INTERACTIVE EFFECTS OF ELEVATED CARBON DIOXIDE AND OZONE ON AN INSECT TRANSMITTED PLANT VIRUS

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INTERACTIVE EFFECTS OF ELEVATED CARBON DIOXIDE AND OZONE ON AN INSECT TRANSMITTED PLANT VIRUS

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Disease epidemics can profoundly shape the ecosystems of which they are a part, affecting productivity, nutrient cycling, community composition, and species interactions. Recent changes to global ecosystems can modify the effects of disease on organisms or ecosystems, but the nature and extent of these modifications are thus far little understood, particularly for plant viruses. Among the important drivers of complex changes in ecosystems are changing levels of atmospheric gases including the greenhouse gas carbon dioxide and the oxidizing agent ozone. I used open top chambers in the field to examine the effects of rising carbon dioxide and ozone levels on the spread and severity of barley yellow dwarf virus (BYDV), an economically and environmentally important pathogen of plants in the grass tribe. I examined the effects on viral fitness and viral spread in monocultures of the epidemiologically important host *Avena fatua* and in mixtures of *A. fatua* and *Setaria lutescens*, a poor host of the pathogen. In monocultures of *A. fatua,* within-host viral fitness declined with ozone concentrations, but addition of carbon dioxide to ozone restored within-host viral fitness to ambient levels. Despite reduced virus concentration in ozonated plants, transmission of the virus did not significantly decrease with gas treatment, suggesting that no abatement of BYDV epidemics is likely under future atmospheric conditions. Ozone also reduced host vegetative and reproductive biomass in monocultures. While adding carbon dioxide to the ozone restored vegetative biomass to ambient levels, it did not compensate for losses in reproduction. If this pattern extended to crop species, yield of major grain crops such as wheat, oats, and barley could be severely reduced. In mixtures of *Avena* and *Setaria*, within-host viral fitness and transmission

rates in both *Avena* and *Setaria* were enhanced. In addition, when *Avena* and *Setaria* were grown in competition, carbon dioxide reduced the benefits to *Avena* of growing in mixtures across infection treatments. Although presence in mixtures significantly suppressed growth in *Setaria*, CO_2 increased reproductive output in infected plants. These results highlight the potential importance of disease in plant competition under changing global atmospheres. Overall, these studies demonstrate the complex interactions between atmospheric conditions and community context that are likely to regulate both disease establishment and plant health under future atmospheric conditions.

BIOGRAPHICAL SKETCH

Emily Pollina has loved the natural world as long as she can remember, but this love came to fruition at Westover School in Middlebury, CT. There she learned to love biology, teaching, and spending time running and hiking in the outdoors, and she credits Westover's Outdoor program for inspiration to become an ecologist. She graduated from Westover in 2000. Emily took this enthusiasm for nature to Swarthmore College in Swarthmore, PA, where she was specifically introduced to a love of plants by Dr. Mark Jacobs and became fascinated with plant defenses in classes with Dr. Colin Purrington. She earned a Bachelor of Arts in biology there in 2004. From Swarthmore, Emily went to Duke University, where she worked for Dr. James Clark's lab as a field assistant and in the Durham Public School system teaching AP Environmental Science. Through these experiences, she developed an interest in the changing global climate, which she explored further in her Master of Science (2006) at Stanford University where she worked with Dr. Chris Field. Uniting her interests in plant defenses and global change, she joined the lab of Dr. Alison Power at Cornell University in 2006. During her years at Cornell (2006-2013) she explored the effects of rising carbon dioxide and ozone levels on an insect-transmitted plant virus. While at Cornell, her teaching interests flourished as well, leading her to research effective methods of teaching primary literature and writing to undergraduates with Dr. Barbara Crawford, Dr. Richard Kiely, and Dr. Kimberly Williams. She hopes to unite her love of ecology and teaching at a liberal arts college in the future.

For my family, whose unconditional love and support have made this work possible

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LIST OF ABBREVIATIONS

A_{max}	Maximal rate of leaf-level photosynthesis
BYDV	Barley yellow dwarf virus
CO_2	Carbon dioxide
DAE	Days after emergence
ELISA	Enzyme-linked immunosorbent assay
O_3	Ozone
RUBISCO	Ribulose 1,5 bisphosphate carboxylase/oxygenase

INTRODUCTION

Diseases have important effects on the structure and function of ecosystems, affecting population dynamics (Power and Mitchell, 2004), biomass and productivity (Mitchell, 2003), nutrient cycling (Tokuchi et al., 1994), community dominance and composition (Power and Mitchell, 2004), and the outcomes of competition (Power and Mitchell, 2004; Dunn et al, 2012). Plant diseases can impact human health not only by reducing food supply but by altering the frequency with which humans encounter disease vectors. For example, sudden oak death has increased the prevalence of major Lyme disease vectors by altering the abundance of small mammals that are the primary hosts for the disease (Swei et al., 2012). Understanding the effects of disease on ecosystems and predicting the effects of disease epidemics in the future may be complicated as the effects of diseases on ecosystems may themselves be altered due to environmental changes occurring throughout the globe. These environmental changes include interacting factors such as rising temperatures, altered gas concentrations in the atmosphere, changes to precipitation regimes, land use changes, nitrogen deposition and species introductions. Among these changes, rising levels of the greenhouse gases carbon dioxide and ozone (Polley et al., 2012; Hayes et al., 2013) may be of particular interest as they are drivers of many of the changes in temperature and precipitation globally.

These atmospheric changes may modify host physiology, pathogen physiology, or (for vectored diseases) vector transmission and preferences. Ozone has profound effects on organismal physiology because it is a potent oxidizer and thus damages membranes and respiratory surfaces (Sanderman, 1996). In plants, ozone damages cell and chloroplast membranes and photosynthetic enzymes, causing stunted growth (Sandermann,1996). Initially, plants may avoid ozone by stomatal closure, but over time ozone damages guard cells, making it difficult for them to close (Sanderman, 1996; Li et al., 2013). In addition, stomatal closure stunts growth further by reducing access to carbon dioxide. Rising carbon dioxide levels may alleviate some of the biomass losses due to stomatal closure, especially for C_3 plants, which become more photosynthetically efficient at high CO_2. In contrast C_4 plants are frequently less

responsive to high CO_2, as their CO_2 concentration mechanisms are not significantly benefited by additional CO_2 (Polley et al., 2012). CO_2 has additional direct effects on plant chemistry and architecture, reducing stomatal densities (Ocheltree et al. 2013) and increasing the C:N ratio of plant tissues (McElrone et al., 2005).

These direct effects on physiology may in turn affect suitability of hosts for pathogens. Studies of the effects of changing atmospheric gases on plant diseases show a wide variety of patterns (Chakraborty et al., 2000; Chakraborty, 2005). CO_2 has been shown to increase some fungal pathogens of plants (Mitchell et al., 2003; McElrone et al., 2010), while decreasing others (McElrone et al., 2005). Ozone shows similar contradictory effects, with some studies demonstrating pathogen increases under high ozone (Woodbury, 1994; von Tiedemann and Firsching, 1991; Karnosky et al., 2002, Percy et al., 2002), while other pathogens show decreases (Yalpani et al., 1994; Bilger et al., 2008; Olbrich et al., 2010). We might predict that the pathogens will respond to changes in host physiology in different ways based on their modes of transmission and life history. In particular, fungal diseases, which frequently enter via the stomata of the plant (Brown, 1997) may be more limited by the lower stomatal density and aperture under high CO_2 and ozone. Viruses, however, which are often vectored by insects (Power and Flecker, 2003), might show different patterns based on the responses of the insects and the effects of the gases on the host. To date most of the work on the effects of ozone and CO_2 on plant diseases has focused on fungal diseases of trees and crop plants (e.g. Mitchell, 2003, Percy et al., 2002; McElrone et al., 2005; Fleishman et al, 2010). Studies of the effects of these gases on viral plant diseases are rare, but suggest that elevated CO_2 may have important effects on plant responses to disease by increasing growth of infected plants (Malmstrom and Field, 1997), and increasing interactions with fungal mutualists (Rúa et al., 2013), while ozone has been shown to reduce viral symptoms (Bilgin et al., 2008). However, due to the interactions between climate change factors, especially physiological effects of CO_2 and ozone (Eastburn et al., 2011, Leuzinger et al., 2011), it is important to study them in combination in order to predict the effects of these gases on diseases (and on ecosystems) under future atmospheric conditions.

In this study, I use barley yellow dwarf virus (BYDV) to examine the interacting effects of rising CO_2 and ozone levels on the spread, host symptom severity, viral performance, and influence on host competition of an insect-vectored virus in natural grassland ecosystems. BYDV is a ubiquitous and damaging pathogen of grass tribe (Poaceae), and infects hundreds of species worldwide, though species vary in the readiness with which they become infected and the severity of their symptoms (D'Arcy, 1995). Because many important crop species are part of this family, BYDV can cause tremendous economic losses (McCurdy and Jones, 2002). In addition to inflicting economic damage, BYDV has caused ecological damage, as it has been implicated in the disappearance of native bunchgrasses in the west coast grasslands (Borer et al., 2007; Malmstrom et al., 2005; Malmstrom et al., 2006). Because BYDV is obligately vectored by aphids and infects a broad range of grass species (D'Arcy, 1995), it is a good model for the effects of CO_2 and ozone on a vectored plant virus. Here I consider the effects of projected atmospheric CO_2 and ozone levels on BYDV epidemics at both the individual and community levels in monocultures of an epidemiologically important host and in simple mixtures. In Chapter 1, I address the effects of factorial combinations of CO_2, ozone, and infection on the growth, reproduction, and leaf-level photosynthesis of *Avena fatua* (wild oats), a C_3 grass that is invasive and abundant in US west coast grasslands (Going et al., 2009). *A. fatua* plays an important role in BYDV transmission, as it substantially increases infection levels in communities of which it is part (Power and Mitchell, 2004). In Chapter 2, I evaluate the effects of the gases on within-host and between host viral transmission in the field, and the effects on the correlation between viral and host fitness. In Chapter 3, I consider simple communities of *Avena fatua*, a C_3 grass and a good host of the virus, and *Setaria lutescens,* a C_4 grass and a poor viral host. I examine the effects of the gases on transmission in communities with hosts of varying suitability for the virus, which is important as grasslands typically contain mixtures of such species. Finally, in Chapter 4, I examine the effects of the gases and infection on the outcome of competition between *Avena* and *Setaria*, to explore the interacting effects of ozone, carbon dioxide, and infection on competition between a C_3 and a C_4 plant sharing a pathogen.

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CHAPTER 1

Elevated carbon dioxide restores vegetative biomass but not plant fitness in ozone-damaged *Avena fatua* infected with BYDV

ABSTRACT

Plant diseases can modify many ecosystem processes from carbon capture to nutrient cycling. The impacts of disease on such ecosystem functions may be modified by atmospheric changes such as rising carbon dioxide and ozone levels, which have the potential to impact disease epidemics. However, the effects of these interacting gases on disease epidemics, especially epidemics of viral plant diseases, have received little attention. We used open-top chambers to factorially elevate CO_2 (700 ppm) and ozone (+70 ppb) to examine the effects of these gases on plant growth and the interactions of the gases with barley yellow dwarf virus (BYDV), an aphid transmitted plant virus. The virus caused significant biomass losses only when high ozone was present, with or without CO_2. In addition, ozone alone had a significant damaging effect on both vegetative and reproductive biomass. Though the treatment with CO_2 and ozone restored vegetative biomass to ambient levels, it did not allow ozonated plants to compensate for losses in reproductive biomass. Nor did CO_2 alone significantly increase biomass. These results suggest the potential for fitness declines in ozonated *Avena* species in the future and for a dominance shift in *Avena*-dominated western grasslands towards species whose recruitment may be less affected by ozone or more strongly responsive to CO_2.

INTRODUCTION

Infectious diseases are important components of every ecosystem and have a fundamental, yet often unacknowledged role in determining many properties important to population, community and ecosystem ecology. Diseases can strongly affect plant fecundity (e.g. Seabloom et al., 2009), ecosystem net primary productivity (Mitchell, 2003), community dominance and evenness (Power and Mitchell, 2004) and interactions with mutualists (Rúa et al., 2013). Plant diseases in particular can cause changes in regional biogeochemistry including cycling of nutrients such as calcium (Jenkins et al., 2007) and nitrogen (Tokuchi et al., 1994).

Beyond the ecological and conservation implications, there is increasing evidence that disease can alter communities in ways that alter human health risk. For example, sudden oak death causes forest changes that alter small mammal communities, and these changes affect the density of infected nymphs and thus Lyme disease risk in western US ecosystems (Swei et al., 2012). Similarly, sudden aspen death causes changes in mammal communities that increase prevalence of Sin Nombre Virus (Lehmer et al., 2012).

Because of the enormous importance of disease, much attention has been focused on understanding the immediate impact of diseases on ecosystem function. There is an increasing interest in understanding how disease dynamics will respond to changes in the current global atmosphere and climate. Current anthropogenic changes to the globe are multifaceted and include true climatic factors such as changes in temperature and precipitation regimes, as well as the drivers of such changes- emissions of greenhouse gases such as carbon dioxide (CO_2) and ozone (O_3) (Chakraborty et al., 2000, Chakraborty, 2005). The atmospheric drivers may be particularly significant for plant diseases, as both gases are taken in by the stomata and may have contrasting effects on plant health (Biswas et al., 2013.) Ozone is a powerful oxidant that can damage cell and photosynthetic membranes (Sandermann, 1996). To defend themselves against it, plants frequently close their stomata to minimize exposure, though damage to the stomata themselves may ultimately prevent this (Sandermann, 1996; Hayes et al., 2013). In contrast, CO_2 is thought to ameliorate some ozone damage by increasing the photosynthetic efficiency of C_3 plants during the periods of the day when stomata are open (Biswas et al., 2013).

Recently there has been substantial interest in understanding how the rising levels of CO_2 (Fleishmann et al., 2010; McElrone et al., 2010; Mitchell et al., 2003) and ozone (Olbrich et al., 2010) will impact diseases of plants in non-agricultural ecosystems. However, though these two gases have interactive effects on plant physiology and thus combinations of CO_2 and ozone may produce responses that are difficult to predict from single factor studies (Leuzinger et al., 2011), few studies have examined the combination of these effects in unmanaged ecosystems (e.g. Percy et al., 2002; Karnosky et al., 2002). Furthermore, of the studies in natural ecosystems

examining the gases singly, most have been on trees (e.g. McElrone et al., 2005; McElrone et al., 2010; Olbrich et al., 2010; Fleishmann et al., 2010). Few studies have been done with either CO_2 or ozone on plants in other biomes such as grasslands (but see Mitchell et al., 2003), leaving open the question of how herbaceous plants and plant diseases in these biomes respond to atmospheric changes. Moreover, most such studies concentrate on fungal pathogens of plants (Mitchell et al., 2003; Mc Elrone et al., 2005, Percy et al., 2002; Karnosky et al., 2002; Olbrich et al, 2010; Fleishmann et al., 2010; McElrone et al., 2010). While fungi are an extremely important pathogenic groups, other groups of pathogens such as viruses can have significant effects on the ecosystems they inhabit (Seabloom et al., 2009) and yet have thus far gone nearly unexamined in a climate change context. Because viruses are obligate biotrophs and most are vectored by insects (Power and Flecker, 2003), viruses may show fundamentally different responses to elevated levels of CO_2 and ozone. In particular, the reductions in stomatal conductance associated with elevated levels of both CO_2 and ozone are unlikely to present an entry barrier for viral pathogens, as they do for some fungal pathogens (Eastburn et al., 2011). Therefore, viruses may continue to be transmitted at high rates as CO_2 and ozone rise, and these gases may modify the viral disease epidemics by changing the severity of infection and the tolerance of plants for the infection. Because ozone damages photosynthetic machinery and therefore nutritionally deprives plants (Sanderman, 1996), viruses that cause phloem damage may have particularly severe fitness consequences for plants under high ozone. However, because CO_2 can increase photosynthetic efficiency even when ozone levels are high (Biswas et al., 2013), elevated CO_2 levels may allow plants to partially compensate for ozone damage. Moreover, as a reactive oxygen species, ozone has been shown to upregulate plant defenses (Sandermann, 1998, Zuccarini et al., 2009), including defenses active against viruses (Yalpani et al., 1994), which might make plants less susceptible to infection under ozone.

To examine the interacting effects of these gases on plant symptom severity and health, we used the PAV species of barley yellow dwarf virus (BYDV-PAV) as a model to examine the effects of elevated CO_2 and ozone on viral disease severity. BYDV-PAV belongs to the genus

Luteovirus, a group of viruses in the family Luteoviridae which infects a wide variety of grasses and has been found on every continent except Antarctica (D'Arcy, 1995; Lister and Ranieri, 1995). The family is of economic importance, causing yield losses in critical crops such as wheat, oats, barley and rice (Lister and Ranieri, 1995; McCurdy et al., 2002). In addition, it has come to increasing prominence for grassland conservationists in the western United States, as BYDV and its confamilial viruses the cereal yellow dwarf viruses have been implicated in the disappearance in the native bunchgrasses in western US grasslands (Malmstrom et al., 2005; Malmstrom et al., 2006; Borer et al., 2007). We used open top chambers to factorially elevate CO_2 and ozone to examine the effects of barley yellow dwarf virus (BYDV) in *Avena fatua* (wild oats). *Avena* fatua is easily infected with and supports high within-host viral levels of the PAV species of BYDV. Because *Avena* not only supports a high virus population but also causes pathogen spillover to poorer hosts, *Avena* is of enormous importance within the community (Power and Mitchell, 2004). In addition, it is one of the introduced grasses highlighted by Borer et al. (2007) and Malmstrom et al. (2006) as a key player in the epidemic among native bunchgrasses in western grassland ecosystems. Because of the importance of *A. fatua* in BYDV epidemics, this study examines the effects of rising carbon dioxide and ozone levels on the symptom severity of BYDV in *A. fatua* in the hope of understanding and forecasting BYDV epidemics under future atmospheric conditions.

METHODS

Planting

We planted monocultures of *A. fatua* on East Ithaca Farm (Cornell University) at the end of the first week in June 2009 in square plots 1.5 m on a side. The soil was sandy loam with rapid drainage. Plots were plowed before planting and fertilized with 20:10:10 NPK fertilizer. *Avena* was seeded at a target density of 600 seeds per square meter. Sub-plots with poor germination at 17 and 18 days after first emergence of plants (DAE) received additional seed to bring those sub-plots nearer to target density.

Gas Treatment

To control CO_2 and ozone, we used square open top chambers 1.5 m on a side and covered with clear vinyl plastic wrap. We arranged chambers in 6 blocks, each of which contained 4 chambers: ambient (A), elevated CO_2- target – 680 ppm (C), elevated ozone- target +70 ppbv (O), and elevated CO_2 and ozone (CO). Such target concentrations are typical of work under elevated gases (e.g. Olbrich et al., 2010, Chakraborty et al., 2000), and are in the middle of the range of IPCC projections (IPCC, 2001). Each chamber was split into 4 sub-plots- open, aphid free, mock inoculated (with release of virus free aphids), and inoculated (with release of virus-carrying aphids). The aphid free chamber was caged in a single net made of white aphid-prevention fabric (Optinet 50% light reduction aphid control fabric), while the mock-inoculated and inoculated chambers shared a single large net with a divider made of fine mesh netting.

Air was circulated through each chamber by an impeller fan which pushed air down a long aluminum duct and out 8 PVC pipes. Gases were introduced to the pipe leading to each chamber and then spread among the sub-plots with aerated pieces of PVC fitted between each of the sub-plots. CO_2 fumigation began at midday on 12 June 2009 (day first germinants emerged), and after that began at sunrise and ended at sunset each day. CO_2 measures were made using a manual NOVA IRGA as often as weather permitted during a very wet summer. Chamber values were generally consistent, though there were occasional drifts as low as 500 in some chambers and up to 700 ppm and a few days of delivery failure to some chambers. Ozone fumigation began 24 DAE and took place 4 hours every afternoon from approximately 12:30-4:30 PM until senescence was observed in sub-plots. Ozone concentrations naturally fluctuate more, but background levels were generally in the 20-35 ppb range, with levels of 80-150 ppb in the elevated chambers. The ozonated plants were released from ozone fumigation during the end of the first week of gas treatment because ozone had drifted very high in the chambers due to monitor failure, and the plants were badly burned. To ensure survival, ozone was turned off until recovery was visible.

Inoculation

Rhopalosiphum padi colonies were maintained on *Romulus* barley in growth chambers at 20 degrees C in 24 hour light. We began the inoculation procedure at 19 DAE as follows: 50 adult *Rhopalosipum padi* per infected sub-plot were transferred to dishes containing *Romulus* barley infected with an NY isolate of BYDV-PAV and were allowed to feed on the barley for 48 hours in order to acquire the virus. Mock-inoculation was performed with 50 adult *R. padi* on uninfected barley at the same time. In the morning at 22 DAE, 50 inoculated aphids were sprinkled around one sub-plot in each chamber. A second sub-plot received 50 mock inoculated aphids, while the final sub-plot remained aphid-free. While aphid densities were not measured in this experiment, a later field experiment confirmed that aphid mortality and growth rates were not significantly affected by gas treatment (Appendix A).

Physiology Measurements

To determine the effects of elevated CO_2 and ozone, maximal rate of photosynthesis (A_{max}) and conductance were measured at 400 ppm and 700 ppm using a Li-6400 Photosynthesis system (Licor, Inc, Lincoln, NE) on 1-3 plants from each sub-plot within a chamber for three blocks. Measurements at 400 ppm and 700 ppm were made at the same plant but the order in which the plants experienced the two concentrations was switched every plant. At each CO_2 concentration, plants were allowed to acclimate to the photosynthesis chamber until conductance readings stabilized, and then three A_{max} measurements were taken and averaged. High mortality did not permit reliable sampling in later blocks. Gas treatment order was randomized, but to reduce the chance of contamination, sampling proceeded from uninoculated to inoculated sub-plots each day.

