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IMPACT OF OZONE ON MILKWEED (*ASCLEPIAS*) SPECIES

A Thesis in

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Abstract

Tropospheric (or ground level) ozone in ambient concentrations can damage vegetation and interfere with the human respiratory system. Plants as bioindicators of ozone are commonly used to detect phytotoxic levels of tropospheric ozone where physical/chemical/electrical monitoring equipment cannot be utilized due to expense, electrical needs, or availability of instruments. *Asclepias syriaca* (Common Milkweed) has been effectively used as a bioindicator for ozone. Visual ozone injury on Common Milkweed is characterized as purple stippling of the upper surface of older leaves as the season progresses, the purple coloration of the upper leaf surface may encompass most of the leaf surface. While sensitivity to ozone has been documented on Common Milkweed, less is known about the ozone sensitivity of other *Asclepias* species and little is known regarding the concentration dose response of Common Milkweed to ozone and timing of visual symptoms.

Of the *Asclepias* species evaluated Tropical Milkweed (*A. curassavica*) had the greatest ozone injury throughout the experiment, suggesting that the species might be a more sensitive bioindicator to ozone than Common Milkweed. However, high levels of leaf loss during the season may negate its usefulness as a bioindicator. Levels of ozone injury on Swamp Milkweed (*A. incarnata*) were not significantly different from the standard Common Milkweed, and with the exception of the afore mentioned, ozone injury values were typically greater than observed on Common Milkweed. This suggests that ozone injury on Swamp Milkweed develops more quickly or at a greater amount than on Common Milkweed. However, because the injury values were not significantly different, Swamp Milkweed may be a useful ozone-sensitive bioindicator species in the field. The other milkweed species Showy Milkweed (*A. speciosa*), Prairie Milkweed (*A. sullivantii*), and Spider Milkweed (*A. viridis*), developed distinguishable ozone injury symptoms (including dark stippling), and non-significantly different injury ratings from Common Milkweed, suggesting that these species are comparable and may be useful as field bioindicators in addition to or in lieu of Common Milkweed.

The amount of injury on Common Milkweed rose at an increasing rate over time and with higher levels of ozone (e.g. 60-90 ppb). Plants exhibited phytotoxic injury at 60 ppb, below the current NAAQS of 70 ppb ozone, which may indicate that the current standard may not be strict enough. Plants exposed to 90 ppb ozone developed injury, although less severe, at the same time as those plants exposed to 90ppb ozone. This result combined with the curvilinear relationship described above indicates that Common Milkweed remains a viable indicator at this level of phytotoxic ozone.

Table of Contents

List of Figures

List of Tables

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Introduction

Ozone is a colorless gas that exists throughout the earth's atmosphere. Stratospheric ozone is considered beneficial, since it filters out dangerous UV light (US EPA 2003, 2012b). In contrast, tropospheric (ground level) ozone is harmful because it damages vegetation and harms people via their respiratory system (US EPA 2003, 2012b). The United States Environmental Protection Agency (EPA) sets national ambient air quality standards (NAAQS) in order to protect against this ground-level pollutant. Other U.S. agencies, such as the USDA Forest Service has programs such as the Forest Inventory Analysis (FIA) program that uses bioindicator plants to detect the presence of phytotoxic levels of ambient ozone. Adaxial stippling, the presence of dark spots on the upper leaf surface, is the specific symptom ozone indicator plants exhibit (Richards et al. 1958). The use of bioindicator plants is a useful inexpensive way to monitor air quality in areas where physical/chemical/electrical monitoring equipment cannot be located.

One plant species commonly used as a bioindicator to detect phytotoxic levels of ozone is *Asclepias syriaca* L. (Common Milkweed). Common Milkweed belongs to the dogbane family Apocynaceae, sub-family Asclepiadaceae, and is native to North America. This subfamily is most known for the milky latex sap contained within milkweed plants. The latex sap contained in milkweed is important for *Danaus plexippus* Linnaeus (Monarch Butterfly) because it contains various glycosides (USDA NRCS 2011b). Monarch caterpillars ingest milkweed and store the glycosides within their tissues indefinitely, which makes them unpalatable to predators (USDA NRCS 2011b). In recent years, populations of Monarch Butterflies declined in the U.S., possibly due to stresses to the milkweed plants. We hypothesize that ozone may be such a stress. In addition, certain milkweed species have been utilized by people for fiber, food, medicine, and tools.

The objective of this study was to evaluate the ozone sensitivity of five milkweed species, and compare the response with Common Milkweed to determine if other milkweed species would also be useful as bioindicators to detect phytotoxic levels of ozone. A second objective was to determine if Common Milkweed could detect phytotoxic concentrations of ozone at low ozone levels, thus serving as a useful bioindicator of very low levels of ambient ozone.

Methods

Two main studies were conducted, in 2013 and 2014. In, 2013, six milkweed species were exposed to ozone concentrations ranging from 0 to 120 ppb and foliar response compared. Species included Common Milkweed, *A. curassavica* L. (Tropical Milkweed), *A. incarnata* L. (Swamp Milkweed), *A. speciosa* Torr. (Showy Milkweed), *A. sullivantii* Engelm. ex A. Gray. (Prairie Milkweed), and *A. viridis* Walter (Spider Milkweed). In 2014, Common Milkweed was exposed to ozone levels ranging from 0 to 90 ppb. In addition, a preliminary study was conducted in 2012, as described below.

Facilities

Experiments were conducted within a greenhouse located on the campus of The Pennsylvania State University, University Park, Pennsylvania (Figs. 1&2). The greenhouse was equipped with a charcoal filtration air supply system, designed to maintain internal, background ozone concentrations at ca. 5 ppb. The greenhouse housed 16 continuously stirred tank reactor chambers (CSTR), which were 1.5 m diameter and 1.5 m high (Heck et al. 1975). Chambers were connected to an ozone generator and computer monitoring system that measured, controlled, and displayed ozone concentrations.

Sunshine professional growing mix #1 (sun gro® Agawam, MA) was used as a soil medium for all plants during both years. Pots and trays were sterilized before use with a 10% bleach solution, and plants were watered as needed. During both years, plants were sprayed with Conserve® SC (Dow Chemical, Indianapolis, IN) and Pylon® SC (BASF, Research Triangle Park, NC) to control thrips (Order Thysanoptera).

Fall 2012 Exposure Study

A preliminary ozone study was conducted during fall 2012 in order to determine the experimental protocol for the main 2013 and 2014 milkweed species exposure studies. In 2012, the following milkweed species were exposed to ozone: *Asclepias exaltata* L. (Poke Milkweed), *A. hirtella* (Pennell) Woodson (Tall Green Milkweed), *A. ovalifolia* Decne, *A. tuberosa* L. (Butterfly Milkweed), *A. verticillata* L. (Whorled Milkweed), Swamp Milkweed, Showy Milkweed, Common Milkweed, and Spider Milkweed were selected for study. Plants were exposed to 0, 75, and 120 ppb ozone to evaluate ozone sensitivity as indicated by adaxial leaf stippling. This initial study revealed that Tall Green Milkweed, Butterfly Milkweed, Whorled Milkweed, and *Veronia* sp. were tolerant to ozone and would not be included in the 2013 and 2014 studies. In contrast, Poke Milkweed, Swamp Milkweed, Olive-leaf Milkweed, Showy Milkweed, Common Milkweed, and Spider Milkweed did exhibit ozone-induced adaxial stippling and premature senescence following exposure to ozone and would be exposed in future studies.

