

## Acute O<sub>3</sub> damage on first year coppice sprouts of aspen and maple sprouts in an open-air experiment

Joseph N. T. Darbah,<sup>\*ab</sup> Wendy S. Jones,<sup>b</sup> Andrew J. Burton,<sup>b</sup> John Nagy<sup>c</sup> and Mark E. Kubiske<sup>a</sup>

Received 28th March 2011, Accepted 22nd June 2011

DOI: 10.1039/c1em10269a

We studied the effect of high ozone (O<sub>3</sub>) concentration (110–490 nmol mol<sup>-1</sup>) on regenerating aspen (*Populus tremuloides*) and maple (*Acer saccharum*) trees at an open-air O<sub>3</sub> pollution experiment near Rhinelander WI USA. This study is the first of its kind to examine the effects of acute O<sub>3</sub> exposure on aspen and maple sprouts after the parent trees, which were grown under elevated O<sub>3</sub> and/or CO<sub>2</sub> for 12 years, were harvested. Acute O<sub>3</sub> damage was not uniform within the crowns of aspen suckers; it was most severe in the mature, fully expanded photosynthesizing leaves. Young expanding leaves showed no visible signs of acute O<sub>3</sub> damage contrary to expectations. Stomatal conductance played a primary role in the severity of acute O<sub>3</sub> damage as it directly controlled O<sub>3</sub> uptake. Maple sprouts, which had lower stomatal conductance, smaller stomatal aperture, higher stomatal density and larger leaf surface area, were tolerant of acute O<sub>3</sub> exposure. Moreover, elevated CO<sub>2</sub> did not ameliorate the adverse effects of acute O<sub>3</sub> dose on aspen and maple sprouts, in contrast to its ability to counteract the effects of long-term chronic exposure to lower O<sub>3</sub> levels.

### Introduction

Tropospheric ozone (O<sub>3</sub>) is one of the serious air pollutants<sup>1</sup> that have negative effects on both plants and animals.<sup>2</sup> Results from large-scale field experiments have shown conclusively that forest trees are adversely affected by O<sub>3</sub> at concentrations as low as 50–60 nmol mol<sup>-1</sup>.<sup>3,4</sup> Due to increasing concentration of O<sub>3</sub> in the troposphere, mainly as a result of anthropogenic activities,<sup>5–8</sup> greater portions of the global forest ecosystem are now subject to toxic O<sub>3</sub>.<sup>9,10</sup> In plants, O<sub>3</sub> toxicity is manifested as visible foliar symptoms such as necrotic lesions,<sup>11,12</sup> decreases in yield<sup>13</sup> and

growth,<sup>14</sup> changes in carbon assimilation and stomatal conductance,<sup>15–20</sup> and total leaf necrosis.<sup>21</sup>

Previously, O<sub>3</sub> damage was thought to be mainly due to external exposure (external contact with air pollutant not uptake),<sup>22</sup> whereas more recently it is understood that leaf O<sub>3</sub> uptake is more directly related to O<sub>3</sub> injury.<sup>23–25</sup> Ozone uptake by leaves occurs through stomatal and non-stomatal pathways.<sup>26–30</sup> Non-stomatal uptake includes O<sub>3</sub> deposition on cuticles,<sup>31</sup> while stomatal uptake is associated with stomatal conductance. Low stomatal conductance observed in Mediterranean plants has been associated with their high O<sub>3</sub> tolerance,<sup>16</sup> as this leads to lower O<sub>3</sub> uptake, and lower O<sub>3</sub> concentration inside the leaf, causing less damage.<sup>18</sup> More recently Fares *et al.*<sup>31</sup> observed that O<sub>3</sub> fluxes inside the leaves were directly affected by stomatal conductance and both decreased with leaf age. Short term acute O<sub>3</sub> exposure does not significantly affect stomatal conductance unlike long-term exposure which can cause significant decreases in stomatal conductance,<sup>32</sup> possibly due to the sluggish nature of stomata under high O<sub>3</sub> levels.<sup>33</sup> Heavy O<sub>3</sub> doses could lead to

<sup>a</sup>USDA Forest Service, Northern Research Station, Rhinelander, WI, 54501, USA. E-mail: jndarbah@mtu.edu; Tel: +1 740 331 0332

<sup>b</sup>School of Forest Resources and Environmental Science, Michigan Technological University, 175 Sam Horner hall, 1400 Townsend Drive, Houghton, MI, 49931, USA

<sup>c</sup>Environmental Sciences Department, Brookhaven National Laboratory, Upton, NY, 11973, USA

