

# Effects of elevated CO<sub>2</sub> and O<sub>3</sub> on leaf damage and insect abundance in a soybean agroecosystem

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**Abstract** By altering myriad aspects of leaf chemistry, increasing concentrations of CO<sub>2</sub> and O<sub>3</sub> in the atmosphere derived from human activities may fundamentally alter the relationships between insect herbivores and plants. Because exposure to elevated CO<sub>2</sub> can alter the nutritional value of leaves, some herbivores may increase consumption rates to compensate. The effects of O<sub>3</sub> on leaf nutritional quality are less clear; however, increased senescence may also reduce leaf quality for insect herbivores. Additionally, changes in secondary chemistry and the microclimate of leaves may render plants more susceptible to herbivory in elevated CO<sub>2</sub> and O<sub>3</sub>. Damage to soybean (*Glycine max* L.) leaves and the size and composition of the insect community in the plant canopy were examined in large intact plots exposed to

elevated CO<sub>2</sub> (~550 μmol mol<sup>-1</sup>) and elevated O<sub>3</sub> (1.2\*ambient) in a fully factorial design with a Soybean Free Air Concentration Enrichment system (SoyFACE). Leaf area removed by folivorous insects was estimated by digital photography and insect surveys were conducted during two consecutive growing seasons, 2003 and 2004. Elevated CO<sub>2</sub> alone and in combination with O<sub>3</sub> increased the number of insects and the amount of leaf area removed by insect herbivores across feeding guilds. Exposure to elevated CO<sub>2</sub> significantly increased the number of western corn rootworm (*Diabrotica virgifera*) adults (foliage chewer) and soybean aphids (*Aphis glycines*; phloem feeder). No consistent effect of elevated O<sub>3</sub> on herbivory or insect population size was detected. Increased loss of leaf area to herbivores was associated with increased carbon-to-nitrogen ratio and leaf surface temperature. Soybean aphids are invasive pests in North America and new to this ecosystem. Higher concentrations of CO<sub>2</sub> in the atmosphere may increase herbivory in the soybean agroecosystem, particularly by recently introduced insect herbivores.

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## Introduction

The increase in atmospheric CO<sub>2</sub> derived from the combustion of fossil fuels and changes in land use (Forster et al. 2007) accelerates the rate of plant photosynthesis, often causing an increase in plant productivity (Drake et al. 1997; Ainsworth et al. 2002; Long et al. 2004), whereas the increasing concentration of tropospheric O<sub>3</sub> (Prather et al. 2001) typically has the opposite effect (Fuhrer 2003; Morgan et al. 2003). Changes in the concentration of these gases in

the troposphere also affect many aspects of leaf structure and chemistry that indirectly affect productivity by changing the relationship between plants and insect herbivores. The magnitude and direction of these indirect effects vary widely (Kopper et al. 2001; Percy et al. 2002; Fuhrer 2003; Kopper and Lindroth 2003; Stiling et al. 2003). To date, only a few experiments have examined how these elements of global change alter the behavior of natural insect populations in agricultural ecosystems (Fuhrer 2003). Understanding how insect feeding behavior is altered by elevated CO<sub>2</sub> and O<sub>3</sub> will be important for predicting crop productivity as well as identifying insect species likely to become pests in the future (Herms et al. 1995; Baker et al. 2000).

Growth in elevated CO<sub>2</sub> generally increases the carbon-to-nitrogen ratio (C:N) of plant tissues (Lincoln et al. 1984; Heagle et al. 1998; Rogers et al. 2004; Hamilton et al. 2005), reducing the nutritional quality for protein-limited insects (Coviella and Trumble 1999). Insects may increase their food intake to compensate for reduced leaf nitrogen content (Coviella and Trumble 1999; Kopper et al. 2001; Holton et al. 2003), although this is not always the case (Kopper and Lindroth 2003; Knepp et al. 2005). Depending on the species and its life stage, nitrogen may not be limiting (Gratton and Denno 2003; Heisswolf et al. 2005), and insects also may respond to other primary and secondary compounds that change in elevated CO<sub>2</sub> (Coviella and Trumble 1999; Ziska et al. 2001; Agrell et al. 2005). Because it reduces stomatal conductance, elevated CO<sub>2</sub> also increases canopy temperature (Rogers et al. 2004; Yoshimoto et al. 2005). Insect generation times are strongly related to temperature (Bale et al. 2002) and rising canopy temperature may shorten the generation time of many pests, increasing the number of generations per season and further exacerbating crop losses (Bale et al. 2002; Fuhrer 2003).

Depending on the dose and duration of exposure, elevated O<sub>3</sub> has been shown to increase or decrease susceptibility to herbivores. By diverting assimilates away from the synthesis of plant defenses, elevated O<sub>3</sub> can increase the susceptibility of plants to insect attack (Endress and Post 1985; Chappelka et al. 1988; Sandermann et al. 1998; Long and Naidu 2002; Percy et al. 2002). In contrast, O<sub>3</sub> also may function as an abiotic elicitor of plant defenses and possibly deter folivorous insects (Sandermann et al. 1998). When plants are exposed to both gases simultaneously, elevated CO<sub>2</sub> reduces the effects of O<sub>3</sub> on photosynthesis (Herms et al. 1996; McKee et al. 2000; Isebrands et al. 2001; Heagle et al. 2003). The combined effect of these gases on leaf chemistry and insect performance has been explored in aspen trees (Kopper et al. 2001; Kopper and Lindroth 2003), but how these gases together affect folivorous insects in an agro-ecosystem is unknown.