2013 Experimental Procedures

In November 2012, Tropical Milkweed, Poke Milkweed, Oval-leaf Milkweed, Showy milkweed, Prairie Milkweed, and Spider Milkweed seeds were purchased from Butterfly Encounters (San Ramon, CA). Common Milkweed and Swamp Milkweed were acquired from Prairie Moon Nursery (Winona, MN). Seeds of each species were placed into 1.2 mL plastic bags containing approximately 20 g soil and moistened with water. Seeds were cold-stratified in a refrigerator at ca. 3°C, for 6 weeks, at which time seeds were placed in 25.4 x 50.8 cm trays for germination. Trays were partially filled with soil, seeds spread on the surface, covered with potting mix, and watered. Trays were placed within plastic bags for 2 weeks to maintain high humidity during germination. After germination, seeds were replanted individually into cell-pack seed trays containing 72 cells per tray. Plants were repotted individually 3 months later into 1.9 L pots. Approximately 1 g of Scotts® Osmocote Plus fertilizer (15-9-12) (Scotts Miracle-Gro, Marysville, OH) was applied to the soil surface. Two weeks after repotting, some plants exhibited slight fertilizer burn; therefore, distilled water was added to reduce excess salts.

To allow for acclimation to the chamber environment, milkweed plants were placed into CSTRs 3 days before the ozone exposure. Exposures were initiated in July 2013, at which time plants were full-grown and near or at flowering. One plant per species was placed in each of the 16 chambers, where they were exposed to 0, 40, 75, or 120 ppb ozone, with four chambers set at each target concentration. The experimental treatments consisted of four concentrations with four replicates of each concentration. Control chambers were set at a target concentration of 0 ppb ozone. The level of 40 ppb was used to mimic normal ambient tropospheric concentrations of ozone. A concentration of 75 ppb ozone was used since it represented the 2013 NAAQS for ozone at the time of the study. The greater 120 ppb concentration was used to ensure the induction of foliar injury symptoms, to allow comparison of foliar injury induced by lower ozone levels.

Ozone exposures were initiated 15 July and continued 5 days per week (Mon. – Fri.) from 8:00 AM to 4:00 PM in a square wave design. After each daily exposure, ozone was then terminated for the next 16 hours. The experiment was run for 4 weeks (i.e., 20 exposure days), and injury symptoms were evaluated and recorded each week as described in the next paragraph.

Ozone induced foliar injury on each plant was visually assessed using a modified Horsfall-Barratt scale (Horsfall and Barratt 1945). Injury classes ranging from 0 to 5. As follows: 0 = no visible injury, 1 = 1-6%, 2 = 7-25%, 3 = 26-50%, 4 = 51-75%, and 5 ≥75% injury. Six leaves (of varying age) per plant were randomly selected and tagged for identification to follow visual evaluations over time. Each of the six leaves was evaluated and rated for leaf-level severity, using the 0-5 scale. To provide a single injury value for statistical analyses, the midpoint of each class was calculated as the mean of the minimum and maximum values. For example, the mid-point for class 2 = (7% + 25%)/2 = 16%. Early leaf abscission was rated as 100% severity. The overall amount of ozone injury on the plant was then determined as the percentage of the six leaves that contained a severity rating of 1 or greater. In order to produce a plant-level injury rating, the overall injury rating (INJ) was calculated as the product of the mean of the six leaf-level severity ratings (SEV) and the amount of injury at the plant level (AMT) as follows: SEV % x AMT % = INJ %. An ANOVA was run, using species as a factor, for each ozone concentration and time period. Dunnett's multiple comparison test (Dunnett 1955) was used to find significant differences (p-value <0.05) in injury values of the milkweed species from the control group Common Milkweed. Data were analyzed using MINITAB® Student Version Release 14.11.1.

2014 Experimental Procedures

In 2014, only Common Milkweed was exposed to ozone. Seeds were prepared and germinated as in 2013, and replanted individually into larger containers. Miracle-Gro® Liquid All-Purpose Plant Food (Scotts Company, Marysville, OH) (12-4-8), was applied weekly to the potting soil at a rate of 0.05 L fertilizer/ 7.5 L water.

Plants were individually re-potted in July 2014 into 1.9 L pots. Plants were full-grown, but not yet flowering. Beginning 17 July, plants were exposed to ozone for 8 hours starting at 8:00 AM in a square wave design. Exposures continued 6 days a week, for 3 weeks (i.e., 18 fumigation days). Treatments consisted of four concentrations with two replicates at each concentration. Control chambers were set at a target of 0 ppb ozone, and 30 ppb was used to mimic background ambient ozone concentrations. The proposed lowered NAAQS ozone concentration, a level of 60 ppb ozone, which was lower than the proposed 2016 NAAQS for ozone was selected (National Ambient Air Quality Standards for Ozone 2010). The greater concentration of 90 ppb ozone was selected in order to ensure symptom development for comparison to that induced by lower concentrations.

Plants were tagged and visually assessed according to the 2013 protocol. A multiple regression analysis was performed using injury values, ozone concentration, and exposure time. Data were analyzed using SAS's Proc Reg (Freund et al 1991).

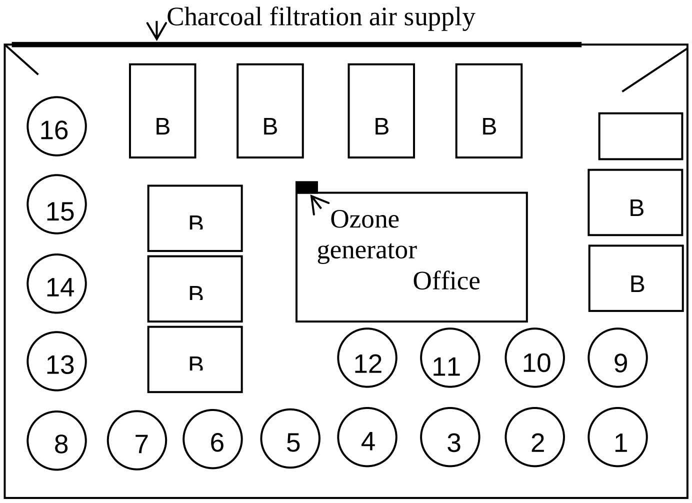

Figure 1. 2012-2013 schematic of greenhouse and ozone exposure system. Numbered circles denote individual CSTRs. B denotes work benches. All 16 chambers were utilized in the 2013 study.

