concentrations of ozone (O₃; 30 and 80 ppb) and carbon dioxide (CO₂; 400 and 800 ppm) to simulate present and future projected levels. Destructive harvests were completed every three weeks to measure productivity. Pollen was collected in polyethylene bags placed around flowers and assessed for pollen number and concentration of the allergenic protein, Phl p 5. We found that elevated CO₂ significantly increased the amount of pollen produced per flower regardless of O₃ level. In addition, the amount of Phl p 5 allergen per flower was significantly increased in plants grown at elevated

CO₂/ low O₃ conditions. We also found that plants grown in both elevated CO₂ and elevated O₃ increased the amount of pollen produced per weight of flower. The Phl p 5 allergen content per pollen grain was significantly reduced by elevated O₃, as was flower length and weight. However, this was partially ameliorated by elevated CO₂. Productivity was affected negatively by elevated O₃ throughout the life cycle. CO₂ increased shoot productivity during the intermediate stages of life and also ameliorated the negative impacts of elevated O₃. We conclude that increasing levels of CO₂ will cause a 2.5 times increase in Timothy grass pollen production thus increasing human airborne pollen exposure. Increases in pollen were likely a result of increased shoot biomass in the stages leading up to reproduction. If Timothy grass is a good model for other grasses, this portends for increased allergy suffering worldwide and an important health impact of global climate change.

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CHAPTER 1

INTRODUCTION AND HYPOTHESIS

1.1 Introduction

Climate change is occurring, with certainty, in part due to anthropogenic increases of greenhouse gases in the atmosphere (Trenberth et al. 2007). While climate change has occurred in the past history of Earth, never has it happened at the rate it is happening now (IPCC 2007). More importantly, never has it happened when the human population was 7 billion (US Census Bureau July 2013). The effect of climate change on human health is something that we need to understand now in order to prepare for the future (Shea et al. 2008; Kinney 2008; Confalonieri et al. 2007).

Allergic disease has a large impact on human health (AAAAI 2000; IPCC 2007). In the United States, health care costs from those suffering from allergic rhinitis (hay fever) is estimated to cost \$18 billion annually, affecting 50 million people, and resulting in 4 million days of lost time and productivity in the workplace and school (AAAAI 2000; Kinney 2008). Worldwide, allergic rhinitis affects 10-30% of the global population and asthma affects more than 300 million (WAO 2011). The prevalence of allergic disease has increased over the last few decades, the cause of which still needs to be determined (Kinney 2008).

Allergenic pollen production is expected to increase with future climate change and may increase both the severity and the numbers of people affected (Beggs 2004; Taramarcaz 2005; Beggs and Bambrick 2005; Confalonieri et al. 2007).

Hence it is important to assess how much pollen production may increase for the major allergenic taxa and the environmental factors that may influence it in the future.

Carbon dioxide is the most important greenhouse gas and is the major driver of climate change (IPCC 2007). Levels of this greenhouse gas are expected to rise to 730-1020 ppm by the year 2100 (Meehl et al. 2007; IPCC 2007). Likewise, background levels of tropospheric ozone, the third most important greenhouse gas (Sitch et al. 2007; Worden et al. 2008), are predicted to rise to 70 ppb or higher due to increasing levels of precursor gases (Thompson 1992; Vingarzan 2004). Increases in these greenhouse gases are mostly a result of the burning of fossil fuels by humans (IPCC 2007). In general, both of these atmospheric gases have been shown to affect plant growth. Carbon dioxide has been well characterized for stimulating photosynthesis and growth in C3 plants (Bazzaz 1990; Ainsworth et al. 2002; Long et al. 2004; Leaky et al. 2009; Reddy et al. 2010). Ozone has been shown to decrease growth due to oxidative damage of photosynthetic components (Lefohn 1992; Darrall 1989; Sandermann 1996; Skelly 2000; Krupa et al. 2001; Fiscus et al. 2005; Ashmore 2005; Booker 2009; Cho et al. 2011).

Here we look at the effects of elevated carbon dioxide and ozone on an important source of aeroallergen, Timothy grass (*Phleum pratense* L.), in an effort to increase the understanding of how allergenic pollen production in grasses will be affected by climate change.

Changes in production and allergen content of pollen in response to elevated carbon dioxide have not been investigated in grasses which are also highly allergenic. This is important knowledge for public health experts in their preparation for climate change effects because grasses are found all over the world and affect large populations of sensitized individuals (Beggs 2004; Confalonieri et al. 2007; Shea et al. 2008). Grass is the cause of allergenic response in approximately 20% of the general population and 40% of atopic individuals (Andersson and Lindholm 2003). Timothy grass was chosen as a model grass species for the ease of recovery of pollen from its flowers, and its ability to flower without vernalization allowing for experiments to be conducted in one growing season and removing the logistical problem of vernalizing under elevated carbon dioxide and/or elevated ozone conditions (Langer 1955; Heide 1982). Furthermore, Timothy grass is the second most allergenic species of grass, after orchard grass (*Dactylis glomerata* L.) (Wodehouse 1935). It is used widely in testing patients for grass allergy (Wodehouse 1935; Andersson and Lindholm 2003) and there is an ELISA assay kit commercially available for quantifying allergens.

In addition we examined the interaction of the oxidative air pollutant, ozone, on pollen production in Timothy grass, which is sensitive to ozone (Pleijel et al. 1996; Mortensen 1997, Mortensen 1999; Danielsson et al. 1999). Ozone has been shown to reduce viable pollen production by reducing anther starch content in *Lolium perenne* resulting in reduced pollen maturity (Schoene et al. 2004). However in the same species, ozone increased the Group 5 allergen, a

group with similar protein structure, content of the pollen (Masuch et al. 1997). Ozone has also been shown to decrease pollen viability through disruption of the cell membrane; however, this disruption has been hypothesized to increase allergenicity of the pollen by allowing cytoplasmic allergen granules to be released, which also contain allergen, thus increasing the exposure to allergen (Majd 2004; Motta et al. 2006; Pasqualini et al. 2011). Finally another experiment showed a decrease in the allergenicity of Timothy pollen through reduced IgE recognition of specific allergens when the pollen, not the plant, was exposed to a mixture of air pollutants: ozone, sulfur dioxide, and nitrogen dioxide (Rogerieux et al. 2007). Here we determine the effects of ozone on both pollen and allergen production when treated with ozone throughout entire life cycle of plant, as would occur in the natural environment. In addition, we also investigate how the possible negative impact of ozone may be ameliorated by elevated carbon dioxide.

Timothy grass is used widely as an agricultural forage crop. Understanding productivity in response to future concentrations of ozone and carbon dioxide is important to maintain high levels of productivity in future climates. Furthermore, whole plant productivity also controls reproduction so understanding the whole plant response is also important for understanding changes in future pollen production.

The effects of carbon dioxide on Timothy growth are not well understood. Research to date has focused on the agriculturally relevant above ground biomass. Results have been conflicting with some showing stimulation in

response (Norton et al. 1999) while others showing a decrease in growth (Saebo and Mortensen 1995; Mortensen and Saebo 1996; Vasseur and Potvin 1998). Biomass allocation to roots has not been evaluated. Crown allocation was evaluated in one experiment and found to increase in response to elevated carbon dioxide (Saebo and Mortensen 1995).

Management strategies for Timothy to date have strived to have a balance between nutritive quality of hay and ability for the plant to overwinter well (Childers and Hansen 1985). Accumulation of fructans in crowns has been shown to be the highest at full bloom (Smith and Groteleuschen 1966). This accumulation is important for re-growth and overwintering. Carbohydrate partitioning was not measured in other plant parts. An early cut of hay can increase the digestibility, protein content, and available metabolic energy (Childers and Hansen 1985). Changes in accumulation in the crown in response to treatments could potentially impact management strategies in the future.

Ozone has been shown repeatedly to decrease shoot biomass at intermediate levels of ozone (Mortensen 1992, Danielsson et al. 1999; Danielsson 2003). Degree of sensitivity across different cultivars is a result of rate of growth and stomata size (Danielsson et al. 1999; Danielsson 2003). However, roots have not been evaluated. The number of flowers has not been affected by ozone treatment (Danielsson et al. 1999).

Studies on the interactive effects of ozone and carbon dioxide together on Timothy indicate that carbon dioxide has the potential to ameliorate the negative impacts of ozone and vice versa (Johnson et al. 1996; Mortensen 1997;

Mortensen 1999; Mortensen 1999b). So far, biomass allocation change within the whole plant has not been evaluated, nor has carbohydrate allocation. Flower numbers have been shown to increase in response to elevated carbon dioxide and the increase was even larger in plants treated with both ozone and carbon dioxide (Johnson et al. 1996).

1.2 Objectives and Hypothesis

1.2.1 Objective 1

This study examines how future predicted levels of carbon dioxide and ozone affect the production and allergen content of the pollen of Timothy grass (*Phleum pratense* L.). Carbon dioxide has been shown to increase pollen production and allergen content in other species; ozone has also been found to affect pollen through reduced production and pollen coat disruption. Predicting changes in pollen amount and potency allows health care providers to prepare for possible changes in sensitized human populations. Furthermore, grass is widely distributed over the globe, thus changes in pollen production and potency could affect a large number of individuals. Our objective is to determine how future predicted concentrations of ozone and carbon dioxide will affect both pollen amount and concentration of the allergenic protein, Phl p 5, in the pollen.

1.2.1.1 Hypothesis 1-1

- Elevated carbon dioxide has been shown to increase plant growth and reproduction in other weedy species that are allergenic to humans. Ozone

is an oxidant and negatively affects plant development and growth. It is unknown how these two gases will interact to affect reproduction in grasses. I hypothesized that elevated carbon dioxide would increase the amount of pollen while ozone will reduce the amount of pollen. I also hypothesized that carbon dioxide would ameliorate the negative impacts of ozone allowing for increased pollen production with increasing carbon dioxide and ozone.

1.2.1.2 Hypothesis 1-2

- Elevated carbon dioxide increased the allergenicity of pollen in other weedy allergenic species. Results regarding ozone affects are less clear with some research indicating increased allergenicity and others reporting decreased allergenicity in pollen. I hypothesize that both elevated carbon dioxide and ozone will increase the Phl p 5 allergen content per pollen grain and will be greater than that found for ambient conditions or elevations of either gas alone.

1.2.2 Objective 2

To date, no one has investigated the effects of ozone and carbon dioxide on the whole plant processes over the life cycle of Timothy grass. Research has focused primarily on single stages of life cycle or only on aboveground biomass. Measured changes in whole biomass and carbohydrate allocation throughout the life cycle could indicate possible avenues of new management techniques for increasing agricultural productivity in changing climates. The objective here is to

determine changes in biomass and non-structural carbon allocation in response to future predicted concentration of ozone and carbon dioxide. We also investigated mechanisms for observed changes in biomass productivity through measurements of photosynthesis rates, stomatal conductance, chlorophyll content, antioxidants, and growth rate. Possible mechanisms by which carbon dioxide may ameliorate any negative impacts of ozone are also investigated.

1.2.2.1 Hypothesis 2-1

- Carbon dioxide and ozone are well characterized for their stimulation and reduction in growth, respectively. I hypothesized that elevated carbon dioxide would increase overall biomass of Timothy, especially in crown and roots, and that ozone would decrease biomass of shoot, crown, and root. I also hypothesized that elevated carbon dioxide will ameliorate the negative impacts of ozone.

1.2.2.2 Hypothesis 2-2

- As a result of elevated carbon dioxide, I hypothesized that there would be an increase in free sugars available in the plant. The excess of free sugars would result carbohydrate accumulation in the crown earlier in the life cycle of Timothy compared to other treatments. I also was expected that ozone would reduce the amount of free sugars available and thus reduce accumulation in crowns and root. I also predicted that carbon dioxide would ameliorate effects of ozone.

1.2.2.3 Hypothesis 2-3

- The mechanism behind the amelioration of ozone injury by carbon dioxide is hypothesized to be the result of increased antioxidant production and reduced stomatal flux. I hypothesize that carbon dioxide treated plants will have reduced stomatal conductance and increased antioxidant production.

1.2.2.4 Hypothesis 2-4

- Chlorophyll content was expected to decrease with ozone injury due to oxidative damage of the leaf tissue membranes. As a result I expected that photosynthesis rates would be higher in the plants not treated with elevated ozone. Furthermore, I hypothesized that elevated carbon dioxide would stimulate photosynthesis rates through increased use of the carboxylation photosynthetic pathway in response to increased intercellular carbon dioxide. Growth rate was hypothesized to increase in response to elevated carbon dioxide early in the life cycle but slow later in life cycle due to feedbacks in response to excess leaf sugars. Ozone was expected to reduce the growth rate throughout the life cycle due to sugar being diverted to defense and repair at the expense of growth.

CHAPTER 2

LITERATURE REVIEW

2.1 Climate Change

Carbon dioxide concentrations are currently higher than they have been in the last 700,000 years (Augustin et al. 2004). This rise in carbon dioxide is closely correlated with a rise in temperature due to the heat trapping abilities of the gas (IPCC 2007); carbon dioxide is the most relevant greenhouse gas in our atmosphere. Relevance of greenhouse gases is determined by their ability to trap heat from escaping to space as well as their abundance and persistence in atmosphere (IPCC 2007). Some greenhouse gases are necessary for the earth to remain at a habitable temperature. Without greenhouse gases trapping some heat and preventing it from reradiating back to space, Earth would be a giant ice ball (IPCC 2007). However, levels of greenhouse gases are on the rise resulting in increases in the amount of heat prevented from reradiating back to space. Since 1970, the temperature of the northeast region of the United States has been rising at a rate of 0.5°C per decade, with winter temperature rising faster than summer temperatures in response to rising carbon dioxide levels (Frumhoff et al. 2006). This excess heat energy being held in the earth's physical system translates into changes in weather patterns, especially hydrological cycles, which over time translate in to changes in climate. While climate change has occurred in the past, it is currently happening at a much faster rate than ever before (IPCC 2007).

The current increases in the greenhouse gases, such as carbon dioxide, are due to human actions both directly and indirectly (IPCC 2007). For billions of years, natural processes have stored carbon deep in the earth. During the Industrial Revolution in the 1600's humans discovered that this stored carbon, called fossil fuels, could be used to power our machines and give us electricity. Through our burning of the fossil fuels we release the carbon to the atmosphere as carbon dioxide and methane. The level of greenhouse gases in the atmosphere has risen exponentially since the Industrial Revolution (IPCC 2007). Furthermore, we are altering the capacity of the earth to sequester the excess atmospheric carbon dioxide through land use changes that involve cutting down forests and removing vegetation, which sequesters carbon dioxide from the atmosphere (IPCC 2007). As our population continues to grow, so do our reliance and use of fossil fuels and our changes to the land surface.

Additionally, our actions are also causing indirect forcing on climate change. Though increases in heat, the glaciers are melting exposing dark rock, which changes the albedo of the surface, and thus absorbs more heat instead of reflecting it, further heating the surface (IPCC 2007). As we replace vegetation with dark asphalt pavement, we are also changing the albedo and absorbing more heat into the earth's surface (IPCC 2007). Warming has also caused the permafrost to melt in the Arctic releasing methane, which is the second most relevant greenhouse gas (IPCC 2007). Methane release can also be found to increase in other anaerobic environments such as bogs and wetlands with

increasing surface temperatures (IPCC 2007). Methane is also released in great quantities from our large piles of buried garbage as it decomposes (IPCC 2007).

Through our burning of fossil fuels, we also release hydrocarbons and nitrogen oxides, which alter the natural photochemical cycle of ozone (Lefohn 1992). Ozone is also a greenhouse gas, third in relevance for its ability to trap heat (IPCC 2007). In addition, ozone is a powerful oxidant.

Historically, climate change has occurred naturally (IPCC 2007). Natural climate change has been caused by volcanic activity, changes in solar-activity, and changes in Earth's orbit around the sun (IPPC 2007). However, the rate and magnitude of which is it occurring now exceeds that of any past record of natural climate change (IPPC 2007). Additionally, past climate change did not occur when the human population on earth was as large as it is now.

Human health is an important factor to consider in preparing for the future. There are over 7 billion people on Earth whose lives are closely linked to current climate conditions and have adapted our water, food, and other necessities to these current climates (US Census Bureau May 2012, IPCC 2007). There are direct effects of climate change on human health associated with heat related mortalities and severe weather mortalities. However there are also indirect effects. Changes in temperature and rainfall can allow for new diseases and vectors of diseases to invade new areas and will also affect water and food security. Climate change has a large impact on human allergic airway disease by allowing allergenic species to invade new areas and by lengthening the pollen

season through a longer growing season (Rosenzweig et al. 2007) in addition to increasing pollen and allergen production.

There are several aspects of climate change that are expected to affect the amount of allergenic pollen (Beggs 2004; Taramarcaz 2005; Beggs and Bambrick 2005; Confalonieri et al. 2007). The stimulating effect of carbon dioxide has been proposed as the main driver of the increase (Ziska and Caulfield 2000; Wayne et al. 2002; Singer et al. 2005; Ziska et al. 2009; Rogers et al. 2006). Temperature is the second (Wan et al. 2002), creating a longer growing and flowering season for allergenic plants (D'Amato et al. 2002; Weber 2002, Beggs 2004; Rogers et al. 2006; Rosenzweig et al. 2007; Ziska et al. 2011), changing the timing of the allergy season (Burr et al. 2003), and allowing invasion of allergenic species into new areas (Voltini et al. 2000; Rybnicek and Jaeger 2001; Asero 2002; Huynen and Menne 2003; Taramarcaz et al. 2005; Cecchi et al. 2006). Land use change is also an important factor; disturbed land allows for quick establishment of weedy allergenic species (Van Vliet et al. 2002; Beggs 2004; Beggs and Bambrick 2005). Finally, air pollution, has been proposed to increase the severity of allergic disease caused by pollen allergy through irritation of the respiratory system (Mudway and Kelley 2000; Hauser et al. 2003; Gilmour et al. 2006, Davila et al. 2007; Shea et al. 2008). Ozone disruption of the pollen surface has also been considered as a factor in altering allergenicity (Masuch et al. 1997; Majd et al. 2004; Motta et al. 2006; Rogerieux et al. 2007; Pasqualini et al. 2011).

2.2 Response of plants to elevated ozone

Ozone affects plants through a number of mechanisms both direct and indirect. However, ozone does not affect all plants equally. The sensitivity of a particular plant both within and between species depends on the genotype of the plant and the developmental stage (Booker et al. 2009) as well as the length and concentration of ozone exposure (Lefohn 1992; Castagna and Ranieri 2009). Plant sensitivity is dependent on the ability of the plant to maintain function while adapting to ozone induced metabolic changes (Cho et al. 2011). It is this variability that makes quantifying universal ozone responses in plants so difficult.

Ozone is a gaseous pollutant and therefore enters the plant through the stomata. Once inside the stomatal cavity, ozone reacts with aqueous apoplastic regions of the cell wall and forms reactive oxygen species (free radicals, peroxides, and singlet oxygen) in what is called an oxidative burst (Sandermann 1996; Pell et al. 1997; Roshchina and Roshchina 2003; Booker et al. 2009; Cho et al. 2011). These reactive oxygen species then activate stress defense pathways (Pell et al. 1997; Mittler 2002). Unlike other plant stressors that also result in the ROS signal cascade, ozone creates a bimodal peak of ROS formation rather than just one peak downstream in signal transduction pathways (Joo et al. 2005; Castagna and Ranieri 2009). It is hypothesized that the initial contact of ozone with the aqueous cell surface is the cause for the first peak located in the cell wall, while the second peak is the result of signal transduction and is found in cytoplasm, peroxisomes, and mitochondria (Pellinen et al. 1999; Castagna and Ranieri 2009). ROS signal transduction pathways and the

oxidative damage that occurs in response activate production of many hormones such as ethylene, salicylic acid and jasmonic acid (Sandermann 1996; Pell et al. 1997; Rao and Davis 1997; Overmyer et al. 2003; Roshchina and Roshchina 2003; Kangasjarvi et al. 2005; Castagna and Ranieri 2009); which then act to turn on/off genes and change plant metabolism. Plants have avoidance and defense mechanisms in place to reduce the level of oxidative burst and thus the impact of ozone injury.

Altering the amount of ozone entering the stomata in an injury avoidance mechanism (Andersen 2003; Fiscus et al. 2005; Roshchina and Roshchina 2003; Crous et al. 2006). Stomatal numbers and sizes control the flux of ozone into the stomatal pore and vary between different species and also within a species (Danielsson 2003; Hetherington and Woodward 2003; Crous et al. 2006; Grulke et al. 2007) as a result of evolutionary pressures (Mansfield 1998; Hetherington and Woodward 2003). Plants growing in a high ozone environment have been shown to change the number and size of their stomata, particularly when sensitive to ozone (Elagoz et al. 2006).

Ozone has been shown to cause partial stomatal closure (Hill and Littlefield 1969; Crous et al. 2006; Wittig et al. 2007). The mechanisms behind this may have to do with altering of the potassium and calcium channels involved in stomatal opening and closing (Torsethaugen et al. 1999; McAinsh et al. 2002) resulting in reduced stomatal aperture. Disruption of these solute channels affects the electrochemical gradient that allows the guard cells to change turgor and open and close the stomatal pore. Hydrogen peroxide induction of abscisic

acid pathways have also been linked to disruption of the calcium status of the cell resulting in aperture reduction (Heath 2008). However, these mechanisms are still largely not understood (Paoletti and Grulke 2005). Ozone, particularly after long-term chronic exposure, has also been shown to make stomata less responsive to changes in the environment thus impairing their function (McAnish et al. 2002; Mills et al. 2009; Paoletti and Grulke 2010; Moldau et al. 2011).