Although many invasive insects are tolerant of a wide range of environmental conditions, elevated CO<sub>2</sub> and the

accompanying increases in global temperatures may facilitate the spread of non-native insect pests, particularly in northern and mid-latitudes (Pimentel et al. 2000; Strand 2000). Already, many ecosystems are affected by non-native insects and at least 40% of insect pest species in agricultural fields in the United States are non-indigenous (Pimentel et al. 2000; Strand 2000; Bale et al. 2002). The absence of natural enemies may contribute to the success of non-native pests, but the vulnerability of agro-ecosystems is exacerbated by their lack of genetic diversity for resistance to multiple stressors (Bale et al. 2002; Baker et al. 2005). Currently, ~\$13.9 billion of the potential value of crops is lost annually to non-native insect pests (Pimentel et al. 2000; USDA 2005). This figure will likely rise in the future as trade between the United States and countries with similar climate such as China continues to increase (Baker et al. 2005).

The objective of this research was to quantify the effect of elevated CO<sub>2</sub> and O<sub>3</sub>, applied singly and in combination, on insect abundance and the loss of leaf area to herbivores in an intact soybean canopy. The Soybean Free Air Concentration Enrichment (SoyFACE) experiment provides elevated CO<sub>2</sub> and O<sub>3</sub> over an intact soybean crop and unfettered access to insect herbivores without altering other aspects of canopy microenvironment (Long and Naidu 2002). Previously, Hamilton et al. (2005) demonstrated that growth under elevated CO<sub>2</sub> increased herbivory of soybean by Japanese beetles (*Popillia japonica*), and increased damage to foliage was associated with elevated concentrations of soluble sugars that serve as powerful feeding stimulants. Using similar methods, we investigated the effects of inter-annual variation in insect populations on the amount of defoliation in soybean grown in elevated CO<sub>2</sub> and O<sub>3</sub>. We predicted that leaf area consumed by herbivores in elevated CO<sub>2</sub> would increase in concert with an increase in leaf C:N. Because both elevated CO<sub>2</sub> and O<sub>3</sub> may reduce the nutritional value of leaves, we expected their effects on herbivory to be additive when both gases are elevated simultaneously.

## Materials and methods

### Site description

This study was conducted at the SoyFACE facility at the University of Illinois, Urbana-Champaign (40°02' N, 88°14' W, 228 m above sea level; [www.soyface.uiuc.edu](http://www.soyface.uiuc.edu)). Each experimental plot was surrounded by pipes that injected CO<sub>2</sub> or O<sub>3</sub> at a supersonic velocity from 300- $\mu$ m pores above the canopy (Miglietta et al. 2001). The target CO<sub>2</sub> concentration was 550  $\mu$ mol mol<sup>-1</sup>; the actual average concentrations for 2003 and 2004 were 552 and 550  $\mu$ mol mol<sup>-1</sup>, respectively, while the ambient plots were ~370  $\mu$ mol mol<sup>-1</sup> CO<sub>2</sub>. The average daily (8 h) O<sub>3</sub>

concentration was 60 nmol mol<sup>-1</sup>, and the elevated O<sub>3</sub> plots were fumigated to 20% above ambient levels. The elevated CO<sub>2</sub> plus elevated O<sub>3</sub> plots received both gases simultaneously. One-minute average CO<sub>2</sub> and O<sub>3</sub> were  $\pm 20\%$  of the target for  $> 95\%$  of the time. The target concentrations for CO<sub>2</sub> and O<sub>3</sub> represent the predicted atmospheric levels by 2050 (Prather et al. 2001; Prentice et al. 2001). A more detailed description of the SoyFACE facility can be found in Dermody et al. (2006).

The experiment consisted of four randomized blocks within a continuous crop of soybean. Each block contained four 20-m diameter octagonal plots (total area 314 m<sup>2</sup>) exposed to ambient air, elevated CO<sub>2</sub>, elevated O<sub>3</sub> or elevated CO<sub>2</sub> plus elevated O<sub>3</sub>. Each block was replicated four times. Soybean (*Glycine max* cv. 93B15, Pioneer Hi-Bred, Johnston, IA, USA) was planted at 0.38-m row spacing in each year. This variety is typical of those grown in commercial production.

#### Leaf area removal

Leaf damage was measured in July and August of 2003 and 2004. The July measurements coincided with the expected peak levels of Japanese beetle (*Popillia japonica*). The August measurements coincided with peak levels of bean leaf beetle (*Cerotoma trifurcata*) and soybean looper (*Pseudoplusia includens*).

To estimate the loss of leaf area to herbivory, 100 plants were randomly selected in each experimental plot and a single leaf was randomly harvested from the top four trifoliates of each plant. Leaves were selected by first randomly selecting the row, then the plant and then finally the nodal position. Randomization was performed in the field by choosing from a set of numbers from 1–30 for row and plant, and from 1–3 for each leaflet on a soybean node. Leaves were cut at the base and photographed against a calibrated background with a high-resolution digital camera (Coolpix 950; Nikon, Melville, NY, USA). The digital photographs were analyzed for tissue lost to herbivory using image analysis software (Scion Image, Beta Version 4.0.2; Scion Corp., Frederick, MD, USA). The size of the leaf was measured and the area before damage was estimated by reconstructing the perimeter. The amount of damage was then converted to a percentage of the estimated original leaf area.