Harvesting and Biomass Measurements

Gas treatment had an observable effect on senescence rate, so rather than choosing a single harvest date, we harvested each plot at the first sign of senescence, as determined by browning leaves or dehiscing seed heads. The first sub-plots to be harvested were in the ozone treatment, and harvested on 55 DAE, while the final sub-plots to be harvested received elevated

CO_2 and were harvested on 73 DAE. Prior to senescence, approximately 0.25 g of leaf tissue was clipped from 15 randomly chosen plants per plot. This tissue was frozen and then sampled for the presence of PAV using double antibody sandwich ELISA (Gray et al., 1991). Plants whose tissue had been clipped for ELISA were allowed to dry naturally for a minimum of 8 weeks. Seed heads were removed for weighing and seed count. Because plants dehisce at different times, some plants still had seed heads covered by leaves, while other plants had begun to dehisce. To correct for this problem, all glumes were counted and scored as full or empty. Total estimated reproduction was calculated as the average weight of full glumes * total number of glumes. Plants that had not opened their seed heads yet were manually opened and their glumes counted. However, plants that broke during harvesting were excluded for analysis. Average seed weight and glume numbers were log-transformed for normality.

Statistical Analysis

Effect of elevated CO_2, ozone, and virus on vegetative biomass of individual plants was assessed using a mixed model ANOVA in JMP 8.1 (SAS Institute) with block as a random effect, while CO_2, ozone, and aphids, and virus were fixed effects. We also modeled all two and 3-way interactions of CO_2, ozone, and virus. We used Tukey's test to compare the LS means of different treatments. Because many plants in the mock-inoculated sub-plot tested positive for virus, we separated plants into aphid exposed but negative (truly mock inoculated) and aphid exposed and positive (inoculated), regardless of the treatment to which the plant had originally belonged. All data points were log-transformed for normal fit. For reproductive biomass, no transformation would produce a normal fit in all treatments. Therefore, we analyzed aboveground vegetative and reproductive biomass in two ways. First, we modeled of the log-transformed data including block as a random effect, while CO_2, ozone, and aphids, and virus were fixed effects. We also included all two and 3-way interactions of CO_2, ozone, and virus, and performed Tukey's test for multiple comparisons on transformed data. In addition, we performed the non-parametric Steel Dwass test, a non-parametric equivalent of Tukey's test, on untransformed data to compare pairs of treatments.

Effect of elevated CO_2, ozone, and virus on photosynthetic rates was modeled using a mixed model ANOVA in JMP 8.1 (SAS Institute) with a random effect, while CO_2, ozone, and virus were fixed effects. All data points were Box-Cox transformed before analysis. Because one treatment at 400 pm CO_2 could not be transformed to normality with a Box-Cox transformation, we also performed a non-parametric Wilcoxon test and compared groups using the Steel Dwass test. The results from the non-parametric analysis confirmed those of the parametric analysis, so the parametric analysis is reported here.

RESULTS

Plant Performance: Aboveground Vegetative Biomass

Elevated CO_2 significantly enhanced end-season plant biomass ($F=19.74$, $p<0.001$), while ozone ($F=111.74$, $p<0.0001$), aphids ($F=7.10$, $p=0.008$) and virus ($F=5.36$, $p=0.02$) significantly reduced aboveground vegetative biomass (Figure 1.1). Reductions due to ozone were significantly steeper under low CO_2 ($F=16.75$, $p<0.001$).

Gas Treatment Effects: Among both infected and uninfected plants, aboveground vegetative biomass was significantly lower in ozone treated plants than in all other gas treatments (Tukey's test, $p=0.05$, figure 1.1). CO_2 alone did not significantly increase vegetative biomass of uninfected plants. However, the biomass of uninfected plants treated with CO_2 + ozone was significantly lower than plants in the ambient treatment but not the high CO_2 treatment (Figure 1.1). In contrast, among plants that tested positive for BYDV, CO_2 significantly increased vegetative biomass, but a combination of CO_2 and ozone reduced biomass to ambient levels (Tukey's test, $p=0.05$, figure 1.1).

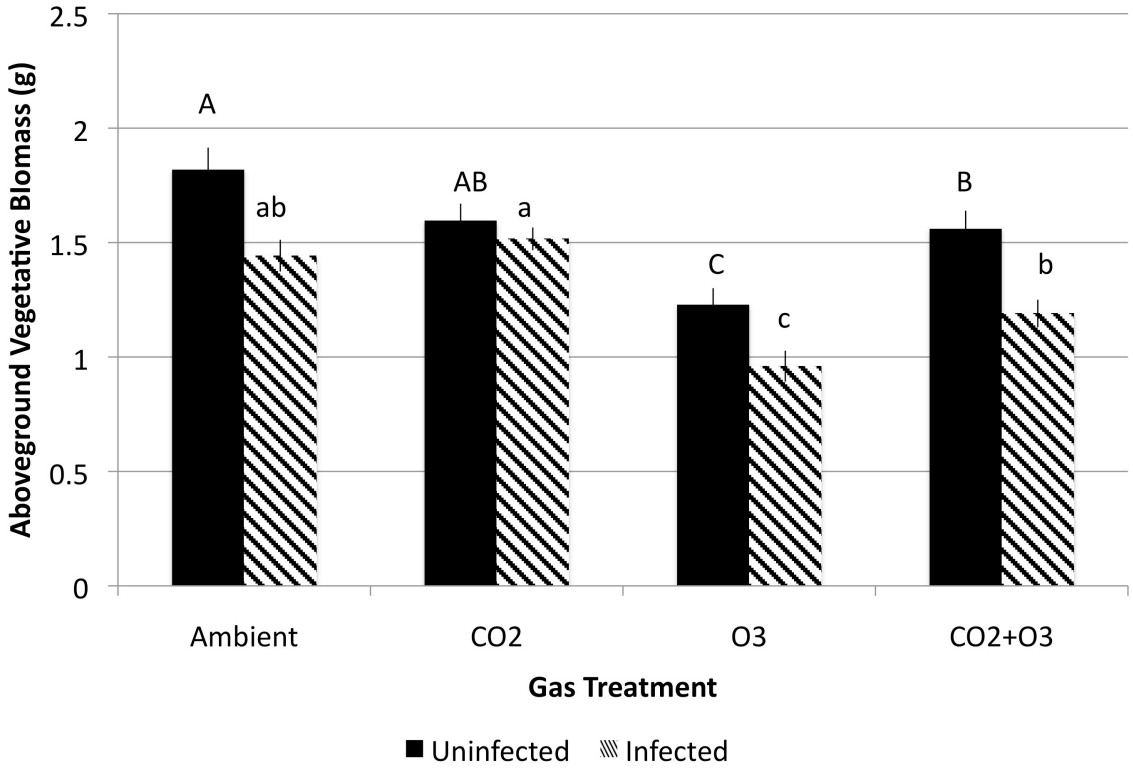

Figure 1.1: Mean stem mass of *Avena fatua* exposed to factorial combinations of elevated CO_2 and ozone. Solid bars indicate uninfected plants, while hatched bars indicate infected plants. Upper case letters indicate uninfected treatments significantly different at p=0.05, while lower case letters indicate infection treatments significantly different at p=0.05. Error bars represent the standard error of the mean.

Disease Effects: While plants in all gas treatments tended to show lower biomass when infected, only in ambient ($z=3.46$, $p=0.03$) and high ozone+CO_2 ($z=3.73$, $p=0.01$) conditions was that difference large enough to be significant (Figure 1.1). There were no significant differences between inoculated plants and mock inoculated plants or between mock inoculated and uninfected plants for any gas treatment.

Plant Performance: Reproductive Biomass

CO_2 did not affect total estimated reproductive biomass ($F=0.25$, $df=1$, $p=0.61$), while ozone ($F= 34.76$, $df=1$, $p<0.0001$) and virus ($F= 6.29$, $df=1$, $p=0.01$) both had significant negative effects on biomass of *Avena.* However, none of the two-way interactions were significant. There was a significant 3-way interaction between CO_2, ozone and virus, such that the gap between infected and uninfected plants was greatest under high CO_2 and ozone ($F=4.05$, $p=0.04$)

Gas effects:

In uninfected plants, ozone significantly reduced total estimated reproductive biomass when compared to ambient biomass ($z=-3.53$, $p=0.02$, Figure 1.2,), high CO_2 alone ($z=-4.05$, $p=0.003$) and CO_2 and ozone combined ($z=-3.36$, $p=0.04$). Neither CO_2, nor CO_2+ozone had a reproductive biomass significantly different from ambient.

Among mock-inoculated plants, both ozone alone ($z=-3.48$, $p=0.02$) and the combination of CO_2 and ozone significantly lowered reproductive biomass below ambient ($z=-5.002$, $p=0.0062$).

In infected plants, ozone significantly suppressed biomass compared to ambient ($z=-5.81$, $p<0.0001$) and high CO_2 ($z=-5.91$, $p<0.001$). The combination of CO_2 + ozone was not significantly different from ozone alone ($z=2.12$, $p=0.13$). However, plants receiving CO_2+ ozone had significantly lower reproductive biomass than those receiving CO_2 alone ($z=-4.25$, $p=0.01$) and those in the ambient gas treatment ($z=-4.21$, $p=0.001$; Figure 1.2).

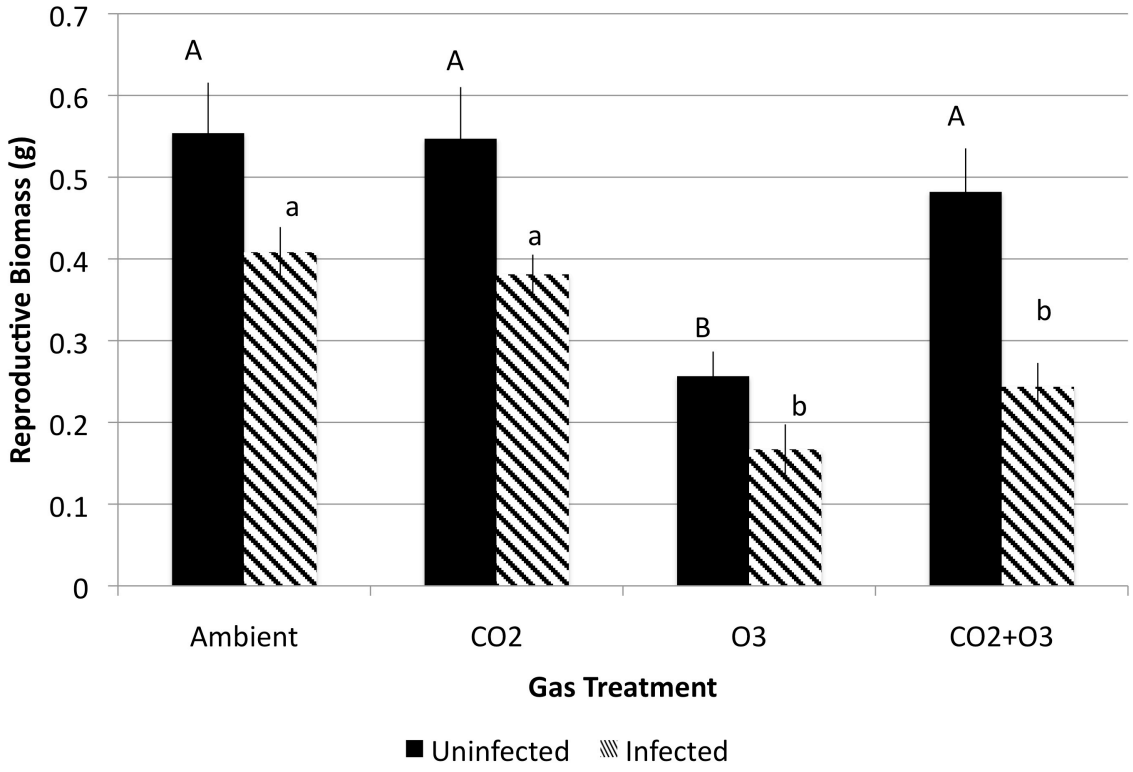

Figure 1.2: Mean estimated reproductive biomass of *Avena fatua* exposed to factorial combinations of elevated CO_2 and ozone. Solid bars indicate uninfected plants, while hatched bars indicate infected plants. Upper case letters indicate uninfected treatments significantly different at p=0.05, while lower case letters indicate infection treatments significantly different at p=0.05. Error bars represent the standard error of the mean.

Infection Effects:

When comparing infected and uninfected plants in the same gas treatment, the only plants that showed significant reductions in biomass due to virus were those that had were exposed to ozone and elevated CO_2 (Figure 1.2, z=4.41, p=0.0006). Plants exposed to ozone alone showed only marginal differences between infected and uninfected plants (F=3.35, p=0.09). No mock-inoculated plants differed significantly from the uninoculated or the inoculated plants in the same gas treatment.

Seed Number and Weight

Number of glumes was significantly increased by CO_2 ($F= 41.96$, $p= <.0001$), and significantly decreased by ozone ($F= 126.78$, $p= <.0001$) and aphids ($F=25.02$, $p<0.0001$), though not significantly by virus. Ozone was significantly less damaging under high CO_2 ($F=24.00$, $p<0.0001$). Infection was significantly less damaging under high CO_2 ($F=5.03$, $p<0.0001$) and significantly more damaging under high ozone ($F=-3.12$, $p=0.0019$), which was also reflected in the 3 way interaction term ($F=29.50$, $p<0.0001$; Figure 1.3A). These overall effects are driven by the strength of particular differences between treatments (Figure 1.3A). For example, there were significantly fewer glumes on plants experiencing high ozone at high CO_2 ($z=3.53$, $p=0.02$) and ambient CO_2 ($z=5.1$ $p<0.0001$). Among uninfected plants, there were no significant differences between gas treatments. In contrast, gas treatment affected infected plants more strongly. Ozone significantly reduced the number of glumes compared to ambient ($z-=-6.24$, $p<0.0001$) and high CO_2 ($z=-6.29$, $p<0.0001$), but CO_2 restored the numbers to ambient levels ($z=-2.49$, $p=0.35$).

Average seed weight was reduced by ozone ($F=133.15$, $p<0.001$) and by the virus ($F=7.99$, $p=0.005$) but was not affected significantly by CO_2 ($F=1.81$, $p=0.18$). Reductions in seed weight due to ozone were significantly steeper under ambient CO_2 ($F=21.79$, $p<0.001$). Among uninfected plants, infection did not significantly reduce average seed weight in any gas treatment (Figure 1.3B). Uninfected plants in all combinations of elevated CO_2 and ozone did show reduced seed weight compared to ambient (Figure 1.3B). Similar patterns were seen in the infected plants, where ozone depressed seed weight below ambient levels ($z=-5.43$, $p<0.0001$) and CO_2 did not restore seed weight ($z=4.42$, $p=0.0006$).

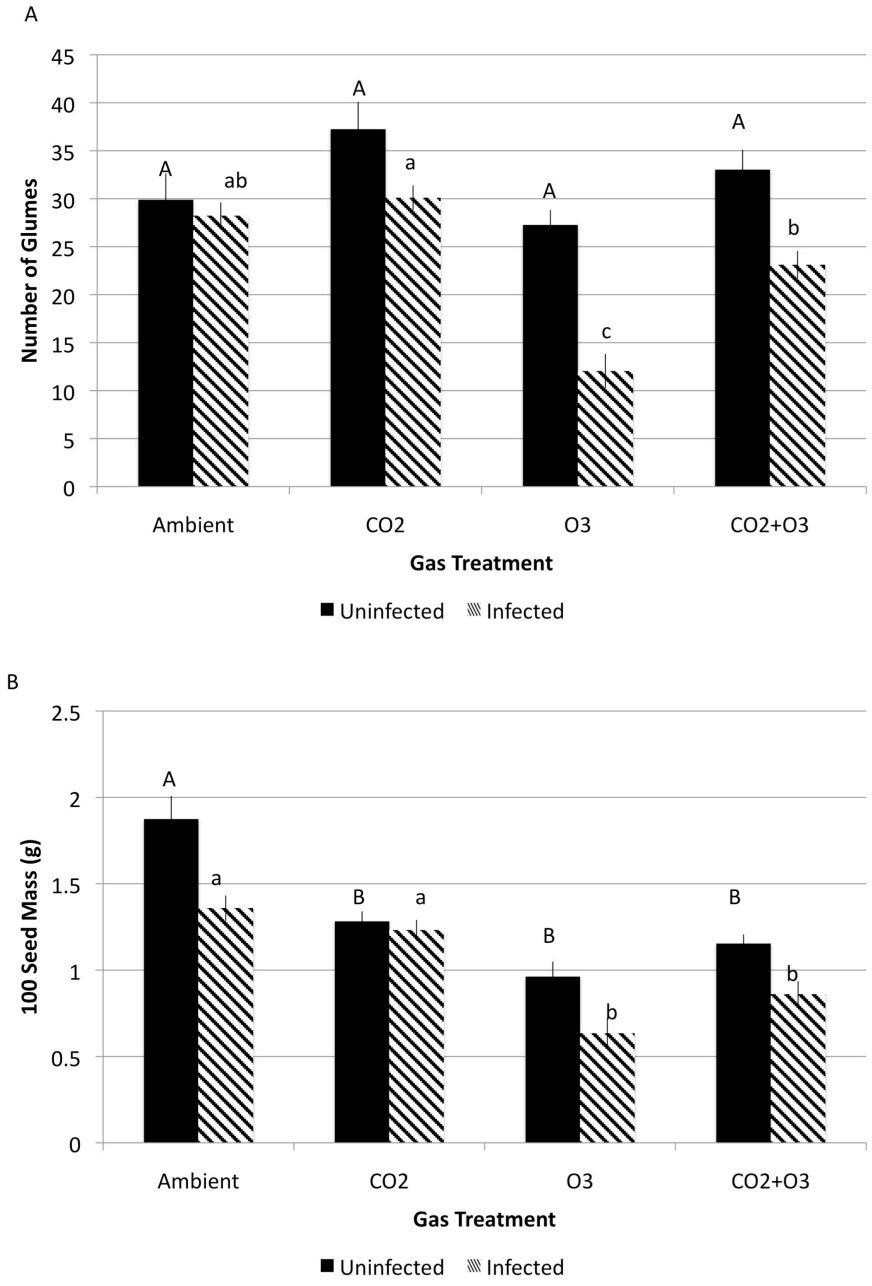

Figure 1.3: Average number of glumes (A) and average one hundred seed weight (B) of *Avena fatua* exposed to factorial combinations of elevated CO_2 and ozone. Solid bars indicate uninfected plants, while hatched bars indicate infected plants. Upper case letters indicate uninfected treatments significantly different at p=0.05, while lower case letters indicate infection treatments significantly different at p=0.05. Error bars represent the standard error of the mean.

Plant Performance: Physiology

When measuring photosynthesis (A_{max}) at 400 ppm CO_2, neither elevated ozone ($F=0.43$, $p=0.52$) nor elevated CO_2 had a significant effect on photosynthetic rate ($F=0.43$, $p=0.51$, figure 1.4A). Virus infection, however, did significantly decrease photosynthetic rate ($F=21.16$, $p<0.0001$). In addition, there was a marginally significant interaction between ozone and virus, where reductions in photosynthesis due to ozone were more precipitous in infected plants ($F=3.91$, $p=0.0504$).

When measuring A_{max} at 700 ppm CO_2, viral infection significantly reduced photosynthetic rates ($F=23.79$, $p<0.0001$). Neither CO_2 ($F=0.17$, $p=0.68$) nor ozone alone ($F=0.05$, $p=0.82$) had a significant effect on the rate of photosynthesis at 700 ppm. In addition, the ozone-virus interaction was not significant at 700 ppm ($F=2.79$, $p=0.1$, Figure 1.4B).

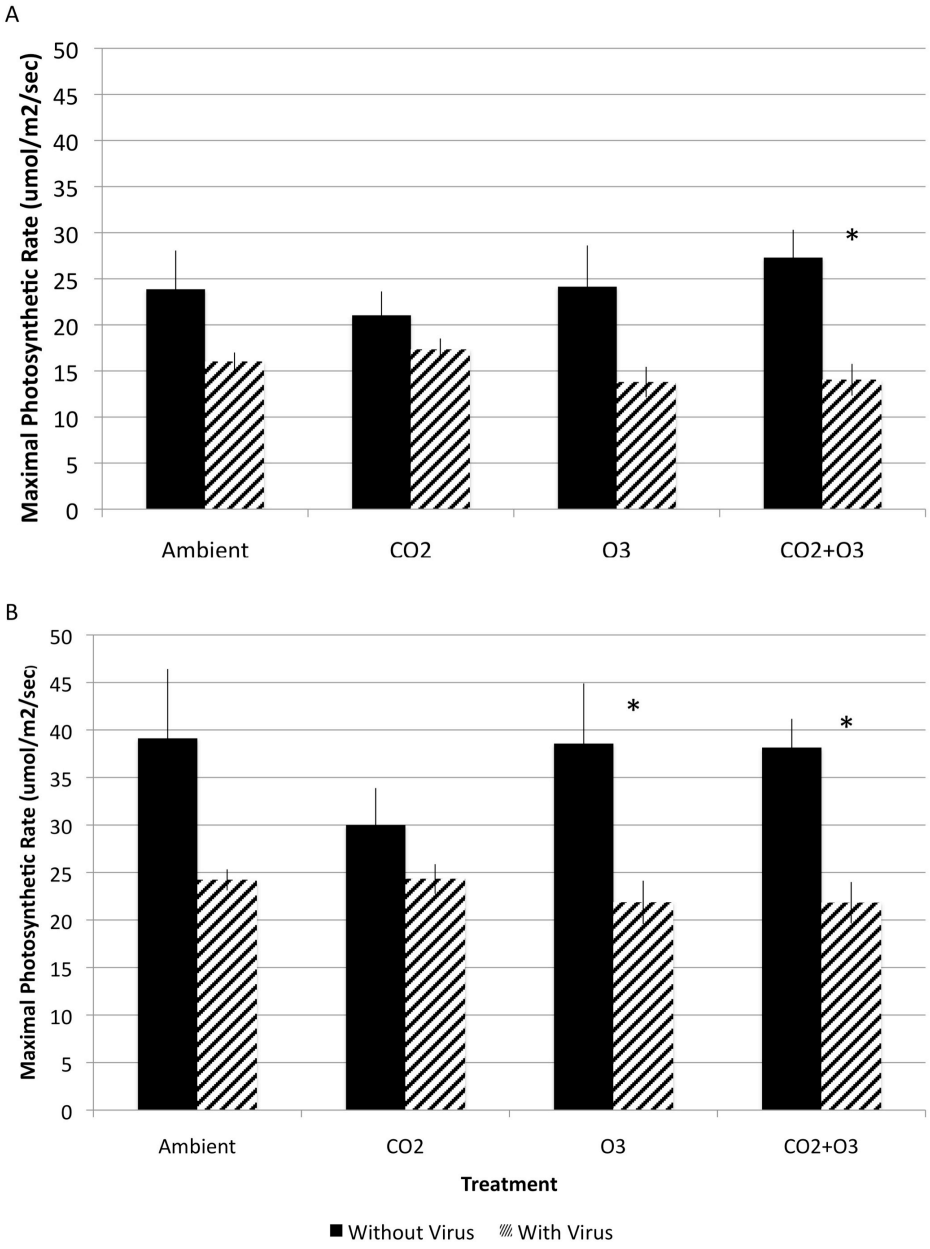

Figure 1.4: Average maximal rate of photosynthesis (A_{max}) of *Avena fatua* exposed to factorial combinations of elevated CO_2 and ozone at 400 ppm CO_2 (A) and 700 ppm CO_2 (B). Solid bars indicate uninfected plants, while hatched bars indicate infected plants. Error bars represent the standard error of the mean. Stars indicate pairs of treatments that are significantly different.