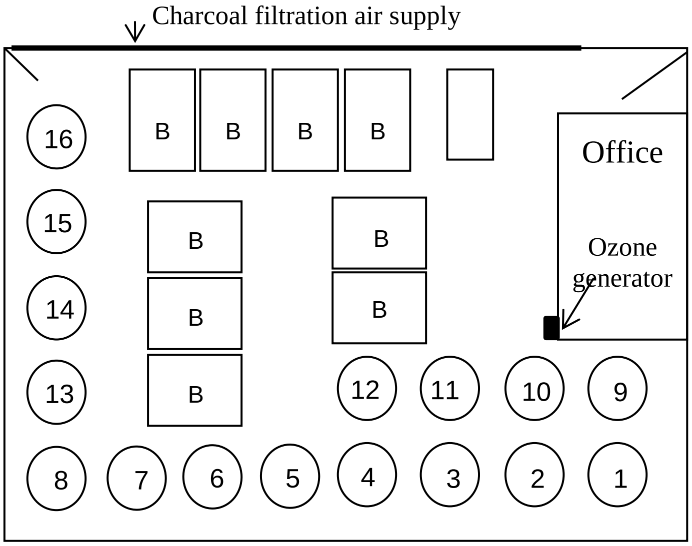

Figure 2. 2014 schematic of greenhouse ozone exposure system after remodel. Chambers 1-8 were utilized in the 2014 study.

Results

2013 Exposure

Tropical Milkweed had the overall greater injury ratings throughout the experiment, as well as the highest defoliation rate (Table 2). No visible ozone-induced injury symptoms were observed on plants in the 0 ppb ozone (control) chambers throughout the study.

After one week of exposure, no symptoms developed on plants exposed to 40 and 75 ppb ozone. Common Milkweed, Showy Milkweed, and Spider Milkweed developed injury symptoms following exposure to 120 ppb ozone (Table 1). However, Showy Milkweed and Spider Milkweed mean injury values were not significantly different from those of the standard comparison Common Milkweed.

After 2 weeks of exposure, injury was still not observed on plants exposed to 40 ppb ozone. All species except Prairie Milkweed developed injury at 75 ppb, but ratings were not significantly different from those of Common Milkweed. All species developed injury at 120 ppb by this point, but none were significantly different from the injury ratings of Common Milkweed.

After week 3 of exposure to 40 ppb ozone Tropical Milkweed developed injury which was significantly different from Common Milkweed. All species developed injury following exposure to 75 and 120 ppb ozone. At 75 ppb, Tropical Milkweed was the only species that had significantly different injury from that of Common Milkweed. At 120 ppb Tropical Milkweed and Swamp Milkweed had significantly different injury values as compared to those of Common Milkweed.

After 4 weeks of exposure, Spider Milkweed was the only species that did not develop ozone injury after exposure to 40 ppb ozone. Tropical Milkweed was the only species that had a mean injury rating significantly different at 40 and 75 ppb from the standard comparison Common Milkweed. No significant differences in injury values among the species to the standard Common Milkweed were found at 120 ppb.

Table 1. The effect of four ozone concentrations on mean foliar injury values of six milkweed species over a 4 week fumigation period.

	Exposure Week for Each Concentration															
Species	**0 ppb**				**40 ppb**				**75 ppb**				**120 ppb**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Common Milkweed	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.002	0.008	0.208	0.006	0.049	0.115	0.553
Tropical Milkweed	0.000	0.000	0.000	0.000	0.000	0.000	0.403*	0.624*	0.000	0.148	0.403*	0.624*	0.000	0.333	0.660*	0.880
Swamp Milkweed	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.293	0.000	0.003	0.169	0.488	0.000	0.021	0.587*	0.587
Showy Milkweed	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.220	0.000	0.112	0.157	0.497	0.006	0.042	0.325	0.463
Prairie Milkweed	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.112	0.000	0.000	0.013	0.144	0.000	0.006	0.012	0.319
Spider Milkweed	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.015	0.117	0.002	0.095	0.122	0.463

Z Means within columns followed by an asterisk are different from Common Milkweed at the 5% level of significance, by Dunnett’s test.

Table 2. The effect of four ozone concentrations over a 4 week fumigation period on mean percentage of weekly leaf abscission of six milkweed species.

Milkweed Species	**Concentration**	**% Leaf Abscission**			
		Week 1	**Week 2**	**Week 3**	**Week 4**
Tropical	0 ppb	0	0	0	0
Swamp		0	0	0	0
Showy		0	0	0	0
Prairie		0	0	0	0
Common		0	0	0	0
Spider		0	0	0	0
Tropical	40 ppb	0	0	46	71
Swamp		0	0	0	33
Showy		0	0	0	25
Prairie		0	0	0	13
Common		0	0	0	4
Spider		0	0	0	0
Tropical	75 ppb	0	8	38	46
Swamp		0	0	13	29
Showy		0	13	0	29
Prairie		0	0	0	4
Common		0	0	0	13
Spider		0	0	0	0
Tropical	120 ppb	0	25	33	54
Swamp		0	0	42	46
Showy		0	0	29	0
Prairie		0	0	0	8
Common		0	0	4	13
Spider		0	0	4	21

2014 Exposure

Common Milkweed, following exposure to a target value of 0 ppb ozone did not exhibit visible ozone-induced injury symptoms throughout the entire experiment (Figs. 3&4). No species developed injury following exposure for one week to any ozone dosage. One plant exposed to 30 ppb developed slight visible injury after week 3, but the overall exposure to 30 ppb did not cause significant injury. Plants exposed to 60 ppb and 90 ppb developed initial foliar injury after 2 weeks of exposure, with injury values of 0.006 and 0.021 respectively. After 3 weeks of exposure, plants exposed to 60 and 90 ppb chambers exhibited more severe symptoms, with injury ratings 0.020 and 0.078 respectively.

Data were analyzed by multiple regression using SAS's Proc Reg (Freund et al. 1991), where injury was the response variable, and weeks after exposure commenced and ozone concentration were the regressor variables. The week by concentration interaction ($P = 0.0077$) and the week^2 by concentration^2 interaction ($P = 0.0001$) were significant ($R^2 = 0.9701$ and adjusted $R^2 = 0.9596$). The predictive model was: injury index = 0.0042 + 0.00459 (week) + 0.0003856 (concentration) -0.000975 (week^2) – 0.00000279 (concentration^2) –0.0003229 (week*concentration) + 0.00000208 (week^2*concentration^2). This indicates that ozone-induced injury increases at an increasing rate over time and with increasing concentration of ozone. The injury at 30 ppb did not increase over time, but following exposure to 60 and 90 ppb ozone, the injury levels increased in a curvilinear manner as exposure time increased.