#### Insect censuses

Insect censuses were conducted to identify species potentially responsible for leaf damage. To minimize disturbance, insect censuses were conducted two days prior to collecting leaves for assessment of leaf area removal. A modified Allen vacuum (Stihl Model BG 85 Blower, Stihl Inc. Virginia

Beach, VA, USA) was fitted with mesh bags (1.4 mm diameter) and each plot was vacuumed for a total sampling time of five minutes per plot. The mesh bags captured the major leaf chewers at SoyFACE. The contents of the mesh bags were freeze-dried for later identification. Similar methods have been employed for other crops (Zalom et al. 1993).

During an outbreak in 2003 we quantified the density of soybean aphids (*Aphis glycines*) on plants grown in ambient and elevated CO<sub>2</sub> and O<sub>3</sub>. In each experimental plot, 50 leaves were selected randomly, using the same method employed for measurement of herbivory (see above), from the top two trifoliates and handled carefully to avoid dislodging aphids. Leaves were taken immediately to a field laboratory, where the number of aphids on a randomly selected 2-cm<sup>2</sup> plot on each leaf was counted, with the condition that the midrib and edge of each leaf were excluded from selection. Aphids were classified as alate (winged), apterous (wingless), or dead. The majority of aphids were actively feeding and apterous, indicating a healthy reproducing population (Rice et al. 2005).

#### Leaf chemistry and temperature

To determine if leaf carbon and nitrogen levels were altered by elevated CO<sub>2</sub> or O<sub>3</sub>, 2-cm<sup>2</sup> discs from six leaves were sampled within two days of the measurements of area loss. Leaves were chosen randomly using the same method as those employed for leaf sampling, i.e. by first randomly selecting the row, then the plant. The nodal position was however kept constant between treatments. Randomization was performed in the field by choosing from a set of numbers from 1–30 for row and plant, and from 1–3 for each leaflet on a soybean node. The leaf discs from each experimental plot were dried, ground to a fine powder using an automatic grinder (BioSpec products, Bartlettville, Oklahoma, USA) and pooled. Leaf carbon and nitrogen levels were determined with an elemental CHN analyzer (ECS 4010, COSTECH Analytical, USA).

Leaf temperatures were measured in ambient and elevated CO<sub>2</sub> plots in 2004 with two non-contact digital infrared thermometers (RadioShack, Fort Worth, Texas, USA). The thermometers were cross-calibrated over non-biological (metal and concrete) and biological (leaves) surfaces. The surface temperature of 25 randomly selected leaves at the top of the canopy was measured in each plot by holding the thermometers 10 cm above the leaf surface; each leaf was measured at two locations for a total of 50 individual leaf temperature measurements per plot. To avoid confounding time with treatment, the measurement of temperature in each ring was paired (e.g. an ambient plot was measured at the same time as an elevated CO<sub>2</sub> plot). Measurements were performed in blocks. Measurements

on the same leaf were averaged and the experimental plot was treated as the independent replicate.