The first line of defense is the presence of antioxidants in the apoplastic region of the leaf. Antioxidants protect plants by quenching ROS, through donation of an electron (Mittler 2002; Roshchina and Roshchina 2003). However, the protection is dependent on the amount of antioxidant produced and level of ozone exposure (Burkey et al. 2006; Cieslek et al. 2009, Gillespie et al. 2011, 2012). Some tolerant plants have been found to have high levels of antioxidants available for immediate quenching of ROS (Robinson and Britz 2000; Burkey et al. 2000; Burkey and Eason 2002; Heath 2008). Other plants must be induced to produce antioxidants in response to stress. Salicylic acid has been determined to be an important mediator in the signal to produce antioxidants (Rao and Davis 1999), known as systemic acquired resistance (Pell et al. 1997; Rao and Davis 1999). If the ozone level is too high in relation to the level of antioxidants the plant is able to produce then ROS will not be quenched and oxidative damage will occur (Heath 2008; Cieslek et al. 2009). Plants that are able to produce enough antioxidants still exhibit changes in their metabolism because making antioxidants requires energy and resources leaving less for growth and other processes.

While there are many types of antioxidants involved, ascorbic acid has been found to be most active in protecting plants against ozone injury (Burkey et al. 2006; Castagna and Ranieri 2009). Ascorbic acid is found in the apoplastic regions as well as within the cell in the cytoplasm, chloroplasts and vacuoles (Burkey 1999). Ascorbic acid acts as a reducing agent to oxygen radicals. Once it has donated its electron it becomes oxidized and is called dehydroascorbate (Robinson and Britz 2000; Roshchina and Roshchina 2003). It can be transformed back to ascorbate by the enzyme dehydroascorbate reductase (Roshchina and Roshchina 2003). Ascorbic acid is superior in protecting lipids against damage than most antioxidants because the products that are formed are less damaging (Chameides 1989).

If the plant is unable to produce enough protective antioxidants or as aging leaves are unable to upkeep needed levels of antioxidants (Pell et al. 1997), ozone and the resulting ROS can cause injury, particularly to the double bonds of lipids and enzymes (Roshchina and Roshchina 2003; Heath 2008). As the cell membranes are broken down by lipid peroxidation, more ROS is generated (Pell et al. 1997; Heath 2008). The break-down of the cell wall releases biologically active compounds that signal the production of other relevant compounds such as ethylene (Pell et al. 1997), salicylic acid (Rao and Davis 1999), and jasmonic acid (Castagna and Ranieri 2009). As damage continues to occur more signals are then sent which results in changes to metabolism and productivity through resources being diverted to defense and repair (Heath 2008). In chronic exposure, this leads to accelerated senescence

of the damaged lower functioning leaves even when there is no visible ozone injury (Castagna and Ranieri 2009). In acute exposure high doses typically over a short period of time plants often exhibit programmed cell death and have visible injury on leaves (Pell et al. 1997). This is known as the hypersensitive response (Pell et al. 1997, Castagna and Ranieri 2009). In the natural environment, plants are often exposed to a mixture of these two exposure types and must create a delicate balance between enough signaling molecules to elicit a gene response without resulting in accelerated leaf senescence or programmed cell death. Ethylene and salicylic acid tend to promote cell death while jasmonic acid acts to stop the spread of programmed cell death to undamaged cells (Kangasjarvi et al. 2005, Castagna and Ranieri 2009). However, jasmonic acid may play a role in the process of accelerated senescence (Overmyer et al. 2003).

Metabolite products released in signaling pathways can alter gene expression. Ethylene production in response to elevated ozone reduces transcription of the small sub-unit of Rubisco, chlorophyll *a/b*, and glyceraldehyde-3-phosphate dehydrogenase (Pell et al. 1997). Salicylic acid up-regulates genes that encode for increased antioxidant production (Rao and Davis 1999). Jasmonic acid up-regulates genes that make receptors that help reduce ethylene induced cell death (Overmyer et al. 2003).

Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) is broken down rapidly in response to ozone exposure; plants exposed to ozone have a lower peak production of Rubisco over their lifetime compared to plants not exposed to ozone (Pell et al. 1992, Pell et al. 1997; Pelloux et al. 2001,

Goumenaki et al. 2010). This is a result of injury to the enzyme by oxidative damage (Pell et al. 1997; Roshchina and Roshchina 2003; Heath 2008; Goumenaki et al. 2010) and reduced transcript of the Rubisco subunits, especially the small subunit encoded by *rbcS* (Pell et al. 1997; Heath 2008; Goumenaki et al. 2010).

As ozone injury affects gene expression and presence of important enzymes, this then translates into changes in plant processes and metabolism. Respiration rates of sensitive plants show an increase in response to ozone exposure (Barnes 1972; Pell and Brennan 1973; Amthor and Cumming 1988; Amthor 1988); as an example bean increased 14-41% (Pell and Brennan 1973). This has been attributed to increased transcription of enzymatic genes involved in maintenance respiration (Gillespie et al. 2012). Photosynthesis rate is decreased (Aben et al. 1990, Coleman et al. 1995); in bean it has been shown to be decreased 13-34% (Pell and Brennan 1973). Damage to photosynthetic components plays a large role. Chlorophyll has been shown to be damaged reducing light harvesting complexes and affecting electron transport chain (Meyer et al. 1997, Krupa et al. 2000, Fiscus et al. 2005; Beltzelberger et al. 2010). In addition to reductions in the enzyme Rubisco, reductions in stomatal conductance have also been considered as a factor because it decreases the available carbon dioxide for the carboxylation in the dark reaction of photosynthesis (Heath 1994). Leaf loss due to accelerated senescence also plays a role in photosynthetic decline (Coleman et al. 1995).

Carbon partitioning and allocation are also affected by ozone injury. Biomass allocation to roots is impaired by ozone exposure, resulting in reduced root growth in comparison to shoot growth (Cooley and Manning 1987; Grantz and Vu 2006). This is a result of several mechanisms. Lower, older leaves are the main source for roots and are often most damaged by ozone, or even senesced in response, due to length of exposure (Cooley and Manning 1987; Andersen 2003, Fiscus et al. 2005). Ozone has also been found to affect phloem loading due to damage of membranes around companion cells (Grantz and Farrar 1999, 2000; Fiscus 2005). Sugars may also just be retained in the leaf due to overall reduced availability and are not available for translocation due to needs for repairs, maintenance, and defense in the leaf (Fiscus et al. 2005; Grantz and Vu 2006).

2.3 Response of plants to elevated carbon dioxide

Elevated levels of carbon dioxide has been shown to have a stimulating effect on the growth of plants that use the C₃ pathway of photosynthesis those that do not have the carbon dioxide concentrating capabilities found in C₄ and CAM pathways. Current ambient concentrations of carbon dioxide are below optimum levels for C₃ pathway plants (Poorter and Narvas 2003). When carbon dioxide is not at optimum levels, oxygen competes with the carbon dioxide for the active site of the Rubisco enzyme in the carbon reaction of photosynthesis (Long et al. 2004, Ainsworth and Rogers 2007; Leaky et al. 2009). This causes the

plant to go through the inefficient photorespiration pathway reducing the rate of carbon assimilation (Taiz and Zeiger 2006).

The degree of stimulation by elevated carbon dioxide is dependent on the plant. The rate of electron transport in the light reactions or the rate of ribulose-1,5- bisphosphate (RubP) regeneration in the carbon reactions can be limiting factors in carbon dioxide stimulation (Makino 1994; Rogers et al. 2004; Ainsworth and Rogers 2007). RubP regeneration has recently been fingered as the major factor limiting carbon dioxide stimulation and is thought to be limited mostly by the availability of ATP for regeneration (Ainsworth and Rogers 2007).

Increasing the carbon dioxide in the sub-stomatal cavity reduces stomatal conductance, while still allowing for efficient assimilation of carbon, and reduces the loss of water through transpiration (Bunce 2004, Leakey et al. 2009). Plants show increased water use efficiency and higher photosynthetic efficiency (Ainsworth et al. 2002; Long et al. 2004; Ainsworth and Rogers 2007; Leaky et al. 2009). Furthermore, carbon dioxide has been shown to increase root growth in some species, which can also lead to more water availability and better water use efficiency (Bazzaz 1990). Water use efficiency is defined as the ability of plants to maintain high rates of photosynthesis while reducing amount of water lost through transpiration.

In the long term, accumulation of storage sugars occurs and plants exposed to carbon dioxide exhibit an acclimation (Ainsworth et al. 2003). The extent of acclimation is dependent on species and environmental conditions and plants can still show a stimulation response but at a lesser degree than in the

short-term (Leakey et al. 2009). Different mechanisms have been determined as to why this occurs rather than the plant continuing to be stimulated by elevated carbon dioxide.

It has been found that both sink capacity and nitrogen supply play a role in the acclimation of plants to elevated carbon dioxide. Elevated carbon dioxide stimulates the production of photosynthate through increasing the carboxylation efficiency of the Calvin Cycle. If there is not an adequate sink for this extra source, then accumulation of foliar carbohydrates occurs (Rogers and Ainsworth 2006). The build-up of carbohydrates due to a reduced sink has been shown to cause plants to down regulate photosynthesis through the feedback inhibition of photosynthetic genes by glucose and sucrose (Rogers and Ainsworth 2006; Pego et al. 2000). These genes regulate the production of Rubisco and thus regulate the rates of carboxylation in the Calvin Cycle portion of photosynthesis (Rogers et al. 1998; Ainsworth et al. 2003). Reduction of chlorophyll content was also measured as a response in the acclimation of photosynthesis in response to carbohydrate accumulation (Delucia et al. 1985). Reduced nitrogen in the plant is thought to exacerbate this by reducing growth of new sinks within the plant (Ainsworth et al. 2007) as well as limiting Rubisco (Long et al. 2004).

Prior to the start of FACE (Free Air CO₂ Enrichment), experiments were conducted in pots and acclimation was seen quite regularly due to root restriction and the “pot effect” (Arp 1991; Stitt 1991; Long et al. 2004; Ainsworth and Rogers 2007; Leakey et al. 2009). Roots are the largest carbohydrate sinks for plants and if the rooting volume is restricted then so is the sink capacity (Arp 1991).

This has been demonstrated in experiments with cotton in different size pots, where photosynthesis rates were reduced when roots were restricted resulting in reduced biomass (Thomas and Strain 1991). A meta-analysis of trees also indicated that acclimation was an effect of pot size (Curtis 1996). It was also demonstrated using soybeans planted at equal densities but in either pots or in the ground (Fiscus et al. 2007); however measurements of reduced photosynthesis in restricted roots were less consistent in this experiment. The smaller root volume of the pots did result in reduced yield. While this is not something that would happen in the natural environment, this idea of reduced sink strength is still important in assessing the effect of elevated carbon dioxide. Plants grown in the city, where root growth is limited could potentially uptake less carbon and this should be kept in mind when determining carbon sequestration in certain areas.

Once FACE was started, plants could be grown with unrestricted root growth in the ground. However, accumulation of carbohydrates was still being observed in response to elevated levels of carbon dioxide (Long et al. 2004; Rogers and Ainsworth 2006). This was tested on soybean plants with indeterminate growth to determine if plants could increase their sink in response to the elevated carbon dioxide; results indicated that there was still significant accumulation of glucose, fructose, and starch in leaves of plants grown in elevated carbon dioxide experiments (Rogers and Ainsworth 2006). Manipulations of the source-sink balance were conducted to see how this affected the carbohydrate accumulation using *Lolium perenne* (Rogers et al.

1998). Here they added in nitrogen as a treatment to make sure that accumulation was not a result of deficient nutrients reducing sink capacity. Every 4-8 weeks about 80% of the above-ground vegetation was removed thus decreasing the photosynthetic capability and making the sink larger than the source. Prior to the harvest, the low N treatment had significantly more accumulated leaf carbohydrate in the elevated carbon dioxide treatments than the high N treatments. However, immediately following the harvest, when the sink was greater than the source, there was no effect of nitrogen treatment. This indicated that N had a part in the accumulation of leaf carbohydrates but only when the plant was limited by its sink capacity. The reduction in accumulation of leaf carbohydrates under elevated CO₂ in response to high-nitrogen supply was also observed in other source-sink manipulation FACE experiments (Isopp et al. 2000) and the same (Rogers et al. 1998) experiment 10 years later (Ainsworth et al. 2003). Trees also exhibit an increased photosynthetic acclimation in response to nutrient stresses (Curtis 1996; Tuba and Litchenthaler 2007).

Nitrogen effects on the acclimation of photosynthesis to elevated carbon dioxide were investigated further. It was hypothesized that plants that are not limited by N due to symbiotic relationship with N₂ fixing symbionts would not acclimate to elevated carbon dioxide. Using *Trifolium repens* this hypothesis was tested and found that acclimation did occur in response to elevated carbon dioxide; however, it was less and stimulation of photosynthesis was still observed at high levels of carbon dioxide (Ainsworth et al. 2003).

The interaction between elevated carbon dioxide and nitrogen nutrition is an important aspect of ecosystem response to future predicted levels of carbon dioxide and must be understood further to fully comprehend the effects of elevated carbon dioxide. As stated previously, it has been well characterized that elevated carbon dioxide reduces photosynthesis via genetic down regulation of Rubisco initiated by an increase in leaf carbohydrate signaling (Leaky et al. 2009; Long et al. 2004; Stitt and Krapp 1999) and this is exacerbated by a reduction in leaf nitrogen (Ainsworth et al. 2007; Ainsworth and Rogers 2007; Ainsworth et al. 2003; Isopp et al. 2000; Leaky et al. 2009). There have been several hypotheses as to why the leaf nitrogen is being reduced.

The first hypothesis is that N concentrations are merely diluted in the leaf in response to the increased levels of carbon assimilation, thus altering the C:N ratio (Taub and Wang 2008; Stitt and Krapp 1999). In addition, increases in sugar can carry over to increases in N metabolism through uptake and assimilation into compounds thus reducing the pool of nitrogen stored in the leaf (Stitt and Krapp 1999). The second has to do with the uptake of nitrogen from the soil. Reductions in transpirational pull due to reduced stomatal conductance, as well as reduced N demand in the leaves due to down regulation of photosynthetic enzymes, result in less N being absorbed through roots; this can be further enhanced by architectural changes in the roots (Taub and Wang 2008; Stitt and Krapp 1999). The third theory is that there is increased exudation of carbon out of the roots, which enhances the microbial community. In response, they are increasing their microbial mineralization of nitrogen compounds resulting

in increased mobilization causing increased leaching of N (Taub and Wang 2008; Diaz et al. 1993; Cheng et al. 2011). Finally, carbon dioxide has been shown to inhibit the assimilation of nitrate into organic compounds within the plant.

Several methods were used to test this hypothesis and in each method demonstrated that there was greater assimilation of nitrate under ambient CO₂ concentrations (Bloom et al. 2010).

2.4 Amelioration of negative ozone impacts by elevated carbon dioxide

As discussed above, elevated carbon dioxide has been shown to stimulate the growth of C3 plants, particularly in annual crop type plants (Fuhrer 2003; Ziska and Bunce 2007) while elevated ozone reduces growth and yield of these plants (Booker et al. 2009; Wilkinson et al. 2012; Krupa et al. 2000; Fiscus et al. 2005). The interaction between these two gases has been studied in trees (Darbah et al. 2008; Riikonen et al. 2008; Kubiske et al. 2007; King et al. 2005; Osaken et al. 2005; Liu et al. 2004; Karnosky et al. 2003; Isebrands et al. 2001), herbaceous wild species (Kanerva et al. 2007; Awmack et al. 2007), and some crops (beans, soybeans, wheat, potato, and radish) (Heagle et al. 2002; Mulholland et al. 1998; Balaguer et al. 1995; De Temmerman et al. 2002; Barnes and Pfirrmann 1992; Heagle et al. 2000; Heagle et al. 1998; Fiscus et al. 1997). While the focus of some of these experiments was competition among sensitive and tolerant individuals (Awmack et al. 2007; Kubiske et al. 2007), the general consensus is that carbon dioxide reduces some of the negative impacts of elevated ozone; however, this reduction is not sufficient enough to allow for

stimulation of growth above control plants (Barnes and Pfirrmann 1992). The reduction is largely dependent on the sensitivity of the species to ozone (Heagle et al. 2002).

The mechanism by which this occurs is still under investigation by plant scientists. However, there are two emerging rationales in the literature that strive to explain this phenomenon. The first deals with the flux of gases into the stomatal pore and the second deals with changes in biochemical defense processes (antioxidants). These are the same defenses found in plants that are tolerant to ozone. It appears that these defenses are magnified in response to elevated carbon dioxide.

Carbon dioxide has been demonstrated to decrease stomatal conductance by increasing the substomatal carbon dioxide concentration (Paoletti and Grulke 2005, Ainsworth and Rogers 2007, Gillespie et al. 2012). In response to elevated carbon dioxide the guard cell membrane becomes depolarized resulting in decreased stomatal aperture. Depolarization occurs through increasing the outward and decreasing the inward potassium channels, which increases the calcium levels inside and stimulates chloride release from guard cell (Ainsworth and Rogers 2007). This smaller aperture limits the uptake of ozone into the plant. This reduced flux has been demonstrated in several experiments (Booker and Fiscus 2005; Fiscus et al. 1997; Gillespie et al. 2012; McKee et al. 1997). Flux appears to play less of a role in trees; acclimation over time results in less of an effect on stomatal aperture in response to elevated carbon dioxide (Onandia et al. 2011; Uddling et al. 2009). It is unknown whether

reduction of stomatal conductance will occur over a long period of time (Paoletti and Grulke 2005; Bernacchi et al. 2006)

Increased antioxidant capacity due to increases in photosynthate by elevated carbon dioxide has also been shown to reduce ozone injury (Booker and Fiscus 2005; Rao et al. 1995; McKee et al. 1997). This method is much harder to characterize as antioxidants are broken down in their quenching of reactive oxygen species (Gillespie et al. 2012, Gillespie et al. 2011; Robinson and Sicher 2004). Furthermore, because of the previous mechanism of reduced flux into the stomata, antioxidant production is sometimes muted in response to the two gases (Gillespie et al. 2011, 2012). However, this quantification has become easier through measuring the up-regulation of the genes creating antioxidant rather than the antioxidant concentration itself (Gillespie et al. 2012; Rao et al. 1995). Elevated ozone increases transcripts for antioxidants, while elevated carbon dioxide alone reduces transcripts (Gillespie et al. 2011). When the two gases are in combination there is an increase in antioxidants, however there is a measured delay in the transcriptional response (Booker and Fiscus 2005; Gillespie et al. 2011, 2012).

2.5 C3 vs C4 Grasses- Evolution, Distribution and Elevated CO₂ Response

The C3 photosynthetic pathway evolved first however it was thought that that C4 pathway evolved following a drop in atmospheric CO₂ concentrations 32-25 million years ago (Christin et al. 2008; Clayton 1981). C4 grasses rose to dominance in some biomes 3-8 million years ago (Edwards et al. 2010). The C4

pathways differs from the C3 pathway in that it uses an enzyme, phosphoenolpyruvate carboxylase (PEPcase), to concentrate CO₂ in the mesophyll cells prior to transporting it for use by Rubisco in the carbon reactions (Christin et al. 2008). This mechanism reduces the oxygenation pathway of the carbon reaction, increasing photosynthetic efficiency in low CO₂ environments.

Today, C4 plants distribution is limited to low latitudes and low elevations, where temperatures are generally warmer (Edwards et al. 2010; Edwards and Still 2008, Clayton 1981). Distribution can also be controlled by rainfall amount and timing, with areas of lower rain favoring C4 species in North America (Paruelo and Lauenroth 1996). About 25 % of the terrestrial biome that photosynthesizes is made of C4 plants, with 60% of these being C4 grasses (Edwards et al. 2010). The C4 grasses dominate the warm-climate grasslands and savannahs (Edwards et al. 2010).

Elevated CO₂ stimulates growth in both C3 and C4 grasses. However, stimulation in C4 grasses is 10% less than C3 grasses (Wand et al. 1999). Furthermore C4 grasses typically showed an increase in leaf area while C3 grasses show increased tillering (Wand et al. 1999).

2.6 Timothy Grass (*Phleum pratense* L.)

Timothy grass (*Phleum pratense* L.) is a member of the grass family, Poaceae (Gramineae), sub family, Pooideae, and tribe, Agrostideae (Lewis et al. 1983). It is a perennial cool season (C3) bunchgrass. The root system is shallow and fibrous. Individual stems are biennial; however it maintains its perennial form

by producing new stems. New shoots form from the base of the crown and these new shoots produce their own crown (Childers and Hanson 1985).

Each shoot is capable of producing one flower. In the northeast, Timothy typically flowers from early summer through early fall (Lewis et al. 1983). Timothy flowering is long day initiated (Langer 1955; Heide 1982).

Timothy grass is a wind-pollinated plant and pollen can travel up to 1 km away (Raynor et al. 1972). It releases its pollen early in the morning. Warm air temperatures and high wind increase the release of pollen while high humidity or rain reduces pollen release (Lewis et al. 1983). Grass pollen allergy is wide spread, in North America it is only second in significance to ragweed allergy. Timothy grass is the second most allergenic grass pollen, after *Dactylis glomerata* L. (Wodehouse 1935) and is one of the worst causes of early summer allergies. Timothy grass is not a quick invader of disturbed areas and so it is contained to agricultural fields where it is grown as a forage crop or in natural grasslands (Lewis et al. 1983; Childers and Hanson 1985).

Early settlers along the east coast from New Hampshire to North Carolina first discovered Timothy grass for agricultural use in the 1700's. It was originally known as Herd's Grass in the north and Timothy in the South; named after the men who discovered it and started cultivating it. It is not native to North America but was likely brought over by early settlers in hay, manure, and ballast cleaned off ships. It is native to Europe and is commonly known as Cat's Tail or Meadow Cat's tail. Timothy produces high quality hay adapted to the cool humid climate of the Northeast (including Great Lakes areas) and Northwest United States

(Childers and Hanson 1985). There are many varieties of Timothy each varying in their growth characteristics, stem and leaf characteristics, head type, flowering time, life span, and winter hardiness (Childers and Hanson 1985).