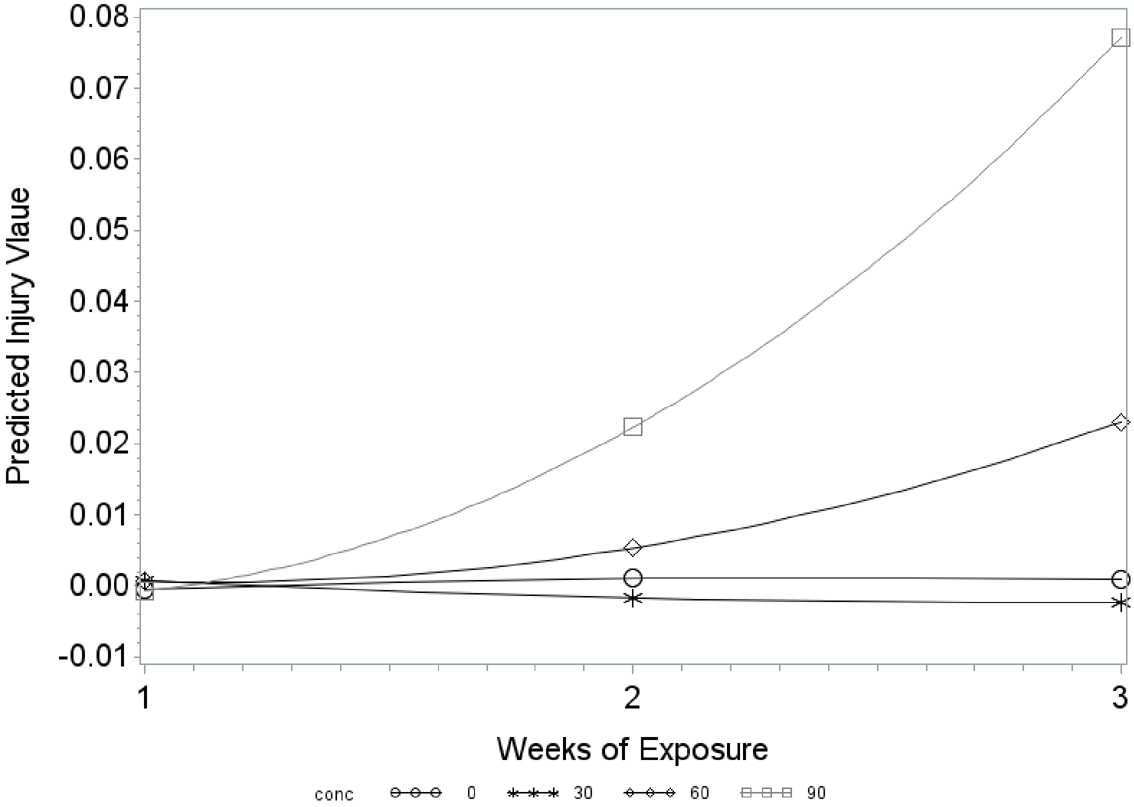

Figure 3. Predicted mean foliar injury values, obtained from multiple regression, on Common Milkweed exposed to 0, 30, 60, and 90 ppb ozone for 1, 2, or 3 weeks in 2014.

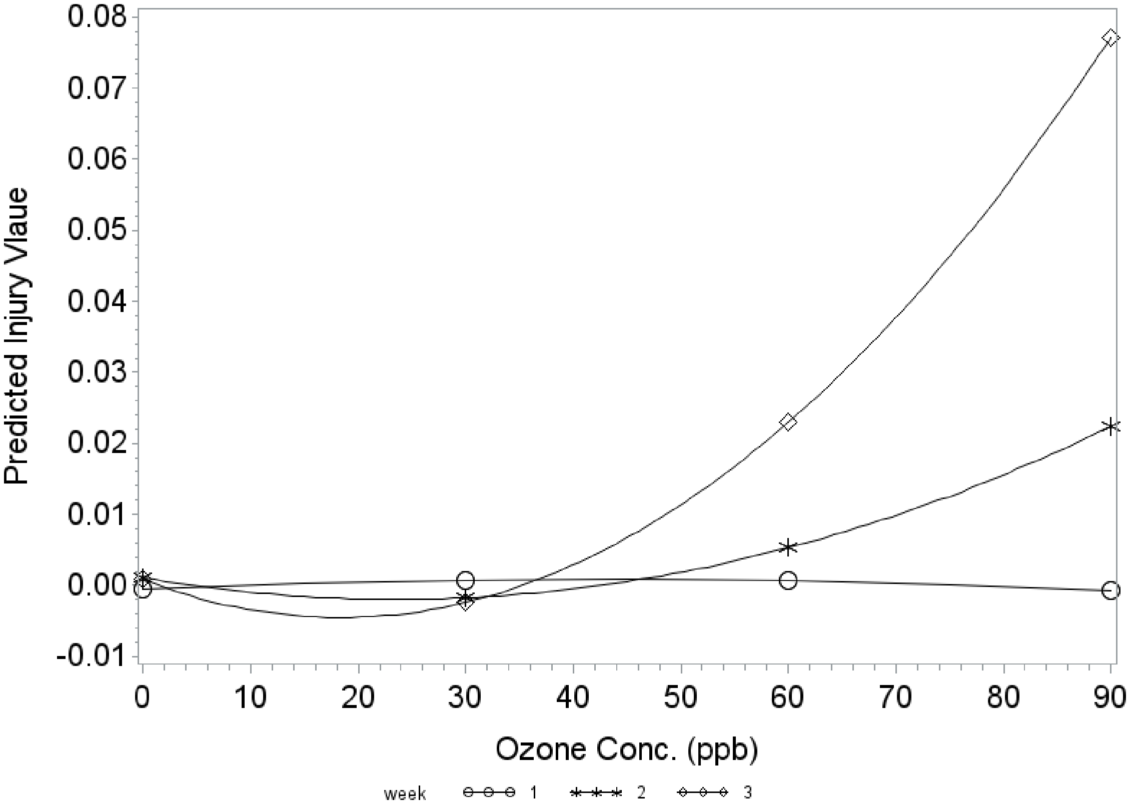

Figure 4. Predicted mean foliar injury values, obtained from multiple regression, of Common Milkweed for week 1, 2, and 3 compared to each ozone concentration 0, 30, 60, and 90 ppb.

Discussion

2013 Exposure

Tropical Milkweed had the greatest ozone-induced foliar injury throughout the experiment, which was significantly greater from Common Milkweed at different points during the experiment. This species also had the greatest rate of defoliation, which contributed to the high injury rating. Defoliation was considered as 100% severity rating for this experiment.

Hughes et al. (1990) conducted a study where Tropical Milkweed was exposed for 15 days, for 7 hours per day. Greater percentages of defoliation occurred at lower levels of ozone exposure in this study (at day 15), as compared to defoliation rates reported by Hughes et al. (1990). They found 20.3%, 26.8%, 52.6%, and 47.5% aborted leaves at 0 ppb, 43 ppb, 84 ppb, and 134 ppb respectively (Hughes et al. 1990). In fact, Hughes et al. (1990) reported that visual injury evaluation of Topical Milkweed leaves, following ozone exposure, could not always be obtained because of premature leaf drop.

Following 15 days of exposure in my study, I observed 0%, 46%, 38%, and 33% aborted leaves at 0 ppb, 40 ppb, 75 ppb, and 120 ppb respectively. Although, my study had a higher rate of defoliation at 40 ppb ozone and it is harder to compare leaf drop of the higher concentrations, it appears that in both studies Tropical Milkweed had a high rate of defoliation. This milkweed species may be too ozone-sensitive to be a viable field replacement for Common Milkweed, since a useful bioindicator becomes injured, but not fatally so by the stressor (Holt and Miller 2011). A possible explanation for the possible difference in ozone sensitivity of Tropical Milkweed and Common Milkweed may be related to differences in metabolic processes following ozone exposure. Bolsinger et al. (1992) studied changes in nutrient composition of ozone-treated leaves as part of a plant herbivore interaction study using Tropical Milkweed and Common Milkweed. They found similar effects in both species for certain metabolites including proteins, phenols, and cardenolides. However, they also reported that sugars decreased in Tropical Milkweed alone, whereas amino acids decreased in Common Milkweed alone.