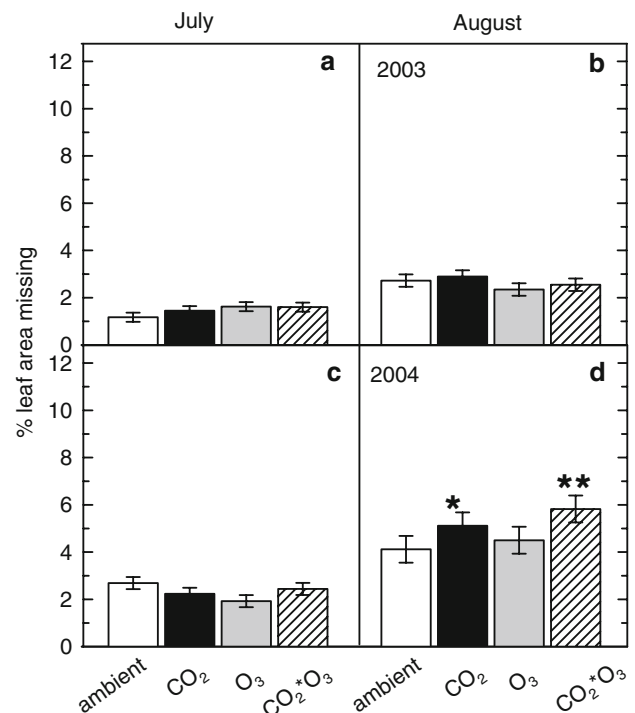
### Statistical analysis

To fulfill the assumptions of ANOVA, the data on leaf area removed by insects were square-root transformed before analysis. Data were separated by year and analyses were performed on the plot means; date and treatment were fixed effects and blocks were included as a random component ( $n = 4$ ; SAS version 8; SAS Institute, Cary NC). A repeated measure ANOVA (PROC MIXED) was used to test for treatment effects on percentage leaf area removed by insects. Values are presented as least squares means with standard errors. The total number of insects and the numbers of individuals of the five main insect species (Japanese beetles, potato leafhoppers, western corn rootworm, soybean nodule fly and multicolored Asian lady beetle (*Harmonia axyridis*) were analyzed in a mixed model ANOVA with treatment and date as fixed effects and blocks as a random component. Leaf carbon and nitrogen contents were analyzed in a mixed model repeated measures ANOVA. A mixed model ANOVA (PROC MIXED; SAS, The SAS Institute; Version 8.1, Cary, NC.) was used to test for treatment effects on leaf temperature. Treatment and day of measurement were fixed effects and blocks were random effects. Post hoc linear contrasts were performed to elucidate treatment effects within interaction terms. All comparisons were made between treatments and the control. Given the limitation on sample size inherent in all FACE experiments, we sought to avoid type II errors by recording results significant at both  $P \leq 0.05$  and at 0.1 as in Hamilton et al. (2004) and Dermody et al. (2005).

### Results

Exposure to elevated  $\text{CO}_2$  increased the area of soybean leaves removed by insect herbivores in 2004 but not in 2003 (August 2003, ambient vs.  $\text{CO}_2$ , F-stat = 0.5,  $P \leq 0.47$ ,  $n = 4$ , August 2004, ambient vs.  $\text{CO}_2$ , F-stat = 5.4,  $P \leq 0.1$ ,  $n = 4$ , Fig. 1). No effect of elevated  $\text{O}_3$  on leaf area removed was evident in either year ( $P > 0.1$ ). There was, however, a significant interaction between elevated  $\text{CO}_2$  and  $\text{O}_3$  in July 2004 (F-stat = 4.4,  $P \leq 0.1$ ,  $n = 4$ , Fig. 1). The amount of leaf area removed was significantly greater for plants exposed simultaneously to elevated  $\text{CO}_2$  plus elevated  $\text{O}_3$ , but the absence of a main effect of  $\text{O}_3$  suggests that this response was driven by exposure to elevated  $\text{CO}_2$ .

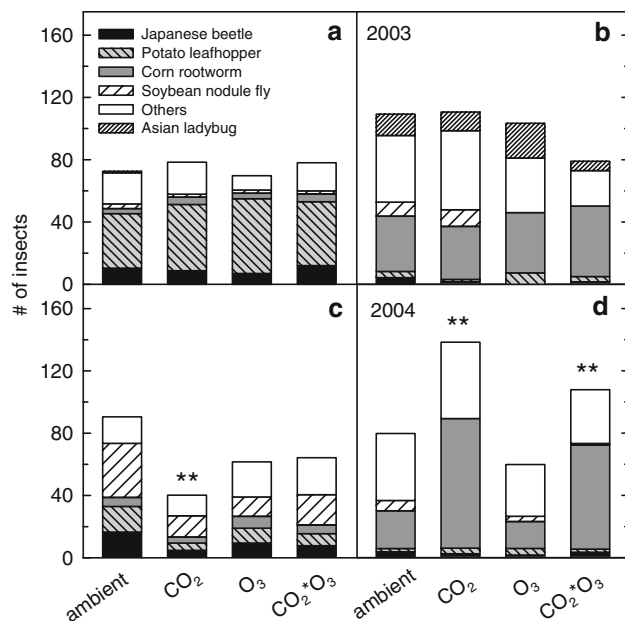
Increased foliar damage to plants exposed to elevated  $\text{CO}_2$  in August 2004 corresponded with greater numbers of insects (Fig. 2). Total numbers of insects were also higher



**Fig. 1** Amount of leaf tissue removed by herbivores as a percentage of the original leaf area, for soybean grown in ambient air (open bars),  $550 \mu\text{mol mol}^{-1} \text{CO}_2$  (black bars) and  $1.2 \times$  ambient  $\text{O}_3$  (grey bars) and elevated  $\text{CO}_2 * \text{O}_3$  (striped bar). The top two panels (a and b) correspond to July and August 2003 and the lower two (c and d) to July and August 2004. Each bar represents the least squared mean  $\pm$  standard error ( $n = 4$ ) calculated from the repeated measures ANOVA. Significant differences ( $P \leq 0.05$ ) between elevated  $\text{CO}_2$  or  $\text{O}_3$  and ambient air are indicated by \*\*, \* indicates differences significant at  $P \leq 0.1$

in elevated  $\text{CO}_2$  and  $\text{CO}_2 * \text{O}_3$  in August 2004. This result was driven primarily by increases in the number of western corn rootworms in elevated  $\text{CO}_2$  plots (ambient:  $15 \pm 11$  rootworms; elevated  $\text{CO}_2$ :  $57 \pm 13$  rootworms, F-stat = 5,  $P \leq 0.04$ ,  $n = 4$ ). There was no detectable effect of elevated  $\text{CO}_2$  or elevated  $\text{O}_3$  on the size of the insect population in 2003 (e.g. July, 2003, ambient:  $17 \pm 3$  insects; elevated  $\text{CO}_2$ :  $18 \pm 3$  insects, F-stat = 0.1,  $P \leq 0.9$ ,  $n = 4$ ; Fig. 2).