Timothy grass is primarily cut at full bloom, as this is when the dry matter yield is the greatest without loss of quality. While fertilizer use, cultivar, and location affect growth; it is the stage at harvest that is the main factor in the quality of the hay. Earlier cut hay can increase the digestibility, protein content, and the available metabolic energy in the hay when compared to hay that is cut later (Childers and Hanson 1985). This timing is also important for re-growth and overwintering. At full bloom, there is rapid accumulation of fructans in the crowns, which is important for re-growth and overwintering (Smith and Groteleuschen 1966).

2.7 Timothy response to elevated carbon dioxide and ozone

As Timothy grass is important agriculturally, growth response to elevated carbon dioxide and ozone has been investigated with respect to agricultural yield and management. Experiments have investigated the effect of ozone, carbon dioxide, and a combination of the two gases on above ground biomass production. However, whole plant response and physiological changes that determine these growth responses have not been measured in detail.

Two papers investigated the effects of elevated carbon dioxide on the growth and regrowth of Timothy grass. Three cultivars of Timothy grass were grown in a cool climate at elevated (680 ppm) and ambient carbon dioxide levels.

Plants were established in a greenhouse and then transferred to outdoor chambers. Three harvests were conducted when the plants were at the desired harvest stage, full bloom. It was found that elevated carbon dioxide did not increase the number of tillers at the first harvest, but the third harvest showed a 29% increase in tillers, due to increased carbohydrate reserves from first two harvests (Saebo and Mortensen 1995). Plant height was reduced by 18-24% in all harvests (Saebo and Mortensen 1995; Mortensen and Saebo 1996). The dry weight of the shoots was decreased by 24% in the first harvest and 17% in the second harvest; there was no significant decrease in the third harvest. However, the plants were 25-64% more dense (unit plant/unit air space) due to the increased tillering and decreased height (Mortensen and Saebo 1996). This compact growth can also result in increased self-shading thus reducing potential growth (Mortensen and Saebo 1996). At the final harvest the stubble (crowns and some roots) was also analyzed and found to be 24% larger under elevated carbon dioxide (Saebo and Mortensen 1995). It is the increased allocation of carbon to this stubble that allowed for better regrowth in subsequent harvests. The reserves in the stubble also are important for winter survival in cool climates with short growing seasons (Saebo and Mortensen 1995). A chamber effect was also observed in this experiment.

FACE experiments allow for elevating the levels of carbon dioxide without creating a chamber effect. Norton et al. (1999) conducted research observing the growth of Timothy grass in an experimental grassland community with several other species. Two harvests were also conducted throughout the two-year

experiment and it was found that the growth of Timothy grass was not significantly stimulated by elevated carbon dioxide (600 ppm), through measurement of dry weight. It was also found that the harvests in the second year showed a significantly less stimulation when compared to the first (Norton et al. 1999).

The next experiment looked at the effects of elevated carbon dioxide on a natural grassland community. Open top chambers were placed over the grassland. Timothy grass was one of the species present in the grassland and showed a positive response to the elevated carbon dioxide in that it was one of the more abundant species in the mixtures as carbon dioxide levels increased (Vasseur and Potvin 1998).

Results from past experiments are interesting. It is unclear whether elevated carbon dioxide does or does not stimulate above ground biomass of Timothy grass initially. However, it appears that growth of the crowns, which are the storage organs, do benefit from elevated carbon dioxide allowing for better regrowth after a harvest. As Timothy grass is harvested for hay, elevated carbon dioxide may benefit agricultural use of this species through allowing for more than one harvest in a growing season.

Timothy grass (*Phleum pratense* L.) is sensitive to ozone. At ozone levels of 55 nmol mol⁻¹ it was found that Timothy grass had a 45% reduction in dry weight shoot biomass (Mortensen 1992). This experiment was conducted in fumigation chambers inside greenhouses.

Experiments have also been conducted outdoors in open top chambers. Several genotypes of Timothy grass from Nordic countries were evaluated for their sensitivity to ozone with respect to biomass accumulation and flowering (Danielsson et al. 1999). It was found that ozone significantly reduced above ground biomass. Ozone injury was correlated with growth rate; genotypes with the fastest relative growth rate also had the most sensitivity to ozone (Danielsson et al. 1999). It was also found that genotypes with the greatest stomatal conductance also showed the greatest injury (Danielsson 2003). Flowering was not significantly reduced by ozone exposure; flowering data was likely confounded by warmer temperatures in response to greenhouse effect in chambers (Danielsson et al. 1999).

Lastly, the effect of ozone on the yield and quality of Timothy grass in a grass clover mixture was investigated (Pleijel et al. 1996). Plants were not assessed separately. While ozone was found to have little effects on yield or quality of the pasture, there was a negative correlation between ozone levels and time of exposure with overall productivity (Pleijel et al. 1996).

The interactive effects of both elevated carbon dioxide and ozone on the growth of Timothy grass has also been investigated briefly. The first experiment investigated the effects of elevated carbon dioxide and ozone on the growth and above ground competition of Timothy grass and alfalfa (Johnson et al. 1996). In this experiment roots were kept separate using Rootainers. It was found that increasing the carbon dioxide from 350 ppm to 700 ppm resulted in stimulation of growth, measured in dry biomass, for both species under low ozone (0.03 $\mu\text{l/l}$).

Under intermediate levels of ozone (.08 $\mu\text{l/l}$ and 0.13 $\mu\text{l/l}$), Timothy grass showed a reduction in biomass. However at the highest level of ozone (0.18 $\mu\text{l/l}$), there was stimulation in growth at both carbon dioxide levels. This was due to the alfalfa being more sensitive to ozone thus having a greater reduction in biomass and allowing increased light for Timothy grass growth and reducing competition. It was also shown that Timothy root production was not altered by elevated ozone (Johnson et al. 1996). Flowering was also evaluated: ozone had no effect on flower numbers, carbon dioxide increased the number of flowers, and the combination of both gases produced the greatest number of flowers (Johnson et al. 1996).

Next, the effect of elevated carbon dioxide was investigated while looking at the interactive effects of ozone, soil type, and temperature on Timothy in a mixture of two other grass species (Mortensen 1997). It was found that carbon dioxide stimulated shoot growth while ozone reduced shoot growth of the entire mixture. Soil types had little effect and temperature showed no interaction with carbon dioxide. Assessment of the plants separately showed that Timothy grass growth was reduced at low carbon dioxide and high ozone; the authors attributed this to the increase in growth of other species in the mixture competing with Timothy. The reduction in Timothy growth by high ozone was ameliorated by elevated carbon dioxide (Mortensen 1997).

Mortensen (1999) then looked at the effects of ozone on Timothy under different levels of light, relative humidity, temperature and carbon dioxide. He found that decreasing the temperature, as well as increasing the relative humidity

both resulted in more ozone injury (Mortensen 1999). On the other hand, he found that increasing the carbon dioxide levels reduced ozone injury (Mortensen 1999). He found that changes in light had little to no effect on ozone injury in Timothy.

Lastly, the effects of carbon dioxide and ozone with relation to day length and irradiance levels were assessed (Mortensen 1999b). Here it was found that Timothy shoot growth was stimulated by elevated carbon dioxide but that there were no interactive effects of ozone, day length, or light levels on the dry biomass of Timothy. However it was found that the number of visible lesions caused by ozone injury was decreased by both increasing carbon dioxide and total irradiance (both day length and level of irradiance). It was also observed that the elevated level of carbon dioxide increased the number of shoots in Timothy (Mortensen 1999b).

2.8 Elevated carbon dioxide effects on other allergenic species

In greenhouse experiments it has been shown that a doubling of current atmospheric CO₂ concentrations increased pollen production by 61% in ragweed (*Ambrosia artemisiifolia* L.), another allergenic plant species (Wayne et al. 2002). Another experiment looked at the effects preindustrial concentrations (280 μmol mol⁻¹), current concentrations (370 μmol mol⁻¹), and future predicted concentrations (600 μmol mol⁻¹) of CO₂ on pollen production of ragweed; found that current levels have increased the pollen by 131% and future levels are expected to increase the pollen by 320% compared to preindustrial levels (Ziska

and Caulfield 2000). They found this increase was due to the number and size of the flowers.

The interactive effects of elevated carbon dioxide and onset of spring on ragweed pollen production were also investigated (Rogers et al. 2006). Coincident with an increase in biomass, were weight per inflorescence, number of inflorescences, an earlier floral initiation, and an increase in pollen production (Rogers et al. 2006). It was found that ragweed pollen increased with an earlier onset of spring, with the earliest onset producing 54.8% more pollen than the latest. With elevated CO₂, the earliest onset did not show a significant increase in pollen production but the latter two showed an increase in pollen of 32% (middle) and 55% (last).

Experiments with ragweed were also conducted under realistic conditions using a natural gradient in CO₂ and temperature found between urban and rural environments (Ziska et al. 2003). Here they used four sites along an urban to rural transect, with the most urban site measured to be 30-31% higher in CO₂ concentrations and 1.8-2.0°C warmer than the most rural site. They found that the ragweed at the urban site grew faster, flowered earlier, produced greater aboveground biomass, and produced more pollen.

While these experiments demonstrated that CO₂ would increase the amount of pollen produced by ragweed plants, they say nothing about whether the allergenic properties of the pollen will change under elevated CO₂. This question was addressed in an experiment that looked at the effects of elevated CO₂ on the level of Amb a 1, ragweed's major allergen, in pollen proteins (Singer

et al. 2005). They found that there were increases in Amb a 1 from pollen of plants grown in future CO₂ concentrations when compared to current and preindustrial concentrations. This increased potency combined with the increase in pollen load results in a much larger increase in allergenic pollen predicted for the future (Ziska and Caulfield 2000).

2.9 Reproductive allocation in wind-pollinated plants

Reproduction is a costly activity for plants (Obeso 2002). However, it is necessary and is an indicator determinate of plant fitness (Reekie and Bazzaz 1987). The plant must balance reproductive success against defense and growth (Karlsson and Mendez 2005; Obeso 2002). This balance shifts depending on strategy of plant. Early successional species, known as colonizers, have ample nutrient available to them due to low competition and will often be able to devote more resources to reproduction than plants from mature communities where competition is more prevalent (Harper 1967).

In areas of high competition, where plant density is high and nutrients are limiting, a hierarchy evolves where there are a few large, dominant plants and many small plants (Harper 1967). Reproductive allocation is less in smaller, subordinate plants. Environmental change, such as elevated carbon dioxide, can affect this hierarchy (Stinson and Bazzaz 2006; Stinson et al. 2006). This has been observed in *Ambrosia artemisiifolia* where subordinate plants in stands increased their reproductive capacity to match that of the dominant plants (Stinson and Bazzaz 2006).

Generally, environmental factors that can increase reproductive allocation are those that will generally increase overall plant growth through increased resources. Light, nutrient availability, soil moisture and temperature all affect plant growth and thus reproduction; levels that are optimum for growth will also be optimum for reproduction (Salisbury 1942). Reekie and Bazzaz (1987) determined that light and nitrogen increased reproductive allocation in *Agropyron repens*, a wind-pollinated plant.

The signal to begin to flower comes from levels of endogenous compounds in the plant. The ratio of C:N in phloem sap has been shown to increase just prior to flowering in *Arabidopsis thaliana* (Corbesier et al. 2002). Elevated carbon dioxide has been found to alter the C: N ratios, thus resulting in earlier flowering in carbon dioxide exposed plants (Taub and Wang 2008). Hormones also play an important role in signaling the plant to begin flowering by regulating gene expression. Levels of ascorbic acid, gibberellins, salicylic acid, and ethylene have been found to be involved in the process of flowering (Barth et al. 2006). Since ozone also triggers production of these hormones, ozone has been found to cause earlier flowering (Sandermann 1996; Pell et al. 1997; Rao and Davis 1997).

Sex allocation is also regulated by hormonal control in response to the environment (Freeman et al. 1981). The ability of the monoecious plant to regulate the sexual dominance of itself through hormonal control is an evolutionary advantage of these plants (Freeman et al. 1981). The main hormones involved in this are auxins, gibberellins, and cytokinins (Freeman et al.

1981). In cucumber, spinach, and hemp plants, gibberellins were found to be associated with maleness (Friedlander et al. 1977; Chailakhyan 1979) while cytokinins and ethylene were correlated to femaleness (Chailakhyan 1979, Rudich et al. 1972).

The consensus is that female reproductive organs require more energy than male reproductive organs due to the requirements of seed production (Case and Ashman 2005). Female flowers need to be maintained for longer periods of time while seeds develop and are at a higher risk for herbivory loss (Case and Ashman 2005). Male flowers typically come first and they do carry an “opportunity cost” in which investment of resources in pollen production takes away from investment in photosynthetic machinery (Case and Ashman 2005; Reekie and Bazzaz 1987). However, most plants need to achieve a minimum size before reproduction can begin so the cost of opportunity is less (Obeso 2002). This differential cost in sex allocation has mainly been demonstrated in dioecious plants where female flowers and male flowers are on separate plants. The increased cost of female reproductive organs is demonstrated in that female plants are often smaller than male plants (Obeso 2002). It has also been shown that dioecious male plants dominate in unfavorable environments, such as low water, altered pH, or low nutrient status (Freeman et al. 1976; Cox 1981; Freeman and Vitale 1985).

In monoecious plants, separating the costs of sex allocation is more difficult (Case and Ashman 2005; Klinkhamer et al. 1997). Here, the male and female organs can occur in the same flower (perfect flower) or in separate

flowers (imperfect flowers) on the same plant. In imperfect flowers, the male and female flowers are often spatially and temporally separate. Male flowers typically appear before female flowers. This influences reproductive allocation through different nutrient requirements of each flower-type and use of resources at different times (Klinkhamer et al. 1997; Case and Ashman 2005). Male flowers produce pollen, which is very protein rich (Roulston et al. 2000) and therefore is more concentrated with nitrogen and phosphorus than seeds (Ashman 1994; Case and Ashman 2005). Female flowers require greater investment in defense compounds to protect seeds against herbivory, which are highly desired due to being rich in carbohydrates, nutrients, and fats (Case and Ashman 2005; Ashman 1994).

Even so, it is still possible to determine differences in ratios of sex allocation between plants in different environments. Similar to total reproductive allocation, sex allocation in monoecious plants is highly influenced by the environment. The ratios of male to female flowers are often a direct result of energy availability within the plant and thus size of plant (Klinkhamer et al. 1997). However, the response of sex allocation to varying environmental conditions or plant size is not the same across wind-pollinated monoecious species and the response is dependent on the individual's reproductive history and genetics (Freeman et al. 1981; Abul-Fatih and Bazzaz 1979; Smith 1981; Willson and Ruppel 1984; McKone and Tonkyn 1986).

Like dioecious plants, some monoecious wind-pollinated trees have been found to have more female flowers when water is readily available in mesic

environments and more male flowers in drier, xeric sites (Freeman et al. 1981). In monoecious wind-pollinated woodland annuals, optimum light and soil moisture caused female reproduction to increase with increasing plant size (Cid-Benevento 1987). At levels outside of optimal conditions, the allocation to male flowers increased but was never more than the allocation to female flowers. This makes sense if the environment of woodland annuals is taken in to account. Wind velocity is relatively low so pollen cannot be dispersed far; providing more energy to the ovules and seed production would increase plant fitness.

Wind-pollinated plants must have a plant architecture that promotes pollen dispersal by wind. Plant fitness depends on the ability for pollen to be released and received by ovules (Lloyd and Bawa 1984). Thus it makes sense that plants that are tall and exposed to higher wind velocities would be better for releasing pollen; alternatively, plants that are smaller and near the ground where wind velocity is reduced would be better receptors. This strategy can be seen in monoecious wind-pollinated trees and annuals by more male flowers on taller plants at the top of the canopy and mainly female flowers on those plants hidden below the canopy (Freeman et al. 1981; McKone and Tonkyn 1986). Abul-Fatih et al. (1979) found that taller plants tended to be more male and this was further selected for by higher seed predation in taller plants of *Ambrosia tiffida*. In *Ambrosia artemisiifolia*, short plants have been observed to produce more female than male flowers and maleness to increase with height (Traveset 1992; Paquin and Aarssen 2004); however, seed predation is higher in small plants so depending on evolutionary pressure of different populations maleness may not

increase with height if pressure is on increased maleness in short plants (McKone and Tonkyn 1986). Increased size and branching also appears to be correlated with an increase in the maleness of another wind pollinated annual, *Xanthium strumarium* (Solomon 1989).

Climate change also appears to have an effect on the maleness of wind-pollinated plants. Elevated CO₂ changed the architecture of *Ambrosia artemisiifolia* by also increasing the number of branches and thus height of smaller individuals (Stinson et al. 2006). Carbon dioxide has also been demonstrated to increase total reproductive effort in ragweed (Rogers et al. 2006). While maleness has not been quantified in response to carbon dioxide, it is safe to hypothesize that this would also lead to increased maleness in this species as maleness has been correlated with size and height in other experiments (Paquin and Aarssen 2004; Traveset 1992; McKone and Tonkyn 1986). Increased maleness in response to elevated CO₂ has also been observed in *Betula papyrifera* (Darbah et al. 2008). It is apparent that much more work needs to be done to understand how climate change will impact maleness and thus pollen production of plants, particularly those that are allergenic to humans.

2.10 Pollen and human allergic response

The human immune system is meant to protect us from foreign substances that could do us harm, such as bacteria and viruses. Since we are frequently exposed to proteins associated with pollen, our immune system recognizes these proteins as foreign and mounts an immune response (Knox

1979; Kaplan 1985; Middleton et al. 1988; Patterson 1985). It is the type of response that determines whether someone gets allergy symptoms upon exposure.

Pollen is the male zygote of plant sexual reproduction. Most plant species that reproduce sexually and each pollen grain must carry a signal to be sure that it matches up with the correct female zygote receptor (Knox 1979, Heslop-Harrison 1975). This signal is carried in the proteins found in the ridges of the outer coat of the pollen grain, called the exine (Solomon and Mathews 1988; Knox 1979). These proteins are produced in conjunction with the inner layer of the pollen coat, the intine (Heslop-Harrison 1975). When the pollen lands on the stigma of the female flower, it will only germinate if the biochemical signal of the stigma matches that of the pollen grain (Solomon and Mathews 1988; Knox 1979; Heslop-Harrison 1975).

When the pollen enters the human body, the proteins on the pollen surface elicit a response by the immune system. Due to the allergic response induced by the immune system, pollen is considered to be an allergen (Knox 1979; Lakin and Strecker 1985; Solomon and Mathews 1988). This response is described to be acquired, specific, and has altered capacity to react depending on susceptible individual (Knox 1979). Acquired refers to previous exposure to the allergen that has stimulated the immune system to develop hypersensitivity and produce antibodies in defense; this is called priming. It is said to be specific due to the fact that the molecular structure of the protein allergen has an exact antibody produced in response. While each pollen type is unique in its protein

structure, there are similarities between pollens in the same family that can create cross-reactivity. Finally, the altered capacity to react comes from the different responses that can occur when the pollen grains enter the body again; either the response can increase, as in hypersensitivity, or decrease as a result of increased immunity or tolerance.

The allergic response to allergens is said to be a type I response in that the response is immediate (Knox 1979; Lakin and Strecker 1985). The immune system arises from lymphoid tissues and stem cells in bone marrow (Knox 1979; Lakin and Strecker 1985; Cohen 1988). There are three cell types that arise from these stem cells when they arrive in their anatomic homopoietic –inducing environments (HIMs) (Lakin and Strecker 1985). These three cells are T-Cells, B-cells, and macrophages; together they create antibodies in defense of the allergen (Lakin and Strecker 1985). The allergen is first ingested by the macrophage leaving portions of the antigen on its surface that is then used by B-cells and T-cells (Cohen 1988). The T-cells, specifically T-helper cells, help the B-cell determine type of immune response (Lakin and Strecker 1985). This intricate reaction of the three cells create a lymphocyte cell which then undergoes blastogenesis and transforms into a mature plasma cell which is responsible for biosynthesis of antibodies in response to allergen (Lakin and Strecker 1985; Cohen 1988). These plasma cells are highly specialized and will only secrete antibodies specific to one allergen and hold a memory for this allergen which induces a quicker and stronger response the next time it recognizes the allergen (Lakin and Strecker 1985).

The plasma cells circulate around the body in the blood serum and when they come into contact with the allergen they release antibodies in response (Knox 1979; Lakin and Strecker 1985). There are several classes of antibodies that can be produced. The majority of the antibodies produced in response to pollen fall in the Immunoglobulin E class (IgE) (Lakin and Strecker 1985; Knox 1979; Conrad 1985); however grasses have also been found to elicit Immunoglobulin G (IgG4) antibodies (Chernokhvostova et al. 1990). These antibodies only last in the blood 2.3 days and are needed to be regenerated to be present in the blood stream; it is estimated that one third of the antibodies are regenerated each day (Zeiss 1985).

Once antibodies are released in to the blood stream, they too circulate around in the blood stream. They have a high affinity for basophil and mast cells and will attach themselves to these cells via a foot piece on the Fc region of the antibody (Knox 1979; Lakin and Strecker 1985; Conrad 1985; Metzger 1985; Li 1988). Hundreds of thousands of antibodies can attach themselves to each mast or basophil cell and IgE antibodies can remain bound there for a week or more while IgG antibodies are shorter lived (Knox 1979; Lakin and Strecker 1985; Pruzansky 1985; Metzger 1985). This primes the basophil and mast cells for an allergic response when allergen is again induced into the body. Each IgE antibody has two arms with terminal recognition sites specific for the allergen that induced their production (Knox 1979; Pruzansky 1985; Lakin and Strecker 1985; Conrad 1985; Metzger 1985; Li 1988). The antibodies communicate with the basophil/mast cell through membrane glycoproteins. When the allergen is

encountered again, it binds to a pair of adjacent IgE antibodies and triggers the mast/basophil cell to rapidly release tissue mediators, such as histamines (Knox 1979; Lakin and Strecker 1985; Pruzansky 1985; Metzger 1985). These tissue mediators are what cause the symptoms of allergic reactions: Itching, inflammation, mucus, wheezing, coughing, and chest tightness.