### Environmental impact

We tested the effect of acute O<sub>3</sub> exposure on regenerating trembling aspen and sugar maple trees in an open-air exposure experiment. We found that aspen leaves in the mid canopy were severely damaged in 3 days, with 100% necrosis. Those leaves had the highest photosynthesis and stomatal conductance rates in the canopy, whereas newly expanding or mature lower canopy aspen leaves, and sugar maple leaves, had low conductances and therefore lower O<sub>3</sub> uptake. Our study confirms that leaf O<sub>3</sub> uptake is a more robust indicator of O<sub>3</sub> damage to plants than is atmospheric O<sub>3</sub> concentration. Also, acute O<sub>3</sub> uptake is not ameliorated by elevated CO<sub>2</sub> unlike chronic exposure at lower concentrations.

rapid O<sub>3</sub> accumulation in intercellular spaces before stomatal closure occurs,<sup>27</sup> resulting in acute O<sub>3</sub> damage.

In addition to O<sub>3</sub> resistance *via* low stomatal conductance, the ameliorative effects of elevated CO<sub>2</sub> concentration on the adverse effects of O<sub>3</sub> have been documented in experiments involving simultaneous exposure to both gases.<sup>17,34–39</sup> Under elevated CO<sub>2</sub> plants often have decreased stomatal conductance, which, in turn, decreases O<sub>3</sub> uptake and therefore reduces O<sub>3</sub> damage.<sup>5,40,41</sup> Recently, Uddling *et al.*<sup>42</sup> reported that the interaction between elevated CO<sub>2</sub> and O<sub>3</sub> at the stomatal level may not necessarily result in a decrease in adverse O<sub>3</sub> effects. This is because elevated CO<sub>2</sub> did not decrease stomatal conductance significantly as much as predicted by various models, and hence, stomatal O<sub>3</sub> uptake is not decreased much especially in northern hardwood forests. Most of the previous reports come from studies involving chronic exposure to moderately high O<sub>3</sub> and not acute exposure to a high dose of O<sub>3</sub> in a short period of time (72–96 h). Hence there is the need to examine the interactive effect of elevated CO<sub>2</sub> and acute dose of O<sub>3</sub> under field conditions.

In this paper we report the effects of a short term, very high dose of O<sub>3</sub> on first-year trembling aspen and sugar maple coppice regeneration from trees that were exposed to elevated O<sub>3</sub> their entire lives (12 years in the former Aspen FACE experiment<sup>43</sup>), harvested, and then allowed to sprout under elevated concentrations of CO<sub>2</sub> and/or O<sub>3</sub>. Our objectives were to determine whether: (1) there were any physiological factors that enable maple to tolerate very high doses of O<sub>3</sub> without visible foliar symptoms compared to aspen; (2) leaf age affects sensitivity to acute O<sub>3</sub> concentration over a short period of time under field conditions; and (3) elevated CO<sub>2</sub> will ameliorate the adverse effects of acute O<sub>3</sub> dose on aspen leaves.

## Materials and methods

### Experimental design

The former Aspen FACE experiment near Rhinelander WI, USA (45.6° N, 89.5° W) laid the foundation for the present study. That experiment site was established in 1997 as the first open-air facility to study the responses of northern forest trees to interacting elevated concentrations of CO<sub>2</sub> and O<sub>3</sub>.<sup>7,43,44</sup> The experiment was designed with three replicates of four treatments: control (C), elevated CO<sub>2</sub> (+CO<sub>2</sub>) of 560 μmol/mol, elevated O<sub>3</sub> (+O<sub>3</sub>) of 1.5 times ambient, and elevated concentrations of both gases (+CO<sub>2</sub> + O<sub>3</sub>). Ozone was generated on-site from pure O<sub>2</sub> gas. The twelve open-air fumigation treatment plots, or “rings”, were 30 m in diameter with treatment gas concentrations monitored at plot center and regulated using FACE technology as described by Hendrey *et al.*<sup>45</sup> Each FACE plot was surrounded by a ring of 32 vertical gas-emitting pipes that dispensed the treatment gasses under computer control. At any one time, only ten of the thirty two vertical emitter pipes operated in each ring depending upon wind direction. The diluted gasses were injected where they quickly mixed with the ambient air, against the wind and were carried into the plots. Further details of the site and experimental design are described in Dickson *et al.*<sup>43</sup>

Each treatment ring was planted in 1997 with six month old rooted cuttings of five clones of trembling aspen (*Populus tremuloides* Michx.) and six-month old seedlings of sugar maple (*Acer*