The composition of the insect community, including folivores and predators, varied from July to August. Japanese beetles (*P. japonica*) and potato leafhoppers (*E. fabae*) were abundant in July (Fig. 2). By August the numbers of individuals of these species had declined and western corn rootworms became dominant. Soybean nodule flies were present in July 2004 in all treatments (Fig. 2). Other species present in August but in low numbers ( $\leq 5$  individuals per plot) included minute pirate bugs (*Orius tristicolor*), soybean loopers (*Pseudoplusia includens*), cucumber beetles (*Diabrotica undecimpunctata howardii*) and bean leaf beetles (*Cerotoma trifurcata*). Of these,

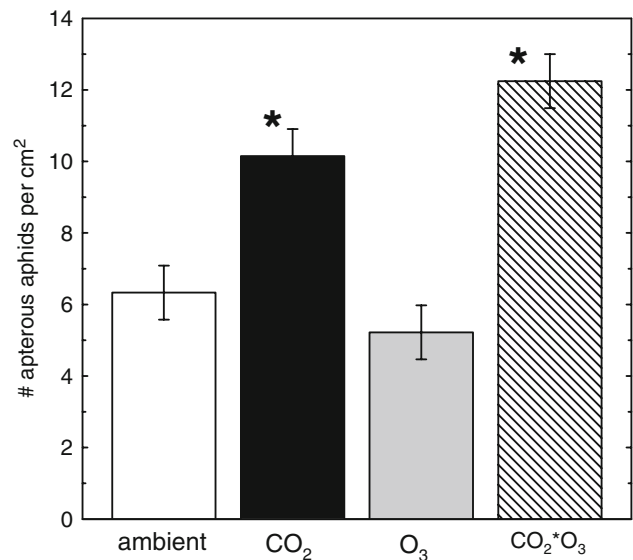


**Fig. 2** Total number of insects captured in soybeans grown in ambient air, 550  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>, 1.2 $\times$  ambient O<sub>3</sub> and elevated CO<sub>2</sub>\*O<sub>3</sub>. Each bar represents the pooled mean of all insect species separated into each of the dominant species. The black represents Japanese beetles, dark grey and striped represent potato leafhoppers, grey represents western corn rootworm, striped represents soybean nodule fly, fine stripes represent Asian lady beetle, and white bars include other species which generally numbered less than five individuals per experimental plot. The top two panels (a and b) correspond to July and August 2003 and the second two (c and d) to July and August 2004. Significant differences ( $P \leq 0.05$ ) between elevated CO<sub>2</sub> or O<sub>3</sub> and ambient air are indicated by \*\*, \* indicates differences significant at  $P \leq 0.1$

Japanese beetles and soybean loopers are folivores and probably were responsible for the greatest proportion of leaf area loss. Because of their low numbers, it is unlikely that various predators contributed to observed differences in leaf damage among the treatments.

During an outbreak in August 2003, soybean aphids responded strongly to elevated CO<sub>2</sub> but not to O<sub>3</sub>. Aphid densities were almost twice as high on leaves in elevated CO<sub>2</sub> and CO<sub>2</sub>\*O<sub>3</sub> plots compared to leaves grown in ambient air (Fig. 3).

In three out of four time periods, exposure to elevated CO<sub>2</sub> reduced the nitrogen content (F-stat = 22.8,  $P \leq 0.01$ ,  $n = 4$ ) and increased the C:N of leaves relative to those grown in ambient air (F-stat = 5.4,  $P \leq 0.03$ ,  $n = 4$ , Table 1). When CO<sub>2</sub> was combined with O<sub>3</sub> this effect was dampened; there was a trend toward lower leaf nitrogen and higher C:N in the CO<sub>2</sub>\*O<sub>3</sub> treatment but it was statistically significant only in July 2004 (Table 1). Apart from an isolated effect on C:N in August 2004, elevated O<sub>3</sub> had no detectable effect on these aspects of leaf chemistry (Table 1).

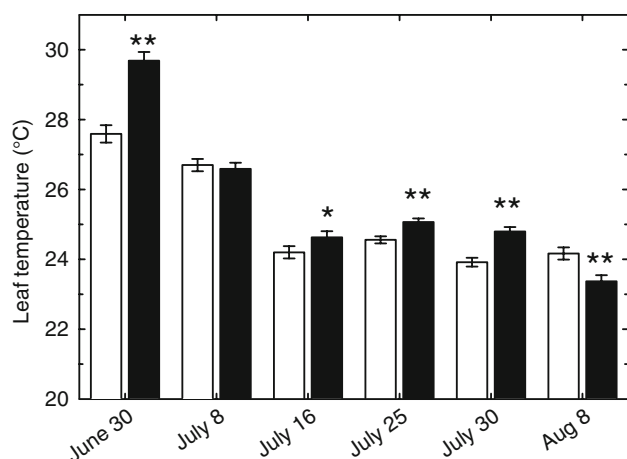


**Fig. 3** The density of apterous aphids on soybean leaves in 2003 grown in ambient air (open bars), 550  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> (black bars) and 1.2 $\times$  ambient O<sub>3</sub> (grey bars) and elevated CO<sub>2</sub>\*O<sub>3</sub> (striped bar). Each bar represents the least squared mean  $\pm$  standard error ( $n = 4$ ) calculated from the repeated measures ANOVA. Significant differences ( $P \leq 0.05$ ) between elevated CO<sub>2</sub> or O<sub>3</sub> and ambient air are indicated by \*

**Table 1** N content ( $\text{mg g}^{-1}$ ), C content ( $\text{mg g}^{-1}$ ) and C:N ratio of soybean leaves measured in mid-July and mid-August of 2003 and 2004.