This response can be prevented in two ways. An antihistamine can be taken to prevent the histamines released from creating the allergic response (Kaplan 1985; Knox 1979; Middleton et al. 1988; Patterson 1985). However, they must be taken prior to the histamine release and taken daily, when exposure will occur, to be effective. The second method is immunotherapy (Middleton et al. 1988; Patterson 1985; Kaplan 1985). Here, doses of the allergen are given on a regular basis to block the environmental allergen and prevent an immune response (Pruzansky 1985). Over a long period of time it can trick the immune system into believing that the allergen is no longer a foreign body. The second method can give a long-term result if the patient keeps up with the therapy until it works.

As mentioned previously, each plant species has pollen with unique proteins that allow it to be recognized by its female counterpart in nature. However, there are similarities in structure and antigen content within some species of the same family but this is not always common (Knox 1979; Solomon and Mathews 1988). Some pollen may have more than one antigen present (Gutman 1985). So, if a patient has become hypersensitive to one type of pollen antigen, there are other pollens with similar antigens that can create an allergic

response due to what is called “cross-reactivity” (Solomon and Mathews 1988; Knox 1979; Metzger 1985). Grasses, trees, and weed pollen are immunologically distinct because they all have different antigens (Gutman 1985). Ragweed in the Ambrosiae tribe of the Asteraceae family contains the most allergenic protein found, antigen E (Heslop-Harrison 1975; Gutman 1985). Grasses have several allergens: group I allergens are common among grasses but not in Bermuda (*Cynodon*) grass, group 2 are common in rye (*Lolium*), fescue (*Fescuta*), orchard (*Dactylis*) and velvet (*Holcus*) but not in timothy (*Phluem*), and timothy contains a unique composition of group B antigens with group I antigens (Solomon and Mathews 1988; Gutman 1985). Interestingly, the relevant allergen in Timothy is cross-reactive with the highly allergenic allergen Amb a 1 found in ragweed (Fischer et al. 1996).

CHAPTER 3

ELEVATED CARBON DIOXIDE AND OZONE EFFECTS ON POLLEN PRODUCTION AND PHL P 5 PROTEIN CONTENT OF TIMOTHY GRASS (*PHLEUM PRATENSE* L.) POLLEN

3.1 Introduction/Background

Allergic disease is an important determinate of human health (AAAAI 2000; IPCC 2007). In the United States, health care costs from those suffering from allergic rhinitis (hay fever) is estimated to cost \$4.5 billion annually, affecting 40 million people, and resulting in 4 million days of lost time and productivity in the workplace and school (AAAAI 2000). World-wide, allergic airway disease has a large impact on human health affecting 10-30% of the global population with allergic rhinitis and more than 300 million with asthma (WAO 2011). Grass, which grows worldwide, is the cause of allergic response in 20% of the general population and 40% of atopic individuals (Andersson and Lidholm 2003). Allergenic pollen is expected to increase with future climate change, thus increasing both the severity and numbers of people affected (Beggs 2004; Taramarcaz 2005; Beggs and Bambrick 2005; Confalonieri et al. 2007). However, it is unknown how much it will increase or how many more people will be affected due to the variability among plants and the environment.

Carbon dioxide is the most important greenhouse gas and is the major driver of climate change (IPCC 2007). Levels of this greenhouse gas are expected to rise to 730-1020 ppm by the year 2100 (Meehl et al. 2007). Likewise, background levels of tropospheric ozone, the third most important greenhouse gas (Sitch et al. 2007; Worden et al. 2008), are predicted to rise to

70 ppb or higher due to increasing levels of precursor gases (Cooper et al. 2010, Meehl et al. 2007; Thompson 1992; Vingarzan 2004). In general, both of these atmospheric gases have been shown to affect plant growth. Carbon dioxide has been well characterized for stimulating photosynthesis and growth in C3 plants, particularly in annual plants such as ragweed (Bazzaz 1990; Ainsworth et al. 2002; Long et al. 2004; Leaky et al. 2009; Reddy et al. 2010). Ozone has been shown to decrease growth due to oxidative damage of photosynthetic components (Lefohn et al. 1988; Darrall 1989; Sandermann 1996; Skelly 2000; Krupa et al. 2001; Fiscus et al. 2005; Ashmore 2005; Booker 2008; Cho et al. 2011; Ainsworth 2012).

The effect of carbon dioxide on the pollen production of grass has not been investigated previously. However, carbon dioxide effects on ragweed has been investigated and found to increase both the pollen amount (Ziska and Caulfield 2000; Wayne et al. 2002; Ziska et al. 2003; Rogers et al. 2006; Stinson and Bazzaz 2006) and the allergenicity of the pollen (Singer et al. 2005).

The effect of ozone on grass pollen production and allergenicity has been investigated briefly. Ozone was found to reduce the amount of viable pollen produced in *Lolium perenne* L. by reducing the amount of starch in the anther where the pollen is produced and preventing pollen maturity (Schoene et al. 2004). In another experiment the level of group 5 allergen content in *Lolium perenne* L. exposed to ozone and levels were found to increase with ozone exposure, making pollen more allergenic (Masuch et al. 1997). Motta et al. (2006) reported an increase allergen content of Timothy grass (*Phleum pratense*

L.), following exposure of pollen to air pollutants. Ozone caused membrane disruption, which allowed cytoplasmic allergen granules to be released and the authors hypothesized that this would result in increased amount of allergens in contact with the human membranes. They only counted the granules however and did not test for allergen recognition on the ozone treated granules. Rogerieux et al. (2006) did investigate ozone treated pollen recognition and indicated Timothy pollen allergen recognition by IgE was decreased by air pollutant exposure. They attributed this decrease in allergen recognition to mechanical damage and post-translational modification of allergens.

Here I investigate the effect of elevated carbon dioxide and ozone on the grass pollen by using Timothy grass (*Phleum pratense L.*) as my model grass. Timothy was chosen for its ability to flower in the first year without vernalization (Heide 1982). Furthermore Timothy grass can be found throughout North America and Europe thus coming into contact with a large portion of the population (Lewis et al. 1983; Childers and Hanson 1985).

Timothy grass is a wind-pollinated plant and pollen can travel up to 1 km away (Raynor et al. 1972). It releases its pollen early in the morning. Warm air temperatures and high wind increase the release of pollen while high humidity or rain reduces pollen release (Lewis et al. 1983). Grass allergy is wide spread, in North America it is only second in significance to ragweed allergy. Timothy grass is the second most allergenic grass pollen, after *Dactylis* (Wodehouse 1935) and is one of the worst causes of early summer allergies. However, it is not a quick invader of disturbed areas and so is found mostly in agricultural fields where is it

grown as forage crop and natural grasslands (Lewis et al. 1983; Childers and Hanson 1985).

Objectives of this research are to determine 1) changes in the amount of pollen produced by Timothy under elevated carbon dioxide, elevated ozone, and a combination of the two and 2) changes in the Phl p 5 protein content of the pollen in response to the same treatments.

3.2 Methods

3.2.1 Plant propagation

Experiments were conducted in the Laboratory of Plant Environmental Biology at the University of Massachusetts, Amherst, USA. Plants were fumigated with carbon dioxide and ozone in specially designed CSTR (Continuously stirred-tank reactors) chambers. The CSTRs operate such that the desired gas concentrations are injected into the top of the chambers via inlet airflow. The air inside the chambers is perfectly mixed using paddles that are proportionally the correct size to the chambers so that all air inside the chambers is uniformly mixed with the desired gas concentrations. Airflow exits through the bottom of the chamber so that the volume of air in the chambers is exchanged several times per minute; gas concentration are kept constant during exchanges (Manning and Krupa 1992). Eight chambers were set up in randomized complete block design using current and future predicted concentrations of ozone: 30 ppb and 80 ppb and two concentrations of carbon dioxide: 400 ppm and 800 ppm. The experiments were conducted three times to produce six

replications. Replications were blocked in analysis to account for variation in temperature and light in the greenhouse during each replication through time. Carbon dioxide treatments were administered continuously while ozone treatments were given 9:00-16:00 daily.

Seeds of *Phleum pratense* L. var. CLIMAX were sown in 10.5 cm X 10.5 cm X 35 cm pots (Treepots™; Hummert international; Missouri, USA) at the rate of 20 seeds per pot using MM 200 growing medium (SunGro; Bellevue, WA). Equal number of pots were placed in each treatment chamber and allowed to grow through maturity. Pots were fertilized weekly with a dilute concentration of Peat Lite Special (1/3 concentration; 15-16-17; Peter's Professional ; Scott's , Ohio, USA) to ensure ample nutrients for flowering (Lambert 1967). Plants were watered as needed. Plants were exposed to natural light as chambers are located in a greenhouse; however, light was supplemented with 400 W metal Halide lights (Metal Arc; Sylvania, Massachusetts, USA): on 12- hours for first five weeks, then switched to 16-hours for remainder of experiment (Heide 1982). Greenhouse daytime temperatures ranged from 17°C- 29°C and night time temperature ranged from 13°C -18°C. Chamber temperature (Figure 3.1) and relative humidity were monitored using hoboware data loggers (Onset Computer Corp, Massachusetts, USA).

3.2.2 Pollen collection and Analysis

To capture the pollen, polyethylene bags were placed over the inflorescence upon emergence and held open with wire and secured with clear

tape. Bag and flower spike were removed following complete dehiscence of pollen and placed in a -20°C freezer until analysis.

Upon analysis, first the protein was extracted from the pollen using PBS-T (Phosphate Buffered Saline with 0.05% Tween 20 pH 7.4). Inflorescence and bags were washed three times with PBS-T; the amount of PBS-T was determined by inflorescence length and measured precisely using a micro-pipette. Pollen was incubated at room temperature for 2 hours during extraction. Pollen was then separated from suspension using a centrifuge (1,500 RPM). PBS-T protein extraction solution was decanted from pollen and placed in a -20°C freezer until protein analysis. Inflorescences were dried at 70°C for 72 hours before being weighed on an analytical scale.

Pollen was then counted by suspending pollen in a precisely measured volume of PBS-T solution and counted using a hemocytometer. Counts were repeated and averaged to ensure accuracy.

Protein content was determined using a direct sandwich enzyme-linked immunosorbent assay (ELISA). The Phl p 5 kit (Indoor Biotechnologies, Inc, Georgia, USA) was created using IgG mouse antibodies. The primary antibody is attached to sites on wells. Unspecified sites in the wells are blocked using a 1% bovine serum albumin in PBS-T. The extracted allergen solution is placed in the wells and subjected to 4 dilutions. A standard curve is run to determine quantity of allergen per sample. The secondary antibody is biotinylated giving it a high affinity for the enzyme Streptavidin-Peroxidase. Finally, the substrate used is

1 mM ABTS (2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid)) in 70 mM citrate-phosphate buffer (pH 4.2).

3.2.3 Data Analysis

The effects of the treatments in this randomized complete block experimental design were tested with analysis of variance (ANOVA) using the generalized linear model procedure in SAS 9.3 (SAS Institute Inc, North Carolina, USA). Differences across treatment means were determined using the Tukey-Kramer test due to uneven replications that were an effect of treatment (Zolman 1993). Linear regressions were performed using analysis of covariance (ANCOVA) to evaluate relationships between Inflorescence weight and length.

3.3 Results

Elevated carbon dioxide significantly increased the amount of pollen produced by each inflorescence. Ozone had no effect on the amount of pollen produced (Figure 3.2).

Due to the increased amount of pollen produced in inflorescences, the concentration of Phl p 5, the major allergen, per inflorescence was also significantly increased by elevated CO₂ under low O₃ levels (Figure 3.3). Elevated ozone significantly reduced the level of Phl p 5 per inflorescence under elevated carbon dioxide. However, this trend was not significant at low carbon dioxide treatments. This relationship can also be demonstrated by looking at the concentration of Phl p 5 per pollen grain (Figure 3.4). Here it is clear that ozone

is reducing the allergenicity of the pollen grain. Elevated carbon dioxide partially ameliorates this effect. Carbon dioxide does not increase the allergenicity of the pollen grain when compared to the control, treatment 1; this infers that increased levels of Phl p 5 at the flower level are a direct effect of increased amount of pollen produced.

While O₃ reduced the Phl p 5 content of the pollen grain, it did not affect the amount of pollen produced by the inflorescences.

Elevated ozone and carbon dioxide also affected inflorescence size. Inflorescence length was significantly reduced by elevated ozone at both levels of carbon dioxide when compared to the control (low ozone, low carbon dioxide). Elevated carbon dioxide showed a trend of decreased inflorescence length but this was not significantly different than the control (Figure 3.5). Elevated carbon dioxide also partially amended reduction by elevated ozone. Inflorescence weight was significantly reduced by elevated ozone compared to the control (Figure 3.6). There was a trend towards reduced weight in response to elevated carbon dioxide but this was not significant. Elevated carbon dioxide did significantly ameliorate weight loss in response to elevated ozone. A linear regression analysis comparing weight to length at each treatment level, or density, determined that the elevated ozone under ambient carbon dioxide significantly produced inflorescences with a smaller overall density (Figure 3.7). There were no significant differences found between the flower densities of the other three treatments.

Using the inflorescence size data and the pollen per inflorescence data, I analyzed the relationships between inflorescence size and pollen production. Plants grown under elevated ozone and carbon dioxide did not produce significantly more pollen per gram of inflorescence than the control (Figure 3.8). Inflorescences under elevated carbon dioxide regardless of ozone level produced significantly more pollen per length of inflorescence (Figure 3.9).

Number of flowering plants per chamber was not significantly affected by elevated ozone or elevated carbon dioxide (Figure 3.10). However, trends indicate that flowering was increased by elevated carbon dioxide. I multiplied the average number of inflorescences produced per treatment by the number of pollen produced per inflorescence in that treatment to determine how the pollen load in the environment will change from current levels of carbon dioxide to future predicted levels (Figure 3.11). Linear regression analysis determined a significant increase in the pollen load from current levels of carbon dioxide to future predicted levels at both ozone treatments. However, there was significantly greater increase in future levels when ozone levels remained at current levels.

3.4 Discussion

Taken together, these results strongly suggest an increase in grass pollen load in the atmosphere under future predicted levels of these two atmospheric pollutants. We can expect a 2.5 times increase in the pollen load of grass pollen under future predicted levels of carbon dioxide (Figure 3.11). If Timothy grass is a good model for other grass response, this implies that atmospheric grass

pollen levels will increase in the future resulting in increased exposure for sensitized individuals.

Elevated carbon dioxide did not increase inflorescence size even though it increased pollen production. This indicates that something else changed in the physiology of the flowers. Other wind-pollinated plants have been found to increase their maleness in response to elevating carbon dioxide (Darbah et al. 2008). This is particularly evident in plants that are monoecious but have separate male and female flowers. Timothy grass is also monoecious but has a perfect flower, male and female on same flower. Separating the male and female components would be difficult. However, one can hypothesize that because the same size flower is producing more pollen this could indicate an inflorescence that is more male. Alternatively, increased pollen amounts could be a result of individual anthers producing more pollen.

While the overall exposure level to the allergen Phl p5 will increase with increasing pollen, the potency of the Phl p 5 allergen in the pollen is dependent on ozone levels. My results indicated reduced potency of Phl p 5 in response to increasing ozone, with elevated carbon dioxide ameliorating some of this effect. Ozone alone impacts human health negatively and has been shown to exacerbate the allergic airway response through irritation of the mucus membrane in the respiratory pathways (Penden et al. 1995). It is unlikely that reduced Phl p 5 potency would have a positive effect on allergy sufferers due to the interaction with high levels of ozone in the respiratory system. Some models predict lower ozone levels in future due to mitigation strategies to reduce

precursors to ozone and also climate feedbacks of altered precipitation (Ainsworth 2012, Kawase et al. 2011, Liao et al. 2009). However, lower levels of ozone mean sustained levels of Phl p 5 allergen in increasing amounts of Timothy pollen and thus still affect human health.

I compared our results regarding O₃ effect on pollen to past research regarding *in vivo and in vitro* grass pollen exposure (Schoene et al. 2004; Masuch et al. 1997; Majd et al. 2004; Motta et al. 2006; Rogerieux et al. 2007). Here I found that pollen number was unaffected by O₃; however, I counted indiscriminately of pollen quality. I did not test the viability of the pollen or quantitate the maturity of the pollen, so I do not know the impacts on its function. I found an increase in pollen number in relation to inflorescence weight, which was not evaluated in previous experiments. I also found reduced allergen content in response to elevated O₃. I used ELISA to specifically determine levels of the Phl p 5 protein. The IgG antibodies used in ELISA are specific to the Phl p 5 protein. Ozone is well known to attack bonds in proteins (Roshchina and Roshchina 2003; Heath 2008); oxidative damage could have altered Phl p 5 protein such that it reduced recognition by the IgG antibodies used in ELISA. Rogerieux et al. (2007) came to similar conclusions, using IgE antibodies, when looking at multiple individual allergens found in Timothy grass. However, Masuch et al. (1997) found an increase of group 5 allergen content in response to ozone. It is not clear why they found an increase when other experiments did not. It could be attributed to the fact that they were measuring allergens at the group level and not individual allergens. I can speculate that changes to

individual allergens within the group in response to oxidative damage could have still allowed recognition at the group level where as it did not when looking at specific allergens within the groups. Majd et al. (2004) also found that pollen that had been exposed to air pollutants resulted in increased allergic response in mice. They attributed this to the measured increase in air-borne pollutant particles and released cytoplasmic granules on the pollen coat. Alternatively, Majd et al. (2004) also found that mature pollen elicited a stronger allergenic response than immature pollen in mice. It is possible that my ozone treated pollen was immature and thus contained less allergen.

Past research on grass flower phenology has indicated that with rising levels of temperature, an earlier and longer flowering season occurs (Frei 1998). I did not investigate phenology; experimental treatments were stopped on the same date, so it is unknown if treatment had an effect on length of flowering time. I also did not manipulate temperature. Research has indicated that Timothy grass produces smaller flowers but an increased number of flowering plants under warming temperatures (Heide 1982). Plants in general have been found to flower earlier under elevated carbon dioxide due to changes in the C:N ratios, which signal a plant to flower (Corbesier et al. 2002; Taub and Wang 2008). Johnson et al. (1996) found that elevated ozone had no effect of flowering while elevated carbon dioxide increased number of flowering plants of Timothy grown in a legume-grass mixture. The greatest numbers of flowering plants were produced in the elevated carbon dioxide and elevated ozone treatments (Johnson et al. 1996). It can be hypothesized that future pollen loads could be

further exacerbated by longer flowering seasons that produce more flowering plants with more pollen per inflorescence as a result of rising carbon dioxide levels and the coinciding rising temperatures.

It is also important to note that this experiment was done under artificial conditions. Grassland ecosystems are very dynamic and this experiment only captured the very basic biology behind this complex system. In this reductionist experiment, I did not determine the effects of nutrient levels or water availability on the system. These are two factors that would have a large impact on the growth and flowering of Timothy grass. In addition, there was little to no competition between individuals.

In conclusion, we can expect an increase in atmospheric levels of Timothy grass pollen with future predicted levels of CO₂ and O₃. These findings can most likely be extended to other grass species, both C3 and C4 as CO₂ stimulation has been shown in both (Wand et al. 1999). Predicting future changes in pollen amount and potency allows health care providers to prepare for possible changes in sensitized populations and allows for better estimation on the true impacts of future climate change.

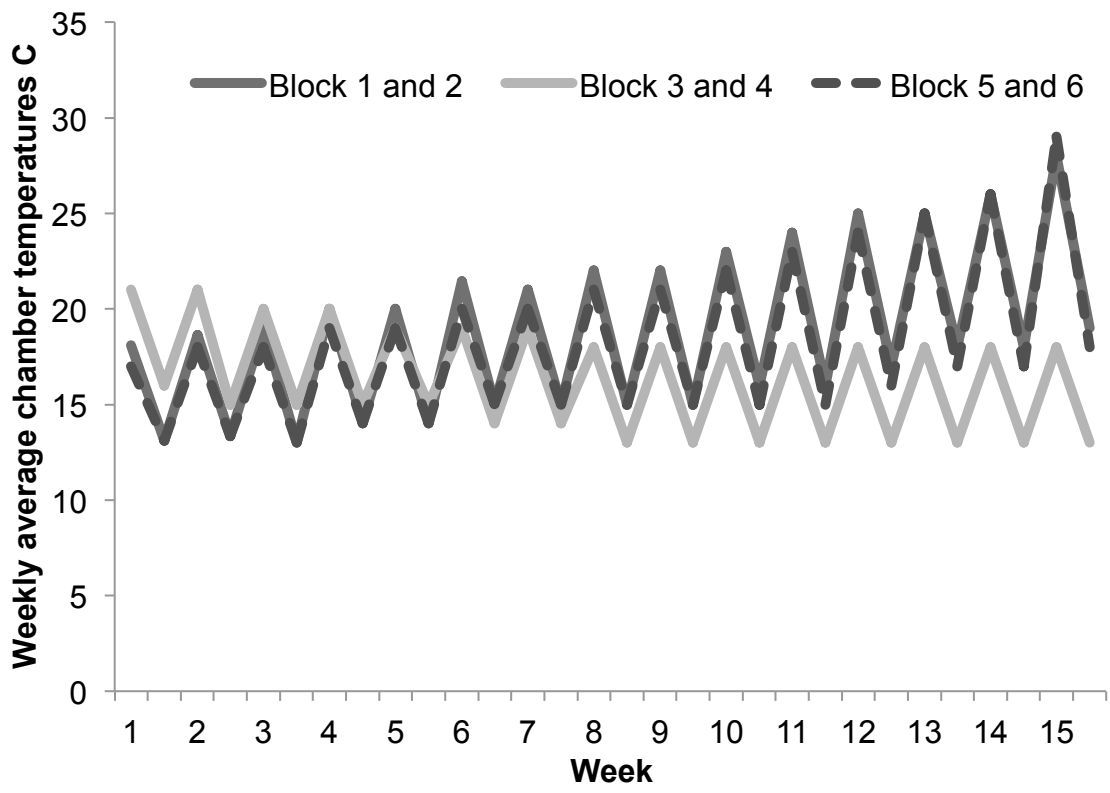


Figure 3.1: Average chamber temperature (°C) per experimental replication.