DISCUSSION

This study demonstrates the severely detrimental effect of elevated ozone, alone and in combination with infection, on plant growth and reproduction. While CO_2 is predicted to help plants compensate for losses due to ozone, this compensation only occurs for some measures of growth and fitness, while other measures remain lower under a combination of high CO_2 and ozone.

Effects of CO_2 and Ozone on Plant Biomass

Plants exposed to ozone had significantly lower biomass (Figure 1.1, Figure 1.2), as expected from prior studies (Sandermann, 1996; von Tiedemann and Firsching, 2000). In vegetative tissue and reproductive tissue, uninfected plants were able to completely compensate for losses due to ozone with increased CO_2, restoring both vegetative and reproductive biomass to ambient levels. In contrast, in infected plants, adding elevated CO_2 to the ozone allowed complete compensation, restoring aboveground vegetative biomass to ambient levels. However, adding CO_2 to the ozone did not allow infected plants to compensate ozone-induced losses in reproductive biomass. While CO_2 allowed plants some recovery from ozone-induced biomass losses, high CO_2 alone did not increase biomass. This is particularly surprising as Malmstrom and Field (1997) reported an increase in biomass in *Avena sativa*, a cultivated relative of this plant, in response to elevated CO_2. However, non-cultivated *Avena* species have been shown to be more refractory to the direct effects of global change than other species. Our results are consistent with those of Zavaleta et al. (2003) who reported that in a complex, multi-year study of CO_2, warming, precipitation and N addition, not only does *Avena barbata* (a close wild relative of *A fatua*) show muted responses to global change compared to other California grassland species, but those responses vary widely from year to year. Additionally, work by Huxman et al. (2001) suggests that seedlings grown from plants in high CO_2 may have reduced growth and photosynthetic rates, further supporting the idea that high CO_2 may not promote growth in annual grasses.

Interestingly, for vegetative tissue, virus infection had particularly strong effects on those

plants in the ambient treatment and those receiving high CO_2+ ozone (Figure 1.1). In contrast, in reproductive tissue, ozone+CO_2 had the strongest effect on differences in infected and uninfected plants (Figure 1.2). Because BYDV is known from many studies at ambient CO_2 and ozone levels to reduce plant size (D'Arcy, 1995; Malmstrom and Field, 1997), this result may be due to limited sample size or low virulence of our strain. However, the fact that CO_2 and ozone had effects strong enough to be detectable even in at low sample size suggests their tremendous importance in regulating the size of *Avena*. Because these CO_2 and ozone levels are in the middle of IPCC (2001) projected ranges for CO_2 and consistent with EPA (2012) data for a moderately polluted city, these results may offer important insight in predicting *Avena* growth under future atmospheric conditions. In mixed communities, *Avena* is often a fast-germinating and fast-growing competitor and may overtake other species. Ozone diminishes this swift growth under current CO_2 conditions, suggesting that competitive dominance of *Avena* might be reduced in high ozone areas. However, because adding CO_2 to ozone restores vegetative growth rates in *Avena,* ozone may have less effect on *Avena* dominance as background CO_2 levels rise.

The difference in response between vegetative and reproductive biomass is also likely to impact community structure. In vegetative tissue, CO_2 produced a complete compensation response, where adding CO_2 restored biomass of ozonated plants to ambient levels. However, in reproductive tissue, ozone was especially damaging and addition of CO_2 was not able to completely restore reproduction in plants. If a similar pattern were observed in cultivated grasses such as oats, wheat and barley, this could suggest that ozone may damage crop yields and rising CO_2 levels may not be able to restore yields.

The two components of reproduction measured here, seed number and seed weight, were both significantly reduced by ozone, and these reductions for both components continued at high CO_2 levels, suggesting that the CO_2 is not capable of compensating for ozone damage for either component of fitness. Seed weight can have significant effects on germination success, but the effects vary widely in magnitude and direction even within the grass family, with some studies reporting a positive correlation between seed size and germination (e.g. Counts and Lee, 1991),

others reporting a negative correlation (Bu et al., 2007). Further work is needed to explore the effects of seed mass on seed germination in *Avena*, however, the small variations in seed mass suggest that differences in *Avena* recruitment are more likely to be driven by differences in seed number.

Seed number is thought to be crucial for recruitment of *Avena*, as *A. fatua* has both high seed set and high mortality in the field, leading to speculation that recruitment and dominance of *Avena* in western grasslands is driven by high seed set (Orrock and Hoisington-López, 2009). If this is true, ozone may initially limit recruitment of *Avena*. However, as CO_2 levels continue to rise, the ozone barrier to *Avena* recruitment among both infected and uninfected plants may decrease, causing increased *Avena* recruitment and restored dominance of *Avena* in western grassland habitats. Because *Avena* is a potent host of BYDV and capable of causing spillover to less susceptible species (Power and Mitchell, 2004), increased dominance of *Avena* may not only have direct negative effects on native grass success due to competition but may also exacerbate the known negative effects of the virus on native grasses in these communities (Malmstrom et al., 2006; Borer et al., 2007).

Effects of Elevated CO_2, Ozone, and Infection on Physiology

The strong negative effect of BYDV on photosynthetic rates at ambient CO_2 levels has been known for some time, and often results from negative feedback on photosynthesis as carbohydrates accumulate in leaves after BYDV damages phloem (Jensen and D'Arcy, 1995). Consistent with this, in measuring at 400 and at 700 ppm, infection exposure, rather than any gas treatment, had the strongest effect on leaf level photosynthesis.

Interestingly, ozone alone did not significantly reduce photosynthetic rates, as it did in previous studies (rev in Sandermann 1996; Hartikainen et al., 2012). Nor was there a compensatory response due to reduced stomatal conductance under high CO_2 (Sandermann, 1996; Malmstrom and Field, 1997; Eastburn et al., 2011). However, because all measurements in the study were taken with ozone fumigation turned off, the effect of ozone represents the lingering effect of ozone on the physiology of the plant and is more likely to be the result of

damage to photosynthetic machinery than instantaneous stomatal conductance.

Ozone virus-interactions are rare in the literature, mainly because the effect of ozone has been documented for few viral species. However Bilger and colleagues (1998) report that in soybean mosaic virus (SMV), ozone eliminates the negative effect of SMV on photosynthetic rate. This is in contrast to our study, in which ozone and virus interacted to produce steeper declines in photosynthetic rates. This suggests, not surprisingly, that ozone-virus interactions vary substantially among host and virus species. The soybean plants in Bilger's study appear to be more tolerant of ozone than the *Avena* in this study, and, in fact, ozone induced some level of virus resistance in the soy plants (Bilger et al., 1998).

Interestingly, unlike Malmstrom and Field (1997), who found that CO_2 helped plants compensate for the damaging effects of BYDV, this study detected no BYDV-CO_2 interaction at either high or low instantaneous CO_2 levels. It is possible that *Avena sativa*, the cultivated species used by Malmstrom and Field, was more responsive to high CO_2 due to breeding for fast growth rates. However, given that *A. fatua* is a fast-growing species in the wild, the difference between these studies merits further consideration. This study detected no three-way interaction between CO_2, ozone, and virus, and would suggest that at present CO_2 levels ozone and virus have strong effects on leaf-level physiology. However, as CO_2 levels rise, the strong effects of ozone, at least, may be less significant, and the effects of the virus will be most important at predicting photosynthetic rates.

Conclusions

The grass family contains many of the world's most important food crops (i.e. wheat, oats, rice, and barley), and *Avena fatua* is closely related to cultivated oats. While crop plants do have different levels of genetic diversity and a different breeding history, our results do suggest that ozone has the potential to be important in cereal yield projections. The world population continues to increase, raising concerns about food supply as the climate continues to change. These results suggest that models of global food production will need to account for ozone as well, as CO_2 and warming, and may need to pay particular attention to the *interactions* of these

effects on yield, as such effects may not be additive, as Leuzinger et al. (2011) have recently emphasized.

In addition, these results have important implications for recruitment of annual grasses such as *Avena* in unmanaged grasslands. Because seed production is strongly affected, annual grasses may be hit especially hard by ozone and may continue to feel the pressure even as CO_2 levels rise. This may result in large scale shifts in dominance, as species whose recruitment is less affected by ozone or species that are longer-lived may come to dominate these grasslands. Moreover, *Avena*'s response to climate change, and its place in the community, may not be driven by direct effects on its physiology but by its response to a disease.

CO_2, ozone, and BYDV have important interactive effects on plant fitness and physiology. Understanding these effects may help predict BYDV epidemics and assist in the conservation of native grasses in communities of which *Avena* is a part. However, to understand BYDV epidemiology further work on aphid vector biology and behavior is needed to more clearly forecast the possibility of BYDV epidemics. This study highlights the importance of a disease in driving a species' response to atmospheric global change and suggests the importance of the disease interactions in predicting individual, and even community responses to global atmospheric change.

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CHAPTER 2

Ozone reduces within-host fitness but not transmission rate of an insect vectored plant virus

ABSTRACT

Rising levels of carbon dioxide and ozone have the potential to influence the spread of disease, but their effects on the fitness and transmission rates of disease-causing organisms has received little attention. In this study, we use open-top chambers to examine the effects of factorial combinations of elevated carbon dioxide and ozone on the within-host viral concentration and transmission success of barley yellow dwarf virus (BYDV), an insect transmitted plant virus, in the highly susceptible host species *Avena fatua*. Within-host viral concentration was significantly reduced by elevated ozone, but elevated levels of CO_2 in combination with ozone were able to restore viral fitness to ambient levels. Moreover, despite ozone-induced reductions in within-host viral concentration, transmission remained high in all gas treatments. This suggests that under current CO_2 levels, infected plants may experience a drop in within-host viral concentration in high ozone areas, but as CO_2 levels continue to rise, any drop in viral within-host viral fitness will be restored.

INTRODUCTION

Rising ozone and carbon dioxide (CO_2) levels are predicted to strongly affect disease prevalence and physiology of both host and pathogen (Chakraborty et al., 2000; Chakraborty, 2005), yet discerning patterns in the disease under these conditions has proven challenging. This difficulty is due at least in part to the diversity of disease responses to those gases, corresponding in many ways to the taxonomic diversity of disease-causing organisms, and to the interactive physiological effects of the gases themselves (Eastburn et al., 2011). Fungal diseases of plants have received the majority of attention in the literature on plant pathogen responses to atmospheric changes (e.g. Chakraborty 2005; Mitchell et al., 2003; Percy et al., 2002; Olbrich et al., 2010). While some early studies suggest ozone induces increased susceptibility to fungal disease (Woodbury, 1994; von Tiedemann and Firsching, 1991; Karnosky et al., 2002, Percy et

al., 2002), other studies highlight the role of ozone as an elicitor of plant defenses, and its disease reducing role (Yalpani et al., 1994; Bilger et al., 2008; Olbrich et al., 2010). Similarly, while elevated CO_2 can reduce some fungal infections of plants (McElrone et al., 2005), other fungal diseases are exacerbated by elevated CO_2 (McElrone et al., 2010). CO_2 can also interact with other conditions to modulate disease changes. For example, Fleishmann et al. (2010) suggest that fungal infections of beech may be most severe when CO_2 is high and N is limiting. Such diversity in disease responses highlights the complexity of the interactions between multiple global changes on host and pathogen physiology. Rising CO_2 levels and ozone levels both reduce stomatal conductance (Eastburn et al., 2011), and ozone has the potential to damage RUBISCO as well (Sandermann, 1996) lowering the plant's ability to capture carbon. However, rising levels of CO_2 make photosynthesis more efficient. They may also induce the formation of C based defenses and increase C:N ratios of plants (Eastburn et al., 2011), on their own or in concert with ozone (Karonen et al., 2006).

Much of the current work examining the effects of elevated CO_2 and ozone on plant diseases has studied fungal diseases of plants. However, plants are also attacked by viruses, and viral diseases have important effects on community structure (Borer et al., 2007, Malmstrom et al., 2006). Viral diseases might be predicted to show fundamentally different responses to elevated gases than fungal diseases because fungal and viral pathogens enter the plant differently. Unlike fungal plant diseases, many of which enter through the stomata (Brown, 1997), most plant viruses are vectored by a piercing-sucking insect such as an aphid or whitefly (Power and Flecker, 2003). Spread of viral plant diseases may therefore not be constrained by the effects of CO_2 and ozone on stomatal density and size (Prichard et al., 1999; Rashidi et al., 2012), and might be driven by the effects of the gases on efficiency of vector transmission, the availability of vectors, or the ability of the pathogen to replicate within the host. Disease severity under rising levels of CO_2 and ozone will therefore be determined by an interaction between the fitness of both the host and pathogen, and by vector fitness in the case of pathogens that are vectored. Of these three, pathogen fitness is among the most difficult to measure as pathogen

biomass may be very difficult to quantify (Mitchell, 2003). Moreover, the fitness of the pathogen will depend on two factors that are themselves interdependent: its ability to replicate within a single host and the ability to move between hosts (Frank and Schmid-Hempel, 2008). Replicating within a host depletes host resources, often shortening the host's life, while raising the probability of moving to a new host (Frank and Schmid-Hempel, 2008). This tradeoff may be especially acute for viruses, as their metabolism is intimately entwined with the metabolism of the host. They are unable to survive at all after the death of the host, and even slowing the growth rate of the host reduces the pool of resources available to the virus from that particular host. This may make the need for high transmission especially acute for virulent viruses. Understanding the effects of gases such as CO_2 and ozone on viral fitness may be crucial to understanding their effects on disease spread, but to date, little work has been done to try to estimate within-host viral fitness, and even the work on between-host viral fitness (transmission) is limited in non-agricultural ecosystems.

In this experiment, we use the barley yellow dwarf virus (BYDV) in the highly susceptible grass host *Avena fatua* as a model to examine the effects of atmospheric gas changes on viral fitness. We examine the effects of ozone, alone and in combination with elevated CO_2 levels, on the transmission success and within-host viral concentration of barley yellow dwarf virus (BYDV) in *A. fatua.*

METHODS

Ozone and BYDV Experiment

System

Avena fatua is an annual grass in the tribe *Avenae*. Though closely related to the cultivated oats (*Avena sativa*), it is itself not cultivated, and is common in grasslands of the western United States (Borer et al., 2007) as well as invading cultivated ecosystems as a weed. It is readily and heavily infected with BYDV, an aphid-transmitted virus inhabiting the phloem tissue of a wide variety of grasses (Power and Mitchell, 2004; Teakle, 1997, Malmstrom and Field, 1997, Miller and Rasochová, 1997). BYDV is actually a complex of at least 6 related

virus species. Though their classification is currently undergoing revision, the group contains at least 2 virus genera (Miller and Rasochová, 1997). Obligately vectored by aphids, each species of BYDV virus can be transmitted by one or more aphid species, and some species of aphids may carry more than one virus type (Miller and Rasochová, 1997). Though rarely fatal (Teakle, 1997), BYDV can cause leaf discoloration (Teakle, 1997; Miller and Rasochová, 1997), photosynthetic disruption (D'Arcy, 1995), and substantial biomass reduction, particularly in root tissue (Malmstrom and Field, 1997). Because it can also reduce reproductive biomass (D'Arcy, 1995), it causes substantial yield loss in crop species (McCurdy and Jones, 2002) and can lead to economic losses if it infects a cultivated species like oats (*Avena sativa*), wheat (*Oryza sativa*) or maize (*Zea mays*), all of which are susceptible to BYDV (Teakle, 1997; Miller and Rasochová, 1997).

Because so many important food crops are susceptible to BYDV and pathogens could easily be exchanged between wild and cultivated species, *Avena*-dominated grassland ecosystems are of particular interest for questions about global climate change and plant disease. *Avena* species are especially good reservoirs for BYDV since they accumulate large virus concentrations, which readily spill over to other susceptible grass species (Power and Mitchell, 2004). In fact, the addition of *Avena fatua* to a plant community can result in epidemics in species that would not normally become infected (Power and Mitchell, 2004).

Experimental Design and Planting

We examined the effects of elevated ozone on two important components of BYDV fitness in *Avena* plants: the success within the plant (within-host viral concentration) and success moving between plants (transmission success). We used a 2x2 factorial design, in which BYDV inoculation is crossed with presence of ozone. The experiment was run twice, once in an open-top chamber in a greenhouse, and once in a fully-enclosed growth chamber. *Avena fatua* seeds purchased from Azlin Seed Co were planted 2 per pot in a 50:50 mix of Metromix and vermiculite in 4-in diameter pots in a greenhouse After germination, plants were thinned to one per pot and were fertilized with water containing (NPK 21:5:20 150 ppm N). All plants were

kept together in a greenhouse except during the hours of gas treatment, when they were in the fumigation chambers themselves.

Gas Treatment

Plants were fumigated in the greenhouse in a 1 m^2 clear plastic open-top chamber for elevating O_3. A pump with a rotometer to control flow circulated air through the chamber. An ozone generator and ozone monitor were used to add O_3 to the chamber at a target concentration of 100-120 ppb for four hours five days per week, a level of ozone found in urban areas during summer days (EPA, 2012). Plants in the ambient treatment remained in a separate greenhouse, as it was impossible to keep from fumigating the entire fumigation greenhouse with ozone. Fumigation times were chosen to minimize the amount of variation in light levels between greenhouses.

BYDV Inoculation

Rhopalosiphum padi aphid colonies were maintained on barley in growth chambers at 20 degrees C in 24 hour light. After 10 days of growth, plants in the inoculation treatments within each gas experiment were inoculated with a NY isolate of the PAV species of BYDV. *R. padi* were fed on infected leaf tissue for 48 hours. Ten aphids were placed on each plant in the infection treatment, and aphids were allowed to feed for 4 days. To avoid aphids moving to the uninoculated control plants, all plants were caged with a fine mesh supported with plastic tubing. Cages remained in place on all plants (including uninoculated) only until the aphids were removed. After 5 days of feeding, aphids were removed from the plants with horticultural oil. Plants in the uninoculated treatment were handled but did not receive aphids. This design did not separate the effects of viral replication from effects of aphid feeding. While direct effects of aphid feeding can be important (Malmstrom and Field, 1997), virus effects are never separate from the effects of the aphid in natural systems, because the virus cannot be transmitted without aphid vectors (Miller and Rasochová, 1997).

Virus Detection

Twelve days after the inoculation period began, approximately 0.25 g of leaf tissue from all plants was clipped and frozen for virus testing by ELISA (an antibody method for detecting the presence of virus in samples, see Miller and Rasochová, 1997, Teakle, 1997). Samples were defrosted and then ground in Bioreba extraction bags (Bioreba Inc,) in 1x PBS (phosphate buffered saline) at a ratio of 1mL buffer per 0.1g fresh weight of tissue. Ground samples were analyzed using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using commercially prepared antibodies (Agdia, Inc) at an antibody dilution of 1:550. In each 96-well plate, we filled the edges with PBS to minimize edge effects. Each plate included 4 wells of healthy *Avena* tissue and 4 wells containing tissue from positive control plants known to be infected with BYDV. Controls were ground at the same ratio of buffer as the samples. Samples were scored as infected or uninfected.

Estimating Viral Fitness

In the second run of the experiment, we extended the study, using a similar design to test for within-host viral fitness. Fourteen days after inoculation, approximately 0.25 g of leaf tissue was harvested per plant and analyzed for infection using double antibody sandwich ELISA (BioReba, Inc). Each plate was allowed to develop until visible yellow coloring showed in positive controls. To estimate average within-host viral concentration we divided each sample's OD value by the average of all 4 positive OD values on the plate to give a standardized OD value.

Ozone, CO_2, and BYDV Study

Experimental Design

We used an open top chamber setup consisting of six blocks (24 chambers) at East Ithaca Farm (Cornell University, Ithaca, NY) to factorially elevate CO_2 (700 ppm) and ozone (+70 bbpv) levels. Each 1.5 x 1.5m open top chamber was divided into 4 sub-plots: one received infected (viruliferous) aphids, one uninfected aphids, and a third no aphids. All three of these sub-plots were shielded from aphid movement from with Optinet fabric cages, while the fourth

was left open to the air. *Avena* was planted in monocultures at a density of 600/m^2.

Gas Treatments

An impeller fan was used to circulate air and gases through the open-top chambers. Fumigations of CO_2 (target concentration 680-700 ppm) began at germination on 12 June 2009 and continued until the plants showed signs of senescence. Ambient concentrations measured 390 to 400 ppm with a manual NOVA IRGA. The 700 ppm concentration falls mid-range in IPCC projections for CO_2 levels in 2100 (IPCC, 2001). Ozone fumigation added 70 ppb to the daily ozone peak, for concentrations near 100-120 ppb), simulating a moderately polluted city (EPA, 2012). Ambient ozone levels fluctuated from day to day but were generally in the 20-30 ppb range. Fumigations with ozone were conducted between 12:30 and 4:30 pm and began 24 days after first germinants emerged (DAE). Further details of field setup and fumigation are provided in chapter 1.

Inoculation

At 19 DAE, aphids were placed on tissue infected with a NY isolate of BYDV and allowed to feed for 60 hours. At 24 DAE, 50 aphids per sub-plot were released into aphid cages in the infected aphid and uninfected aphid sub-plots. Aphids were gently sprinkled into the sub-plots to distribute them as evenly as possible.