Year	Month	Treat	N ( $\text{mg g}^{-1}$ )	C ( $\text{mg g}^{-1}$ )	C: N
2003	July	Ambient	57.77 (1.57)	446.73 (1.77)	7.75 (0.21)
		CO <sub>2</sub>	<b>53.62 (2.83)</b>	<b>451.96 (2.56)</b>	<b>8.50 (0.42)</b>
		O <sub>3</sub>	60.93 (2.85)	444.29 (1.14)	7.34 (0.34)
		CO <sub>2</sub> *O <sub>3</sub>	55.17 (0.59)	448.54 (2.58)	8.13 (0.10)
2003	August	Ambient	58.64 (0.87)	459.93 (0.83)	7.85 (0.13)
		CO <sub>2</sub>	<b>52.51 (1.67)</b>	456.36 (1.12)	<b>8.72 (0.30)</b>
		O <sub>3</sub>	56.80 (0.46)	458.84 (1.71)	8.08 (0.05)
		CO <sub>2</sub> *O <sub>3</sub>	55.39 (2.80)	461.22 (3.23)	8.39 (0.41)
2004	July	Ambient	51.58 (0.87)	452.72 (5.26)	8.78 (0.12)
		CO <sub>2</sub>	47.67 (1.23)*	453.48 (2.48)	9.53 (0.26)*
		O <sub>3</sub>	49.67 (1.46)	442.14 (2.05)	8.92 (0.24)
		CO <sub>2</sub> *O <sub>3</sub>	<b>41.37 (2.11)</b>	441.70 (9.07)	<b>10.74 (0.48)</b>
2004	August	Ambient	46.11 (2.44)	463.90 (1.22)	10.15 (0.57)
		CO <sub>2</sub>	44.33 (0.70)	457.03 (5.42)	10.31 (0.15)
		O <sub>3</sub>	<b>51.18 (0.77)</b>	459.97 (8.69)	<b>8.98 (0.05)</b>
		CO <sub>2</sub> *O <sub>3</sub>	44.47 (2.1)	457.72 (5.21)	10.35 (0.44)

Soybean was grown in ambient air, 550  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>, 1.2 $\times$  ambient O<sub>3</sub> and CO<sub>2</sub>\*O<sub>3</sub>. Leaf discs were sampled from the same section of the canopy in which herbivory was surveyed. Leaves were sampled within two days of photographing the leaf area removal by herbivores. The least squared means of 4 experimental plots are presented with standard error of the mean in parentheses. Significant differences ( $P \leq 0.05$ ) between elevated CO<sub>2</sub> or O<sub>3</sub> or CO<sub>2</sub>\*O<sub>3</sub> and ambient air are in bold, \* indicates differences significant at  $P \leq 0.1$



**Fig. 4** Mean temperature of leaf surfaces in a soybean canopy grown in ambient air (open bars) and  $550 \mu\text{mol mol}^{-1}$   $\text{CO}_2$  (black bars). Leaf temperature was measured between 11 am and 2 pm on June 30th, July 8th, July 16th, July 25th, July 30th and August 8th in 2004. Each bar represents the least squared mean  $\pm$  standard error ( $n = 4$ ) calculated from the repeated measures ANOVA. Significant differences ( $P \leq 0.05$ ) between elevated  $\text{CO}_2$  or  $\text{O}_3$  and ambient air are indicated by \*\*, \* indicates differences significant at  $P \leq 0.1$

With the exception of August, when senescence of the soybean canopy was more advanced in the ambient plots (Dermody et al. 2006), and July 8th, exposure to elevated  $\text{CO}_2$  increased leaf temperature (F-stat = 25.1,  $P \leq 0.01$ ,  $n = 4$ , Fig. 4). The average increase was  $\sim 1^\circ\text{C}$  across all measurement dates.

## Discussion

In August 2004, plants grown under elevated  $\text{CO}_2$  had significantly more leaf damage and in 2003 the populations of chewing insects and phloem-feeding aphids were greater than for plants grown in ambient air (Figs. 1 and 2). In the year prior to this study, Hamilton et al. (2005) also observed a substantial increase in leaf damage caused by greater numbers of Japanese beetles in these same elevated- $\text{CO}_2$  plots. Taken together, these results suggest that future increases in atmospheric  $\text{CO}_2$  will increase the susceptibility of soybean to insect pests. However, in a cool and wet July 2004, the total number of insects was reduced by growth in elevated  $\text{CO}_2$ , indicating how climate variability may complicate any predictions of the effects of elevated  $\text{CO}_2$  and  $\text{O}_3$  on insect pests. Damage to soybean foliage was caused primarily by increased populations of non-indigenous insects (e.g. Japanese beetles and soybean aphid) that have only recently appeared in Midwest fields (Potter and Held 2002; Ragsdale et al. 2004; Rutledge et al. 2004). The spread of invasive pests is an increasingly important threat to agro-ecosystems, and the increased

abundance of these species on plants grown under elevated  $\text{CO}_2$  suggests that these two elements of global change may act synergistically to reduce crop yields in the future.