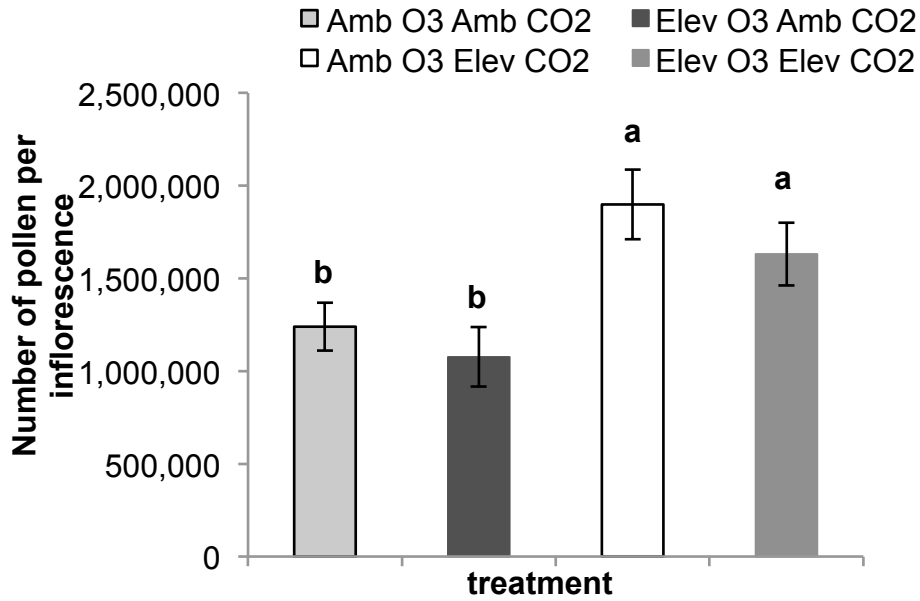


Figure 3-2: Pollen count per flower. AMB O₃= 30 ppb ozone; ELEV O₃= 80 ppb ozone; AMB CO₂= 400 ppm carbon dioxide; ELEV CO₂= 800 ppm carbon dioxide. Significant differences ($p < 0.05$) denoted by letters above bars using Tukey-Kramer test.

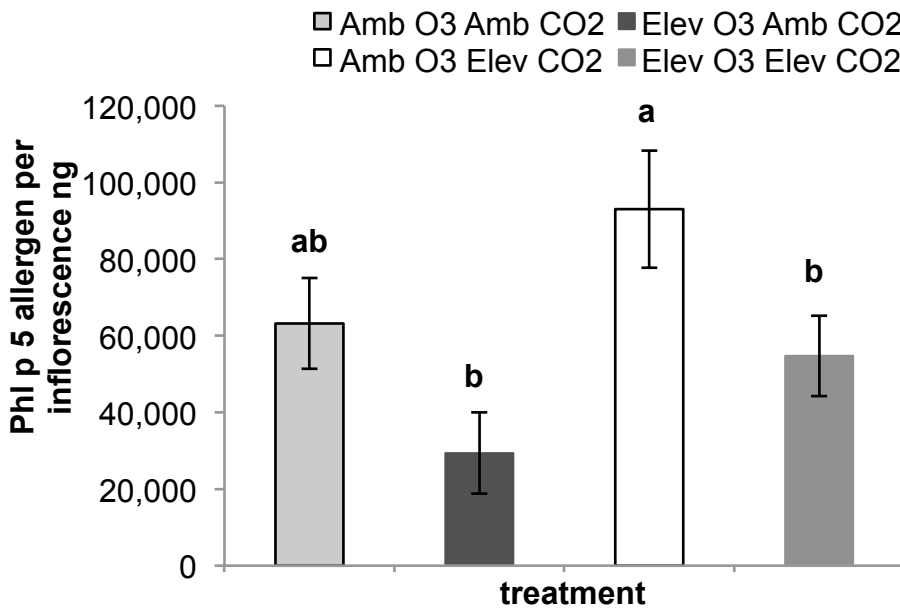


Figure 3-3: Concentration of Phl p 5 per inflorescence. AMB O₃= 30 ppb ozone; ELEV O₃= 80 ppb ozone; AMB CO₂= 400 ppm carbon dioxide; ELEV CO₂= 800 ppm carbon dioxide. Significant differences ($p < 0.05$) denoted by letters above bars using Tukey-Kramer test.

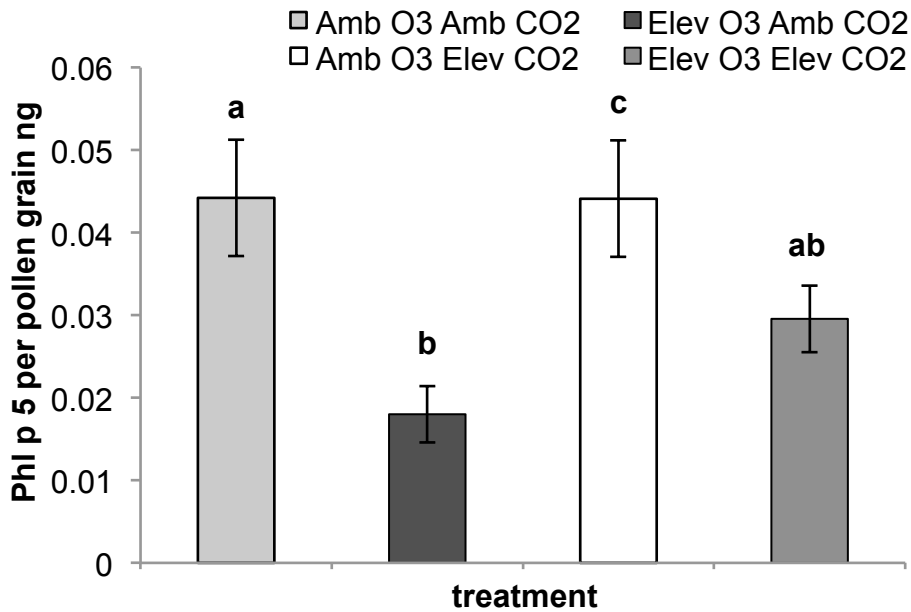


Figure 3-4: Concentration of Phl p 5 per pollen grain. AMB O₃= 30 ppb ozone; ELEV O₃= 80 ppb ozone; AMB CO₂= 400 ppm carbon dioxide; ELEV CO₂= 800 ppm carbon dioxide. Significant differences ($p < 0.05$) denoted by letters above bars Tukey-Kramer test.

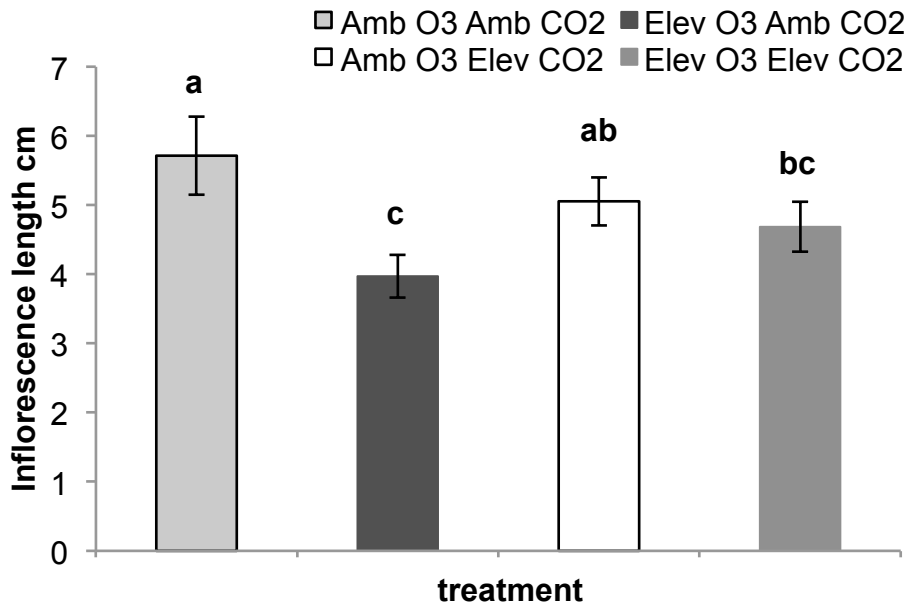


Figure 3-5: Inflorescence Length (cm). AMB O₃= 30 ppb ozone; ELEV O₃= 80 ppb ozone; AMB CO₂= 400 ppm carbon dioxide; ELEV CO₂= 800 ppm carbon dioxide. Significant differences ($p < 0.05$) denoted by letters above bars using Tukey-Kramer test.

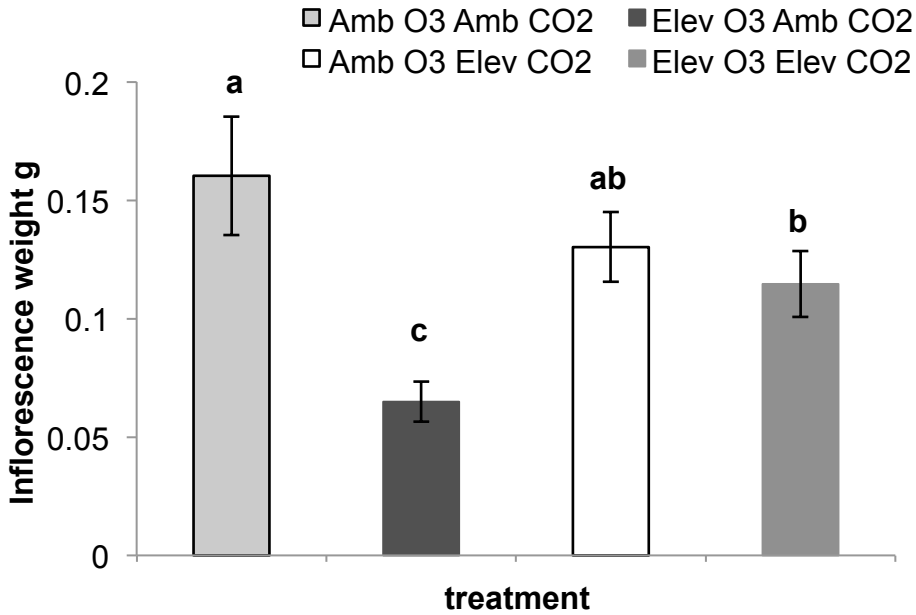


Figure 3-6: Inflorescence weight (g). AMB O₃= 30 ppb ozone; ELEV O₃= 80 ppb ozone; AMB CO₂= 400 ppm carbon dioxide; ELEV CO₂= 800 ppm carbon dioxide. Significant differences ($p < 0.05$) denoted by letters above bars using Tukey-Kramer test.

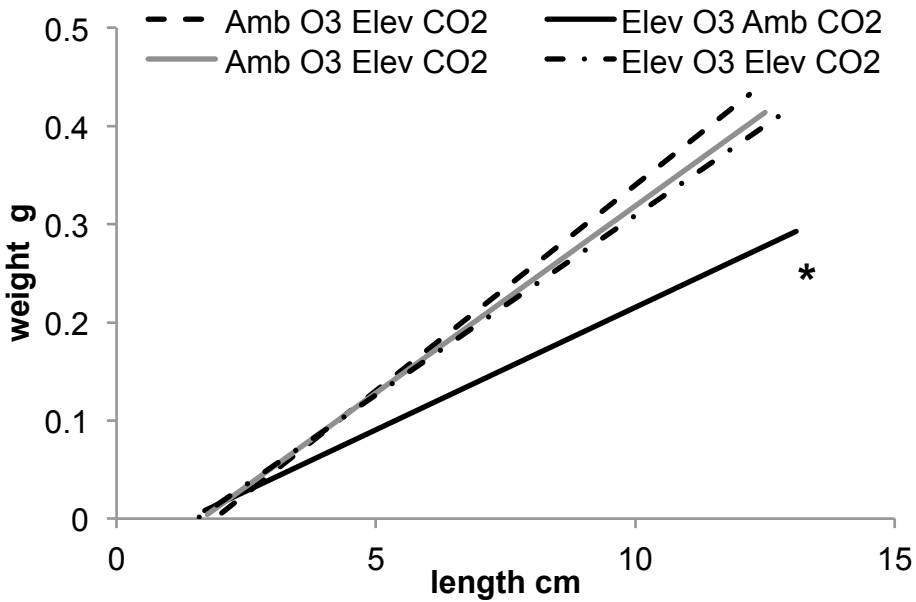


Figure 3-7: Inflorescence density. ANCOVA regression analysis on relationship between weight and length of inflorescence shows that only elevated ozone under ambient carbon dioxide significantly reduced inflorescence density. **Treatment 1 (dashed line): 30 ppb O₃, 400 ppm CO₂ (control) Treatment 2 (black line)= 80 ppm O₃, 400 ppm CO₂ Treatment 3 (grey line): 30 ppb O₃; 800 ppm CO₂ Treatment 4 (dashed dot line): 80 ppb O₃; 800 ppm CO₂.**

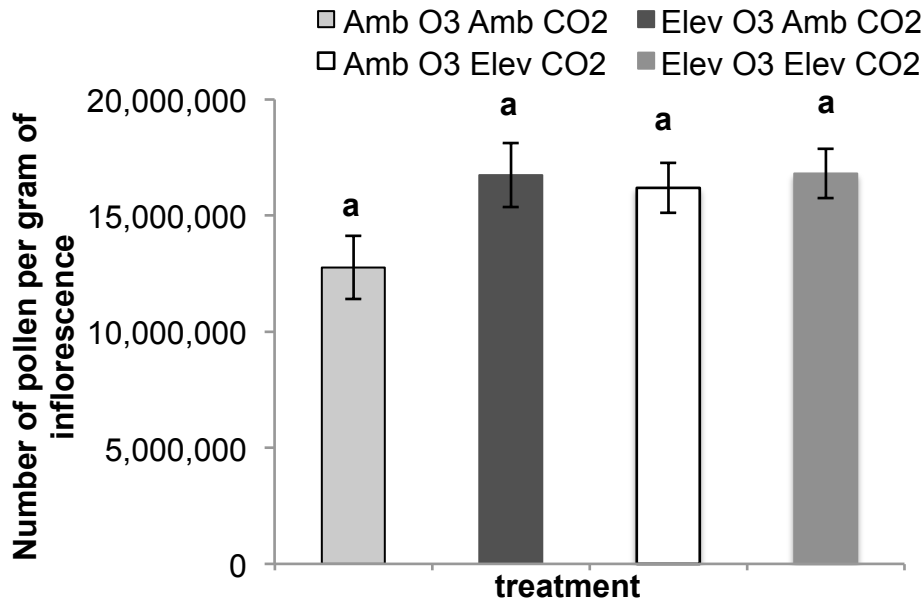


Figure 3-8: Pollen count per inflorescence weight (g). AMB O₃= 30 ppb ozone; ELEV O₃= 80 ppb ozone; AMB CO₂= 400 ppm carbon dioxide; ELEV CO₂= 800 ppm carbon dioxide. Significant differences ($p < 0.05$) denoted by letters above bars using Tukey-Kramer test.

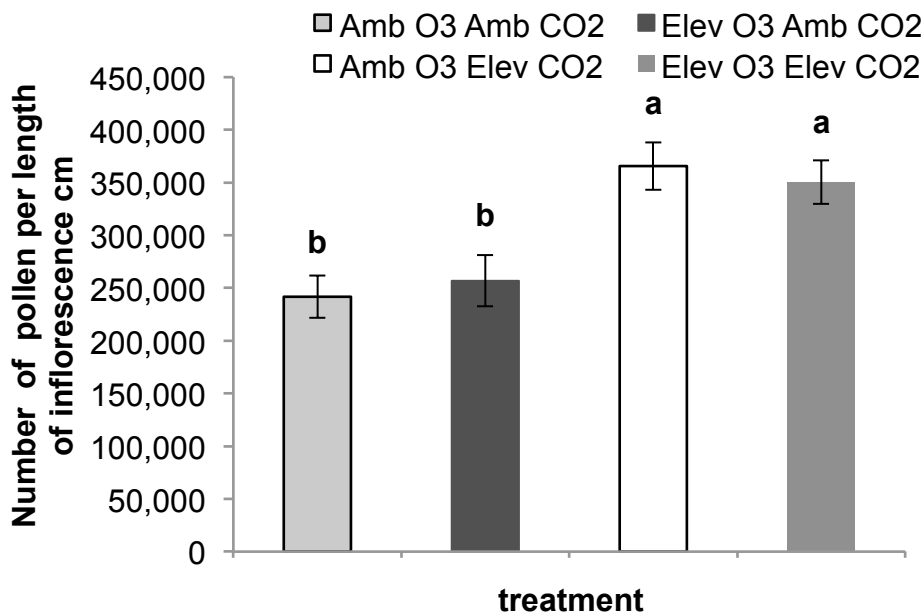


Figure 3-9: Pollen count per inflorescence length (cm). AMB O₃= 30 ppb ozone; ELEV O₃= 80 ppb ozone; AMB CO₂= 400 ppm carbon dioxide; ELEV CO₂= 800 ppm carbon dioxide. Significant differences ($p < 0.05$) denoted by letters above bars using Tukey-Kramer test.

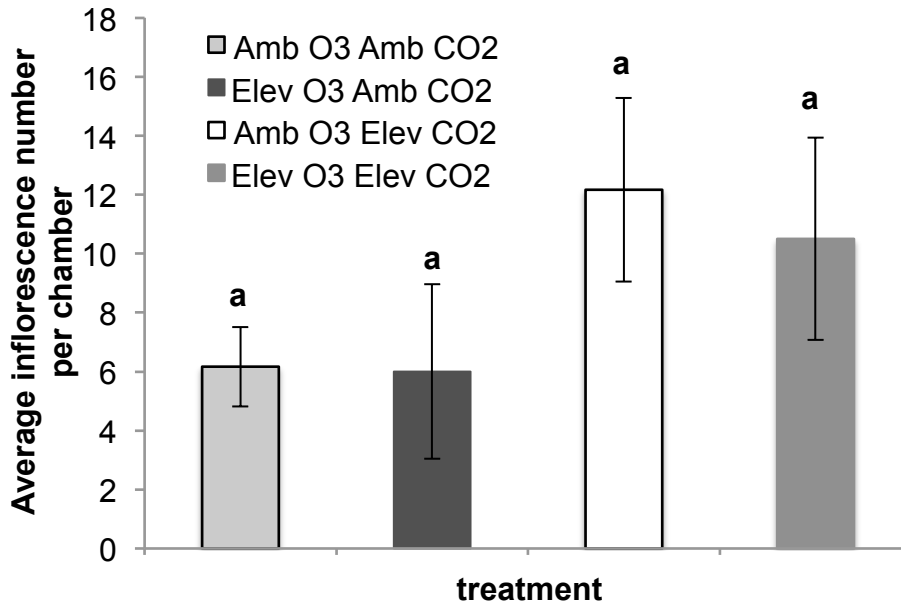


Figure 3-10: Average number of inflorescences per treatment. AMB O₃= 30 ppb ozone; ELEV O₃= 80 ppb ozone; AMB CO₂= 400 ppm carbon dioxide; ELEV CO₂= 800 ppm carbon dioxide. Significant differences ($p < 0.05$) denoted by letters above bars using Tukey-Kramer test.

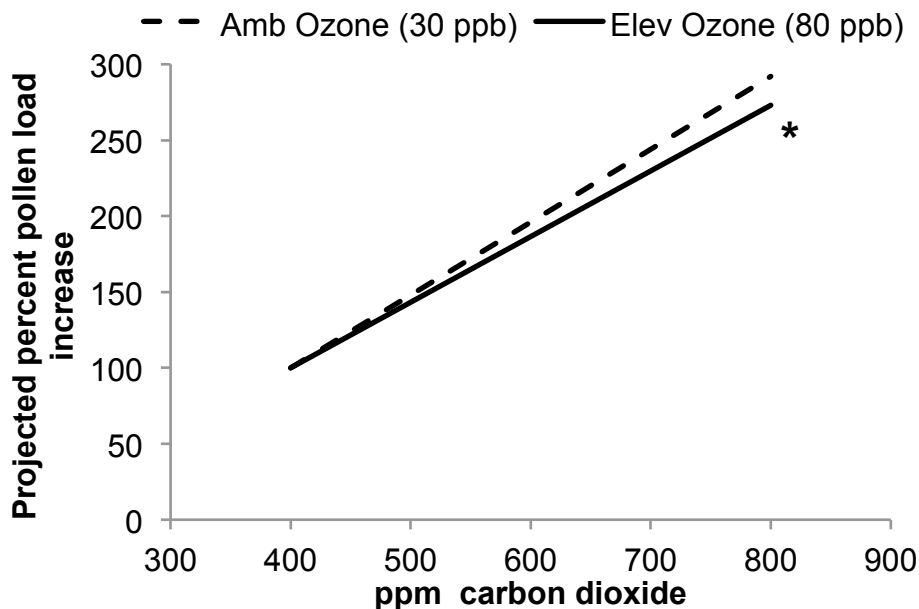


Figure 3-11: Predicted increase in pollen exposure load. Calculated by multiplying the average number of inflorescence in each treatment by the number of pollen produced per inflorescence. Expressed as a percentage, with current levels at 100%. Solid line determines trend at current ambient level of ozone (30 ppb). Dashed line is future elevated level of ozone (80 ppb). Lines were determined to be significantly different using ANCOVA ($p = 0.05$); as indicated by *. Increase from low to high carbon dioxide of both lines is also significant.

CHAPTER 4

GROWTH AND PHYSIOLOGY OF TIMOTHY GRASS (*PHLEUM PRATENSE* L.) IN RESPONSE TO FUTURE PREDICTED LEVELS OF CARBON DIOXIDE AND OZONE

4.1 Introduction/Background

Climate change is occurring, with certainty, due in part to anthropogenic increases of greenhouse gases in the atmosphere (Trenberth et al. 2007). While climate change has occurred in the past history of Earth, never has it happened at the rate it is happening now (IPCC 2007). More importantly, never has it happened when the human population was 7 billion (US Census Bureau July 2013). Changes in productivity of the agroecosystem are an important factor in understanding the full effects of climate change on humans and the environment.