Virus Sampling

Because senescence varied by gas treatment, harvesting was performed at the first signs of senescence in each sub-plot. Ozone treated plots were the first to senesce and were harvested beginning on 55 DAE, while the final plants to be harvested received elevated CO_2 and were harvested on at 73 DAE. At the onset of senescence of each sub-plot, we sampled 15 plants from each quadrant in each chamber for virus. Sampling was random throughout the sub-plot, but effort was made to sample the sides and the center at equal frequency, in case of gradients in gas diffusion. We clipped a piece of green leaf tissue of approximately 0.25 g from each plant. However, in cases where 0.25g was difficult to obtain, we used a whole leaf, and included a piece of stem if needed. This was particularly likely to happen in the ozone-treated plots, as the

ozone fumigated plants were small and many had visibly burned leaves. Samples were weighed and then frozen for later analysis. Contamination in the aphid-free sub-plots was low. Mean proportions (+/-standard error) were 0.08+/0.03 in ambient, 0.03+/-0.02 in high CO_2, 0.12+/-0.04 in high ozone, and 0.04+/- 0.02 in the high CO_2 and ozone treatment.

Virus Detection

Virus detection procedures were done with quantitative ELISA as in the ozone and BYDV study. For each block, samples were randomly distributed onto the plates. Two replicate plates were run in which the samples were distributed in different order on the plates. To estimate infection, all optical denstity (OD) values were graphed without labels and the sharp upward inflection point on the graph was used as the defining point for positive samples. ELISA values were standardized by dividing by the average of the four positive controls on the plate. To estimate the within-host viral concentration, we averaged the standardized ELISA value from both replicate plates.

Statistical Analysis

To compare the average standardized ELISA values (our measure of within-host viral fitness) in the ozone-BYDV study, we used one-way ANOVA (JMP Pro 9.1, SAS Institute). For the within-host viral fitness in the CO_2, ozone and BYDV field study, we used a linear regression with block as a random effect and CO_2, ozone, and the interaction of CO_2 and ozone as fixed effects. Data were log-transformed before analysis in JMP (JMP Pro 9.1, SAS Institute). Proportion data used a similar model using arcsine square-root transformed data.

To test for correlations between host fitness and within-host pathogen fitness, we used the Spearman rank correlation test to test the sign and strength of the host aboveground biomass and the within-host viral concentration of plants from the infected treatment using R (R core development team).

RESULTS

<u>Ozone and BYDV Study</u>

Elevated ozone did not change the transmission success rate; transmission success was

100% in both elevated and ambient ozone. While the percent of plants infected did not change with gas treatment, elevated ozone significantly depressed average index of viral concentration by about 20% (Figure 2.1, F= 4.3937, df=1, p= 0.0484).

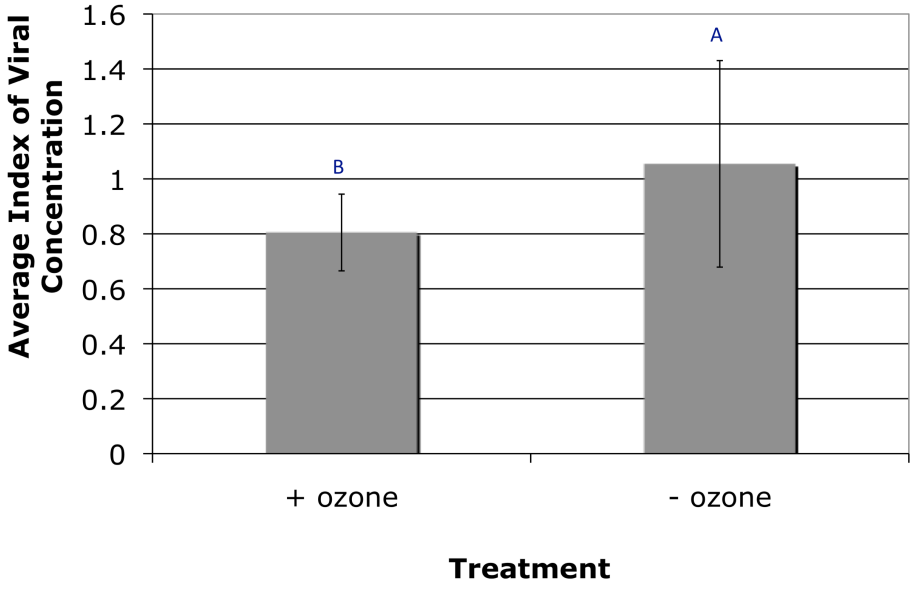

Figure 2.1: Within-host viral concentration of plants in greenhouse study. Different letters indicate differences significant at p=0.05. Ozone significantly reduces within-host viral concentration.

Ozone, CO_2, and BYDV Experiment

Viral Performance

As in the ozone and BYDV experiment, viral performance was similarly evaluated in two ways- as transmission success in the inoculated sub-plots and as virus concentration detectable in

plant tissue. Under ambient CO_2, within-host viral fitness of plants exposed to ozone was significantly lower than all other treatments, but adding CO_2 to the ozone restored within-host viral fitness to ambient levels ($F=10.9898$, $df=1$, $p=0.0010$; Figure 2.2) .

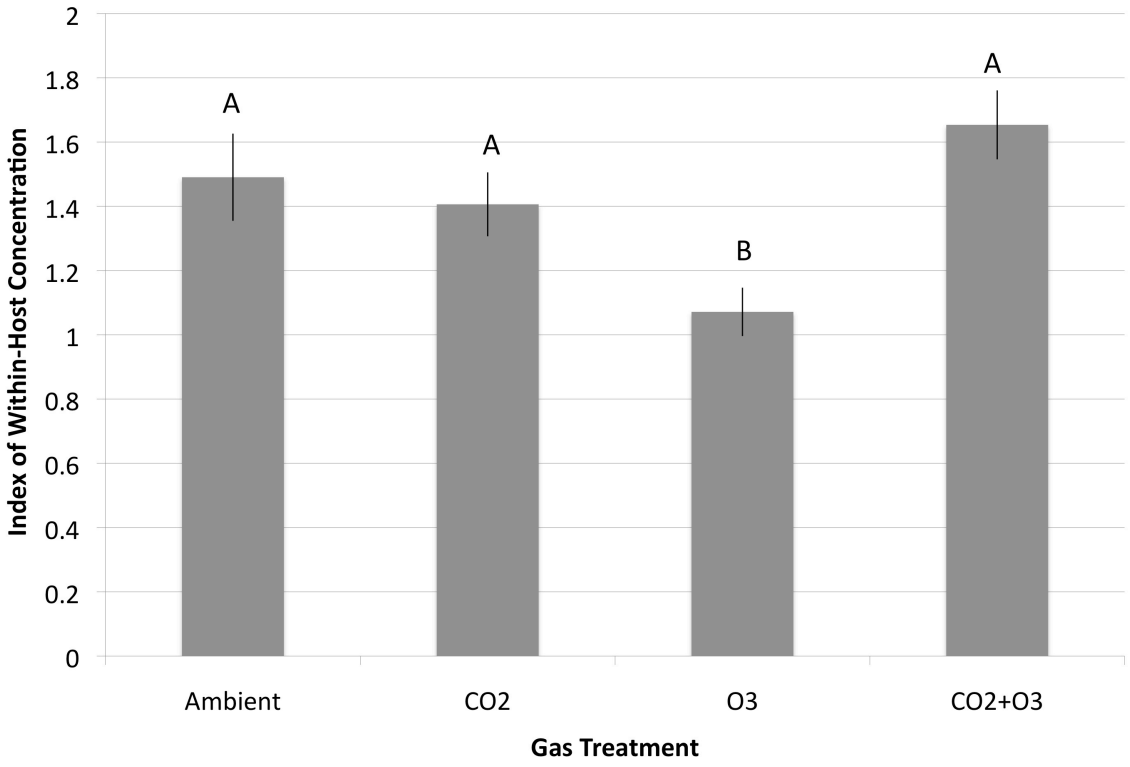

Figure 2.2: Average index of within-host viral concentration in plants exposed to factorial combinations of CO_2 and ozone. Letters indicate treatments significantly different at p=0.05 (Tukey's Test). Plants in the ozone treatment had significantly lower within-host viral fitness but adding CO_2 to the ozone restores viral fitness to ambient levels.

Interestingly, despite lower detectable viral concentrations in ozone-fumigated host plants, gas treatment did not significantly affect the proportion of plants infected in the inoculation treatments (Figure 2.3). There was, however, a non-significant trend towards lower inoculation levels in the ozone plots, and this trend was reversed with the addition of CO_2 and

ozone combined. CO_2 had a marginally significant effect in increasing infection (F=3.5082, df=1, p=0.0807), but neither the main effect of ozone (F=0.8102, df=1, p=0.3823) nor the interaction of CO_2 and ozone (F=0.2871, df=1, p=0.5999) were significant.

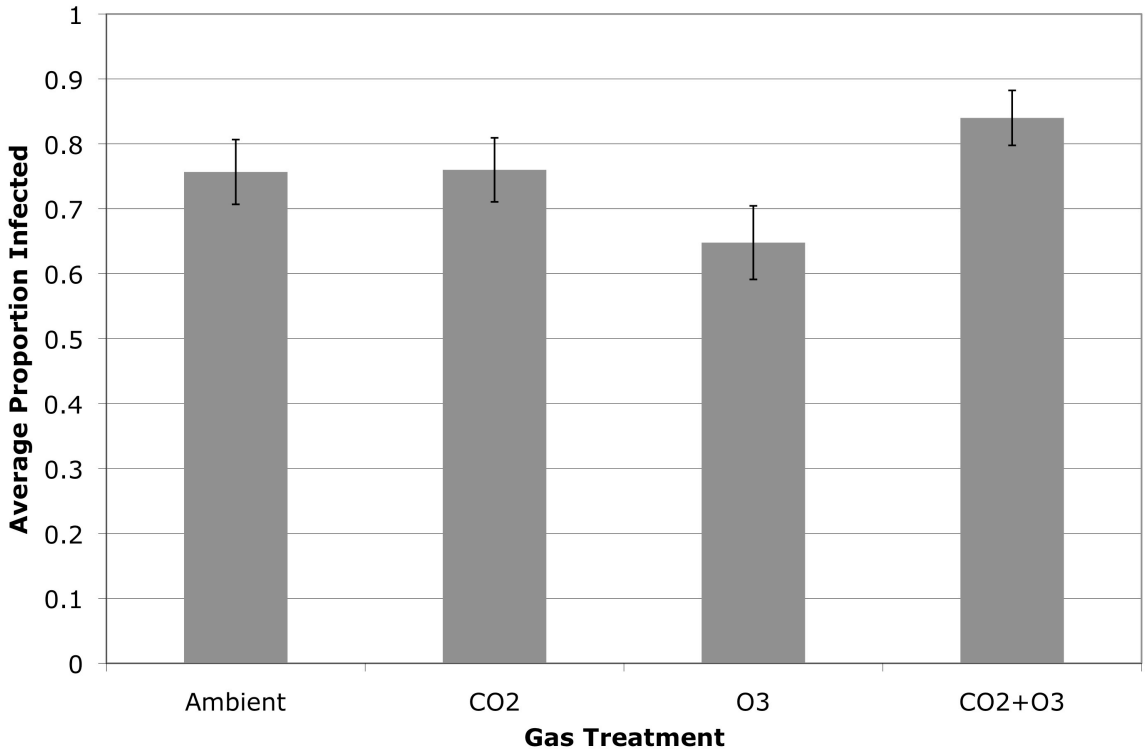

Figure 2.3: Proportion of plants infected in plants exposed to factorial combinations of CO_2 and ozone in open-top chambers in the field. Ozone shows a non-significant trend towards reducing transmission, while CO_2 restores transmission to ambient levels.

For plants in the ozone treatment, total host biomass was significantly negatively correlated with within-host viral fitness (Figure 2.4; r=-0.52, p=0.0097). There was no relationship between within-host viral fitness and plant biomass for the ambient, high CO_2 and high CO_2+ozone treatments (Table 2.1).

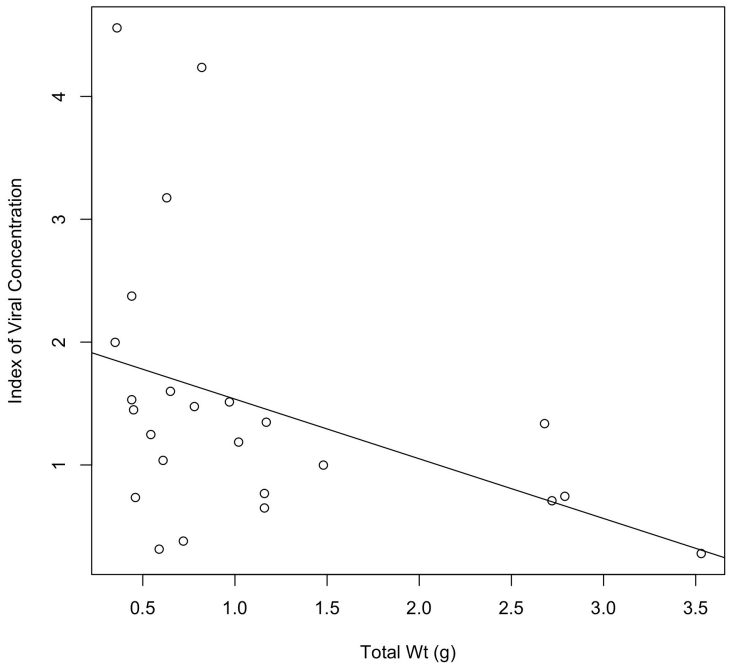

Figure 2.4: Correlation between total host biomass and within-host viral fitness for plants exposed to ozone.

Table 2.1: Correlations between within-host viral fitness and aboveground host plant total and vegetative biomass.

Gas Treatment	Total Host Biomass		Vegetative Biomass	
	Correlation coefficient	p-value	Correlation coefficient	p-value
Ambient	-0.21	0.23	-0.14	0.41
CO_2	-0.18	0.20	-0.09	0.54
Ozone	-0.51	0.01*	-0.49	0.02*
CO_2+Ozone	-0.04	0.78	-0.06	0.71

A similar pattern is shown when looking for the correlation between aboveground host vegetative biomass and within-host viral fitness. Ozone alone showed a significant negative correlation between aboveground host biomass and within-host viral fitness (Table 2.1).

DISCUSSION

While BYDV transmission remains high in all combination of gas treatments, viral success within the plant is lower and negatively correlated with plant size under ozone. Adding CO_2 to the ozone, however, reverses these patterns.

Effects of Ozone and CO_2 on BYDV Replication Within Hosts

Ozone reduced within-host virus concentration by 20-33% in *Avena* in the absence of additional CO_2. The reduction in viral concentration could be caused by one or more of at least three possible factors: reduction in plant quality, increased defenses, or reduced transport rates. Because viruses are biotrophic pathogens, when they reduce the ability of their host to capture resources (in this case photosynthesize), they also reduce the pool of resources which they can exploit, in turn affecting their own fitness. Because viral metabolism is so intimately intertwined with its host, measuring host quality independent of viral fitness is technically challenging. An interesting hint, however, comes from a recent study on BYDV in western grasslands, in which phosphorus, a limiting nutrient for many rapidly growing species such as viruses, significantly increased BYDV infection rates (Borer et al., 2010). BYDV is known to reduce root mass (Malmstrom and Field, 1997 and refs therein), which may reduce the ability to capture phosphorus. Since ozone induces a reduction in photosynthesis, plants may also be carbon-stressed, inhibiting their ability to put energy towards root development and phosphorus capture, and this may in turn be reducing the viral performance. However, this study found a negative correlation between within-host viral fitness of BYDV and biomass of *Avena* under high ozone and no relationship between within-host viral success and plant fitness in other gas treatments, indicating some decoupling of host and viral fitness. The significant negative correlation between host fitness and within-host viral success suggests that under high ozone

plants, already carbon-stressed due to ozone-induced reduction in photosynthesis, may be unable to compensate for the losses caused by BYDV.

A second possibility is that ozone itself is capable of priming the defenses of the plant. Ozone is a reactive oxygen species, and is thus downstream in the salicylic acid signaling pathway active against pathogens (rev in Sandermann et al., 1998, Zuccarini, 2009). Therefore, ozone may genuinely increase the resistance of the plant to viral replication itself, despite its large cost in terms of photosynthesis. While an explicit connection between ozone and plant defense levels has not yet been demonstrated for BYDV, ozone has been shown to reduce the fitness of several common crop viruses by inducing their defensive mechanisms. For example, in tobacco, ozone induces salicylic acid production and the accumulation of pathogenesis-related (PR) proteins, leading to poorer infection of tobacco with tobacco mosaic virus (TMV) (Yalpani et al., 1994). Similarly, Bilger et al. (2008) report that plants exposed to soybean mosaic virus and elevated ozone levels turn on many of the same genes- including genes involved in the hypersensitive response- and that ozone is able to reduce the speed at which the virus colonizes the soybean, though at some cost in soybean growth and yield.

These explanations are by no means mutually exclusive. In fact, if there is a cost to activating the SA pathway, the induction of the pathway by ozone may divert resources into that pathway, leaving fewer available for growth. Therefore, host plant quality may be reduced not only by damage to the photosynthetic machinery but by the building of resistance proteins. The possibility of ozone reducing viral concentration within the host has interesting implications for the communities of which plants are a part. If ozone does reduce the within-host fitness of the virus, this may reduce the transmission rates to poorer hosts of the virus if poorer hosts need a larger quantity of inoculum to become infected. Lower transmission to poorer hosts could reduce the effects of BYDV on bunchgrasses in California, as many of those grasses are not preferred hosts of the vectors (Cronin et al., 2010). Vectors may choose to spend less time feeding on the bunchgrasses, and may therefore be less likely to pass on the virus to the bunchgrasses in areas with high ozone.

However, this potential for increased production in the native bunchgrasses of the western US is unlikely to continue as carbon dioxide levels rise. In our study, we simulated atmospheric conditions that fall within the middle of the range of IPCC projected CO_2 concentrations (IPCC, 2007) and EPA reports of ozone concentrations in a moderately polluted city (EPA, 2012). Adding CO_2 to the ozone restored the within-host BYDV concentrations to ambient levels, which suggests that over the next fifty to one hundred years, viral fitness will rebound to current levels as ozone and CO_2 levels continue to rise. This may in turn increase spillover to poor viral hosts in ozone-prone areas.

The long-term outcome of this interaction is likely to be influenced by the evolutionary responses of both host and virus to the ozone itself, which represents and important, and largely unexamined aspect of this system. Classical theory suggests that if within-host and between-host fitness are positively associated, virulence should increase, while if the association is negative, a less virulent virus should evolve (Anderson and May, 1983; Frank and Schmid-Hempel, 2008). On the plant side, the negative correlation of viral and host fitness suggests that ozone reduces the ability of plants to tolerate infection. Ozone may increase selective pressure for evolution of resistance in these hosts. However, as Bilger et al. (2008) point out, the evolutionary response to virus-induced stress is likely to be more difficult in crop plants, which are often extremely inbred compared to wild versions, and would have to have genes for resistance to virus or ozone introduced. Interestingly, while CO_2 restored viral fitness in ozonated plants, CO_2 alone did not further increase viral levels within a host,suggesting CO_2 may not have direct benefits for host or within-host viral fitness.

Effects of Ozone and CO_2 on Field Transmission Of BYDV

Given the reduction in within-host virus concentration in ozonated plants, we might expect that the lower viral concentration would result in reduced spread. Because BYDV does not replicate within the aphid, the amount of BYDV acquired and available for transmission should depend on the amount of time the aphid spends feeding and the concentration of virus the aphid encounters. However, we found no significant differences between proportions of plants

infected in the inoculated sub-plots. There was, however, a trend towards lower infection under elevated ozone, and we might speculate that constraints on the number of plots limited the statistical power to detect this difference. However, it is also possible that the selection of the host played a role. Aphid transmission success in a monoculture is a function of at least 3 factors: aphid ability to take in the virus, aphid movement (long enough on one plant to acquire the virus and then choosing to move to an uninfected plant) and aphid ability to deliver the virus to a new plant. Interestingly, Menéndez et al (2009) point out that the ozone-induced salicylic acid defenses that should defend against the virus should also be effective against aphids, yet aphids feed readily on *Avena* even under elevated ozone, suggesting that the SA-pathway defenses are not completely effective at deterring aphid feeding and BYDV transmission. In addition, host susceptibility to the virus may also play a role in maintaining high transmission in all gas treatments. *Avena fatua* is a particularly good host of the PAV species of BYDV (Power and Mitchell, 2004). It is possible that aphids are so efficient at acquiring the virus from *Avena* and resistance of the host is so minimal that even with lower amounts of virus present in the tissues, transmission success from *Avena* to *Avena* is still high.

Conclusions

Overall, these results highlight an important disconnect between the within-host viral fitness and the transmission rate for a vectored virus, namely, that lowering within-host viral concentrations is not necessarily enough to reduce transmission if the vector is efficient enough. This may have important implications for the control of vector-borne diseases, as it implies that the reducing the infection rates across host populations will require completely clearing the host's infection or reducing vector abundance. Furthermore, while ozone may reduce within-host viral success at current CO_2 levels, within-host viral fitness and viral transmission will likely remain high under future levels of CO_2 and ozone, and thus there is likely to be no relief from BYDV epidemics under future atmospheric conditions. In addition, in high-ozone areas under current CO_2 levels, antagonism between within-host viral replication and plant growth is particularly severe. These results highlight the complexity of the interactions between the

multiple global changes now occurring and reinforce the need for studies of interactive effects to accurately predict disease rates in the future.