Swamp Milkweed was the only other milkweed species besides Tropical Milkweed to have significantly different injury values, as compared to those of Common Milkweed. However, for Swamp Milkweed injury values were significantly different only following 3 weeks of exposure to 120 ppb ozone. The injury ratings were similar and not significantly different for these species by week 4 of exposure. Although injury values for Swamp Milkweed were not significantly different from the standard Common Milkweed, with the exception of the afore mentioned, the injury values were typically greater than those of Common Milkweed. This suggests that ozone injury on Swamp Milkweed develops more quickly or at a greater amount, as compared to Common Milkweed. However, because the injury values are not significantly different from the standard Common Milkweed, Swamp Milkweed may be a useful ozone-sensitive bioindicator species in the field.

The other milkweed species (Showy, Prairie, and Spider) developed distinctive ozone injury symptoms (including dark stippling), and non-significantly different injury ratings from Common Milkweed suggesting that these species are comparable and may be used as field bioindicators in addition to or in lieu of Common Milkweed.

2014 Exposure

Overall, the 2014 results indicate that the amount of ozone-induced injury on Common Milkweed will rise at an increasing rate over time and with higher levels of ozone of at least at 60 to 90 ppb. This pattern is generally accented because plants exposed to ozone may result in higher energy expenditure on cellular protection and repair, which over time causes stress and/or permanent damage such as necrosis of tissues (Krupa et al. 2001, Skelly 2000, US EPA 2006). Therefore, ozone damage is cumulative over time. Also, injury is expected at higher concentrations of ozone because plants have to repair and defend themselves against a higher amount of ozone molecules per unit time (Skelly 2000). Skelly (2000) reported that injury symptoms accumulated more quickly over time at higher concentrations of ozone (greater than 80 ppb) and slower at lower concentrations of ozone (less than 40 ppb). By examining both variables, a curvilinear relationship between time and ozone concentration.

The ozone concentration of 60 ppb was chosen for this study because the US EPA proposed to lower the NAAQS standard from 75 ppb to a level between 60 and 70 ppb (National Ambient Air Quality Standards for Ozone 2010). As of December 2015, the US EPA lowered the primary and secondary NAAQS of ozone to not exceed 70 ppb for the 3 year average of the fourth highest daily maximum 8 hour average ozone concentrations (National Ambient Air Quality Standards for Ozone 2015). Plants in this study exhibited phytotoxic injury at 60 ppb, below the current NAAQS of 70 ppb ozone, which may indicate that the current standard may not be sufficient. Plants exposed to 60 ppb ozone developed injury, although less severe, at the same time as plants exposed to 90 ppb ozone. This result combined with the curvilinear relationship described above indicates that Common Milkweed remains a viable indicator at this level of phytotoxic ozone. These findings also illustrate that 60 ppb is phytotoxic and capable of causing ozone injury under the conditions of this study. Additionally, the plants exhibited symptoms at each ozone dose, but not fatally so, further supporting Common Milkweed as a good bioindicator of tropospheric ozone (Holt and Miller 2011). However, caution must be exercised in the interpretation of each results, since these studies were conducted in chambers within a greenhouse. Nevertheless, results appear to be fairly realistic.

Although one plant became slightly injured at 30 ppb ozone by the conclusion of the study, no other injury occurred at this level. No or little injury is expected at this concentration level, chosen to mimic a natural background concentration of ozone. Thus, this anomalous result may be an outlier. However, additional studies would be required to prove or disprove this.

Appendices

Appendix A: Literature Review

A. Air Pollution

The United States Environmental Protection Agency (EPA) defines an air pollutant as any gas, liquid, or solid particles that when airborne and occur in high enough concentrations are harmful to people, animals, vegetation, or structures (US EPA 2009). Air pollutants can be classified as either primary pollutants (directly emitted from a source) or secondary pollutants (formed through reactions of primary pollutants in the atmosphere) (US EPA 2009). Sulfur dioxide is an example of a primary pollutant emitted by coal burning power plants, while ozone is an example of a secondary pollutant formed from the primary pollutants hydrocarbons and nitrogen oxides.

The EPA sets National Ambient Air Quality Standards to regulate air pollutants, in accordance with the Clean Air Act. Current standards are set for six pollutants: ozone, particulate matter, carbon monoxide, lead, nitrogen dioxide, and sulfur dioxide (US EPA 2012c). Each pollutant has two categories of standards. Primary standards are set to protect human health, while secondary standards are set to protect welfare (including visibility, animals, crops, and structures) (US EPA 2012c).

B. Ozone

Ozone is a triatomic oxygen molecule that exists as a colorless gas with a sweet-to-acrid odor (Skelly 2000, US EPA 2008). Ozone exists throughout the Earth's atmosphere and depending upon its location within the atmosphere it can either be protective or detrimental to life forms (US EPA 2012b, 2003). Ozone that is beneficial for life is in the stratosphere (outer layer of the atmosphere) approximately 10- 50 km above the Earth (US EPA 2012b, 2003). This is where ozone absorbs ultra violet (UV) radiation which can stress or even cause cancer at high enough doses (US EPA 2003). Ozone that is potentially injurious to life is and is considered a major pollutant is in the troposphere (lower half of the atmosphere) approximately 0-10 km above the surface of the Earth. Tropospheric ozone is a major constituent of urban smog, and plays a minor role as a greenhouse gas.

Ozone is formed from the precursor chemicals nitrous oxides (NOx) and volatile organic compounds (VOCs) that react in sunlight-driven photochemical reactions. NOx and VOCs are produced by a mix of natural and anthropogenic activities (Krupa et al. 2001, Skelly 2000). Natural or ambient ground level concentrations of ozone can range from 10-40 ppb (Krupa et al. 2001, Skelly 2000). Ozone can also occur in the troposphere from lightening or stratospheric intrusion (mixing of stratospheric ozone) (Krupa et al. 2001, Skelly 2000).

Human activities contribute to tropospheric ozone formation by the release of NOx and VOCs from fossil-fuel combustion, vehicle use, industrial processes, and electric utilities, among other sources. Thus, the greatest concentrations of tropospheric ozone are formed in urban areas. Although the majority of ozone is formed in urban areas it can and is easily transported by air masses and wind to rural areas such as agricultural and forested areas (US EPA 2012b, US FS 2007).