Carbon dioxide is an important greenhouse gas and is one of the major drivers of climate change (IPCC 2007). Levels of this greenhouse gas are expected to rise to 730-1020 ppm by the year 2100 (Meehl et al. 2007; IPCC 2007). Likewise, background levels of tropospheric ozone, also an important greenhouse gas (Sitch et al. 2007; Worden et al. 2008), are predicted to rise to 70 ppb or higher due to increasing levels of precursor gases (Thompson 1992; Vingarzan 2004). Increases in these greenhouse gases are mostly a result of the burning of fossil fuels by humans (IPCC 2007). Carbon dioxide concentrations are currently higher than they have been in the last 700,000 years (Augustin et al. 2004). This rise in carbon dioxide and other greenhouse gases are closely correlated with a rise in temperature due to the heat trapping abilities of these

gases (IPCC 2007). Since 1970, the temperature has been rising at a rate of 0.5°C per decade, with winter temperature rising faster than summer temperatures in response to rising carbon dioxide levels (Frumhoff et al. 2006).

In general, both of these atmospheric gases have been shown to affect plant growth. Carbon dioxide has been well characterized for stimulating photosynthesis and growth in C3 plants (Bazzaz 1990; Ainsworth et al. 2002; Long et al. 2004; Leaky et al. 2009; Reddy et al. 2010). Ozone has been shown to decrease growth due to oxidative damage of photosynthetic components (Lefohn 1992; Darrall 1989; Sandermann 1996; Skelly 2000; Krupa et al. 2001; Fiscus et al. 2005; Ashmore 2005; Booker 2009; Cho et al. 2011).

Here we look at the effects of future predicted levels of the two atmospheric gases, ozone and carbon dioxide, on the growth and physiology of the forage crop Timothy grass (*Phleum pratense* L.) over the whole life-cycle of the plant.

As Timothy is important agriculturally, growth response to elevated carbon dioxide and ozone has been investigated with respect to agricultural yield and management. A handful of experiments have investigated the effect of ozone, carbon dioxide, and a combination of the two gases on shoot biomass production. However, these experiments have focused only on the agriculturally significant shoot and during the flowering stage of the growth cycle when Timothy is typically harvested. The effects of these gases on whole plant biomass allocation and growth throughout the life cycle have not been investigated. This is important for understanding the full implications of climate change on the

agroecosystem. Furthermore, past research has been conducted under colder temperatures (11-13°C), which are unlikely to persist in the future with the observed and predicted concomitant increases in greenhouse gases and temperature.

Past experiments have found that elevated CO₂ (680 ppm) at 11-13°C favored tillering over shoot elongation (Saebo and Mortensen 1995; Mortensen and Saebo 1996). It was found that elevated carbon dioxide caused up to a 29% increase in tillers and plant height was reduced by 18-24% in the different subsequent harvests (Saebo and Mortensen 1995, Mortensen and Saebo 1996). The plants were 25-64% more dense (unit plant/unit air space) as a result the increased tillering and decreased height (Saebo and Mortensen 1995). Conflicting results were reported on the effects of elevated CO₂ on the dry weight of above ground biomass with one reporting a 17-24% decrease (Saebo and Mortensen 1995) and the other reporting a 14-51% increase (Mortensen and Saebo 1996). The crowns and some roots were analyzed at the third harvest of one experiment and found to be 24% larger under elevated carbon dioxide indicating a shift in biomass allocation preference to crowns over shoots (Saebo and Mortensen 1995). All harvests were done in the flowering stage and the authors indicated that more research was needed to understand physiological changes in the tillering stage to better understand these effects observed in the flowering stage.

Timothy (*Phleum pratense* L.) is sensitive to ozone. At ozone levels of 55 nmol mol⁻¹ it was found that Timothy had a 45% reduction in dry weight shoot

biomass (Mortensen 1992). Several genotypes of Timothy from Nordic countries were evaluated for their sensitivity to ozone with respect to biomass accumulation (Danielsson et al. 1999). It was found that ozone significantly reduced above ground biomass. Ozone injury was correlated with growth rate; genotypes with the fastest relative growth rate also had the most sensitivity to ozone (Danielsson et al. 1999). It was also found that genotypes with the greatest stomatal conductance also showed the greatest injury (Danielsson 2003).

The interactive effects of both elevated carbon dioxide and ozone on the growth of Timothy has also been investigated. However, past research has only investigated the interaction of these gases on Timothy grass growth while grown in mixtures of plants and therefore competition may have masked true effects of these gases. Johnson et al. (1996) investigated the effects of elevated carbon dioxide and ozone on the growth and above ground competition of Timothy and alfalfa. It was found that increasing the carbon dioxide from 350 ppm to 700 ppm resulted in stimulation of growth, measured in dry biomass, for both species under low ozone (0.03 $\mu\text{l/l}$). Under intermediate levels of ozone (.08 $\mu\text{l/l}$ and 0.13 $\mu\text{l/l}$), Timothy showed a reduction in biomass. However at the highest level of ozone (0.18 $\mu\text{l/l}$), there was stimulation in growth at both carbon dioxide levels. This was due to the Alfalfa being more sensitive to ozone thus having a greater reduction in biomass and allowing increased light for Timothy growth. It was also shown that Timothy root production was not altered by elevated ozone (Johnson et al. 1996).

The effect of elevated carbon dioxide was investigated while looking at the interactive effects of ozone, soil type, and temperature on Timothy in a mixture of two other grass species (Mortensen 1997). It was found that carbon dioxide stimulated shoot growth while ozone reduced shoot growth of the entire mixture. Soil types had little effect and temperature showed no interaction with carbon dioxide. Assessment of the plants separately showed that Timothy grass growth was reduced at low carbon dioxide and high ozone; the authors attributed this to the increase in growth of other species in the mixture competing with Timothy. The reduction in Timothy growth by high ozone was ameliorated by elevated carbon dioxide (Mortensen 1997).

Mortensen (1999) then looked at the effects of ozone on Timothy in another mixture under different levels of light, relative humidity, temperature and carbon dioxide. He found that decreasing the temperature, as well as increasing the relative humidity both resulted in more ozone injury (Mortensen 1999). On the other hand, he found that increasing the carbon dioxide levels reduced ozone injury (Mortensen 1999). He found that changes in light had little to no effect on ozone injury in Timothy.

Lastly, the effects of carbon dioxide and ozone with relation to day length and irradiance levels were assessed (Mortensen 1999b). Here it was found that Timothy shoot growth was stimulated by elevated carbon dioxide but that there were no interactive effects of ozone, day length, or light levels on the dry biomass of Timothy. However it was found that the number of visible lesions caused by ozone injury was decreased by both increasing carbon dioxide and

total irradiance (both day length and level of irradiance). Here, It was also observed that the elevated level of carbon dioxide increased the number of shoots in Timothy (Mortensen 1999b).

Presented here are the effects of elevated carbon dioxide and ozone on the total biomass allocation to root, crown, and shoot throughout the entire life cycle of Timothy grass. Changes in biomass and total non-structural carbohydrate allocation were measured periodically through flowering to identify responses during different stages of the life cycle. In addition, some physiological responses that may explain observed changes were also measured.

We hypothesized that elevated carbon dioxide would stimulate biomass accumulation, especially the crown tissue. It was also predicted that an increase in concentration of total non-structural carbohydrates would occur in response to elevated carbon dioxide and that it would decline in response to energy needs during reproduction. Ozone was predicted to have the opposite effect, with overall decreases in plant biomass, particularly in root and crown. Total non-structural carbohydrates were expected to be reduced in the plant due to a higher demand for maintenance and repair processes in response to oxidative injury. It was expected that carbon dioxide would ameliorate the negative impacts of ozone through either reduced flux into the stomata or increased antioxidant production.

4.2 Methods

4.2.1 Plant propagation and Experimental Design

Experiments were conducted in the Laboratory of Plant Environmental Biology at the University of Massachusetts, Amherst, USA. Plants were fumigated with carbon dioxide and ozone in specially designed CSTR (Continuously stirred-tank reactors) chambers. The CSTRs operate such that the desired gas concentrations are injected into the top of the chambers via inlet airflow. The air inside the chambers is perfectly mixed using paddles that are proportionally the correct size to the chambers so that all air inside the chambers is uniformly mixed with the desired gas concentrations. Airflow exits through the bottom of the chamber so that the volume of air in the chambers is exchanged several times per minute; gas concentration are kept constant during exchanges (Manning and Krupa 1992). Eight chambers were used and set up in randomized complete block design using 2 concentrations of ozone: 30 ppb and 80 ppb and two concentrations of carbon dioxide: 400 ppm and 800 ppm. Carbon dioxide treatments will be given continuously while ozone treatments will be given 9:00-16:00 daily. The experiment was repeated through time to increase the replications.

For biomass allocation study, seeds of *Phleum pratense* L. var. CLIMAX were sown in 10.5 cm X 10.5 cm X 35 cm pots (Treepots™; Hummert international; Missouri, USA) at the rate of three seeds per pot using MM 200 growing medium (SunGro; Bellevue, WA). Pots were then thinned to 1 plant per pot after germination. Fifteen pots were placed in each treatment chamber.

For carbohydrate determination, chlorophyll content, and antioxidant production, seeds of *Phleum pratense* L. var. CLIMAX were sown in 10.5 cm X 10.5 cm X 35 cm pots (Treepots™; Hummert international; Missouri, USA) at the rate of 20 seeds per pot using MM 200 growing medium (SunGro; Bellevue, WA). Twelve pots were placed in each treatment chamber and allowed to grow through maturity.

Pots were fertilized weekly with a dilute concentration of Peat Lite Special (1/3 of recommended concentration; 15-16-17; Peter's Professional; Scott's , Ohio, USA). Plants were watered as needed. Plants were exposed to natural light, as chambers are located in a greenhouse. However, light was supplemented with 400 W metal Halide lights (Metal Arc; Sylvania, Massachusetts, USA): on 12- hours for first five weeks, then switched to 16- hours for the remainder of experiment (Heide 1982). Greenhouse daytime temperatures ranged from 17°C- 29°C and night time temperature ranged from 13°C -18°C. Chamber temperature (Figure 4.1) and relative humidity were monitored using Hoboware data loggers (Onset Computer Corp, Massachusetts, USA).

4.2.2 Measurements

Destructive harvests were completed at 3, 6, 9, 12, and 15 weeks to measure biomass growth. Data was collected for total plant leaf area (LI-3000, Li-Cor) and dry weight of roots, crown, and shoot tissue. Tissue was dried in a 70°C forced air oven for 72 hours prior to obtaining dry weights.

Tissue from roots, crowns, and shoots were harvested at 6, 9, 12, and 15 weeks to determine total non-structural carbohydrate (TNC) content. Tissue was dried according to Smith (1973), at 100°C for 24 hours to stop enzymatic activity and respiration that can reduce dry matter and increase the percentage of reducing sugars. Tissue was then stored until ground at 70°C to prevent further enzyme activity that can occur with moisture content above 4%. Tissue was ground using a Wiley Mill and then stored in airtight container until analysis. Each tissue was analyzed for its total non- structural carbohydrate content using methods described in Ting (1956), Smith (1981a), and Fu and Dernoeden (2009). The method described here determined total nonstructural carbohydrate content through oxidation of sugars by an alkaline solution. Storage sugars were first decomposed into sucrose and fructans using the enzyme, α - amalyase. Sucrose and fructans were then hydrolyzed to reducing sugars using Hydrochloric acid. Reducing sugar levels were determined by adding the oxidizing agent alkaline ferricyanide and the coloring agent arsenmolybdate and read using a spectrophotometer (Ting 1956).

Chlorophyll (chl a, chl b, and carotenoids) was measured at 6, 9 and 15 weeks (Barnes et al. 1992; Wellburn 1994). Fresh tissue (50 mg) was placed in 20 ml Di-Methyl Sulfoxide (DMSO) in the dark for 5 days and then measured using a spectrophotometer (Spectronic Genesys 2; Thermo Electron Corporation; Massachusetts; USA).

Photosynthesis and stomatal conductance were measured at 9 weeks and 12 weeks using a photosynthesis machine (CIRAS-2; PP-Systems;

Massachusetts; USA) on the youngest fully expanded leaf at the top of canopy on three pots per chamber. Measurements were taken from 12:00- 15:00. A halogen light attachment set at 600 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ was used to keep light levels stable during measurements. Instantaneous water use efficiency was calculated for each plant using the photosynthesis rates and stomatal conductance data.

Total antioxidant content of the leaf tissue was assessed at 9 and 15 weeks (Re et al. 1999). This method uses ABTS (2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid)), which is first oxidized by potassium persulfate, creating a dark color. Antioxidants are extracted from tissue in 95% ethanol in the freezer for 48 hours. Tissue is then homogenized and removed via the centrifuge. Then 1 ml ABTS/potassium persulfate solution is added to 50 μl the supernatant and incubated for 2.5 minutes. The antioxidants in the sample then reduce or quench the oxidized solution and in the process remove the color. The color is read using a spectrophotometer (Spectronic Genesys 2; Thermo Electron Corporation; Massachusetts; USA) at 734 nm and the amount of antioxidants is determined based on calculations.

4.2.3 Data Analysis

Data from this randomized complete block was analyzed for statistical significance using SAS 9.3 (SAS Institute, Inc.). Main effects and their interactions were tested using analysis of variance with the generalized linear model procedure. To determine differences across treatment means we used the Tukey method ($p < 0.5$).

4.3 Results

Effects of these atmospheric pollutants on biomass allocation and total biomass were highly dependent on timing within the life cycle.

Early in the life stage (0-3 weeks) elevated carbon dioxide (CO₂; 800 ppm) did not increase total plant biomass significantly. However, elevated ozone (O₃; 80 ppb) did negatively impact biomass under ambient CO₂ (400 ppm). Elevated CO₂ partially ameliorated this effect (Figure 4-2a). Measurements of biomass allocation between root, crown, and leaf show that elevated ozone significantly reduced shoot and crown growth while the root growth was not significantly less than plants in the other treatments (Figure 4-2b). The root: shoot ratio (RSR=root/ shoot) was not significantly affected by treatments at this stage (data not shown). The relative growth rate (RGR) of the plants from week 0 (planting) to week 3 was significantly less in the ambient carbon dioxide/ elevated ozone treatments (Figure 4-2c). Relative growth rate is a measure of the amount of biomass accumulated during a time period ($RGR = \frac{\ln \text{weight}_2 - \ln \text{weight}_1}{\text{time}_2 - \text{time}_1}$). Plants at this time period had only the main shoot and 2-3 leaves.

Ozone continued to reduce biomass growth in ambient carbon dioxide treatments over the next three weeks (3-6 weeks). Elevated carbon dioxide partially ameliorated the negative impacts of ozone on total biomass growth (Figure 4-3a). Allocation to leaves was significantly higher in the elevated carbon dioxide/ ambient ozone (30 ppb) treatments. Plants in ambient carbon dioxide/ elevated ozone had significantly reduced biomass allocation to leaves and roots (Figure 4-3b). Elevated carbon dioxide ameliorated the negative impacts of

elevated ozone and plant allocation to leaves, crowns, and roots were maintained at the same levels as the control plants (ambient ozone, ambient carbon dioxide). The root: shoot ratio was not significantly different across treatments (data not shown). Relative growth rate was not found to be significantly different across treatments during this time period from 3-6 weeks (data not shown). Leaves were large enough to measure leaf area at this time; allowing us to measure other components such as Specific Leaf Area, Leaf Area Ratio and Net Assimilation rate. Specific Leaf Area (SLA) is a measurement of leaf density ($SLA = \text{leaf area} / \text{leaf weight}$). At 6 weeks the elevated carbon dioxide/ elevated ozone treated plants had a significantly smaller SLA (more dense) than the control plants (Figure 4-3c). The other treatments (elevated carbon dioxide/ambient ozone and ambient carbon dioxide/elevated ozone) while showing a trend of decreased SLA were not significantly different from the control. Leaf Area Ratio (LAR) is a measure of the photosynthetic area (leaf area) in relation to the whole plant biomass ($LAR = \text{leaf area} / \text{whole plant biomass}$). No significant differences were found for LAR at 6 weeks (data not shown). Net Assimilation Rate (NAR) is a measurement that relates the RGR to the LAR (RGR / LAR); it indicates photosynthetic efficiency through estimating the possible photosynthetic capacity through LAR and relating it to that used for growth (RGR) versus what may have been lost in other physiological processes (secondary compound production, maintenance respiration, etc). At 6 weeks we found no significant differences in NAR across treatments (data not shown). Total non-structural carbohydrates (TNC) were also measured for the first time at

6 weeks. There were no significant differences across treatments for leaf or crowns. However, we did see a significant reduction in the free sugars in the roots of elevated carbon dioxide/ elevated ozone treatment compared to the elevated carbon dioxide/ ambient ozone treatment (Figure 4-3d). At this time, most plants were still producing leaves on a main shoot only. Some plants were starting to produce tillers. There was no affect of treatment on growth stage.

As time progressed elevated carbon dioxide had an increasing stimulatory effect on total biomass production. By week 9, plants in elevated carbon dioxide/ ambient ozone treatments had significantly more biomass than all other treatments (Figure 4-4a). Elevated carbon dioxide also ameliorated the negative impacts of elevated ozone such that the elevated carbon dioxide/elevated ozone treated plants were equal in total biomass to the control. Ambient carbon dioxide/ elevated ozone treatments significantly reduced total biomass below all other treatments. Roots and shoots of the ambient carbon dioxide/ elevated ozone treatment were significantly smaller than all other treatments; however, the crown biomass was only significantly smaller than the elevated carbon dioxide/ ambient ozone treatment (Figure 4-4b). Biomass of the roots and crowns in the elevated carbon dioxide/ ambient ozone treatment were significantly higher than the control; shoot biomass was significantly higher than all other treatments. At 9 weeks the root: shoot ratio of the ambient carbon dioxide/ elevated ozone treated plants were significantly smaller than both of the elevated carbon dioxide treatments (Figure 4-5a). This indicates reduced root growth in relation to shoot growth. There was no significant effect of treatment on SLA at this stage (data

not shown). Elevated carbon dioxide/ambient ozone had a significantly smaller Leaf Area Ratio (LAR) than the ambient carbon dioxide/ elevated ozone treatments specifying a smaller leaf area in relation to whole plant biomass (Figure 4-5b). Analysis of the RGR from 6 to 9 weeks showed a significantly smaller growth rate in the ambient carbon dioxide/ elevated ozone treatment compared to all other treatments (Figure 4-5c). Elevated carbon dioxide/ ambient ozone treated plants had the significantly greatest NAR over this time period (Figure 4-5d). Ambient carbon dioxide/ elevated ozone treatments had a significantly smaller NAR compared to the two elevated carbon dioxide treatments. The elevated carbon dioxide treatments also had significantly greater levels of TNC in the leaves than the two ambient carbon dioxide treatments (Figure 4-4c). Most plants were producing tillers at this time.

By week 12, elevated carbon dioxide, elevated ozone treated plants were not significantly different than the elevated carbon dioxide, ambient ozone treated plants or the control (Figure 4-6a). The ambient carbon dioxide/ elevated ozone treated plants had significantly less total biomass than the two elevated carbon dioxide treatments but not the control treatment, although it was reduced it was not found to be significant. A similar trend was found in the biomass allocation to roots, shoots, and crowns (Figure 4-6b). The RSR, SLA, LAR, RGR, and NAR were not significantly affected by treatments at 12 weeks (data not shown). In the leaf tissue, TNC was significantly higher in the elevated carbon dioxide/ ambient ozone than the ambient carbon dioxide/ elevated ozone treatments (Figure 4-6c). There were no significant differences in TNC levels of the root or

crown tissue. All plants were producing tillers at this time and some plants were starting to elongate stems in preparation of flowering.

Finally, at week 15, there were no significant differences between the control or both elevated carbon dioxide treatments (Figure 4-7a). However, ozone still significantly reduced the total biomass under ambient carbon dioxide conditions. This can be attributed mostly to the significantly smaller shoot biomass as the crown and root allocation was not significantly smaller than the control or elevated carbon dioxide/elevated ozone treated plants (Figure 4-7b). The RSR, SLA, and LAR were not significantly affected by treatments at 15 weeks (data not shown). For the growth period between 12 to 15 weeks ambient carbon dioxide/elevated ozone treated plants had significantly reduced RGR compared to the control and elevated carbon dioxide/ambient ozone treatment but not the elevated carbon dioxide/ elevated ozone treatment (Figure 4-7c). Treatments did not significantly affect NAR during the time period from 12 to 15 weeks (data not shown). There were also no significant differences in TNC for leaf, crown, and roots (Figure 4-7d). Some plants were flowering at this stage and most plant had elongating stems.

There were two possible mechanisms for the amelioration of ozone by carbon dioxide: antioxidant production and reduced stomatal flux. Total antioxidant levels were measured at 9 weeks and 15 weeks and were not found to be significantly different across treatments (data not shown). Photosynthesis rate and stomatal conductance were measured at 9 weeks and 12 weeks (Figure 4-8, 4-9).

At 9 weeks, photosynthesis rates were significantly higher in the elevated carbon dioxide/ ambient ozone treated plants (Figure 4-8a). Elevated ozone significantly reduced the photosynthesis rate in the ambient carbon dioxide treatment compared to both of the elevated carbon dioxide treatments. Elevated ozone significantly reduced the photosynthesis rate in the elevated carbon dioxide treatment, but this was not reduced below control levels. Both elevated ozone and elevated carbon dioxide reduced stomatal conductance but this was only significantly less than the control in the elevated carbon dioxide/elevated ozone plots (Figure 4-8b). Using the photosynthetic rate and stomatal conductance it is possible to calculate the instantaneous water use efficiency. At 9 weeks, there was significantly greater instantaneous water use efficiencies in the elevated carbon dioxide plots, with the ambient ozone being significantly higher than the elevated ozone in the elevated carbon dioxide treatments (Figure 4-8c).