*saccharum* Marsh.) and paper birch (*Betula papyrifera* Marsh.) from open-pollinated seed sources. All trees were planted at a 1 × 1 m spacing. The east half of each treatment ring was a random distribution of the 5 aspen clones, the northwest quadrant was a 50/50 alternating arrangement of aspen (only clone 216) and maple, and the southwest quadrant was a 50/50 alternating arrangement of aspen and birch. The trees were allowed to grow under the fumigation treatments and natural climatic conditions from 1998 through 2008. In July and August 2009, the subplots within each FACE ring were experimentally harvested to fully assess the final above- and below-ground distribution of biomass, C and N. All remaining trees were removed (cut to ground level) during the winter of 2009–10. Beginning in spring, 2010, the rings were allowed to naturally regenerate with aspen root suckers and maple and birch stump sprouts, under the previously established treatments (*Aspen FACE, Phase II*). Thus, this was the first open-air facility to study how forest vegetative regeneration will be affected under a future, modified atmosphere.

Per the established treatment protocols, neither CO<sub>2</sub> nor O<sub>3</sub> were dispensed at night or when the maximum daytime temperatures were projected to be less than 15 °C for O<sub>3</sub> (since O<sub>3</sub> formation declines below this temperature) and 4.5 °C for CO<sub>2</sub> (since photosynthetic activity is insignificant at this temperature). In addition, O<sub>3</sub> was not dispensed during periods when leaf surfaces were wet (from fog, dew, or rain), or when sustained winds exceeded 5 m s<sup>-1</sup> (for both treatments).

### Field measurements

During the brief period of this study (DOY 197, 198 & 200), wind directions were predominantly from the northwest and the combined effects of these low winds and the ozone treatments were the basis for the study. Our measurements focused on the northwest quadrant of the elevated O<sub>3</sub> and elevated CO<sub>2</sub> + O<sub>3</sub> treatment rings, which consisted of the mixed aspen and maple community. Acute O<sub>3</sub> injury was observed by visual inspection. Leaf blade of length 15mm was regarded as Leaf plastochron index (LPI) of zero. Nine leaves per species were selected (from upper [LPI 1–10 ± 2], mid-canopy [LPI 11–20 ± 5 depending on height] and lower canopy [lowest 10 green leaves] from each treatment for measurements of instantaneous photosynthesis and stomatal conductance and photosynthetic light response using a portable photosynthesis system (Model LI-6400, LiCor, Lincoln, NE, USA). All measurements were conducted between 0900 and 1200 h local daylight savings time.

Aspen and maple sprouts were 1.5–2.0 m and 1.0–1.5 m tall respectively, at the time of the measurement. Yet, these differences in height between the aspen and maple sprouts did not affect our results. Measurements were taken from plants within 5 m of the pipe vents and not the core section of the quadrant. Gas exchange measurements were taken twice (first on DOY 194) before visible necrotic symptoms were observed and (a second one on DOY 202) after the visible symptoms were seen. Fumigation was carried out only on DOY 197, 198 and 200 between the two measurement days (DOY 194 and 202) making a total exposure days of 3.

Clear nail polish was applied to the lower side of the leaves (both upper and lower canopy leaves) and allowed to air dry.

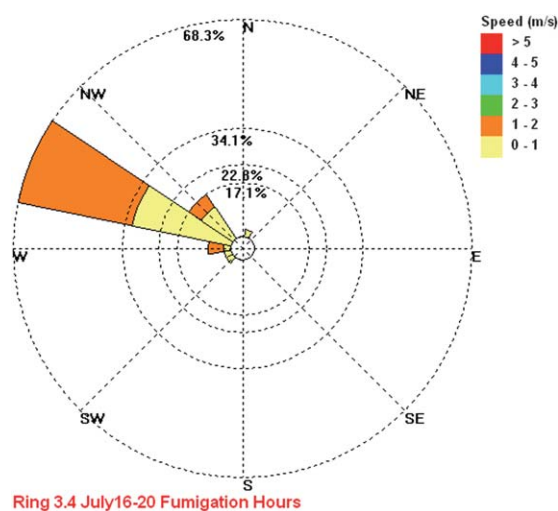
Then a clear tape was applied on the dried nail polish and peeled off to capture the stomatal imprints for microscopic examination. From these stomatal imprints, stomatal aperture and density were measured and counted using an upright biological microscope (Motic BA 400 EPI-Fluorescence, Ted Pella Inc., Redding, CA, USA). These leaves were then harvested and their leaf area measured with a portable leaf area meter (LI-3100C, LiCor Inc., Lincoln, NE, USA). Leaf discs of 2 cm<sup>2</sup> were taken from these leaves (2 discs per leaf and 3 leaves per ring for each species, oven dried at 65 °C for 72 h and weighed to compute specific leaf area.