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CHAPTER 3

Amplification of an aphid vectored disease in mixed plant communities under elevated CO_2 and ozone

ABSTRACT

Increasing diversity frequently reduces disease rates in communities, but the effect of diversity may depend on individual responses of host and vector species to environmental conditions. For plants, the increases in carbon dioxide and ozone levels observed since the industrial revolution have the potential to alter the frequency and severity of disease outbreaks. We used open top chambers in the field to factorially elevate CO_2 (700 ppm) and ozone (+70 ppb) in monocultures of the competent grass host *Avena fatua,* monocultures of the non-competent host grass *Setaria lutescens, and* 50:50 mixtures of the two host species. We found that ozone reduced the amount of barley yellow dwarf virus within a host but did not depress transmission rates in a competent host. In contrast, adding CO_2 and ozone restored amount of virus within a host to ambient levels. Because the amount of virus per plant was lower under elevated ozone, we predicted that high ozone levels would reduce spillover to a less competent host, while CO_2, which restores within-host viral fitness, should also restore spillover levels. Spillover from the highly competent host *Avena fatua* to *Setaria lutescens* was uniformly high across gas treatments, despite lower concentrations of the virus in *Setaria* tissue under elevated ozone. The proportion of *Avena fatua* infected was higher in mixtures than in monocultures, regardless of gas treatment. Furthermore, the amount of virus in *Avena* tissue was also higher in mixtures than in monocultures across gas treatments. This suggests that growing *Avena* with a non-competent host results in pathogen amplification, increasing disease levels in both competent and non-competent hosts.

INTRODUCTION

Generalist pathogens can profoundly affect the ecosystems of which they are a part, especially in cases where the hosts vary in susceptibility to the pathogen or in ability to build up large quantities of pathogens in their bodies. Such pathogens have been shown to alter

productivity (Power and Mitchell, 2004), community evenness (Power and Mitchell, 2004), and interactions with mutualists (Rúa et al., 2013), to reduce seedling recruitment (Malmstrom et al., 2005; Beckstead et al, 2010), and to facilitate invasions (Malmstrom et al. 2005, 2006, Borer et al. 2007; Kelly et al., 2009).

In turn, pathogen epidemics are strongly influenced by properties of the communities in which they occur. A great deal of attention has been focused on the role of diversity, especially species diversity, in regulating epidemic spread, and this topic has been the subject of several excellent reviews (e.g. Ostfeld and Keesing, 2006; Ostfeld et al., 2008; Ostfeld and Keesing, 2012; Wood and Lafferty, 2013). The effects of diversity on disease in communities with hosts of varying quality for the pathogen are generally classified by the focus of the effect (poor hosts vs. good hosts/communities) and the direction of the effect (increasing or decreasing disease prevalence). For poor hosts, the focus is on the incidence of pathogen spillover, which occurs when excellent hosts of the pathogen build up a high pathogen load in the community. Some of that large inoculum makes its way onto poorer hosts, causing an epidemic in hosts that would not otherwise be able to sustain an epidemic (reviewed in Power and Mitchell, 2004; Kelly et al., 2009). The effect of spillover on the host community itself depends on the relative abundances of the two hosts and their respective tolerances for the pathogen. If the poor host is common and spillover rates are high, infection may allow the good host to persist and even outcompete the poor host, becoming in the process the dominant host in the community. Such a disease-mediated facilitation has been suggested as a contributor to the invasion of the western US grasslands by annual grasses. A model by Borer et al. (2007) suggests that the presence of barley yellow dwarf virus allows annual grasses outcompete native bunchgrasses. In contrast, if the good host is already dominant and tolerant of infection, the pathogen could prevent invasion by a less tolerant poor host (Ostfeld and Keesing, 2012). Moreover, understanding the incidence of spillover and the species that cause it has played a crucial role in management of important diseases as diverse as tuberculosis (Nugent, 2011) and soybean rust (Fabiszewski et al., 2010).

The effects of diversity on disease levels in good hosts (or in whole communities) are

classified according to the direction of the effect: dilution -a reduction in disease as diversity increases- **(**reviewed in Ostfeld, 2008; Keesing et al., 2006; Ostfeld and Keesing, 2012) or amplification -an increase in disease with increased diversity (Ostfeld and Keesing, 2012). The dilution effect has been explored in both managed and unmanaged ecosystems, and examples include vector-borne animal diseases and plant diseases (Ostfeld and Keesing, 2012; Cardinale et al., 2012; Pagan et al., 2012). Modeling and empirical studies have identified several conditions under which a dilution effect may be expected. One such condition is that hosts must vary in their suitability for infection, with poor hosts becoming more common as diversity increases, leading to reduced numerical dominance of good hosts in the community (Ostfeld and Keesing, 2012). However, in addition to host quality, some models highlight the role of pathogen transmission mode. A model by Dobson (2004) suggesting dilution is more likely than amplification in vectored pathogens, and that amplification may be likely for directly transmitted diseases whose transmission depends on host density, because of the potential for vectors to spend a portion of their limited lifespan on non-competent hosts. Another condition favoring dilution would be higher vector mortality on poor hosts, or vector preferences for poor hosts as a food source (Ogden and Tsao, 2009: Ostfeld and Keesing, 2012).

In contrast, much less attention has been paid to systems in which biodiversity results in increased disease, and reports of amplification effects in the literature are comparatively rare (Ostfeld and Keesing, 2012; Cardinale et al., 2012). However, both theoretical (Ogden and Tsao, 2009) and empirical work (Becker and Zamudio, 2011) have reported amplification effects. Disease amplification may occur under conditions in which the additional species in diverse communities are poor hosts or in which diversity extends the length of the epidemic season by including susceptible hosts whose abundance peaks at different times of the year.

Pathogen dilution, amplification and spillover are determined by the epidemiological properties of the host and the pathogen such as host and vector growth rates (Cronin et al, 2010), and within- and between- host transmission rates (Dobson, 2004). Therefore, altering these epidemiological properties has the potential to change spillover and dilution rates substantially.

These epidemiological properties may in turn be influenced by the effect of environmental changes on host and pathogen physiology. For plants, the physiological changes triggered by rising levels of carbon dioxide and ozone may alter host physiology in several ways that change the epidemiology of the disease (Mitchell et al., 2003). Ozone has several complementary direct physiological effects on the plant. First, it reduces the carbon pool of the plant by damaging photosynthetic tissue and limiting stomatal aperture (Sanderman, 1996; von Tidemann and Firsching, 2000). Limiting the plant's photosynthetic capacity limits the pool of resources the pathogen is able to exploit. Second, ozone acts as an immunostimulator, upregulating the defenses which attack viral and bacterial pathogens (Sandermann et al., 1998; Yalpani et al., 1994; Zuccarini, 2009, Bilger et al., 2009). Both of these mechanisms might limit the reproduction of the virus within the host, as we have observed in monocultures of the highly competent host *Avena fatua* (Chapter 2). Because host species may vary in their sensitivity to ozone, their photosynthetic losses to ozone and the strength of defenses upregulated may vary, and this in turn may change the suitability of the host for the pathogen.

Like ozone, rising levels of carbon dioxide can change a plant's carbon uptake rate, C:N ratio, and the stomatal conductance, (McElrone et al., 2005), which can in turn affect a host plant's suitability for the virus. In addition, carbon dioxide can increase the C:N ratio in the phloem, making aphid feeding more challenging and affecting aphid preferences and movement patterns (Finlay and Luck, 2011). An initial burst of rapid growth triggered by CO_2 might create a pool of resources for a virus to exploit. In addition, carbon dioxide can mitigate the damaging effects of ozone on photosynthesis by making photosynthesis more efficient at lower concentrations of carbon dioxide (Biswas et al., 2013).

We used the barley yellow dwarf virus (BYDV) system to study the effects of rising carbon dioxide and ozone on pathogen dilution and spillover in plant communities. BYDV is an obligately aphid-vectored generalist luteovirus that infects over 150 species of grasses, but host grasses vary widely in their suitability for viral reproduction and their ease of transmission (D'Arcy, 1995). The two model species we used were *Avena fatua*, an excellent grass host of

the virus, and *Setaria lutescens*, a poor grass host that has difficulty sustaining an epidemic in monoculture but becomes infected via spillover processes when grown with *Avena* (Power and Mitchell, 2004). Because previous work from our lab suggested that ozone lowers the within-host viral concentration in *Avena* (see chapter 2), we hypothesized that this might lead to lower spillover rates to *Setaria*. However, because adding CO_2 to the ozone restores within-host viral fitness to ambient levels, we expected a similar restoration of spillover levels in the combined treatment. Since BYDV is a vectored virus whose vectors can use both species (though they prefer the good host *Avena)*, we expected to see lower infection rates in *Avena* when grown with *Setaria*, consistent with modeling work in this area (Dobson, 2004; Ostfeld and Keesing, 2012).

METHODS

Experimental Design and Planting

We used open-top chambers in the field to factorially elevate carbon dioxide (target concentration 680-700 ppm) and ozone levels (target +70 ppb). Chambers were arranged in 6 blocks, each of which had an ambient chamber, an elevated CO_2 chamber, an elevated ozone chamber, and a chamber with both gases elevated. Each chamber was 2.25 m by 1.5 m and was divided into six equally sized sub-plots. Two sub-plots were planted with *Avena,* two with *Setaria,* and the final two with a 50:50 mixture of *Setaria* and *Avena* seeds. Planting took place at East Ithaca Farm, NY (Cornell University) at the beginning of the first week in June 2010. The soil of the plots was sandy loam with rapid drainage. Plots were plowed before planting and fertilized with 20:10:10 NPK fertilizer. Plants were seeded with a target total density of 600 plants per square meter.

Gas treatment

We used rectangular open top chambers the size of the whole plot and covered with clear vinyl plastic wrap to elevate the gases. The sub-plots were individually caged with nets made of white aphid-prevention fabric which helped to control both the aphid movement and the temperature elevations that are common side effects of open top chambers. However, in each plot, two of the aphid-free sub-plots shared a single large net due to equipment limitations.

Air was circulated through each chamber by an impeller fan which pushed air down a long aluminum duct and out 8 PVC pipes. Gases were introduced to the pipe leading to each chamber and then spread among the sub-plots with aerated pieces of PVC in the center of the sub-plots. CO_2 fumigation began at midday on 6 June 2010 (day first germinants emerged) and ended at sunset each day. CO_2 measures were made using a manually carried NOVA IRGA each dry day. Chamber values were generally repeatable. Ozone fumigation began on 25 days after first germinants emerged (DAE) and took place 4 hours every afternoon from approx 12:30-4:30 PM and fluctuated more, but background levels were generally in the 20-35 ppb range, with levels from approx. 80-150 ppb in the elevated chambers.

Inoculation

Colonies of *Rhopalosiphum padi* were maintained on Romulus barley in growth chambers at 20 degrees C in 24 hr light. At 14 DAE we began the inoculation procedure by transferring 50 adult *Rhopalosipum padi* per sub-plot to dishes containing *Romulus* barley known to be infected with a New York isolate of the PAV species of BYDV and allowing the aphids to feed on the barley for 72 hours. At 27 DAE, we gently sprinkled the aphids randomly around the inoculated sub-plots.

Virus Sampling

We chose two sampling periods for the experiment: early season (38-40 DAE) and late season (57-60 DAE). At 38-40 DAE, we sampled and marked 15 plants of every species planted in that sub-plot, except where plants were too small to sample. Though we sampled both species at each time point, sampling for *Setaria* at the early season time point was difficult due to small plant size, and thus only late season sampling (57-60 DAE) for *Setaria* is reported here. Sampling was random throughout the sub-plot, but we made an effort to sample the sides and the center at equal frequency, in case of gradients in gas diffusion. We clipped a piece of green leaf tissue of approximately 0.25 g from each plant. However, in cases where 0.25g was difficult to obtain, we used a whole leaf, and included a piece of stem if needed. Samples were weighed and then frozen for later analysis.

During late season sampling (57-60 DAE), we repeated the procedure, locating the marked plants for a second sampling. In cases where the original plant had died or could not be found, we chose a new plant and gave it a new ID number. We performed the same tissue clipping and freezing procedure as the early season sample. In addition, at the final harvest we clipped tissue from 15 plants in the uninoculated sub-plots to check for any potential contamination.

Virus Detection

When each block was ready for analysis, we defrosted samples and then macerated them in Bioreba extraction bags (Bioreba Inc,) in 1x PBS (phosphate buffered saline) at a ratio of 1mL buffer per 0.1g fresh weight of tissue. Macerated samples were analyzed using double antibody sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) using commercially prepared antibodies (Agdia, Inc) at an antibody dilution of 1:550. In each 96-well plate, we filled the edges with PBS to minimize edge effects. Each plate included 2 wells of healthy control tissue from the host species present on that plate and 4 wells containing tissue from infected plants maintained in our laboratory. Both were ground at the same ratio of buffer as the samples. Samples and controls were randomly distributed throughout the plate to reduce any artifacts of placement. In addition, a second plate was analyzed with the same samples in a different order. Within each block, treatments of each species were randomly distributed to plates, so that each plate contained a mixture of monocultures and mixtures of a single species. Gas treatments were also randomly distributed on the plates.

Each plate was allowed to develop until visible yellow coloring showed in positive controls, then read at 410 nm using an EL800 universal microplate reader. To estimate within-host viral concentration we divided each sample's OD value by the average of all 4 positive control OD values on the plate to give a standardized OD value. We then averaged the two standardized OD values to give an estimate of within-host viral concentration.

Data Analysis

Because the proportions of plants infected in late season *Setaria* sampling and the early season *Avena* sampling had a large number of zeros, the proportions of plants infected for each species was modeled in two ways (Fletcher et al, 2005), as probability of disease establishment and as amount of spread given establishment. Probability of establishment in the sub-plot was computed as the number of inoculated sub-plots in which in which at least one infection was detected. It was modeled using logistic regression with block, CO_2, ozone, and mixture as main effects. Amount of spread given establishment was measured for plots with at least one infection and was the number of positive plants over total plants sampled. It was modeled using a binomial GLM with CO_2, ozone, mixture and block as predictors. All sub-plots showed some *Avena* infection in August, and therefore the proportion of plants infected per sub-plot was modeled using a binomial model with block CO_2, ozone, and mixture and all interactions of CO_2, ozone and mixure as fixed effects. Models were compared using likelihood ratios, and the chi-square values for these terms are reported.

Because no transformation of the within-host virus data was normally distributed, data were analyzed by the non-parametric Kruskal-Wallis test and the Steel Dwass test for multiple comparisons (a non-parametric equivalent of Tukey's Test). All analyses were carried out using JMP Pro 9 (SAS Institute).

RESULTS

Early Season Infections

Early season infections were driven mainly by *Avena,* as *Setaria* infections were rare. The probability of disease establishment in *Avena* at 38-40 DAE was significantly affected by gas treatments and mixtures (Overall model χ^2= 24.24, df=12, p= 0.02). Specific effects on probability of infection included block (likelihood ratio (LR) χ^2= 12.89, df=5, p=.0245), the interaction of CO_2 and O_3 (LR χ^2= 7.46319159, df=1, p= 0.006, and the three-way interaction of CO_2, ozone, and mixtures (LR χ^2= 4.10, df=1, p= 0.042). In ambient plots, the probability of disease establishment was the same in monocultures and mixtures (Figure 3.1). However, in

high CO_2 plots, mixtures had a higher probability of disease establishment in the absence of ozone and a lower probability of disease establishment in the presence of ozone (Figure 3.1).

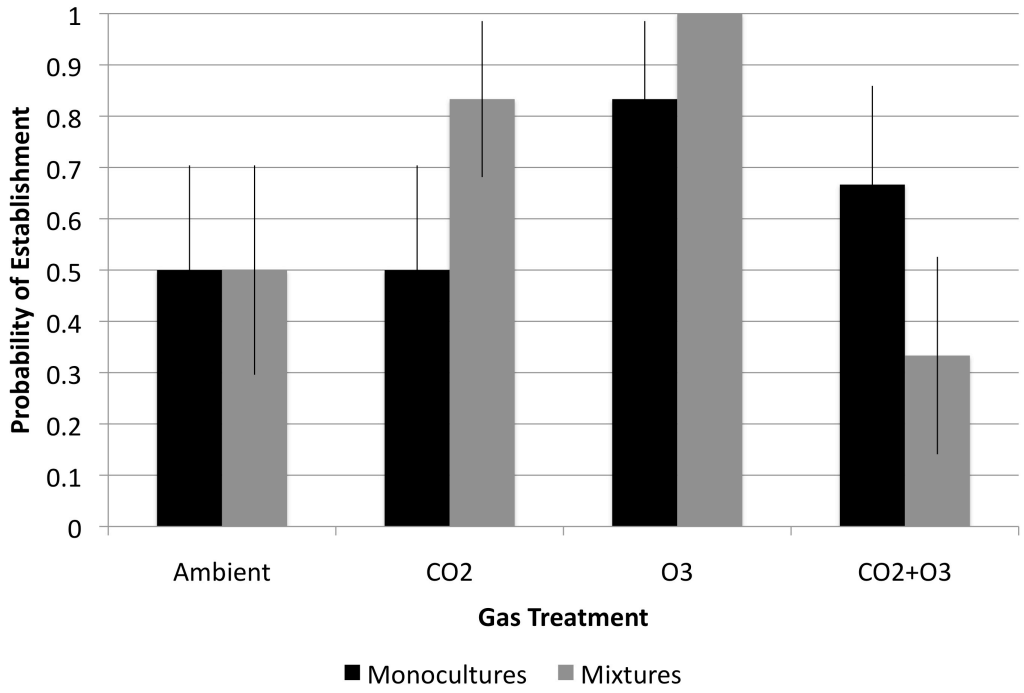

Figure 3.1: Probability of disease establishment in *Avena* at early season sampling in monocultures (black) and mixtures (grey) across gas treatments. In the absence of ozone, CO_2 confers a higher probability of disease establishment in mixtures than in monocultures. In the presence of ozone, CO_2 confers a lower probability of disease establishment in mixtures.

In sub-plots where disease establishment occurred midseason, *Avena* in mixtures showed a significantly greater proportion of plants infected than monocultures of *Avena* (LR χ^2= 7.23, df=1, p= 0.0069, Figure 3.2).

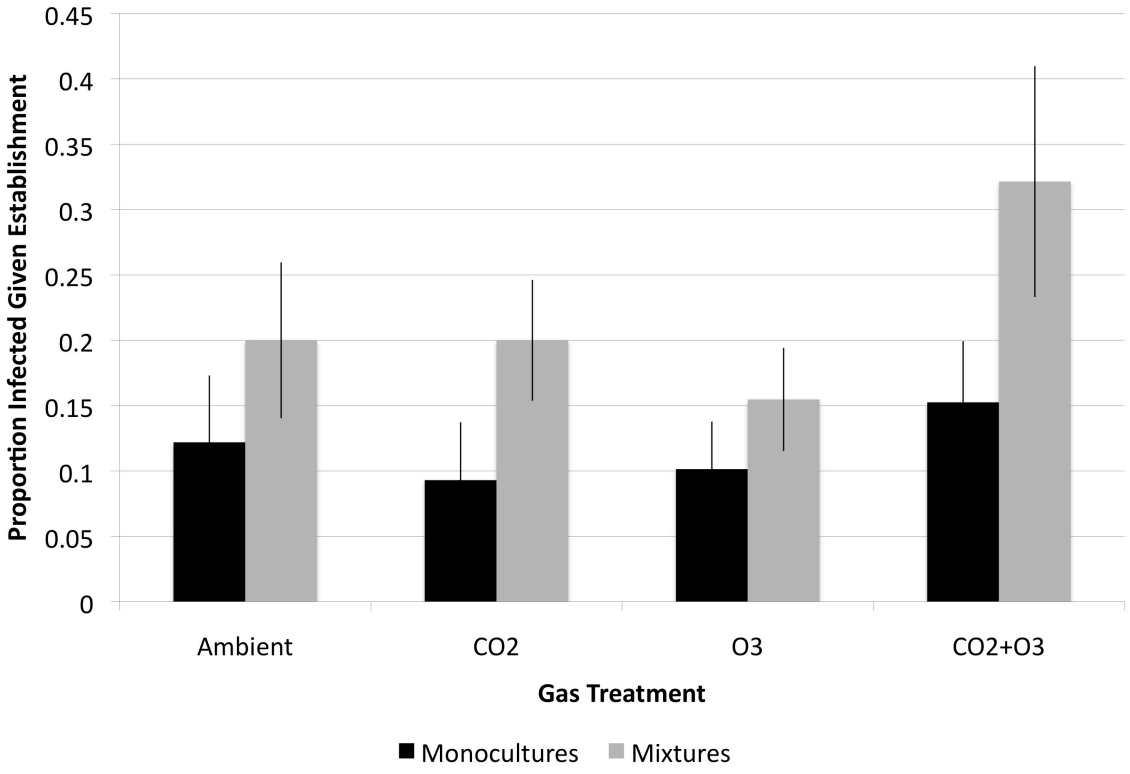

Figure 3.2: Amount of virus spread in early season *Avena* sampling: proportion of *Avena* plants infected per gas treatment in monocultures (black) and mixtures (grey).

Late Season Infections

By the late season sampling date (57-60 DAE), all *Avena* sub-plots (both mixtures and monocultures) had infected *Avena*, and virus prevalence was high (85-97%). (Overall model: χ^2=32.77, df=12, p=0.0010, OD=1.62). There was a significant effect of block (LR χ^2= 24.06, df=5, p=0.002). However, no combination of gas treatment or mixture had a significant effect on prevalence (Figure 3.3).

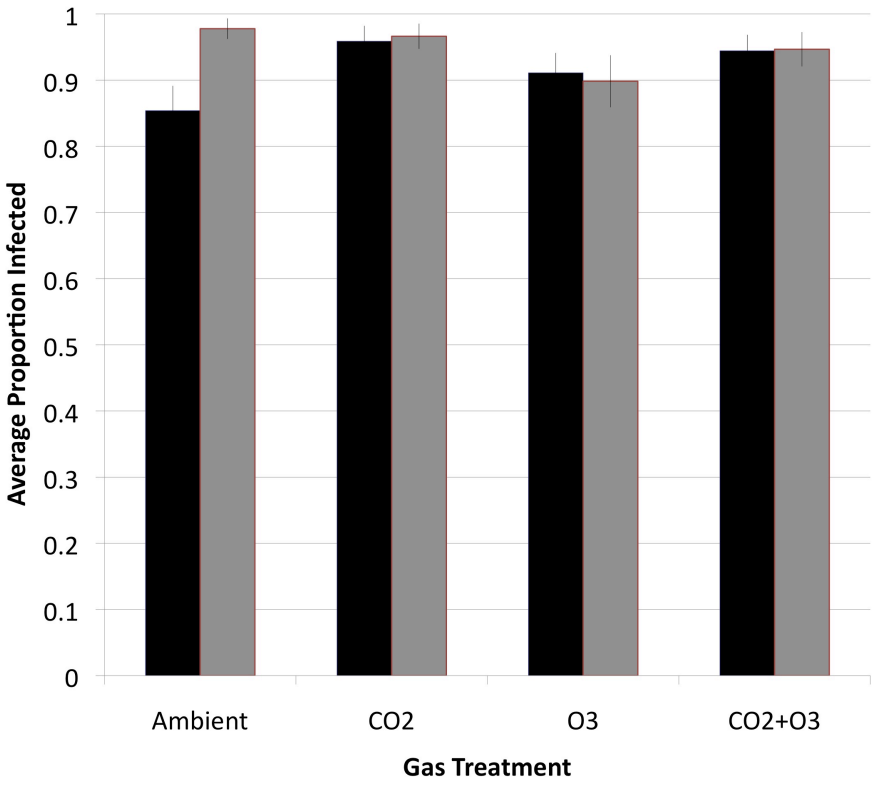

Figure 3.3: Infection rates in *Avena* (late season). Proportion of *Avena* infected in monocultures (black) and mixtures (grey) across gas treatments. CO_2 and mixture significantly increased prevalence.