At 12 weeks, elevated carbon dioxide increased the photosynthesis rate at both ozone levels compared to the ambient carbon dioxide treatments (Figure 4-9a). There was no effect of ozone on the photosynthetic rate. Stomatal conductance was reduced by elevated ozone but this was only found to be significant when comparing the ambient and elevated ozone of the two elevated carbon dioxide treatments (Figure 4-9b). These measurements resulted in a calculation of instantaneous water use efficiency that was significantly greatest in the elevated carbon dioxide/ elevated ozone treatments. The elevated carbon dioxide/ ambient ozone treated plants were significantly greater than the two

ambient carbon dioxide treatments but significantly less than the elevated ozone/elevated carbon dioxide treatment (Figure 4-9c).

Chlorophyll content (chl-a, chl-b, and carotenoids) was not altered significantly by treatments (data not shown).

4.4 Discussion

Results indicate that elevated ozone has a negative impact on plant productivity at all stages of the life cycle. The negative impact of reduced biomass growth affected shoots, crowns and, roots equally. It was expected that we would see reduced root:shoot ratio in response to elevated ozone due to reduced sugar availability and damage to phloem loading mechanisms (Cooley and Manning 1987; Grantz and Vu 2006; Andersen 2003; Fiscus et al. 2005; Grantz and Farrar 1999, 2000). However, there were no significant differences in root: shoot ratio across treatments, except at week 9, where RSR was reduced in the elevated ozone/ambient carbon dioxide treatment. At this stage plants were starting to tiller, so it is likely that root growth was reduced while sugars were being used to produce secondary shoots and crowns. Elevated carbon dioxide did ameliorate the negative impacts of ozone such that root, shoot, and crown growth were equal to the control throughout all stages of the life cycle.

Elevated carbon dioxide did not stimulate productivity immediately and played the greatest stimulatory role during the vegetative tillering stage of the life cycle. Later on in the life cycle, during the start of reproduction, carbon dioxide

had less of a stimulatory effect on root, shoot, and crown. This can most likely be attributed to energy being diverted from vegetative growth to reproductive needs.

Additionally, stimulation by elevated carbon dioxide occurred first in the shoot tissue and continued to be greatest in shoot tissue compared to other treatment. Roots were not found to be significantly different across treatments (excluding ambient carbon dioxide/ elevated ozone). This may indicate that Timothy favored shoot growth over root growth in this experiment. This could have been an affect of management. Plants were watered well and provided ample nutrients; it has been shown elsewhere that root growth is often stimulated by drought and nutrients deficiencies (Marschner et al. 1995; Jupp and Newman 1987). Due to water and nutrient that were not limiting, the plants were able to increase shoot growth while still receiving requirements of water and nutrients.

Elevated carbon dioxide did not stimulate crown growth as previously reported during the flowering stage (Saebo and Mortensen 1995). Crown growth stimulation by elevated carbon dioxide above ambient carbon dioxide treatments occurred during weeks 9-12. However, early in life cycle (up to week 6), there was no stimulation in the crown above the control but there was compared to the ambient carbon dioxide/elevated ozone. We did not see increased free sugar accumulation during flowering either as was expected due to accumulation of fructans in crown at flowering (Smith and Groteleuschen 1966). Elevated carbon dioxide did ameliorated negative impact of ozone on crown growth. This was especially evident in the early stage of establishment (week 3) and during tillering (week 9-12). It is likely that our warmer experimental temperatures led to favor

shoot growth over other growth and reduced sugar accumulation in the crowns compared to the cooler temperatures of past experiments (Smith and Groteleuschen 1966, Saebo and Mortensen 1995; Mortensen and Saebo 1996).

Total nonstructural carbohydrates did not show the significant differences that were hypothesized in response to the treatments. Starting with week 6, there were no differences in available free sugars (TNC) in the shoots or crowns. Free sugars in the root of elevated carbon dioxide/ elevated ozone were significantly reduced below elevated carbon dioxide/ ambient ozone however this did not have a negative impact on root growth. This reduced sugar level is an indication of the high ozone stress; the plant was able to keep up its rate of growth but did not have excess sugars left in tissues after growth. During the same time period we did not see the same reduction in free sugar content of the ambient carbon dioxide/elevated ozone treated but there was reduced biomass accumulation at this time. Plants in all ozone treatments appeared to be most sensitive to ozone during this early stage of the life cycle; we did not see changes in free sugar content in roots or crowns throughout any other time point in this experiment. However, as discussed above, there were differences in biomass. This indicates that free sugars in the ambient ozone and elevated carbon dioxide/ elevated ozone treatments were being used for growth, resulting in increase biomass. While the sugars in the ambient carbon dioxide/ elevated ozone were either not being made at same levels or being diverted to other mechanisms such as maintenance and/or repair, resulting in reduced growth

(Barnes 1972; Pell and Brennan 1973; Amthor and Cumming 1988; Amthor 1988).

We did see significant increases in free sugars in the leaves of the elevated carbon dioxide treatments at week 9. In the elevated carbon dioxide/ ambient ozone treatment, this is supported by increased biomass accumulation and elevated photosynthesis rates. However, in the elevated carbon dioxide/ elevated ozone the relationship is not clear. We do see an increase in biomass in the growth period from 9-12 weeks but photosynthesis rates at 9 weeks were not higher than ambient carbon dioxide treatments; however, photosynthesis rates were only measured once and it is likely that they could have been higher previous to harvesting plant material resulting in the elevated free sugars. The elevated free sugars measured at week 9 could have contributed to the increased growth over the 9-12 week time period.

At week 12, there were significant increases for all treatments above the ambient carbon dioxide/ elevated ozone treatment. This correlates with measured increases of photosynthesis at high carbon dioxide and also available free sugars resulting in biomass accumulation. Increased sugar at week 12 resulted in increased relative growth rate for the two ambient ozone treatments from week 12-15 compared to the ambient carbon dioxide/elevated ozone. Elevated carbon dioxide/ elevated ozone did not have a significant increase in RGR over the ambient carbon dioxide/elevated ozone possibly indicating a stress response to elevated ozone resulting in some of the excess free sugars being diverted to other processes than growth, such as repair or reproduction.

As expected the plants in the two elevated carbon dioxide treatments had an increased instantaneous water use efficiency at the two times measured during week 9 and 12. This was a result of increased photosynthesis rates and decreased stomatal conductance. This would be beneficial to the plants in the natural environment where water would be limiting. It probably did not play an important role here because we watered all plants well.

Through out the experiment we saw amelioration of the negative impact of ozone by elevated carbon dioxide. We tested total antioxidants and stomatal conductance as two mechanisms for this amelioration. Stomatal conductance was reduced in elevated carbon dioxide treatments. This reduced aperture thus resulted in reduced flux of ozone into the plants in the elevated carbon dioxide/ elevated ozone treatments resulting in reduced ozone impact. We found no significant change in total antioxidants across treatments. However, this does not mean that they didn't play a role in protection. It is likely that excess antioxidants in the elevated ozone treatments were already used up in their quenching of increased ROS in response to ozone (Gillespie et al. 2012, Gillespie et al. 2011; Robinson and Sicher 2004). Furthermore, ascorbic acid has been delegated as the main antioxidant used in ozone defense (Burkey et al. 2006; Castagna and Ranieri 2009). It is possible that by measuring total antioxidants, measureable increases in ascorbic acid were masked by the total antioxidant pool.

In comparing our results to past experiments, we support findings in regards to reduction in total biomass in response to elevated ozone (Mortensen 1992, 1997; Danielsson et al. 1999, 2003; Johnson et a 1996). In addition, we

also support some past findings regarding carbon dioxide effects on productivity. Mortensen and Saebo (1996) reported that carbon dioxide enrichment was most significant on plant tillering. We found similar results here with our greatest stimulation occurring during the tillering stage at weeks 9 through week 12. However, another experiment found that the greatest stimulation was in the stubble left behind after harvest of the shoots and there was negative a negative response of shoot biomass to elevated carbon dioxide (Saebo and Mortensen 1995). Here we did not observe the stimulation in crown growth and our greatest stimulation occurred in the shoot tissue. Differences between experiments can most likely be attributed to different experimental growing conditions. We had warmer temperatures and lower light, while other experiment had cooler temperatures and high light.

In conclusion, it is unlikely that future predicted levels of elevated carbon dioxide would have a positive affect on the agricultural productivity of Timothy grass if Timothy grass continues to be harvested at full bloom. While we did see stimulation in the shoot tissue this was not the case throughout the entire life cycle, particularly in the flowering stage when Timothy is traditionally harvested (Childers and Hanson 1985; Smith and Groteleuschen 1966). Warmer temperatures could interact with rising levels of carbon dioxide to increase productivity slightly however there are other negative implications that come with warmer temperatures, such as drought, that are unlikely to help productivity. However, we did observe increased instantaneous water use efficiency in plants treated with elevated CO₂ so it is possible that plants could maintain productivity

through initial periods of warming and drought. In addition we did not see sufficient stimulation in the crown or roots, both of which would be important for agricultural use in terms of regrowth and overwintering (Smith and Groteleuschen 1966). However, our well-watered and ample nutrient applications may not have produced realistic rooting growth. Furthermore, carbon dioxide did protect Timothy from the negative impacts of ozone. As a result elevated carbon dioxide could benefit agricultural productivity in a high ozone future.

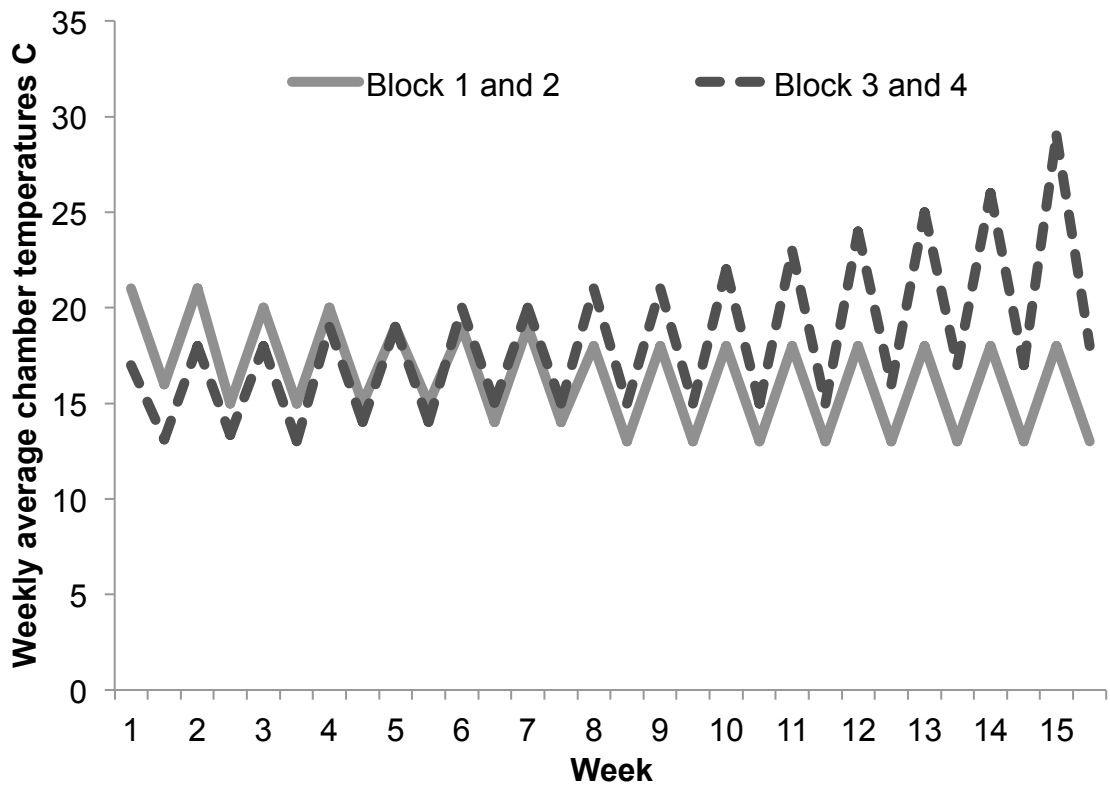


Figure 4-1: Average chamber temperatures (C) during each experimental replication.

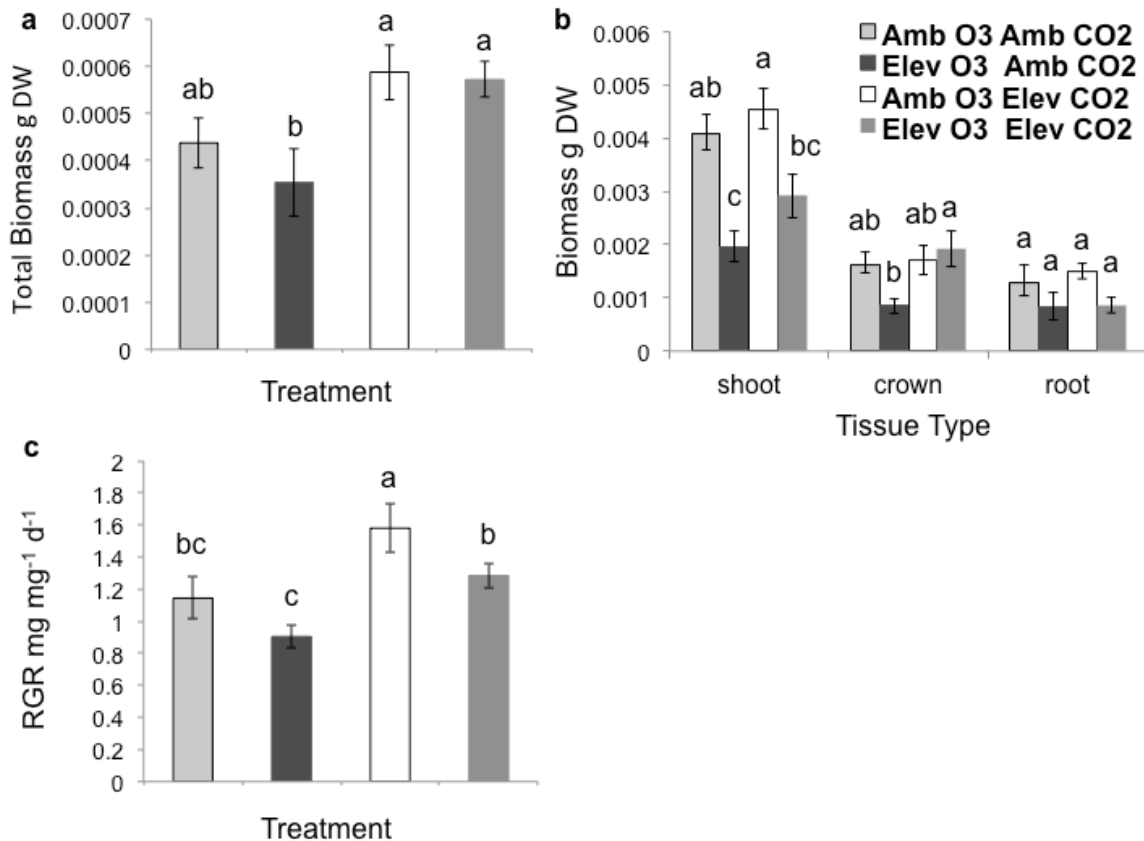


Figure 4-2: Growth at 3 weeks. a) Total Biomass at 3 weeks. Letters denote significant differences across treatments and was determined using the Tukey test ($p < 0.05$) **b) Biomass allocation to shoot, crown, and root at 3 weeks.** Letters denote significant differences across treatments within each tissues type only: shoot, crown, root. Significance was determined using the Tukey test ($p < 0.05$) **c) Relative Growth Rate from 0 weeks (planting) to 3 weeks.** Significance determined using the Tukey test ($p > 0.05$) and is indicated by letters. **AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide.**

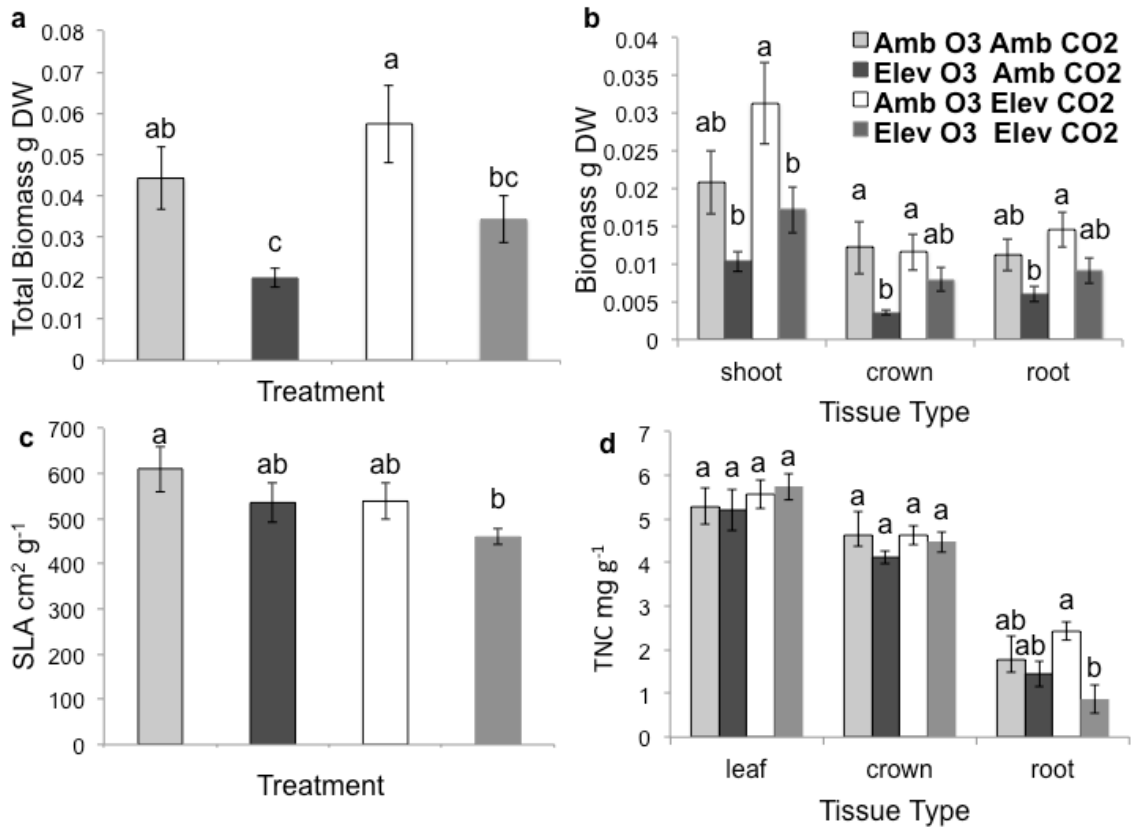


Figure 4-3: Growth at 6 weeks. a) Total biomass at 6 weeks. Letters denote significant differences across treatments and was determined using the Tukey test ($p < 0.05$). **b) Biomass allocation at 6 weeks.** Letters denote significant differences across treatments within each tissues type only: shoot, crown, root. Significance was determined using the Tukey test ($p < 0.05$). **c) Specific Leaf Area at 6 weeks.** Significance determined using the Tukey test ($p > 0.05$) and is indicated by letters. **d) Total non-structural carbohydrates at 6 weeks.** Letters denote significance across treatments within each tissue type. Significance determined using the Tukey test ($p > 0.05$). **AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide.**

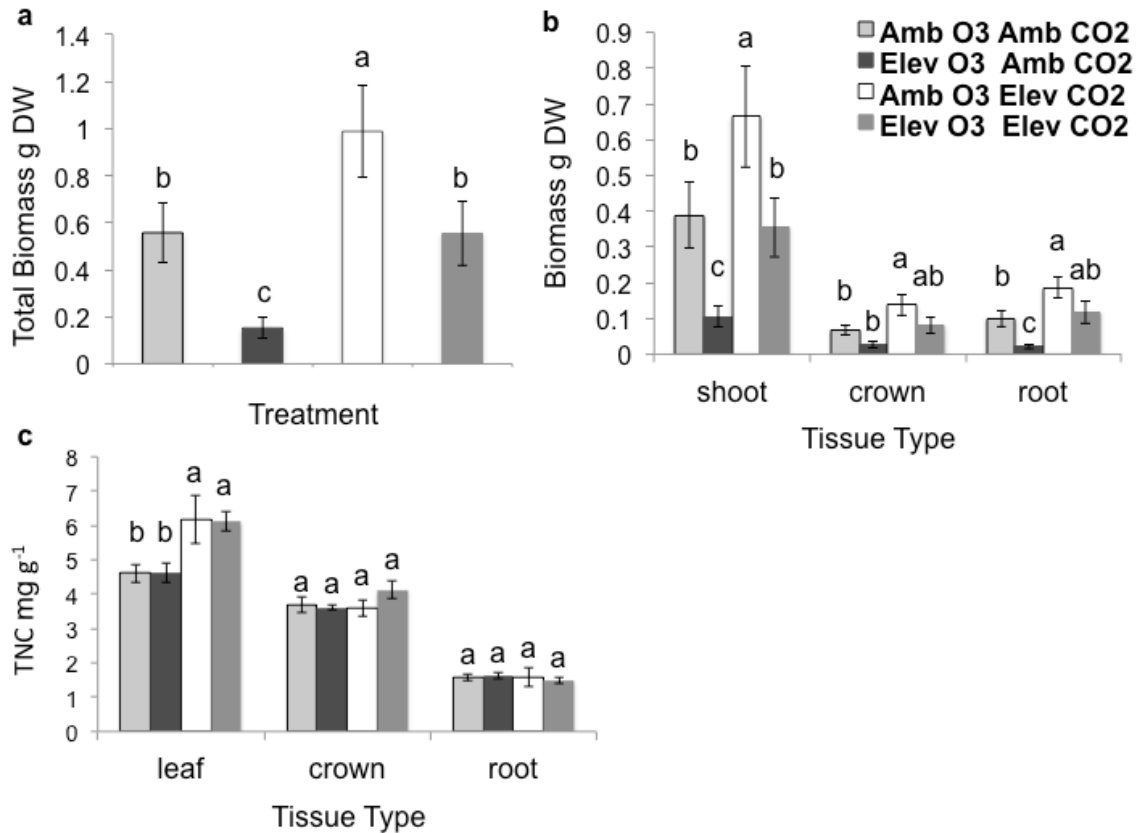


Figure 4-4: a) Total biomass at 9 weeks. Letters denote significant differences across treatments and was determined using the Tukey test ($p < 0.05$). **b) Biomass allocation at 9 weeks.** Letters denote significant differences across treatments within each tissues type only: shoot, crown, root. Significance was determined using the Tukey test ($p < 0.05$). **c) Total non-structural carbohydrates at 9 weeks.** Letters denote significance across treatments within each tissue type. Significance determined using the Tukey test ($p > 0.05$). **AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide.**