Growth in elevated  $\text{CO}_2$  changes many facets of plant metabolism that potentially alter the feeding behaviour of insects. In this study, increased numbers of insects and leaf area loss to herbivory were associated with greater leaf C:N in elevated  $\text{CO}_2$  (Table 1, Figs. 1 and 2). Most phytophagous insects are limited by nitrogen and greater C:N may have stimulated herbivory. Additionally, nitrogen metabolite pools can change following exposure to elevated  $\text{CO}_2$  (Stitt and Krapp 1999); for instance Ainsworth et al. (2007) measured decreased levels of ureides, specifically allantoin in developing leaves growing in elevated  $\text{CO}_2$  at SoyFACE. As a storage form of N, allantoin is not an optimal N source for folivorous insects on soybean (Wilson and Stinner 1984), so it is possible that increased susceptibility of leaves to herbivory in elevated  $\text{CO}_2$  reflected a preference of insects for these lower ureide levels. The nutritional requirements of insects differ depending on life stage, with actively growing larval forms often limited by nitrogen and reproducing adults limited by energy sources (Cloutier et al. 2000; Jacob and Evans 2000; Kopper et al. 2001; Wheeler 2003; Scheirs et al. 2004). As reproductive adults, Japanese beetles and western corn rootworm may respond more strongly to the substantial increase in soluble carbohydrates evident in soybean leaves grown under elevated  $\text{CO}_2$  (Hamilton et al. 2005). Insect herbivores also may have responded to changes in leaf secondary compounds in plants grown in elevated  $\text{CO}_2$ . Specifically, increased lifespan and fecundity of Japanese beetles are associated with higher levels of individual flavonols (quercetin and kaempferol) that have potent antioxidant properties (B.F. O'Neill, unpublished), as well as with reduced levels of defensive protease inhibitors (Zavala et al. 2008) in leaves grown under elevated  $\text{CO}_2$ . The relative contribution of changes in primary versus secondary metabolism on herbivory of soybean under elevated  $\text{CO}_2$  is not yet known.

Chronic exposure to  $\text{O}_3$  did not affect the susceptibility of soybeans to herbivory (Fig. 1). Growth in elevated  $\text{O}_3$  induces many biochemical changes in leaves, including multiple defence pathways; however, these effects are strongest in older leaves that develop at high levels of  $\text{O}_3$  (Reid and Fiscus 1998; Sandermann et al. 1998; Booker et al. 2005; Riikonen et al. 2005). Many herbivorous insects preferentially feed on young upper canopy leaves (Bale et al. 2002; Potter and Held 2002), which show few symptoms of  $\text{O}_3$  damage after chronic exposure (Ewert and Porter 2000; Morgan et al. 2004). This phenomenon may explain why our results contrast with those of Endress and Post (1985) and Chappelka et al. (1988), who, in closed systems and with acute exposure, found that leaf area

removal increased for soybean foliage grown in elevated O<sub>3</sub>. In previous experiments, the effects of elevated O<sub>3</sub> on leaf chemistry and thus on herbivory may have been overestimated by artefacts associated with enclosed systems, e.g. unrealistically high concentrations of O<sub>3</sub> at the base of the canopy at the site of O<sub>3</sub> injection into the chamber (Long and Naidu 2002). FACE technology avoids these artefacts and allows a chronic treatment, more representative of the predicted scenario for major agricultural areas such as the Midwest United States (Prather et al. 2001). However, the effects of O<sub>3</sub> may be more severe in agricultural areas heavily influenced by cities (Ashmore et al. 2006). In aspen trees exposed to elevated CO<sub>2</sub> and O<sub>3</sub> in a FACE system, the effects of the treatments varied depending on which insect herbivore was examined; however, overall effects on leaf chemistry and insect performance were relatively small (Kopper et al. 2001; Lindroth et al. 2001; Kopper and Lindroth 2003).

The density of western corn rootworms was greater on soybean plants grown in elevated CO<sub>2</sub> relative to those in ambient air (Fig. 2). Western corn rootworm is the most important pest of corn in the United States, and, until recently, annually rotating corn with soybean was the principal management strategy to control its numbers (Onstad et al. 2003). Some populations of western corn rootworm circumvent this crop rotation strategy by ovipositing in soybean fields (Onstad et al. 2003; Crowder et al. 2005), and nutritional stress increases the oviposition rate of western corn rootworm (Mabry et al. 2004). Soybean foliage is not an optimal food for western corn rootworm, and higher C:N of soybeans in elevated CO<sub>2</sub> relative to ambient air may have increased nutritional stress, thereby increasing the number of eggs (Schroeder et al. 2006). Although CO<sub>2</sub> is an attractant for western corn rootworm larvae in soil, the adult rootworms are not attracted to CO<sub>2</sub> (Bernklau and Bjostad 1998; Hammack and Petroski 2004). Like many other folivorous insects, western corn rootworms are attracted to plants and stimulated to feed by green leaf volatiles (Hammack 2001; Pare et al. 2005; Pophof et al. 2005). Greater production of green leaf volatiles measured in soybeans grown in elevated CO<sub>2</sub> relative to ambient air (B.F. O'Neill, unpublished) may have contributed to the higher numbers of western corn rootworm. Elevated CO<sub>2</sub> also increased the emission of green leaf volatiles in other systems (Staudt et al. 2001; Vuorinen et al. 2005).