The O<sub>3</sub> flux equation used by Wieser and Havranek<sup>46</sup> was adopted here to calculate O<sub>3</sub> flux into the leaves:

$$F = [O_3] * gO_3 \quad (\text{eqn 1})$$

Where F is the flux or uptake rate of O<sub>3</sub> (mol m<sup>-2</sup> s<sup>-1</sup>), [O<sub>3</sub>] is the ozone concentration in the ambient air (nmol mol<sup>-1</sup>) and gO<sub>3</sub> is the stomatal conductance (mol m<sup>-2</sup> s<sup>-1</sup>) for O<sub>3</sub>. Stomatal conductance for O<sub>3</sub> was calculated by multiplying the conductance for water vapor by the ratio of diffusivities of water vapor and O<sub>3</sub> (0.613).<sup>46</sup> This method for calculating O<sub>3</sub> uptake has previously been found to be in good agreement with measured O<sub>3</sub> fluxes into leaves.<sup>46-48</sup>

Ozone concentrations at the treatment gas emitters were computed using the ozone generator output (3.25 g m<sup>-3</sup>), conversion from concentration to mixing ratio (510 000 nmol mol<sup>-1</sup> (g m<sup>-3</sup>)<sup>-1</sup>, the estimated fan rate (100 000 L min<sup>-1</sup>), and the 1-minute sample oxygen carrier gas flow rate reading in L min<sup>-1</sup>. If the systematic uncertainty in each of these four factors were 10%, then the overall systematic uncertainty in the vent mixing ratio when combined in quadrature, *i.e.* uncorrelated, would be 20%. Based on these calculations, the daily average O<sub>3</sub> concentration coming out of the vents when ozone treatment was active ranged between 110 and 490 nmol mol<sup>-1</sup> over the three exposure days and six treatment plots. Occasional brief peaks reached 1990 nmol mol<sup>-1</sup> (the average time for this maximum value is in the order of 1 s with a recording interval of 1 min). Day-to-day variation in ambient ozone mixing ratio affected all plots the same way. The micrometeorological conditions in each plot also



**Fig. 1** Distribution of hourly wind speed and direction measured at 2 m above ground inside the rings. Wind speed measured on site during the high dose exposure period (DOY 197, 198 and 200 in 2010) at NFEE site, Rhinelander WI, USA.

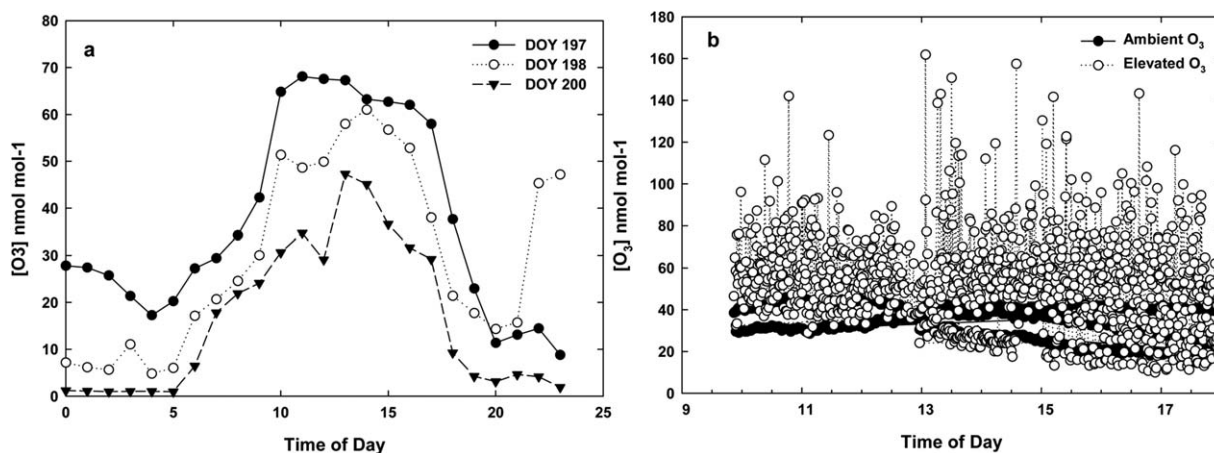
had a strong influence on O<sub>3</sub>, and this varied day-to-day and plot-to-plot.