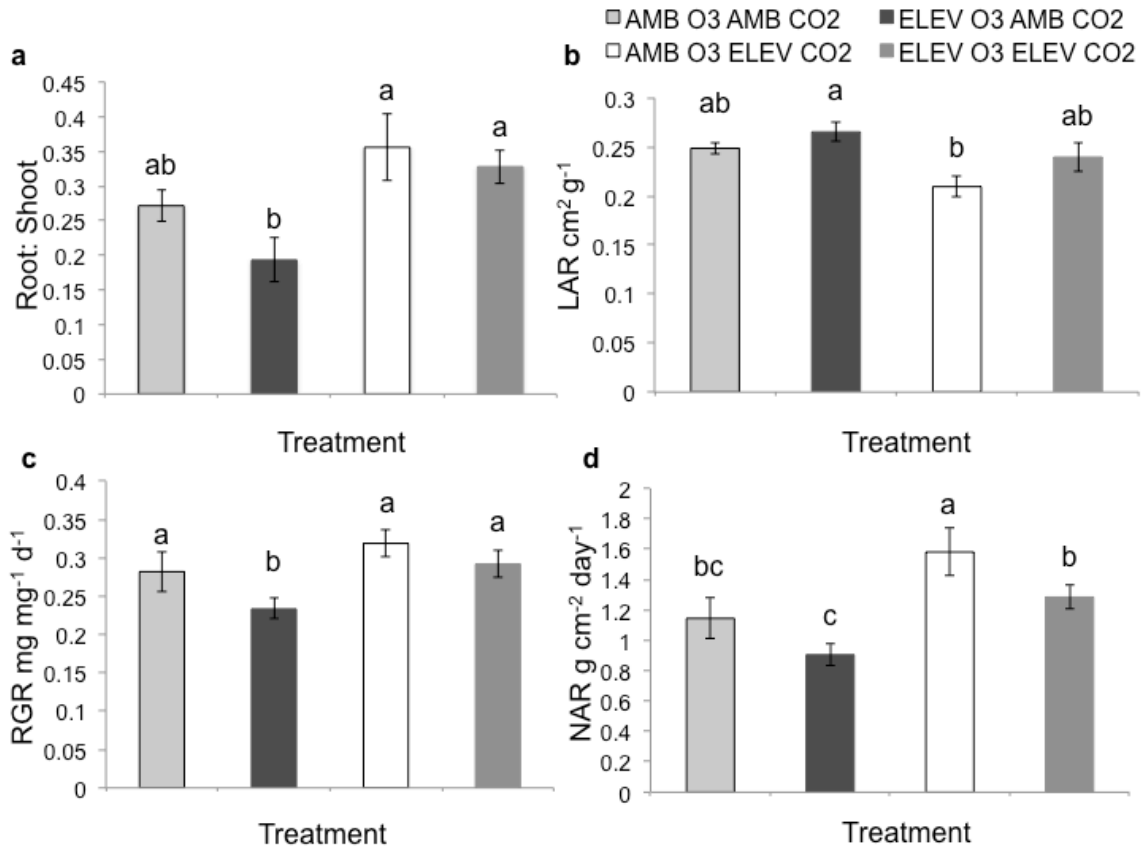


Figure 4-5: a) Root Shoot Ratio at 9 weeks. Letters denote significant differences across treatments and was determined using the Tukey test ($p < 0.05$). Significance determined using the Tukey test ($p > 0.05$) and is indicated by letters. **b) Leaf Area Ratio at 9 weeks.** Significance determined using the Tukey test ($p > 0.05$) and is indicated by letters. **c) Relative Growth Rate from 6 to 9 weeks.** Significance determined using the Tukey test ($p > 0.05$) and is indicated by letters. **d) Net assimilation Rate from 6 to 9 weeks.** Significance determined using the Tukey test ($p > 0.05$) and is indicated by letters. AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide.

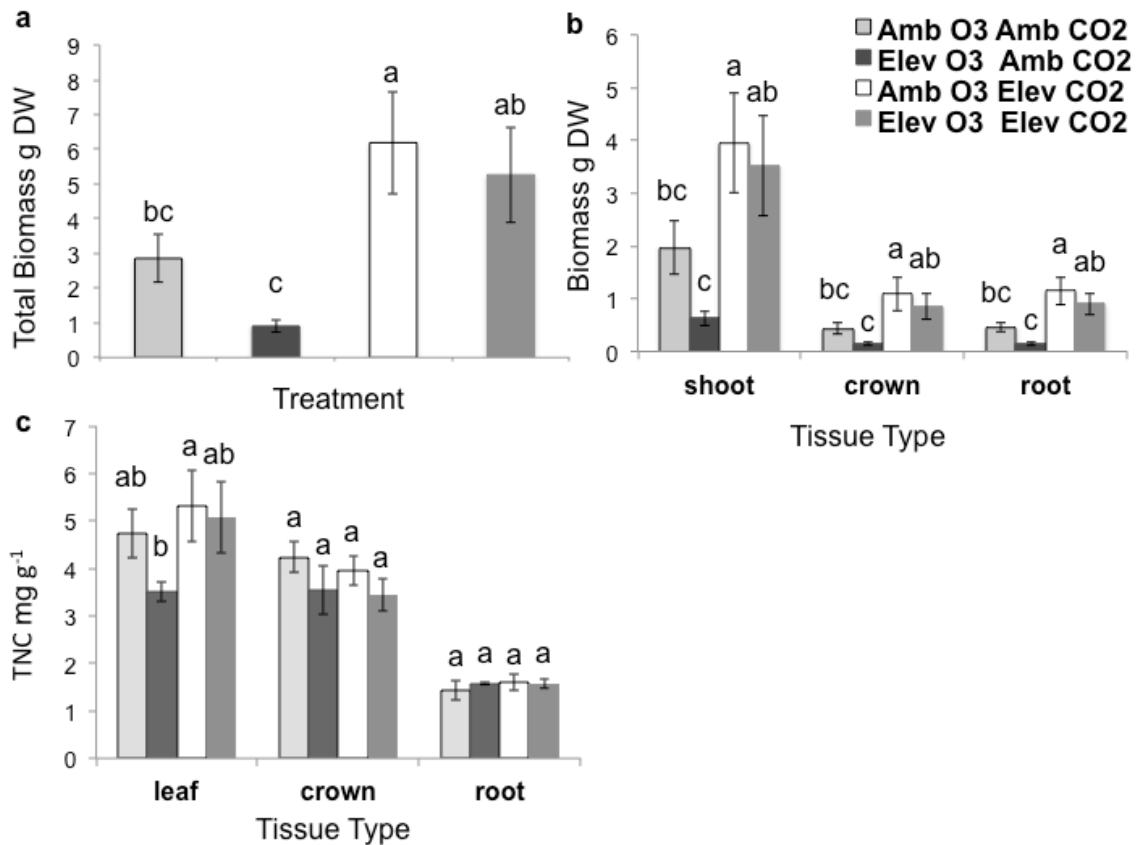


Figure 4-6: a) Total biomass at 12 weeks. Letters denote significant differences across treatments and was was determined using the Tukey test ($p < 0.05$). **b) Biomass allocation at 12 weeks.** Letters denote significant differences across treatments within each tissues type only: shoot, crown, root. Significance was determined using the Tukey test ($p < 0.05$). **c) Total non-structural carbohydrates at 12 weeks.** Letters denote significance across treatments within each tissue type. Significance determined using the Tukey test ($p > 0.05$). **AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide.**

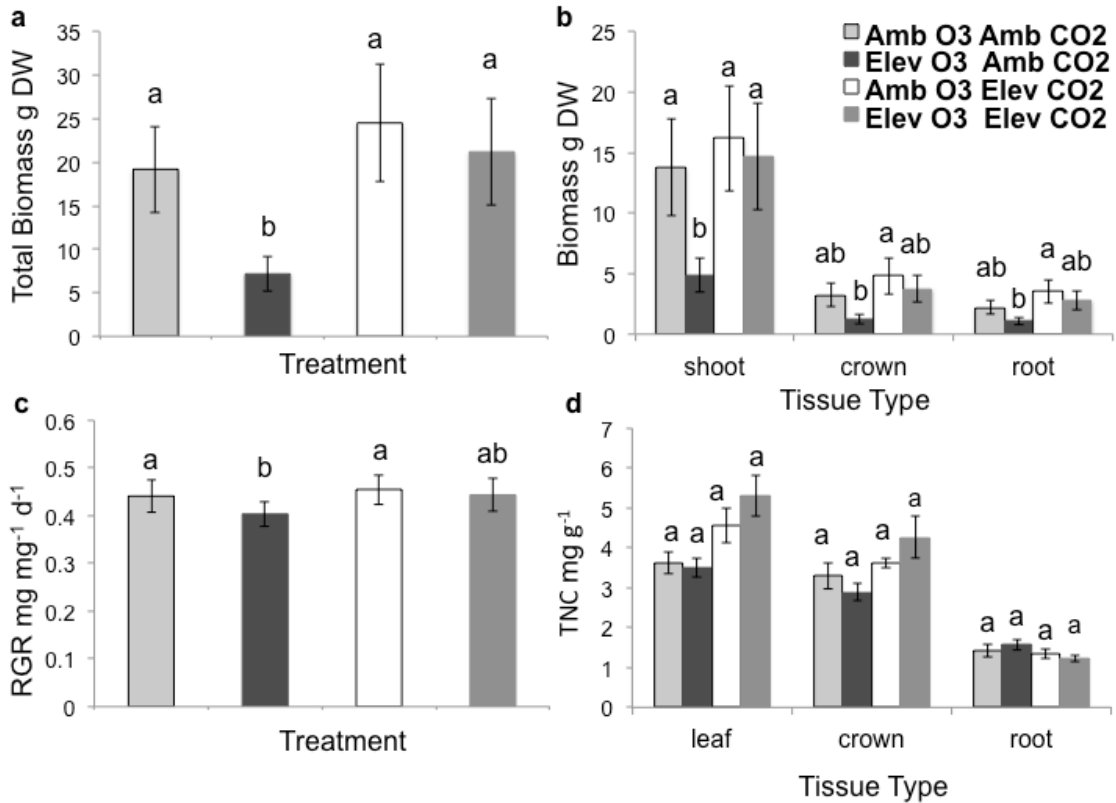


Figure 4-7: a) Total biomass at 15 weeks. Letters denote significant differences across treatments and was determined using the Tukey test ($p < 0.05$). **b) Biomass allocation at 15 weeks.** Letters denote significant differences across treatments within each tissues type only: shoot, crown, root. Significance was determined using the Tukey test ($p < 0.05$). **c) Relative Growth Rate from 12 to 15 weeks.** Significance determined using the Tukey test ($p > 0.05$) and is indicated by letters. **d) Total non-structural carbohydrates at 15 weeks.** Letters denote significance across treatments within each tissue type. Significance determined using the Tukey test ($p > 0.05$). **AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide**

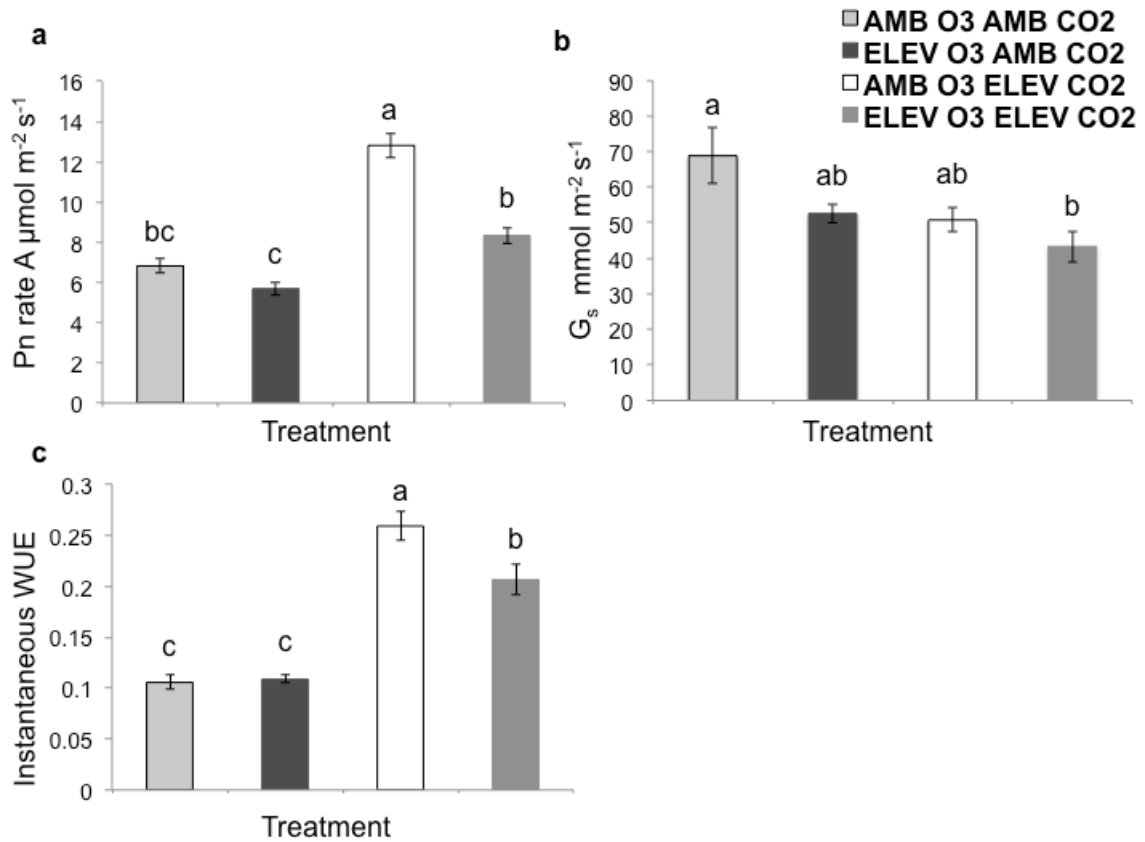


Figure 4-8: Gas Exchange Measurements at week 9. a) Photosynthesis rate at 9 weeks. b) Stomatal Conductance at 9 weeks. c) Instantaneous water use efficiency at 9 weeks. Letters denote significant differences across treatments and was determined using the Tukey test ($p < 0.05$). AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide.

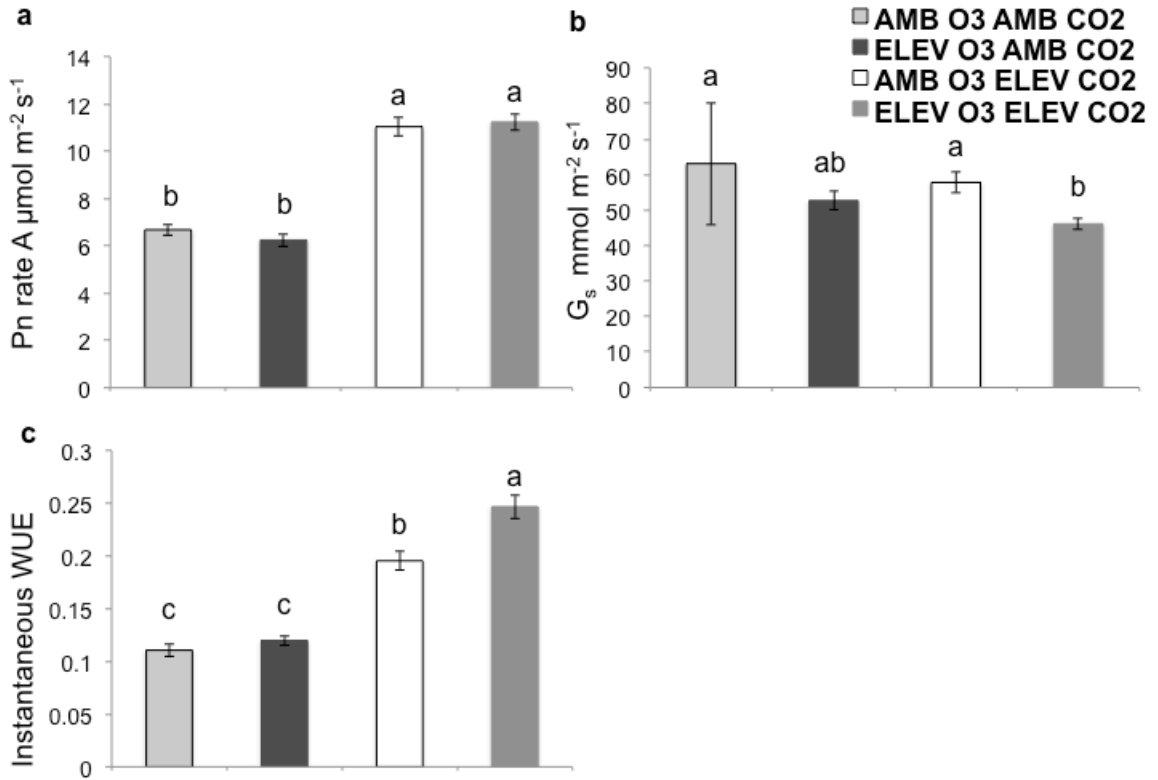
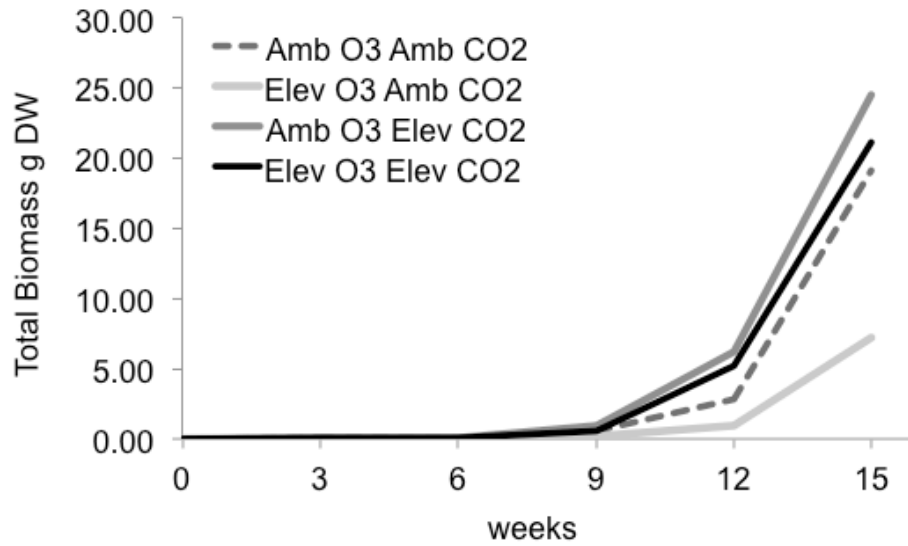


Figure 4-9: Gas Exchange Measurements at week 12. a) Photosynthesis rate at 12 weeks. b) Stomatal Conductance at 12 weeks. c) Instantaneous water use efficiency at 12 weeks. Letters denote significant differences across treatments and was determined using the Tukey test ($p < 0.05$). **AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide.**



TOTAL BIOMASS (g)	week									
	3		6		9		12		15	
Amb O3 Amb CO2	0.0070	ab	0.0443	ab	0.5578	b	2.8557	bc	19.1953	a
Elev O3 Amb CO2	0.0036	c	0.0200	b	0.1523	c	0.9035	c	7.1778	b
Amb O3 Elev CO2	0.0078	a	0.0575	a	0.9884	a	6.1898	a	24.5056	a
Elev O3 Elev CO2	0.0057	bc	0.0342	b	0.5538	b	5.2668	ab	21.1906	a

Figure 4-10: Total Biomass through life-cycle. Letters denote significant differences across treatments and was determined using the Tukey test ($p < 0.05$). **AMB O3= 30 ppb ozone; ELEV O3= 80 ppb ozone; AMB CO2= 400 ppm carbon dioxide; ELEV CO2= 800 ppm carbon dioxide.**

CHAPTER 5

CONCLUSION

In conclusion, we can expect an increase in Timothy grass pollen load in response to predicted levels of carbon dioxide and ozone. This is in part a result of growth stimulation by elevated carbon dioxide over the life cycle of the Timothy grass. We observed increases in above ground biomass in the elevated carbon dioxide treated plants and this increased photosynthetic tissue likely played a large role in the observed increases in pollen production.

Due to the plant increasing reproduction at expense of leaf biomass growth during the reproductive stage, it is unlikely that elevated carbon dioxide will result in increased agricultural productivity in future climates. This is particularly true if management practices continue to harvest at full bloom. Other factors of climate change, such as temperature and precipitation are likely to further affect productivity of this forage grass. However, indications of increased water use efficiency could help with productivity in future climates. Our root data was confounded by our application of ample nutrients to encourage flowering. It is necessary to investigate these findings under more natural conditions that allow for more natural roots responses. Grassland ecosystems are very dynamic and their response to climate change is not well understood yet.

Even so, these findings regarding pollen production can most likely be extrapolated to other grass species, both C3 and C4. Predicting future increases

in pollen load allow health care providers to prepare for changes in sensitized populations and allow for a better estimation of the true impact of climate change on human health.

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