Probability of disease establishment in *Setaria* late in the season was highest in mixtures (Overall model: χ^2=19.81472, df=12, p=0.0707, mix: LR χ^2 =10.41, df=1, p=0.0013; figure 3.4). Neither CO_2 (LR χ^2=1.626832, df=1, p= 0.2021) nor ozone (LR χ^2= 0.29989153, df=1, p= 0.5840) altered the probability of disease establishment.

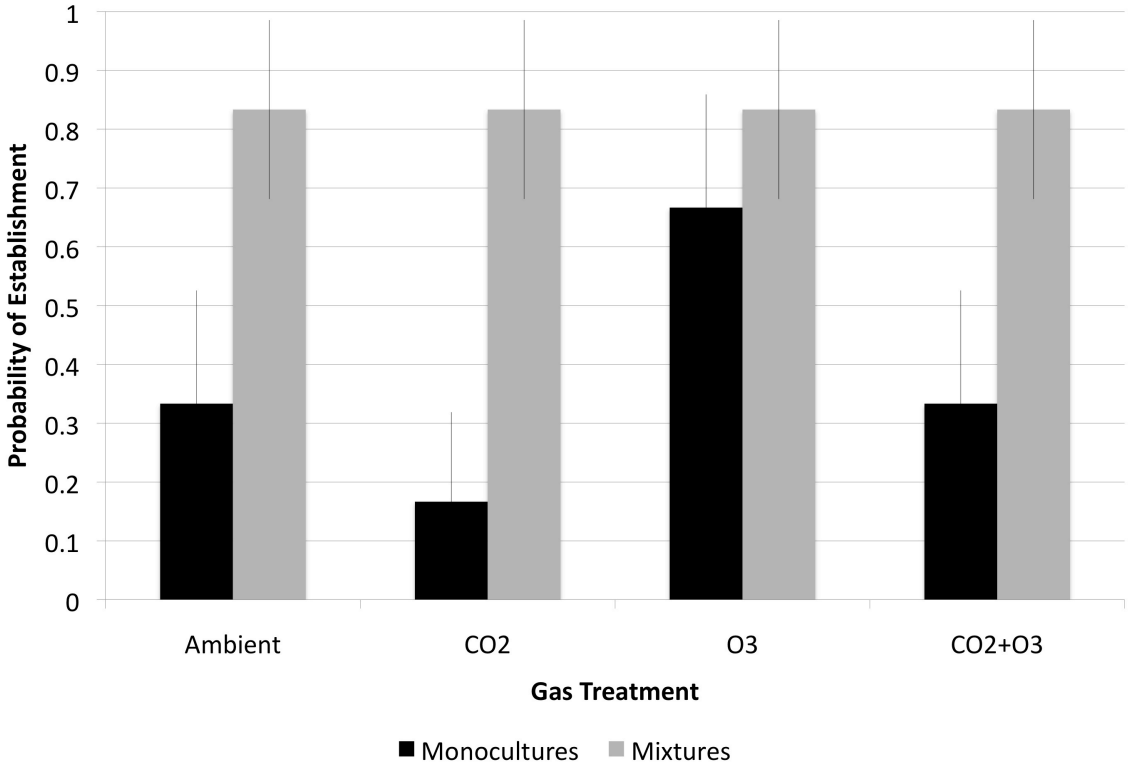

Figure 3.4: Probability of disease establishment in *Setaria* late in the season (57-60 DAE) in monocultures (black) and mixtures (grey). Presence of *Avena* significantly increases the probability of an establishment in *Setaria.*

The amount of spread late in the season in *Setaria* was significantly greater in mixtures (mix: LR χ^2 =25.68, df=1, p<0.001, Figure 3.5). No gases had a significant effect on proportion of plants infected, though there was a significant block effect (Overall model: χ^2 =62.12, df=12, p<.0001, OD=4.74, block effect: LRχ^2 = 18.57, df=5, p=0.002, figure 3.5).

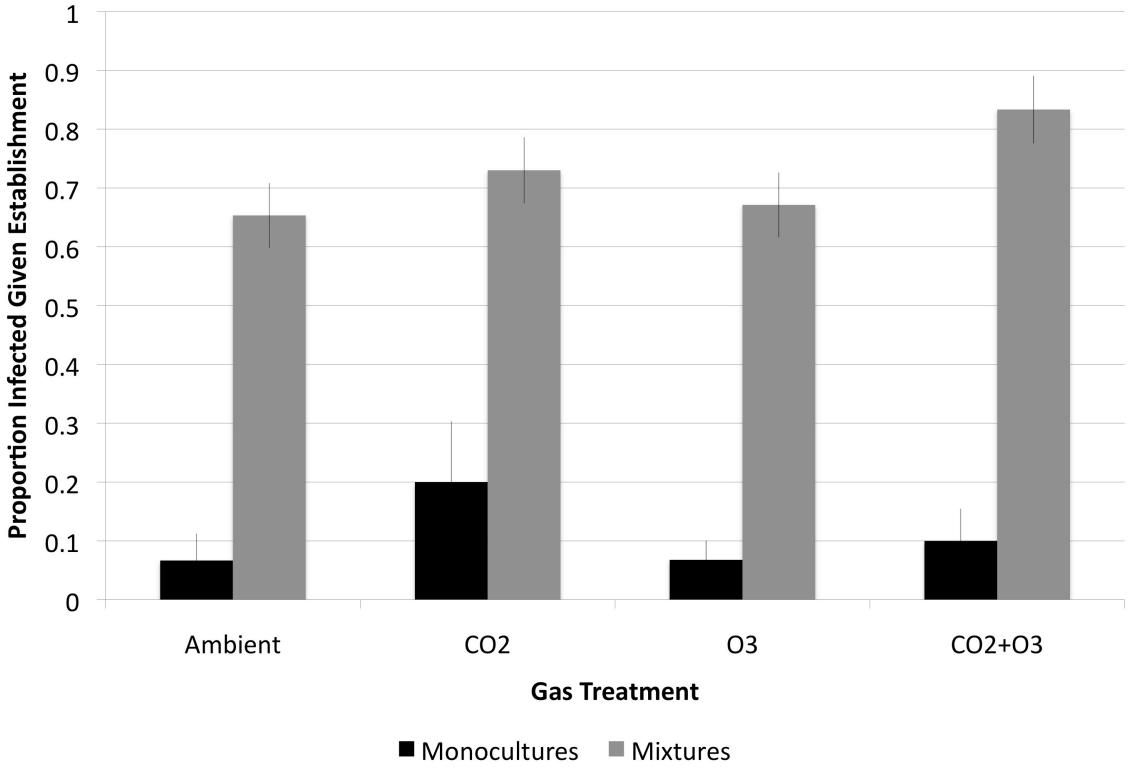

Figure 3.5: Pathogen spillover from *Avena* to *Setaria* late in the season (57-60 DAE) Proportion of plants infected in sub-plots showing disease establishment in *Setaria* monocultures (black) and mixtures with *Avena* (grey).

Within-Host Viral Fitness

Within-host viral fitness in *Setaria* was evaluated at the late season sampling point. Because so few plants in monocultures became infected, data from monocultures and mixtures were pooled for analysis. Gas treatment had a highly significant effect on within-host viral concentration (χ^2 =18.2909, df=3, p=0.0004). Adding CO_2 to ozone raised the within-host viral fitness above the within-host viral fitness of plants experiencing ozone alone (z= -3.50858, p= 0.0025; Figure 3.6). CO_2 did not significantly raise within-host viral fitness above ambient,

(z= -3.50858, df=1, p=0.2562; Figure 3.6).

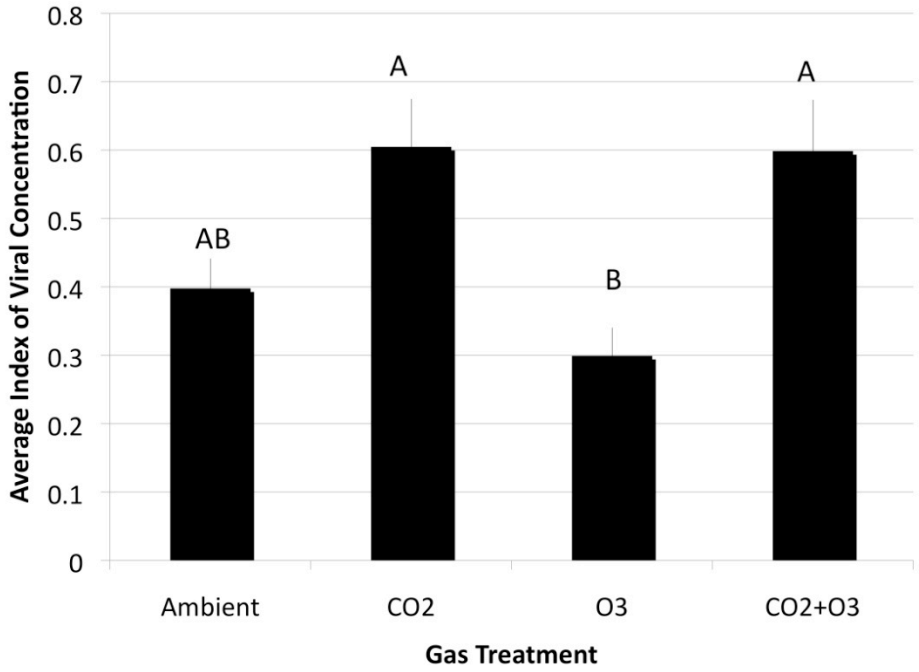

Figure 3.6: Within-host viral fitness of BYDV in *Setaria* across gas treatments. Measures are standardized such that positive control plants are scored as 1. Letters indicate treatments significantly different at p=0.05.

Within-host viral fitness for *Avena* was evaluated at the early season sampling date, as by the late season sampling date many plants had started to senesce and within-host viral titers had dropped. Mixtures significantly raised the within-host viral fitness (χ^2 =7.18, df=1, p=0.0074; Figure 3.7), but there was no significant effect of gas treatment on within-host viral fitness in *Avena*.

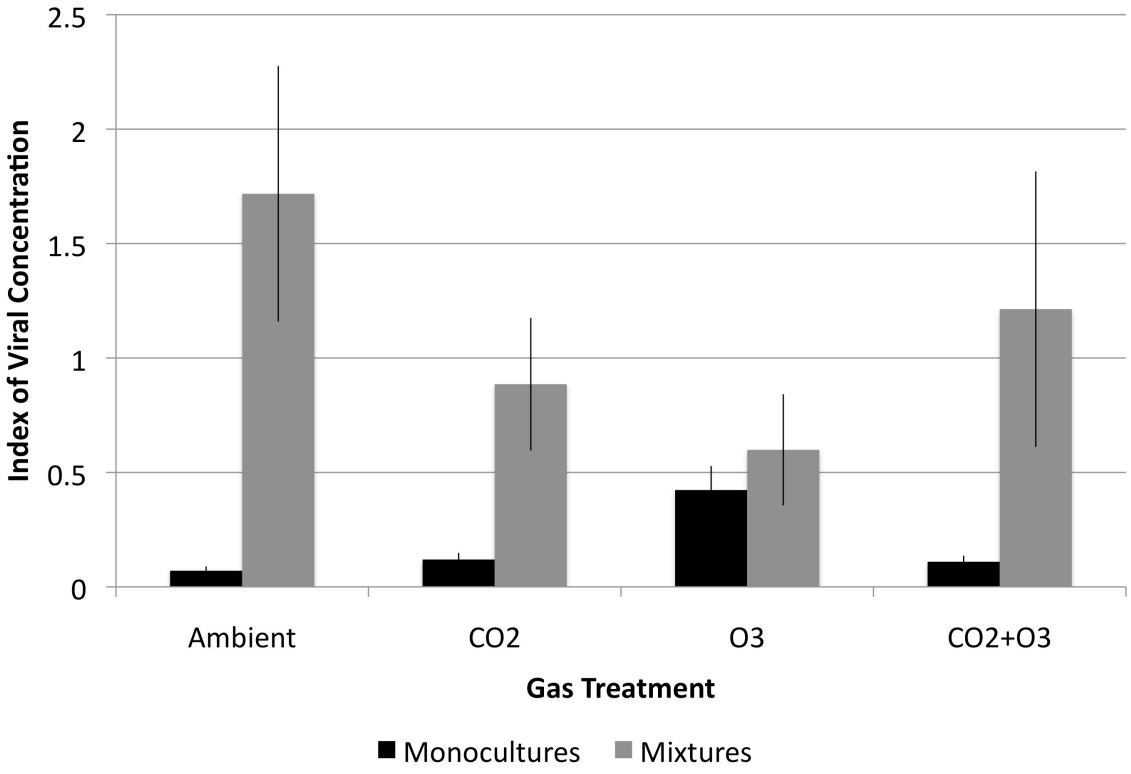

Figure 3.7: Within-host viral fitness of BYDV in *Avena* across gas treatments in monocultures (black) and mixtures (light). Measures are standardized such that positive control plants are scored as 1. Growing *Avena* in mixtures significantly increased within-host viral fitness in all gas treatments.

DISCUSSION

Amplification, dilution, and spillover may be assessed in two ways- changes in probability of disease establishment in a community or changes in the extent of disease spread once established. Carbon dioxide and ozone had different effects on these measures of disease pressure, suggesting that different processes may be controlling them (Fletcher and Villouta, 2005).

Early-Season Epidemics

Early season epidemics were dominated by infections in *Avena*, as expected. Interestingly, the probability of disease establishment but not the extent of spread once established was influenced by elevated CO_2 (Figure 3.1, Figure 3.2). In the absence of ozone, CO_2 raises probability of establishment in mixtures, but in the presence of ozone, CO_2 decreased the probability of establishment in mixtures (Figure 3.1). Aphid behavior might contribute to an explanation of these results. Under high CO_2, *Rhopalosiphum padi* tend to spend more time feeding on a single plant, because CO_2 reduces plant quality for aphids (Finlay and Luck, 2011). In monocultures of a good host such as *Avena*, aphids may move less, resulting in lower infections, while they may take more time to settle on a compatible host in mixtures, resulting in higher infection rates. It is puzzling, however, that this trend does not continue when ozone is added.

Given disease establishment in a sub-plot, we predicted that the inclusion in the community of poor hosts like *Setaria* would divert some virus-bearing aphids onto the poor host, reducing the number of infections to *Avena*, the competent host, as predicted by models of mixed-species communities of vectored diseases (Dobson, 2004). However, the opposite was the case: rather than lowering the viral infection rates and loads in the community, presence of *Setaria* tended to amplify the proportion of *Avena* infected in communities where BYDV became established (Figure 3.4, Figure 3.5). While the dilution effect is often invoked to explain disease reductions as an ecosystem service provided by intact communities rather than simple mixtures (Ostfeld 2008; Keesing et al., 2006, Ostfeld and Keesing, 2012), our results suggest that disease amplifications could result from changes in diversity in other systems. While amplification effects are much rarer in the literature than dilution effects (Haas et al, 2010; Ostfeld and Keesing 2012; Cardinale et al., 2012), several have been reported. For example, higher host diversity resulted in greater fungal disease levels in frogs (Becker and Zamudio, 2011). Similarly, in the Lyme disease system removal of a poor host (western fence lizards) actually

decreased the number of infected tick vectors and the disease risk they pose to humans, suggesting that in intact systems, the presence of the poor host keeps tick infection levels and disease levels high (Swei et al., 2011). In our study, we may be observing a similar trend, where the presence of the poor host *Setaria* allows an increased number of infected aphids to persist in the system. It is likely that vector behavior may play a large role in the amplification of the epidemic. Because *R. padi* strongly prefer *Avena* over *Setaria*, the aphids may have tasted *Setaria* and then moved back to the smaller number of *Avena* in the mixed sub-plots. In addition, it is possible that aphids moved more freely between plants in those sub-plots. A similar effect was observed in the west nile virus system, in which the competent host robins amplified WNV infection in mosquitoes, and few infections in the less competent hosts (humans) were observed until after the robins had left for the season (Kilpatrick et al., 2006). In our system, infections in the less competent host became common late in the season, when the good host had begun to leave the plot by senescence. The results of Kilpatrick et al. (2006) also suggest that amplifications in a good host, such as the one we observed here, may be of serious consequence to epidemics in grasslands with sensitive native grasses. Because *Avena* senesces earlier than many other species, the amplification effect reported here produces a large population of BYDV-positive aphids, which may then move late in the season to poor hosts, causing an outbreak later in the season. Our results also suggest the potential for amplification over time as well as across space, which may be a fruitful investigation in amplification vs. dilution theory in disease ecology.

Late Season Epidemics

Amplification in *Avena* was impossible to detect late in the season, because virus epidemics were detectable in all inoculated sub-plots and prevalence was very high. This is expected, as *Avena* is an excellent host for the pathogen and the vector. In addition, because vector movement was constrained by cages, infection levels may be increased as the ability of the vector to disperse at high density was limited. It is therefore possible that the amplification effect would still have been detectable late in the season in a larger, less constrained plot.

Consistent with this idea, spillover to the poor host *Setaria* was very high in this system. In monoculture, *Setaria* was not able to sustain an epidemic of BYDV, consistent with previous work with this species (Power and Mitchell, 2004). In fact, we had very low infection rates in all gas treatments, and did not find any aphids in the *Setaria* monoculture sub-plots at harvest. In mixtures with *Avena*, we had hypothesized that the lower concentrations of the virus might reduce spillover rates of BYDV from the excellent host *Avena* to the poor host *Setaria.* In fact, gas treatment did not affect probability of BYDV establishment (Figure 3.4). In sub-plots where an infection occurred, spillover was high across all sub-plots and we detected no significant difference in the rates of spillover across gas treatments (Figure 3.5). This suggests no reduction in spillover levels is likely in the future as CO_2 and ozone levels rise. If other species also show this trend, it could be of some concern to managers and conservationists, because pathogen spillover can cause severe recruitment problems in western native bunchgrasses (Malmstrom et al., 2005) and can exacerbate difficulties in restoring bunchgrasses in the *Avena*-dominated western grasslands (Malmstrom et al., 2005; Borer et al., 2007).

Within-Host Viral Fitness

Despite the inability of *Setaria* to sustain an epidemic of BYDV on its own, the virus did replicate in *Setaria.* Detected quantities of virus were small, relative to the quantities found in barley controls and *Avena.* Nonetheless, the virus was clearly detectable in more than 60% of the plants from mixed sub-plots (Figure 3.6). As in monocultures of *Avena* (Chapter 2), ozone depressed within-*Setaria* concentrations in the ozone-fumigated plots, and adding carbon dioxide restored the within-host viral concentrations to near or above ambient levels (Figure 3.6). This drop in fitness due to ozone may be a result of carbon stress induced by stomatal closure (Sandermann, 1996) or an ozone-induced increase in defenses against BYDV, similar to those observed against other plant viruses such as soybean mosaic virus (Bilger et al., 2008) and TMV (Yalpani et al., 1994). In addition, it is possible that CO_2 may alter the physiology of the plant in a way favorable to viral replication or may reduce expression of disease defenses. Favorable alterations in host physiology may be more likely, as some evidence suggests that plant defenses

effective against viruses in dicots are increased rather than decreased by elevated CO_2 (Sun et al., 2011). These results suggest that further investigation of the regulation of defensive gene expression by elevated CO_2 in grass plants could be fruitful.

Though gas treatments did not significantly affect within-host viral fitness in *Avena*, growth in mixtures with *Setaria* did cause an increase in within-host viral fitness in *Avena* (Figure 3.7). Plants in mixtures were larger than plants in monocultures (Chapter 4) and it is possible that the larger plants in mixtures had faster growth rates and so were able to support a larger population of virus. This result also raises the possibility that over time, growing the good host *Avena* in mixtures could select for increased virulence of BYDV in mixtures, as suggested by classical virulence-transmission theory (Alizon et al., 2013).

Conclusions

These results suggest that no waning of BYDV epidemics in *Avena*-dominated systems is likely as the atmospheric gases carbon dioxide and ozone continue to rise. Because spillover to poor hosts occurs with amplification of infection in good hosts, the overall strength of infection in the whole community may therefore be increased. More broadly, these results have important implications for the study of disease ecology. They highlight the fact that environmental variables have different effects on the probability of disease establishment and the amount of spread once established. This suggests that it may be fruitful to examine the influence of environmental factors on probability of having an epidemic and size of epidemics separately rather than lumping them into a single parameter quantifying transmission. These results also highlight the potential for amplification (or dilution) not only within space but across time, an aspect of dilution and amplification that has so far seen little attention (but see Becker and Zamudio, 2011). Different peak infection times across species could lengthen the amount of time an epidemic affects a community and therefore increase the impact that disease has on a community (Becker and Zamudio, 2011), and deserves further research.