During an outbreak in 2003, the number of aphids per unit leaf area was considerably higher for soybeans grown under elevated CO<sub>2</sub> than in ambient air (Fig. 3). In addition to altering leaf chemistry, growth under elevated CO<sub>2</sub> may have improved the canopy micro-environment for aphids. By decreasing stomatal conductance, exposure to elevated CO<sub>2</sub> increases canopy temperature (Rogers et al. 2004),

which may in turn shorten the generation time of aphids (Bezemer et al. 1998; Holopainen and Kainulainen 2004; Newman 2004) and contribute to their higher densities on soybeans. Although the effect varied across the season, we measured an average increase of 1°C in leaf surface temperature in 2004 (Fig. 4). The exception in August was associated with a change in phenology; plants in the ambient plots had initiated canopy senescence at this time (Dermody et al. 2006) and presumably were operating at lower conductances and hence had greater leaf temperatures than those growing under elevated CO<sub>2</sub>. In this system, continuous measurements over three growing seasons revealed that leaf temperature was consistently 0.2°C higher during the day and 0.5°C higher at mid-day under elevated CO<sub>2</sub> (Bernacchi et al. 2006).

Nutritional factors also may have contributed to increased aphid numbers in soybeans grown under elevated CO<sub>2</sub>. Soybean aphids preferentially feed on young soybean leaves (Ragsdale et al. 2004), and in elevated CO<sub>2</sub> higher maximum leaf area index and delayed senescence contributed to an abundance of young leaf tissue (Dermody et al. 2006). Aphids rely on free amino acids in phloem fluid (Helden et al. 1994) and their response to elevated CO<sub>2</sub> may be governed by interactions with leaf nitrogen (Newman et al. 2003). Leaf nitrogen content is high in soybean (Dermody et al. 2006) and was probably sufficient to allow continued growth of aphid populations, even with increased leaf C:N in elevated CO<sub>2</sub> (Hoover and Newman 2004).

Soybean aphids were first recorded in the United States in 2000 (Wedburg 2000) and as newly invasive pests their populations are not regulated by specialist predators. However, soybean aphids are vulnerable to predation by generalists, including multicolored Asian lady beetles and minute pirate bugs (Fox et al. 2004). The multicolored Asian lady beetle responds rapidly to increased populations of soybean aphids and is a well known aphid predator in its native range (Koch 2003; Mignault et al. 2006). At SoyFACE, Asian lady beetles co-occurred with soybean aphid, although we were unable to detect any effects of elevated CO<sub>2</sub> and CO<sub>2</sub>\*O<sub>3</sub> on their populations. Asian lady beetles are becoming an integral part of the pest management strategy for soybean aphid (Rutledge et al. 2004), but the higher density of aphids in the elevated CO<sub>2</sub> plots suggests that the feeding activity of Asian lady beetles may not compensate for faster population growth rates of aphids in elevated CO<sub>2</sub> and CO<sub>2</sub>\*O<sub>3</sub>. Aphid feeding can reduce instantaneous rates of leaf level photosynthesis by as much as 50% (Macedo et al. 2003) and can lower subsequent soybean yields by as much as 28% (Wang et al. 1996). Aphid outbreaks are highly episodic and difficult to compare across years; however, in 2003, aphid densities were highest when soybean was in the reproductive phase and at

its most vulnerable to herbivore damage (Haile et al. 1998). If this pattern is repeated in the future, the productivity gains expected in elevated CO<sub>2</sub> and CO<sub>2</sub>\*O<sub>3</sub> (Morgan et al. 2005) may be reduced by aphid outbreaks.

A variety of factors, including increased leaf C:N and warmer leaf surfaces, were associated with higher susceptibility of soybean to herbivory in elevated CO<sub>2</sub> and CO<sub>2</sub>\*O<sub>3</sub>. In addition to the effects on leaf area removal, the fecundity of Japanese beetles that were fed on leaves grown in elevated CO<sub>2</sub> was greater than those that fed on leaves grown under ambient conditions (O'Neill et al. 2008). Greater fecundity suggests that the rate of population increase for this species will be even higher under elevated CO<sub>2</sub> than it is today. The effects of elevated CO<sub>2</sub> on aphid densities were particularly notable, given the capacity of this pest to reduce soybean yields (Wang et al. 1996; Rice et al. 2005). Predicting the response of insect herbivores to elevated CO<sub>2</sub> and O<sub>3</sub> is difficult, and will depend on feeding guild, life stage and adaptability of both the plant and insect to changing atmospheric conditions (Bezemer et al. 1998; Hughes and Bazzaz 2001; Newman et al. 2003; Chen et al. 2004). Because we were unable to quantify insect movement between plots and the role of diseases and predators in driving population dynamics, our results highlight the net effects of elevated CO<sub>2</sub> and O<sub>3</sub> on leaf area loss to herbivory. It is possible that insects preferentially move to elevated CO<sub>2</sub> plots given their preference for tissue grown in elevated CO<sub>2</sub> (Hamilton et al. 2005). Despite this limitation, our results suggest that greater insect damage may be one factor contributing to lower than expected yield gains when soybean is grown under elevated CO<sub>2</sub> in open-air FACE experiments compared to partially enclosed chambers that reduce the movement of pests (Long et al. 2006).

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