Ozone concentrations typically vary daily and seasonally in the Northeastern US. A diurnal pattern of ozone concentration is typically observed as sunlight is required to drive the ozone photochemical reaction. Ozone concentrations are highest in late afternoon, and lowest in the early morning. Annual cycles are also observed as ozone formation is highest in the summer when there is a greater day length, greater angle of incidence of sunlight (resulting in higher light intensities), and higher temperatures. (Krupa et al. 2001, Skelly 2000, US EPA 2003)

C. Effects on Biota

Acute exposure effects of ozone on humans can include throat irritation, congestion, coughing, inflamed lung tissue, and general breathing difficulty (US EPA 2008, 2003). Ozone can induce scar tissue in lungs, the result of chronic tissue inflammation. This can reduce lung capacity and function which can lead to heart attack, stroke, and increased susceptibility to infection (US EPA 2008, 2003). Children and the elderly are at greater risk for health problems due to exposure. Persons with previous chronic lung conditions often experience worsened symptoms with increased exposure to ozone (US EPA 2008, 2012b, 2003).

Ozone damages vegetation by stunting growth, damaging leaves, and accelerating phenology (US EPA 2008, 2003). Ozone damage to crops reduces yields, resulting in an approximate economic loss of $500 million dollars per year in the US (US EPA 2003). Broader ecosystem impacts through vegetation damage include loss of species diversity, quality of habitat water and food, and nutrient cycling (US EPA 2012b).

During the summer months the greatest potential for ozone exposure coincides with the vegetative growing season for most crops. Metabolizing plants assimilate the greatest amount of CO_2 from the atmosphere during this time, and concurrently take up other gasses such as ozone. Therefore, the highest rate of ozone injury on plants is often reported during the growing season (US EPA 2012c, US FS n.d.).

D. Plant injury

Plant injury occurs when ozone enters the leaf through stomata (small openings on leaf surfaces for gas exchange), Ozone reacts with water inside the plant, and results in the products of hydrogen peroxides, superoxides, hydroxyl radicals, and peroxy radicals (which are more mobile throughout plant tissue). These products are classified as free radicals because they have free valence electrons and can affect membrane function, signal transduction, proteins, and ultimately photosynthesis (Krupa et al. 2001, US EPA 2006).

Plants can respond to free radical species by producing antioxidant chemicals or by closing stomata (US EPA 2006). Plants may expend more energy on cellular protection and repair to free radicals (Krupa et al. 2001, US DOI 2003, US EPA 2006) resulting in plant growth reductions and yield. Stomata closure limits gas exchange resulting in lower photosynthesis rates. Reduced photosynthesis may cause growth stunting, chlorosis, lowered water use efficiency, changes in food storage and allocation to different parts of the plant, defoliation, and premature senescence (Krupa et al. 2001, Skelly 2000, US EPA 2006).

Dark spots or stippling of the upper (adaxial) leaf surface (the most recognized ozone visible injury on broadleaf plants) may be observed as the result of the ozone induced free radicals oxidizing mesophyll cells (US DOI 2003, US EPA 2006). This is characteristically only observed on the upper leaf surface of broadleaf species (Krupa et al. 2001, Skelly 2000, US EPA 2003). Since the primary cause of stippling is damage in mesophyll cells, stippling only occurs in plant tissue between veins and veinlets, with symptoms usually occurring more on older leaves (Skelly 2000). These stippling characteristics help to distinguish injury from ozone from injury from other biotic or abiotic stressor injury symptoms such as nutrients, pathogens, and other pollutants (Skelly et al. 1987, Skelly 2000). Chlorosis (the loss of green chlorophyll pigment) and upper leaf red/ purple tinges to the leaf are other visible symptoms to ozone (Krupa et al. 2001, Skelly 2000, US DOI 2003).

Acute exposure injury symptoms on broadleaf species (greater than 80 ppb) are visible hours to a few days after exposure, and include signs such as stippling or flecking (Skelly 2000). Symptoms of chronic exposure (less than 40 ppb), which is most common, progress over days or weeks and can appear as upper leaf stipple, premature senescence, and leaf drop (Skelly 2000). Chronic exposures can leave plants more vulnerable to other biotic and abiotic stressors such as disease and weather events (Krupa et al. 2001, US EPA 2012c).

E. Bioindicators

Bioindicators are any biotic factor (organisms) in an environment that can be used to show changes in quality of the system (such as an environmental factor) (Holt and Miller 2011). Certain attributes are necessary for a biotic factor to be a bioindicator. This includes being sensitive, but not fatally so, to the particular stressor of question (Holt and Miller 2011). In addition, helpful characteristics include easy identification, moderate tolerance to environmental stress, moderate abundance, and public interest or need (Holt and Miller 2011, US DOI 2003).

Plants have been shown to exhibit specific symptoms to a stressor and have been successfully used as bioindicators (Holt and Miller 2011). Plant bioindicators of air pollutants is often more cost effective and in some cases more practical than electronic monitoring in remote forested regions (Holt and Miller 2011, Skelly 2000). Particularly, good characteristics of a plant bioindicator for ozone is that it develops distinct injury or symptoms on leaves that are distinguishable from other disease symptoms (US DOI 2003, US FS 2007). The foliar symptom used for identification of ozone injury on broadleaf plants is upper leaf surface stippling (US FS 2007). Plant species previously established as sensitive and/or bioindicator species for ozone include *Nicotiana tabacum* (Tobacco), *Vitis vinifera* (Grape), *Asclepias syriaca* (Common Milkweed), *Rubus* spp. (Blackberry), and *Prunus serotina* (Black Cherry) (Kline et al. 2008, Krupa et al. 2001).

Biomonitoring projects such as the US FS Forest Inventory Analysis Program (FIA) utilize plant bioindicators at approximately 1,130 sites in 45 states to detect presence and changes in ozone concentrations (US FS 2007). Injury assessments are done during the growing season. In the eastern US there is a prime 3 week period from late July through mid-August, and in the western US, because of more variable conditions, there is a 5 week period at the height of the growing season (US FS n.d.).

F. Milkweed

The milkweed sub-family, Asclepiadaceae, is a group of plants comprised primarily of perennial herbs with paired opposite leaves, simple umbellate flowers with five petals, and a milky latex sap (Fayaz 2011, Kirkpatrick 1992). This family is native to

North America with a broad range throughout most of the US (except Alaska) and throughout Canada (Common Milkweed 2007, USDA NRCS 2002). There are about 120 species that belong to the genus *Asclepias* (Fayaz 2011). Milkweed reactions to ambient ozone include dark, purple, black, and/or brown, adaxial stipple of leaves, especially lower older leaves, and premature senescence (Kline et al. 2008, 2009, Skelly 2000).

Asclepias spp. contain a variety of chemicals within their sap such as resinoids, glycosides, and alkaloids (USDA NRCS 2011b). Cardiac glycosides are poisonous to humans and livestock, and if ingested may impart symptoms such as weakness, high body temperature, rapid or weak pulse, and difficulty breathing (USDA NRCS 2011b, 2011d). These glycosides are, however, important for *Danaus plexippus* (Monarch Butterfly), which depend on milkweed plants for survival (USDA NRCS 2011b). Monarch caterpillars ingest milkweed and store the glycosides within their bodies for the remainder of their lives, which makes them unpalatable to predators (USDA NRCS 2011b). There is concern about Monarch Butterfly numbers declining, and many home gardeners are starting to plant milkweeds for Monarch Butterflies to utilize.