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CHAPTER 4

Community context alters response to biotic and abiotic stressors and competition between a C_3 and C_4 grass

ABSTRACT

Changes in global atmospheric conditions have the potential to alter competitive interactions between species. Such alterations may be mediated by changes in resources, or by modification of biotic and abiotic stresses. Rising levels of CO_2 increase carbon availability and moisture levels, particularly benefiting C_3 plants. In contrast, rising ozone levels may limit carbon uptake and damage plants. In this paper, we show one of the first examples of the interacting effects of rising CO_2 and rising ozone on direct competition and virus-mediated competition between a C_3 and C_4 grasses sharing a pathogen. We used open top chambers in the field to factorially elevate CO_2 and ozone, and grew *Avena fatua* (a C_3 grass) and *Setaria lutescens* (a C_4 grass) in infected and uninfected monocultures and 50:50 mixtures. *Avena* had higher growth rates and reproductive output in mixtures than in monocultures, but the benefits of mixtures were attenuated under high levels of CO_2. In comparison to *Setaria* growing in monocultures, competition from *Avena* significantly reduced *Setaria* biomass in mixtures across gas treatments. However, CO_2 enhanced reproduction in infected plants. This suggests that the outcome of competition may be altered by changing atmospheric conditions and by pathogens.

INTRODUCTION

Worldwide introductions of species due to human movement patterns, along with uneven movement of species assemblages poleward in response to global warming (Gray and Hamann, 2013), may result in novel competitive interactions, or alterations to competition between coexisting species (Pautasso et al., 2010). Classical competition theory suggests that the outcome of these competitive interactions may be regulated by one or both of two types of processes: bottom up regulation- the efficiency with which the two species obtain resources and convert them into biomass, and top-down regulation -tolerance to or avoidance of either predation or physiological stress (Hairston et al., 1960; Tilman, 1989). For rapidly growing

plants such as grasses, limiting resources typically include space, nitrogen, phosphorus, water, and light (Tilman, 1989), while stressors may include gaseous pollutants such as ozone or biotic stressors such as pathogens or herbivores.

The resource concentrations that may affect the outcome of competition are themselves changing globally. Among those changes, worldwide concentrations of CO_2 have risen nearly 80 ppm since the industrial revolution (Polley et al., 2012). High concentrations of CO_2 are predicted to favor plants with C_3 photosynthesis over plants with C_4 photosynthesis, as high CO_2 levels reduce losses to photorespiration (Polley et al., 2012). In addition, high CO_2 tends to increase soil moisture content, which diminishes the advantage C_4 plants have in water use efficiency (Polley et al., 2012).

The outcome of competition will be affected not only by the acquisition of resources but also by tolerance to environmental stressors. Among those environmental stressors, the atmospheric gas ozone has been on the rise since the industrial revolution (Li et al., 2013). Ozone causes substantial damage to the cell membranes, photosynthetic membranes and pigments of plants (Sandermann, 1996; Li et al., 2013). While plants can close their stomata to avoid ozone, this reduces photosynthetic efficiency. Elevated CO_2 levels are predicted to help offset the costs of avoiding ozone by stomatal closure (Eastburn et al., 2011). In addition, ozone damages stomatal guard cells, ultimately defeating this measure of avoidance (Sandermann, 1996; Hayes et al., 2012). Ozone can have strong effects on competition, and may even intensify the competitive interactions between weeds and important crops such as wheat (Li et al., 2013). Because CO_2 and ozone have interactive effects on physiology, they may jointly regulate the outcome of competition and have been shown to do so in both grasslands (Polley et al., 2012) and forests (Zak et al., 2012).

Top-down regulation of competitive interactions may be determined not only by responses to abiotic stresses such as ozone but by responses to biotic stresses such as pathogens (Dunn et al., 2012). For species that share generalist pathogens, tolerance or resistance to a pathogen can allow a weaker competitor for resources to coexist with a stronger competitor

(Dunn et al., 2012; Power and Mitchell, 2004). However, the reverse is also possible, with pathogens facilitating the invasion of a stronger competitor (Malmstrom et al., 2006; Beckstead et al., 2009). While pathogen-mediated competition may be predicted to interact with competition for resources (Dunn et al., 2012), this interaction has received relatively little attention in the literature. In plants, changing levels of carbon dioxide and ozone may affect the susceptibility to disease, which may in turn affect disease-mediated competition. Carbon dioxide and ozone levels may modify susceptibility directly, or, in the case of ozone, indirectly by priming the defensive systems of the plant (Yalpani et al., 1994; Zuccarini, 2009).

In this study, we explored the effects of both top-down and bottom-up processes on competition in grasses. We examined the effects of rising carbon dioxide and ozone levels on the competitive interactions between two annual grass species sharing a pathogen: the rapidly-growing C_3 plant *Avena fatua* and the slower-growing C_4 plant *Setaria lutescens.* In field experiments we tested the effects of additional resources supplied by CO_2, the abiotic stressor elevated ozone, and the biotic stressor barley yellow dwarf virus (BYDV).

METHODS

Experimental Design

Open-top chambers in the field were used to factorially elevate carbon dioxide (target concentration 680 ppm) and ozone levels (target +70 ppb). Chambers were arranged in 6 blocks, each of which had: 1) a control chamber open to ambient CO_2 and ozone, 2) an elevated CO_2 chamber (target concentration 680-700 ppm) 3) an elevated ozone chamber (target concentration +70 ppb), and 4) a chamber with both gases elevated. Each 2.25 m by 1.5 m chamber was divided into six equally sized sub-plots. Sub-plot treatments included infection status (with and without BYDV) crossed with three plant communities: 1) *Avena* monocultures, 2) *Setaria* monocultures, and 3) a 50:50 mixture of *Setaria* and *Avena*. Further experimental details are provided in Chapter 3. All communities were planted at a total density of 600 plants per square meter on East Ithaca Farm, NY (Cornell University) at the beginning of the first week in June 2010. The soil of the plots was a rapidly-draining sandy loam. Plots were tilled before planting

and fertilized with 20:10:10 NPK fertilizer.

Gas Treatment

Rectangular open top chambers the size of the whole plot and covered with clear vinyl plastic wrap were used to elevate the gases. The sub-plots were individually caged with nets made of white aphid-prevention fabric to control both the aphid movement and reduce the temperature elevations that are common in open top chambers. In each sub-plot, two of the aphid-free sub-plots shared a single large net.

Air was circulated through each chamber by an impeller fan which pushed air down a long aluminum duct and out 8 PVC pipes. Gases were introduced to the pipe leading to each chamber and then spread among the sub-plots with aerated pieces of PVC fitted in the center of the sub-plots. CO_2 fumigation began when the first germinants were detected- at midday on 6 June 2010. Thereafter, gas treatments began at sunrise and ended at sunset each day until harvest. CO_2 measurements were made using a manual NOVA IRGA each dry day. Chamber values were generally repeatable. Ozone fumigation began 25 days after emergence (DAE) and took place 4 hours every afternoon from approx 12:30-4:30 PM. Ozone levels fluctuated more than CO_2 levels, but background levels were usually in the 20-35 ppb range, with levels from approx. 80-150 ppb in the elevated chambers.

Inoculation

Colonies of *Rhopalosiphum padi* were maintained on Romulus barley in growth chambers at 20 degrees C in 24 hr light. We generated infected aphids 14 DAE by transferring 50 adult *R. padi* per sub-plot to dishes containing *Romulus* barley infected with a New York isolate of the PAV species of BYDV and allowing the aphids to feed on the barley for 72 hours. At 17 DAE, virus-treated sub-plots were inoculated by gently sprinkling the aphids randomly throughout the sub-plot.

Virus Sampling

Plants in the inoculated sub-plots were sampled twice for virus during the experiment: 38-40 DAE and 57-60 DAE. At 38 DAE, we sampled and marked 15 plants of every species

planted in that sub-plot, except where plants were too small to sample. Sampling was random throughout the sub-plot, but we made an effort to sample the sides and the center at equal frequency, in case of gradients in gas diffusion. We clipped a piece of green leaf tissue of approximately 0.25 g from each plant. However, in cases where 0.25g was difficult to obtain, we used a whole leaf, and included a piece of stem if needed. Samples were weighed and then frozen for later analysis. We also mock-clipped a selection of uninfected plants to control for the effects of tissue removal.

At 57 DAE, we repeated the procedure, locating the marked plants for a second sampling. In cases where the original plant had died or could not be found, we chose a new plant and gave it a new ID number. We performed the same tissue clipping and freezing procedure as the 38 DAE sample. In addition, at the final harvest we clipped tissue from 15 plants in the uninoculated sub-plots to check for any potential contamination. Proportion of plants infected and within-host viral fitness are reported in Chapter 3.

Harvesting

Between 57 and 60 DAE, marked plants were removed from the sub-plot. Though considerable natural drying occurred by harvest, a final drying was conducted for 12 hours at 60 deg. C. For each plant, vegetative and reproductive tissue was separated and weighed to +/-0.1g. Because some *Setaria* plants had very low biomasses (<0.01 g), we conservatively coded them as 0.009 for analysis.

Statistical Analysis

Avena and *Setaria* aboveground vegetative and reproductive biomasses were Box-Cox transformed and modeled using least squares regression with block as a random effect and CO_2, ozone, mixture, and infection as fixed effects. Models also included all 2, 3, and 4 way interactions of the fixed effects. Although some *Setaria* treatments were difficult to transform to normality, the results of the model were supported by non-parametric Steel Dwass tests. All analyses were carried out in JMP Pro 9.1 (SAS Institute).

RESULTS

Aboveground *Avena* Biomass

Aboveground vegetative biomass in *Avena* was significantly increased by elevated CO_2 ($F=6.10$, $p=0.01$), and by growth in mixture ($F=52.66$, $p<0.0001$). However, the positive response to mixture was decreased under elevated CO_2 ($F=5.07$, $p=0.02$; Figure 4.1). Singly, neither ozone ($F=1.98$, $p=0.16$) nor infection ($F=1.77$, $p=0.18$) had a significant effect on aboveground biomass. In contrast, there was a significant interaction between mixture and infection, such that infected plants grew proportionally better in mixtures than in monocultures ($F=5.25$, $p=0.02$; Figure 4.1).

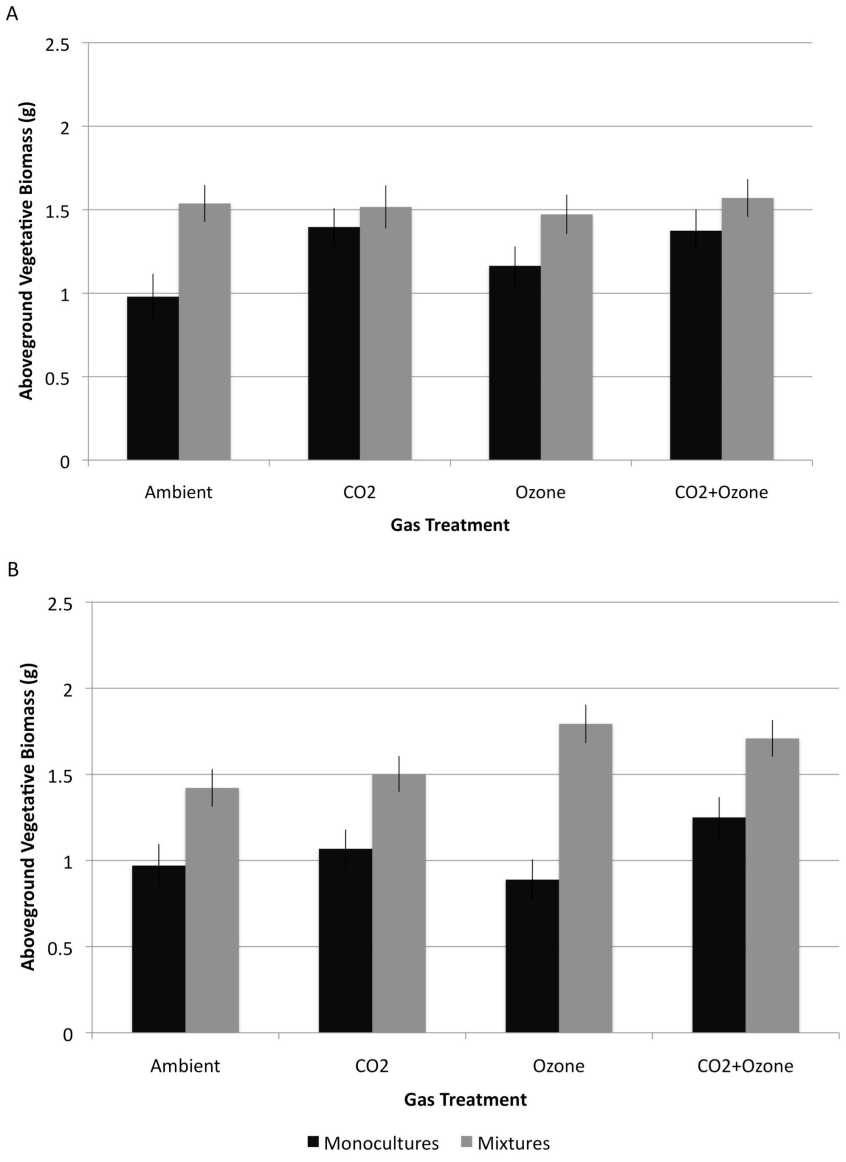

Figure 4.1: Aboveground vegetative biomass in *Avena* in uninfected plants (A) and infected plants (B). Black bars indicate vegetative biomass in monocultures while gray bars indicate biomass in mixtures. While growth in mixtures significantly enhances aboveground vegetative biomass, the growth benefits of mixtures are least under high CO_2. Infected plants grow proportionally better in mixtures.

Reproduction in *Avena*

In contrast to vegetative biomass, elevated CO_2 had no significant effect on reproductive biomass in *Avena* ($F=0.44$, $p=0.50$), while ozone had a slight but significant negative effect ($F=4.08$, $p=0.04$). Adding CO_2 to the ozone reversed this pattern ($F=9.18$, $p=0.003$). Similar to the vegetative biomass, growth in mixtures significantly enhanced reproduction in *Avena* ($F=18.78$, $p<0.0001$), but elevated CO_2 diminished this benefit ($F=10.39$, $p=0.001$). Ozone modified the effect of mixtures and infection: under elevated ozone, there was a greater difference between infected and uninfected plants in mixtures than under ambient ozone ($F=6.30$ $p=0.01$, Figure 4.2).

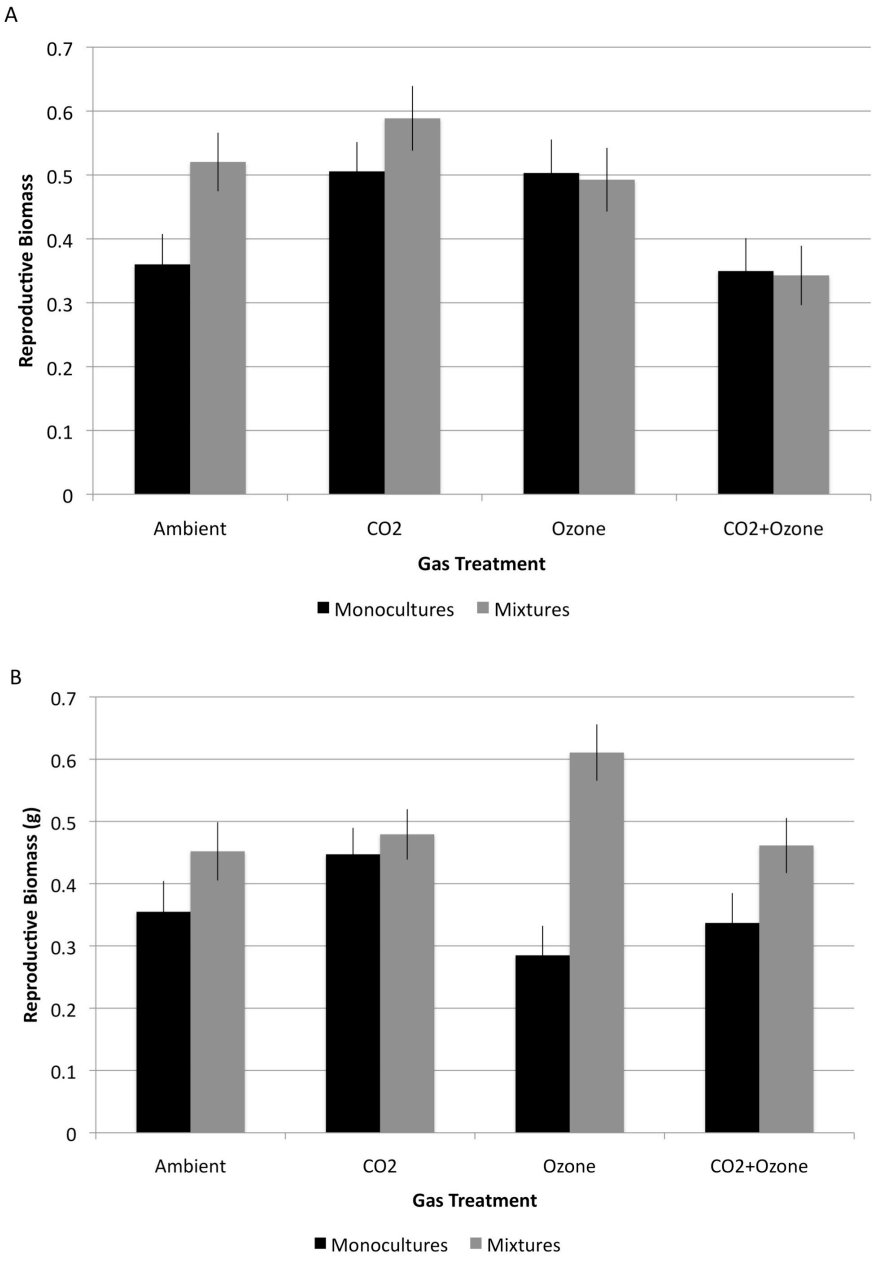

Figure 4.2: Reproductive biomass in *Avena* in uninfected plants (A) and infected plants (B). Black bars indicate reproduction in monocultures while gray bars indicate reproduction in mixtures. Growth in mixtures significantly increases reproduction. While CO_2 reduces the reproductive benefits of mixture, ozone enhances the benefits of mixture among infected plants.

Aboveground *Setaria* Biomass

Growth in mixtures significantly reduced aboveground vegetative biomass in *Setaria* ($F=164.54$, $p<0.001$, Figure 4.3). Elevated CO_2 and infection did not significantly affect aboveground vegetative biomass. There was a marginally significant trend of reduced biomass with elevated ozone ($F=3.60$, $p=0.0577$). None of the interactions between factors significantly affected aboveground biomass.

Reproductive biomass in *Setaria* was also significantly reduced in mixtures ($F=186.63$, $p<0.001$, Figure 4.4). High CO_2 significantly increased the fitness of infected plants ($F=3.89$, $p=0.049$; Figure 4.4), but adding ozone eliminated this pattern, as demonstrated by the significant 3 way interaction between CO_2, ozone, and infection ($F=4.15$, $p=0.042$).

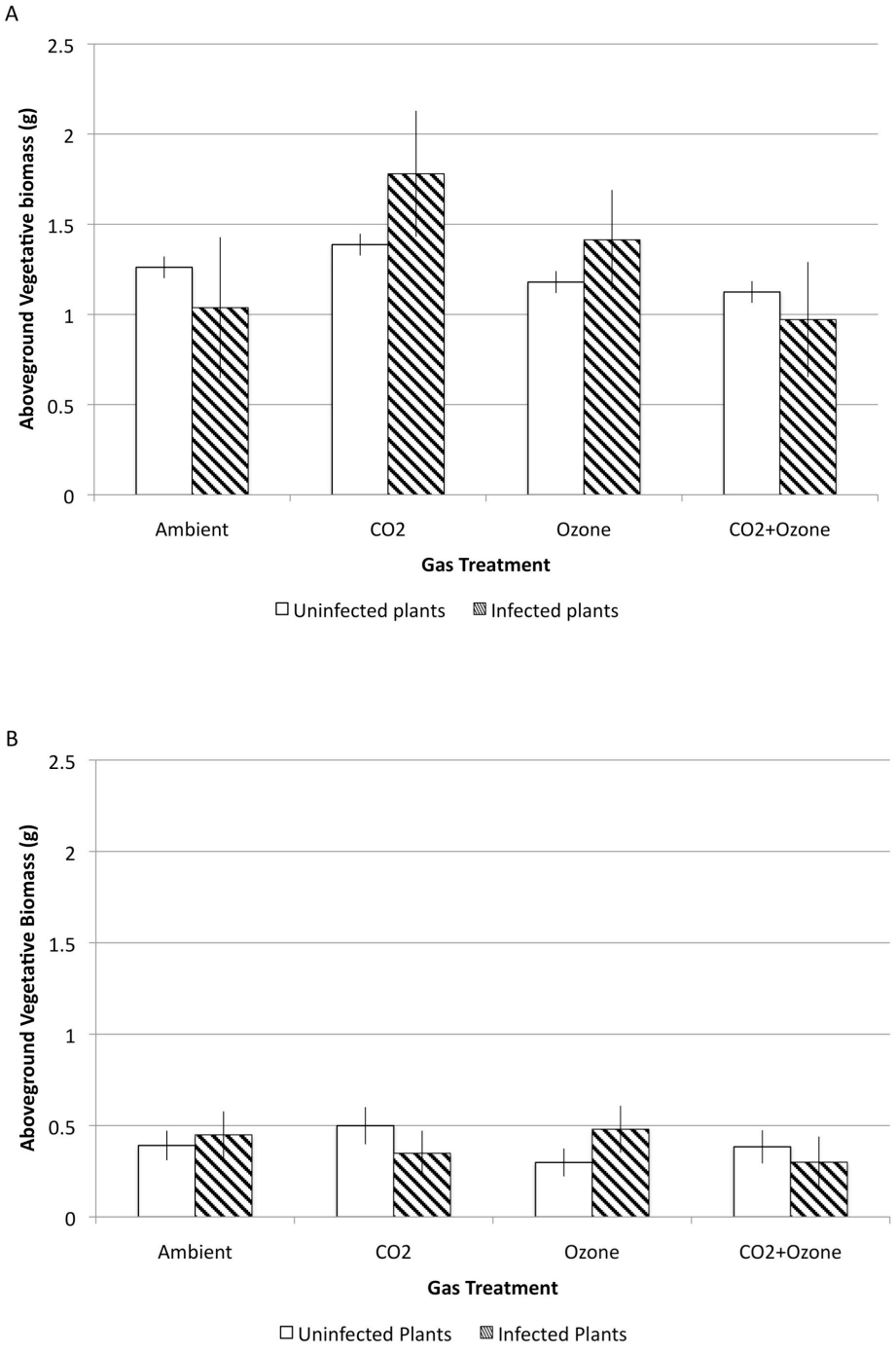

Figure 4.3: Aboveground vegetative biomass in *Setaria* in monocultures (A) and mixtures (B). Open bars indicate uninfected plants while hatched bars indicate infected plants. Growth with *Avena* significantly reduces *Setaria* biomass.

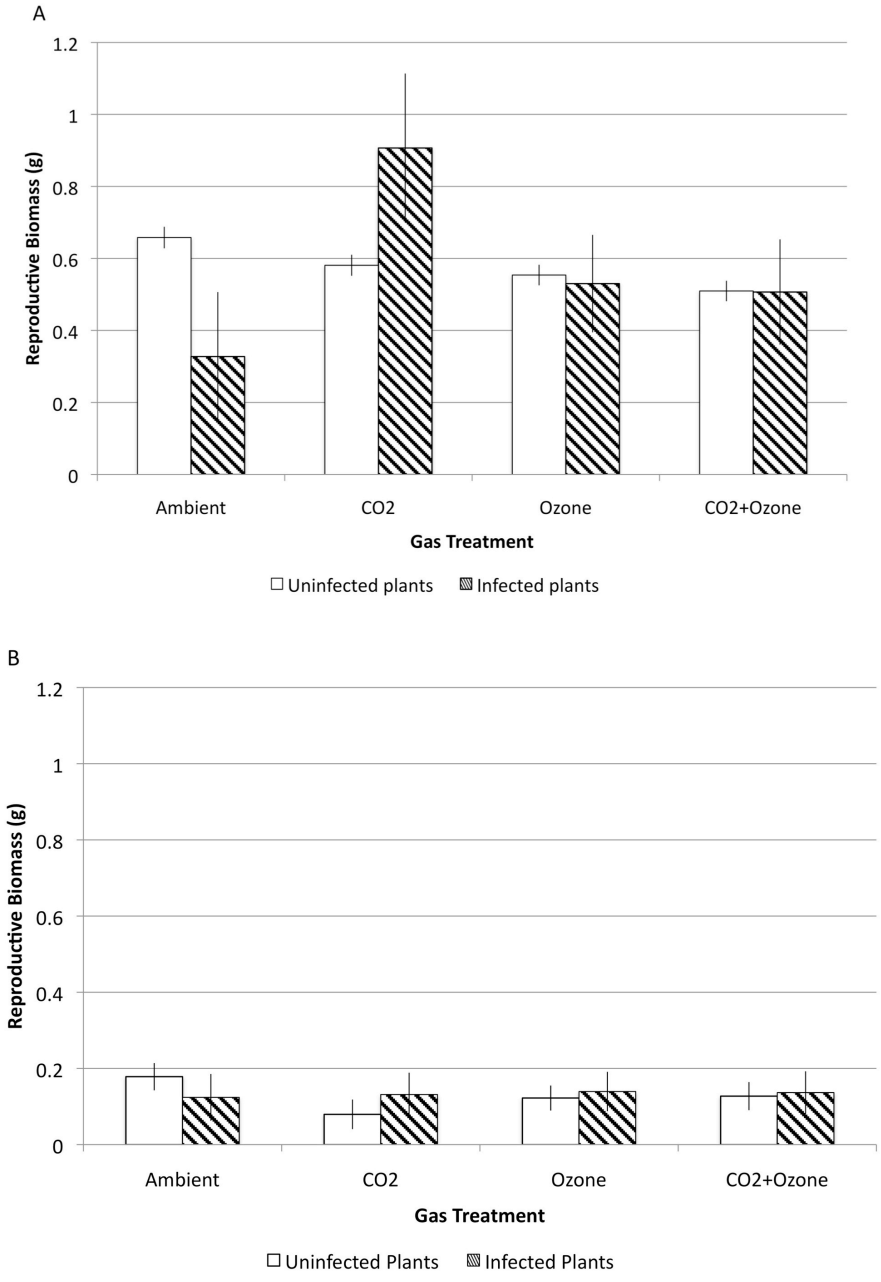

Figure 4.4: Reproductive biomass in *Setaria* in monocultures (A) and mixtures (B). Open bars indicate uninfected plants while hatched bars indicate infected plants. Growth in mixtures significantly reduced reproduction. However, under high CO_2, infected plants had higher reproductive output than uninfected plants, for both monocultures and mixtures.

DISCUSSION

Top-down and bottom up processes may regulate the outcome of competition between a C_3 and C_4 plant under current and future atmospheric conditions. Biomass of *Avena* and *Setaria* was significantly affected by interactions between competition, infection, and atmospheric gas treatment.

Effects of Competition on *Avena*

Avena individuals grew larger in mixtures, regardless of infection (Figure 4.1), suggesting that interspecific competition from *Setaria* is less intense than intraspecific competition. This idea is consistent with models of competition that predict that organisms of the same species and occupying the same ecological niche will compete intensely for resources within that niche (Tilman, 1987). This pattern is also consistent with work suggesting that plants in polycultures are more productive than in monocultures because they partition resources effectively (e.g. Postma and Lynch, 2012). However, in successful agricultural polycultures, both species benefit from the resource partitioning, whereas *Setaria* did not benefit from growing in mixtures with *Avena* (Figure 4.3). Interestingly, for vegetative biomass, growing in mixtures had a smaller beneficial effect on growth under elevated CO_2, though overall CO_2 had a positive effect on vegetative growth. This is surprising as we expect a benefit to C_3 plants, including *Avena,* under high CO_2, (Ziska, 2001; Polley et al., 2012), and, in fact, weeds such as *Avena* often benefit from CO_2, especially when grown with a C_4 crop (Ziska, 2001). While there is increasing evidence from grasslands that the benefits of CO_2 to grasses vary substantially from year to year (Zavaleta et al., 2003; Polley et al., 2012; Leadley et al., 1999), this reduction in the benefits of mixtures under high CO_2 suggests that other resources become limiting in mixtures. While CO_2 itself may not be limiting in *Avena* monocultures, water availability might be (Tilman, 1987). Water availability is enhanced under high CO_2 in coarse textured soils (Polley et al., 2012) such as the soil in this study, raising the possibility that water is less limiting in monocultures under high CO_2, and therefore reducing competition for water in mixtures under high CO_2 has less effect on growth. Consistent with these results, elevated CO_2 facilitated

annual grass invasion in a desert ecosystem during a very wet year (Smith et al., 2000). More broadly, these results suggest that the effects of elevated resources such as CO_2 depend on the community context.

Equally surprising was the minimal effect of ozone on the vegetative and reproductive biomass of *Avena*. However, a recent study suggests that weedy species such as *Avena* may do better under high ozone conditions, especially when in competition with other species (Li et al., 2013). In this study, ozone enhanced the gap between reproduction in mixtures and monocultures of infected plants (Figure 4.2). This suggests that there may be a synergistic interaction between the three stressors of ozone, infection, and intraspecific competition. Reducing intraspecific competition then has a proportionally larger effect on infected biomass under ozone, further emphasizing the importance of community context in regulating plant responses to combinations of biotic and abiotic stressors.

Community context alters not only response to resources but also response to infection, as vegetative biomass in infected *Avena* was higher in mixtures than in monocultures. This may indicate that the stress imposed by intraspecific competition can exacerbate the effects of a virus that plants can tolerate in a more diverse community. The rapid growth of *Avena* in mixtures could have important ecosystem-level consequences. Given that *Avena* is a common invader of western grasslands (Going et al., 2009), its high vegetative biomass in mixtures could disrupt ecosystem fire regimes, as the large amount of fuel it produces could amplify the frequency and intensity of fires, as has been demonstrated for cheatgrass (*Bromus tectorum*), another rapidly growing annual grass in the western US (Brooks et al., 2004). Our work suggests that this may be of particular concern in partially invaded areas where *Avena* is still growing with some slower-growing competitors.

Effects of Competition on *Setaria* Biomass

Setaria individuals were significantly smaller in mixtures (Figure 4.3) across all gas and infection treatments, as might be expected for a slow-growing C_4 plant unlikely to benefit from an increase in CO_2 (Ziska, 2001). *Setaria's* growth was not significantly stunted by elevated

ozone nor benefited by CO_2 alone, as we might expect given the lower stomatal conductances for a C_4 plant (Ocheltree et al., 2012).

Setaria reproduced more poorly in mixtures, suggesting that its slow growth makes it a poor competitor with fast-growing species like *Avena*. Interestingly, high CO_2 did not significantly reduce *Setaria*'s competitive ability, despite the expectation that high CO_2 will favor C_3 plants (Polley et al.,2012; Ziska, 2001). Indeed, under high CO_2 there was an increase in fitness due to infection in monocultures but not in mixtures, though adding ozone to the CO_2 eliminated this pattern (Figure 4.4). This raises the possibility that *Setaria* may become less reproductively limited in high infection years as the global CO_2 level rises. Moreover, because *Setaria* only sustains an epidemic when grown with a good viral host such as *Avena* (Chapter 3), this gap in fitness is only likely to be observed when *Setaria* is grown with a good host. Therefore, this increase in fitness due to infection may enhance *Setaria's* ability to compete reproductively with *Avena* under future high global levels of CO_2, especially in low ozone areas such as the rural areas and unmanaged grasslands where *Setaria* is common. This pattern could enhance *Setaria*'s competitive ability at the establishment stage by increasing its contribution to the seed bank and thus promote coexistence between the two species. This suggests that an interaction between atmospheric change and infection could supply an important mechanism for maintaining diversity.

Conclusions

These results highlight the way that resources and stressors can modify the effect of community context on competition, suggesting the importance of studying the interactions between biotic and abiotic changes for determining the outcome of species competition under climate change. In addition, they suggest that under future atmospheric conditions, competition between a C_3 and a C_4 grass may be regulated not only by their respective responses to CO_2 but by their tolerances to other environmental pollutants and to shared predators or pathogens. Under future atmospheric conditions, pathogens are likely continue to play a large- and perhaps even increasing – role in determining the outcomes of such competitions. As species continue

to shift ranges due to warming, predicting the outcome of competition under future atmospheric conditions will require attention to effects on both biotic and abiotic stressors on species interactions.

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APPENDIX

Effects of elevated CO_2 and ozone on aphid mortality and population growth rates

Because elevated CO_2 and ozone have been shown to affect aphid abundance in some species of aphids (Finlay and Luck 2011; Percy et al., 2002), I conducted a study to determine the effects of the gases on aphid growth and mortality rates.

METHODS

Experimental Design

Open-top chambers in the field were used to factorially elevate carbon dioxide (target concentration 680 ppm) and ozone levels (target +70 ppb). Chambers were arranged in 6 blocks, each of which had: 1) a control chamber open to ambient CO_2 and ozone, 2) an elevated CO_2 chamber, 3) an elevated ozone chamber, and 4) a chamber with both gases elevated. Each 2.25 m by 1.5 m chamber was divided into six equally sized sub-plots. Sub-plots were enclosed with white aphid-prevention fabric (Optinet).

Planting

On 17 to 19 August we filled 6 in standard greenhouse pots with soil from East Ithaca Farm. We planted 3 seeds per pot, and placed 3 pots in each of the 144 aphid-net enclosed sub-plots mentioned above.

Gas treatment

Rectangular open top chambers the size of the whole plot and covered with clear vinyl plastic wrap were used to elevate the gases. The sub-plots were individually caged with nets made of white aphid-prevention fabric. In each sub-plot, two of the aphid-free plots shared a single large net.

Air was circulated through each chamber by an impeller fan which pushed air down a long aluminum duct and out 8 PVC pipes. Gases were introduced to the pipe leading to each chamber and then spread among the quadrants with aerated pieces of PVC fitted in the center of each of the sub-plot. CO_2 fumigation began at sunrise and ended at sunset each day. CO_2

measurements were made using a portable NOVA IRGA each dry day. Chamber values were generally repeatable. Ozone fumigation occurred for 4 hours every afternoon from approx 12:30-4:30 PM . Ozone levels fluctuated more than CO_2 levels, but background levels were usually in the 20-35 ppb range, with levels from approx. 80-150 ppb in the elevated chambers.

Inoculation

Colonies of *Rhopalosiphum padi* were maintained on Romulus barley in growth chambers at 20 degrees C in 24 hr light. We generated infected aphids on 3 September 2010 by transferring 200 adult *R. padi* per sub-plot to dishes containing *Romulus* barley infected with a New York isolate of the PAV species of BYDV and allowing the aphids to feed on the barley for 48 hours. We also transferred aphids to uninfected barley for 48 hours and allowed them to feed. On 5 September 2010, plants were transplanted to 3 per pot. In each pot, one plant received an infected aphid, one an uninfected aphid, and one no aphid at all. Aphids were gently placed on plants with a paintbrush and then caged on the plants. Cages were made of narrow plastic tubes with mesh on the top to allow gas exchange.

Aphid Counts

Fifteen days after aphids were placed on plants, I counted the number of adults and nymphs on each plant. If no aphids were found, I assumed that the aphids had died on the plant in the interval. I computed mortality as the number of plants on which no aphid was found at final harvest.

Statistical Analysis

Numbers of adults and numbers of nymphs for all plants where aphids survived were each modeled using a Poisson distribution. The model contained Block (which cannot be a random effect in these models), CO_2, ozone, and infection as fixed effects, and also examined the two and three way interactions of CO_2, ozone, and infection. Main effects are evaluated using likelihood ratio χ^2 tests. Proportion of plants where the aphid died used a similar model, but this model used a binomial distribution to model the proportion dead. All statistical analyses were carried out in JMP Pro 9 (SAS Institute).

RESULTS

Number of adult aphids after two weeks was not significantly affected by CO_2 (χ^2=2.15, p=0.14) or ozone (χ^2=0.14, p=0.71; Figure A1A). Infection did significantly reduce number of adults (χ^2=6.59, p=0.01). None of the two or three way interactions had a significant effect on number of adult aphids. Neither CO_2 (χ^2=0.1, p=0.76), nor ozone (χ^2=1.8, p=0.18), nor infection (χ^2=3.7, p=0.054) had a significant effect on number of nymphs (Figure A1B). None of the two or three way interactions had a significant effect on number of nymphs produced.

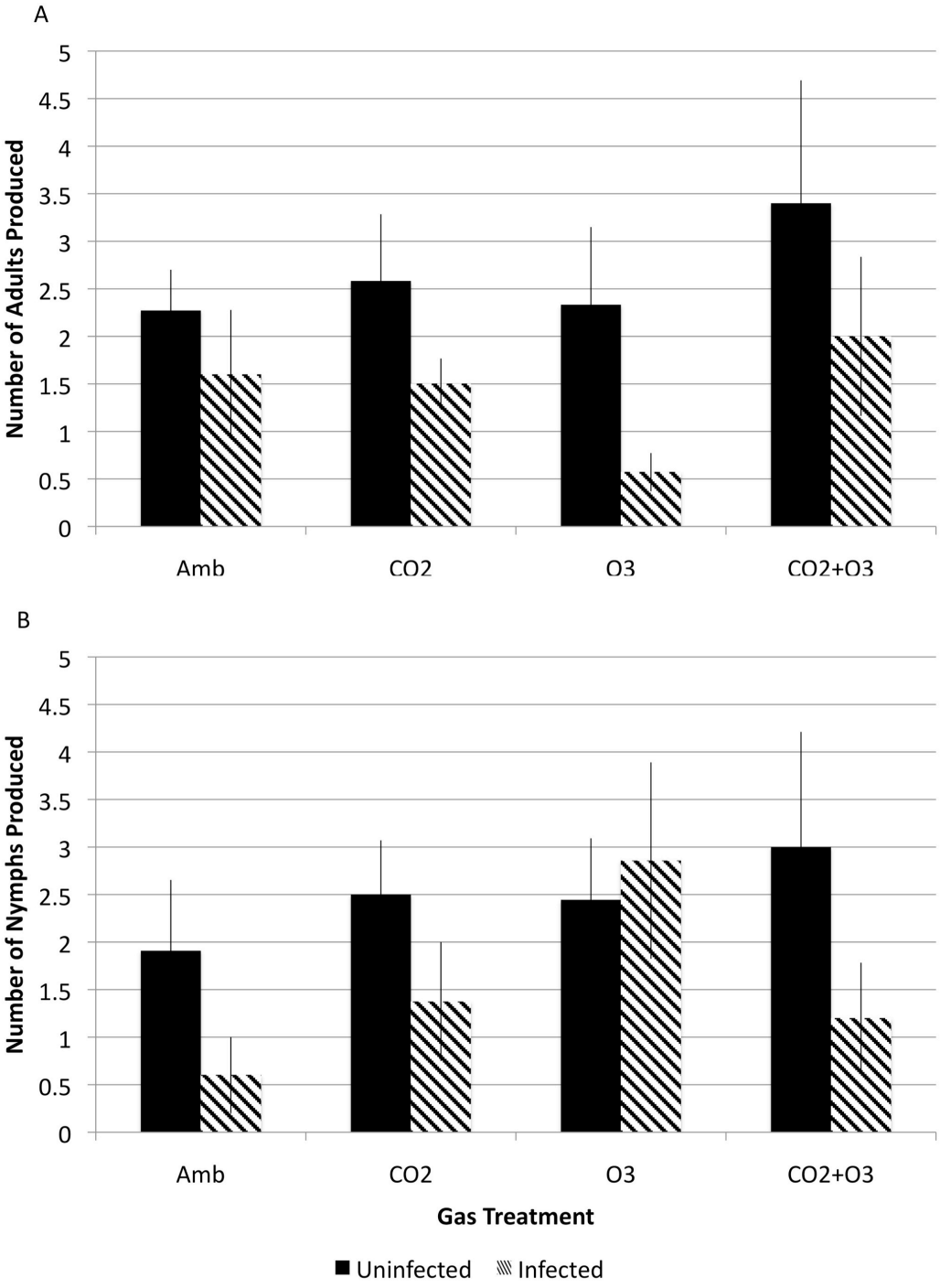

Figure A1: Number of adult aphids (A) and number of nymphs (B) in surviving colonies across gas and infection treatments. Solid bars indicate uninfected aphids while hatched bars indicate infected aphids. Error bars represent the standard error of the mean.

Proportion of plants where the aphid died was not significantly affected by CO_2 (χ^2=0.07, p=0.8) or ozone (χ^2=0.0005, p=0.99; Figure A2). Infection significantly increased the proportion of colonies in which the colonizing aphid died (χ^2=11.53, p=0.0007).

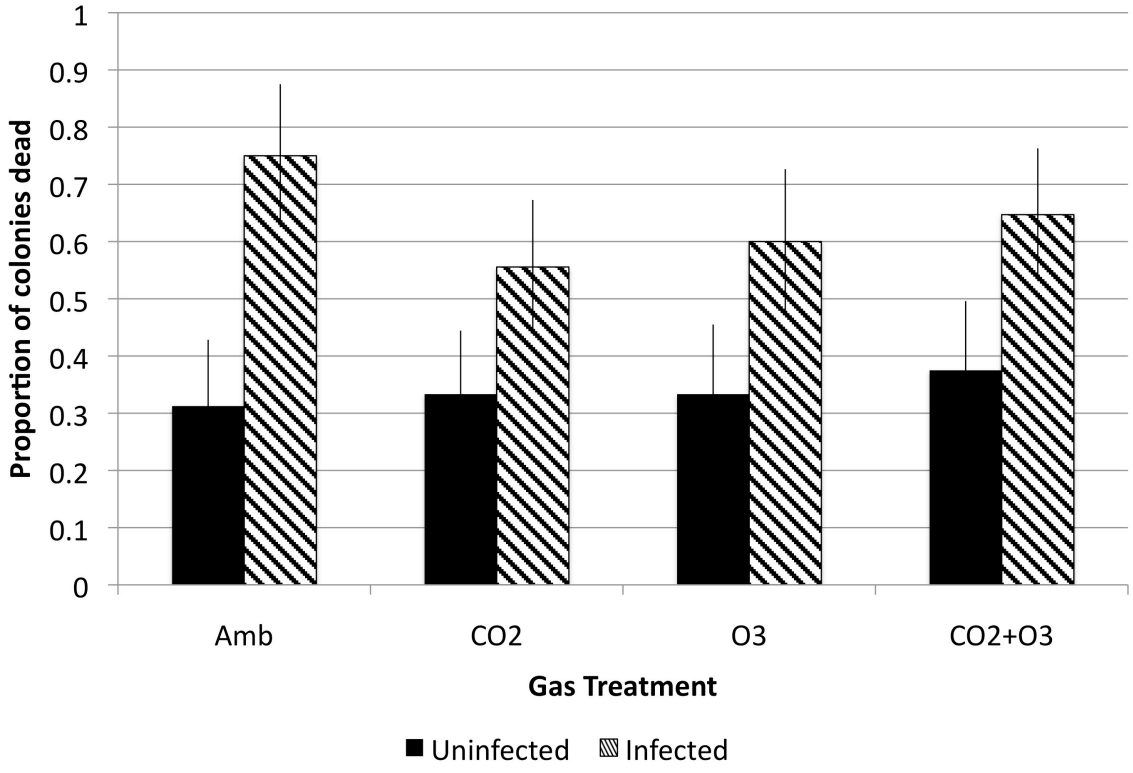

Figure A2: Proportion of aphid colonies dying out on plants. Solid bars indicate uninfected aphids while hatched bars indicate infected aphids. Error bars represent the standard error of the mean.

DISCUSSION

Unlike Percy et al.'s results (2002), which showed some aphid species more abundant in both high CO_2 and high ozone, our results suggest no significant effects of gas treatment on aphid growth or mortality. This may not be surprising, as aphid responses to elevated CO_2 do

vary across species (Newman et al, 2003). Even *Rhopalosiphum padi* the species used in this study, shows variation in responses across studies (Finlay and Luck, 2011). Infection did seem to increase mortality and reduce growth rates. However, as only two people were available for inoculation in the field, one person handled infected aphids and the other uninfected aphids, to reduce contamination. While the person handling infected aphids had more experience, the potential for bias due to aphid handling causes me to treat these results cautiously. These results do raise the possibility that the infection could negatively impact the aphids. However, such a negative effect is surprising, given reports that aphids benefit from growth on infected plants (Bosque-Pérez and Eigenbrode, 2011). In view of these considerations, I think it is unlikely that the transmission patterns observed in Chapters 2 and 3 were a result of aphid growth or mortality differences.

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