Historical uses of various milkweed species are extensive. Indigenous peoples throughout the US and Canada used milkweed species for fiber, food, medicine, and tools. Certain milkweed species were cultivated in America during WWII to utilize the seed comas, which are the white hairs attached to the seeds, as lifejacket and pillow stuffing because of its buoyant and thermal properties (USDA NRCS 2011a). Today milkweed species, such as Showy Milkweed, are cultivated in order to utilize seed comas as a hypo-allergenic down alternative (USDA ARS 2009, USDA NRCS 2011b). Milkweed seed oil is used in cosmetics, and is currently being investigated for use as a sunscreen (USDA ARS 2009).

Certain milkweed species are sought after by wild food enthusiasts for consumption, and includes Common Milkweed, Showy Milkweed, and Swamp Milkweed as edible species. The shoots, flower buds, flowers, and follicles of are edible when harvested at the correct time and properly prepared by boiling in at least three changes of water (Elias and Dykeman 2009, Zachos 2013).

Asclepias syriaca L.

Asclepias syriaca is a perennial herb propagating from either seeds or rhizomes and can grow to be 0.5-2 m in height (USDA NRCS 2011b, Zachos 2013). Leaves are opposite, ovate to elliptic, and range from approximately10-20 cm long (USDA NRCS 2011b). Flowers have five petals and are arranged in simple umbels, ranging from rose to purple in color (USDA NRCS 2011b). Spindle shaped follicles develop containing the seeds with silky hairs (USDA NRCS 2011b). Common names for *A. syriaca* include Common Milkweed, Virginia Silk, and Silky Swallowwort. (USDA NRCS 2011b)

Common Milkweed prefers full to partial sun and can grow in sandy, clayey, or rocky soils along banks, forest edges, prairies, roadsides, and waste places (USDA NRCS 2011b, Zachos 2013). This species has a wide distribution throughout North America. This includes southeastern Canada south to Texas (excluding Florida), and throughout the US east coast and plains region (USDA NRCS 2011b).

Common Milkweed is the most recognized *Asclepias* species to be affected by ozone and has been used for biomonitoring by the US FS (US FS n.d.). Common Milkweed is on the National Park Service (NPS) and US Fish and Wildlife Service

(FWS) ozone sensitive and field bioindicator plant lists, and has been used widely as a bioindicator for tropospheric ozone (US DOI 2003).

***Asclepias curassavica* L.**

Asclepias curassavica is a perennial herb that may grow 1-2 m tall (Fayaz 2011, USDA NRCS n.d.-a). Leaves are opposite, lanceolate, and 1-4 cm long, and flowers are cymes that may range from reds, oranges, to yellows (Fayaz 2011, Missouri Botanical Garden n.d.-a). Follicles range from 1-1.5 cm (Fayaz 2011). Common names include Tropical Milkweed, and Bloodflower.

Tropical Milkweed prefers moist areas, banks, fields, and roadsides in light well-drained soil preferably with full sun (Fayaz 2011, Missouri Botanical Garden n.d.-a, USDA n.d.-a). This South American native has spread and established to other tropical/ subtropical areas, and can be found in found in Florida, Louisiana, Texas, California, and Hawaii (Fayaz 2011, Missouri Botanical Garden n.d.-a, USDA n.d.-a).

Tropical Milkweed injury to ozone exposure has been documented in a plant-herbivore interaction study (Bolsinger et al. 1991). Injury symptoms documented were purple stippling in leaves (Bolsinger et al. 2009).

Asclepias incarnata L.

Asclepias incarnata is a herbaceous perennial, and may grow from 1 to almost 2 m tall (USDA NRCS n.d.-b, 2011a). Leaves are lance shaped arranged in opposite pairs, and flowers form in clusters of simple umbels ranging from white to pink or purple (USDA NRCS n.d.-b, 2011a). Seeds with coma form within tear shaped seed pods, about 10 cm long (USDA NRCS n.d.-b, 2011a). Alternative names for this species include Swamp or Rose Milkweed.

Swamp Milkweed prefers full sun to partial shade and moist fine-textured soils that may be slightly acidic, although it is tolerant of well drained soils (USDA NRCS n.d.-b, 2011a). Swamp Milkweed can be found in wetlands, along banks and floodplains, wet meadows or woods, fields, and roadsides. This species is native to North America and is found in eastern Canada and the majority of the mainland US (with the exception of the western coastal states) (USDA NRCS n.d.-b, 2011a).

Swamp Milkweed has been reported to start with tan stippling, which turns into a darker stipple (Kline et al. 2008). This species is considered ozone sensitive, but is not on the bioindicator plant species list by the NPS and US FWS (US DOI 2003). This species is currently utilized for wetland rehabilitation (USDA NRCS 2011a).

***Asclepias speciosa* Torr.**

Asclepias speciosa is a herbaceous perennial that can grow to heights of 0.5-1.5 m tall (Kirkpatrick 1992, USDA NRCS 2011d). Leaves are opposite ovate to elliptic blades about 10-18 cm long with rounded tips, and flowers are rose-purple that grow in large loose clusters (Kirkpatrick 1992, USDA NRCS 2011d). Follicles grow from 8-12 cm in length, and produce seeds with coma (Kirkpatrick 1992, USDA NRCS 2011d). *A. speciosa* adapts well and can be found in fields, clearings, roadsides, and anywhere there is full sun and well-drained soils (Kirkpatrick 1992, USDA NRCS 2011d). This species ranges from southwestern Canada and the entire western half of the US (Kirkpatrick 1992, USDA NRCS 2011d). *A. speciosa* is commonly known as Showy Milkweed.

Showy Milkweed is reported to be ozone sensitive, however specific injury is not documented.

Asclepias sullivantii Engelm. ex A. Gray

A. sullivantii is a herbaceous perennial native to the US (Missouri Botanical Garden n.d.-b, USDA NRCS n.d.-d). This plant grows 0.5 m to almost 1 m tall, and has ovate-lanceolate leaves about 20 cm long. Flowers range from light pink to light purple, arranged in simple umbels, and form seed pods about 5-10 cm long (Missouri Botanical Garden n.d. -b). This species prefers full sun in moist prairies and fields in the plains region of the US and south central Canada (Missouri Botanical Garden n.d.-b). This species is known as Prairie Milkweed. There is no known documentation of ozone sensitivity or injury.

Asclepias viridis Walter

A. viridis a perennial herb that can grow to 0.5 m, or slightly taller (Missouri Botanical Garden n.d.-c, USDA NRCS n.d.-f). Leaves are ovate-lanceolate oppositely arranged about 5-12 cm long (Missouri Botanical Garden n.d.-c, USDA NRCS 2011c). Flowers are clusters of simple umbels with petal color varying from white –pale green and may have tinges of pinks and purples (Missouri Botanical Garden n.d.-c, USDA NRCS 2011c). Follicles form approximately 12 cm long, containing seeds with silky coma (Missouri Botanical Garden n.d.-c). *A. viridis* may also propagate by underground rhizomes (Missouri Botanical Garden n.d.-c). Spider, Green, or Green Antelopehorn Milkweed are common names for this species.

Spider Milkweed prefers full sun in dry to moist loamy soils, and is drought tolerant (Missouri Botanical Garden n.d.-c, USDA NRCS 2011c). Found in prairies, fields, glades, and roadsides; this species is the most abundant milkweed found in Texas (Missouri Botanical Garden n.d.-c, USDA NRCS n.d.-f). The range for this North American native is the central and southern US from Ohio west to Nebraska, growing as far south as Florida and Texas (Missouri Botanical Garden n.d.-c, USDA NRCS n.d.-f). Spider Milkweed has been surveyed in an ozone injury evaluation study, but with inconclusive results (Davis 2002).

Appendix B: Additional Tables and Figures

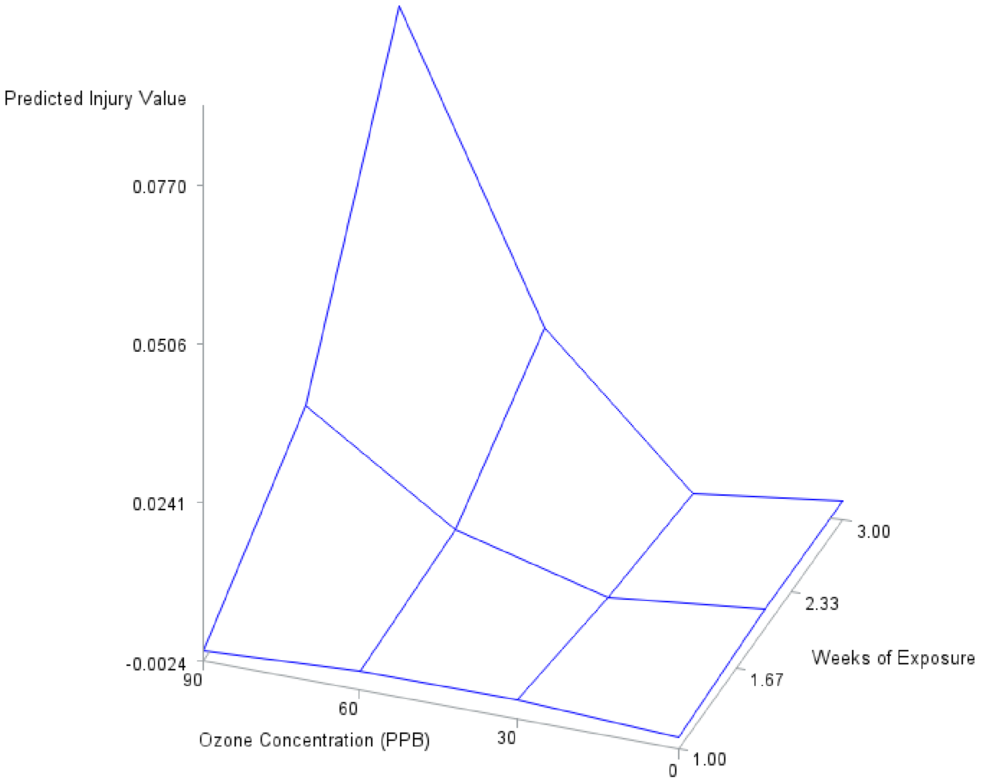

Figure 5. Predicted mean foliar injury values, obtained from multiple regression, on Common Milkweed for each concentration and time for duration of experiment.

Appendix C: Additional Work

Evaluation of non-milkweed species

Eruca vesicaria sativa, Ocimum basilicum, and *Verbena stricta* were also included in the 2013 exposure study.

Ocimum basilicum L. cv. Genovese Gigante

Genovese Basil, family Lamiaceae, is a cultivar of *Ocimum basilicum* L. an annual herb. Genovese Basil has a square stem, and can grow to 0.5 m or more (Tucker and Debaggio 2009, *Ocimum basilicum* n.d.). Leaves are ovate and grow in opposite pairs (Tucker and Debaggio 2009). Flowers are white, and grow in small clusters. Basil species are native to Asia, Africa, Central & South America, and the Mediterranean region, and grows best in full sun and moist conditions (*Ocimum basilicum* n.d., Tucker and Debaggio 2009). Genovese Basil is introduced to North America, and is present in 16 states/ territories of the U.S. including the commonwealth of Pennsylvania (USDA Plants Profile *Ocimum basilicum* n.d., Simon et al 1992, Jayasinghe et al. 2003).

Genovese Basil is the most used species of basil for culinary, medicine, pharmacy, and perfume uses commercially (Simon et al 1999, Jayasinghe et al 2003, Tucker and Debaggio 2009). Plants in the family Lamiaceae contain antioxidants, and basil specifically contains the antioxidants cinnamic acid, caffeic acid, sinapic acid, ferulic acid, and phenolic compounds (Jayasinghe et al 2003). Genovese Basil was added to this study because of its commercial importance, antioxidant activity, and because there are no known studies in which this species has been exposed to ambient ozone.

Genovese Basil did exhibit foliar damage when exposed at 75 and 120 ppb ozone. Foliar injury was in the form of grey or tan stippling on the upper leaf surface. Further investigation, namely exposure at higher levels of ozone, is needed to in order to confirm foliar damage symptoms.

Verbena stricta Vent.

Verbena stricta is a member of the family Verbenaceae (vervain), and is known as Hoary or Wooly Verbena because of its dense hair covering the plant (Kirkpatrick 1992, USDA Plants Profile *Verbena stricta* n.d., *Verbena stricta* n.d.). Hoary Verbena is a herbaceous perennial, and may grow to just over 1 m high (*Verbena stricta* n.d., Kirkpatrick 1992). The leaves are ovate with incised edges, and grow to be about 2.5-10 cm long (Kirkpatrick 1992, *Verbena stricta* n.d.). The flowers form on spikes at the top of the plant, are very small and tightly packed, and are a blue-purple color (Kirkpatrick 1992, *Verbena stricta* n.d.). This North American native grows best in dry well-drained and sandy soils with full sun, and can be found in fields, waste places, thickets, and roadsides (Kirkpatrick 1992, *Verbena stricta* n.d.). Hoary Verbena is a prolific plant is drought tolerant, and ranges from southeastern Canada, and the majority of the U.S. mainland with the exception of some coastal states (Kirkpatrick 1992, USDA Plants Profile *Verbena stricta* n.d., *Verbena stricta* n.d.).

Hoary Verbena did not develop any visible injury symptoms when exposed at various levels of ozone up to 120 ppb.

***Eruca vesicaria* (L.) Cav. subsp. *sativa* (Mill.) Thell.**

Eruca vesicaria sativa is an annual of the Brassicaceae family, known as arugula or rocket salad (USDA Plant Profile *Eruca vesicaria* n.d., *Eruca sativa* n.d.). Arugula grows best in full sun and moist soils, and may grow to just under 1 m high (*Eruca sativa* n.d.). This European native has been introduced to North America and can be found in the southern provinces of Canada, and many parts of the U.S. where it is cultivated for use as a culinary green (*Eruca sativa* n.d.).

Arugula did not develop any visible injury symptoms when exposed at various levels of ozone up to 120 ppb.

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