### Statistical analysis

Data were analyzed in a randomized block ANOVA, using Tukey's HSD post hoc test for multiple comparisons of treatment means. Treatment effects were regarded as significantly different at P ≤ 0.05. The statistical test was performed using the SigmaStat version 3.5 by Systat Inc.

### Results

During the study period (DOY 197, 198 and 200; there was no fumigation on DOY 199 due to weather conditions), 68% of hourly wind directions were from 304 to 326°, and 80% were from 281 to 349° (Fig. 1). Moreover, wind speeds during this period were unusually low with an hourly mean of 0.1 m s<sup>-1</sup>.



**Fig. 2** (a) Mean hourly [O<sub>3</sub>] recorded in the middle of the rings for DOY 197, 198 and 200 (b) Ozone concentrations recorded every minute showed peaks of up to 160 nmol mol<sup>-1</sup> in the middle of the rings. These measurement were taken on DOY 197, 198 and 200 at the NFEE site Rhinelander WI, USA.



This low wind speed decreased diffusion and transport rate of the emitted  $O_3$  treatment gas which, in turn, caused higher  $O_3$  around the sprouting aspen and maple trees in the ring quadrant that were closest to the vertical vent pipes and outside the core section where long-term measurement are taken. Occasionally,  $O_3$  peaks of up to  $160 \text{ nmol mol}^{-1}$  were recorded by the monitor at plot center over the study period (Fig. 2b).

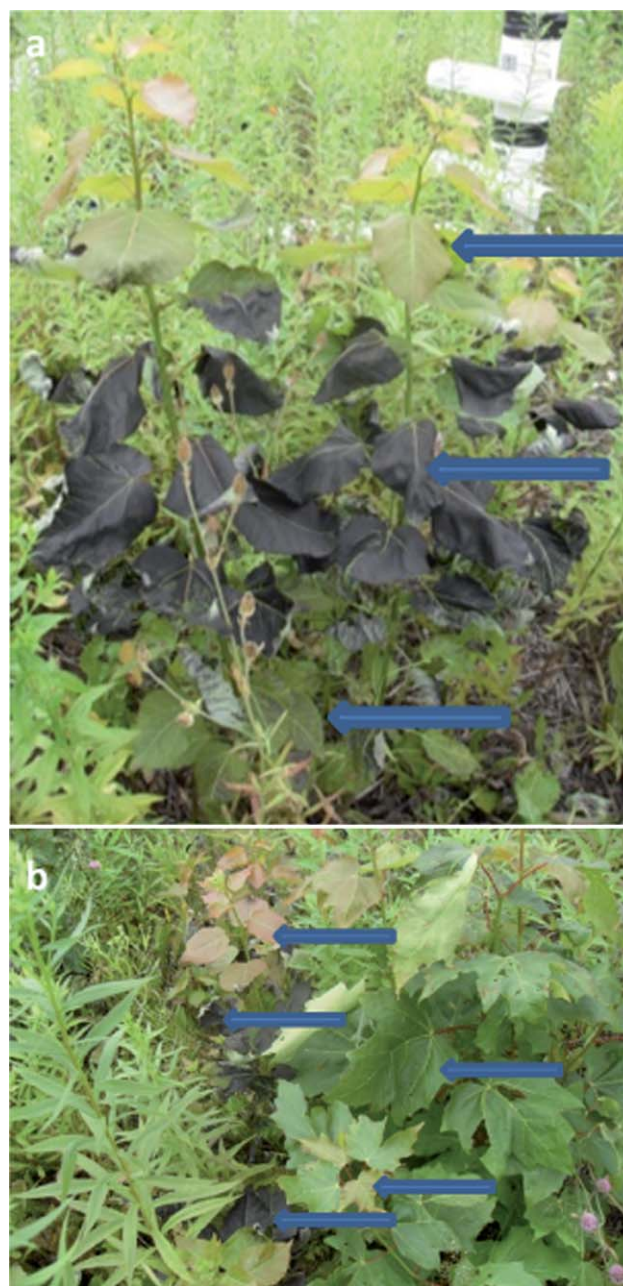
Mid canopy leaves of plants close to the emitters (less than 4 m) had 100% necrosis, while those 8 m away had 50% and those 14 m away had hardly any (<5%) necrotic spots on their mid-canopy leaves. We also observed that acute  $O_3$  damage was not uniform on the young aspen canopy and that leaves of different ages and species responded differently. Expanding and recently expanded aspen leaves of leaf plastochron index (LPI)<sup>49</sup>  $1-10 \pm 2$  observed during the study period did not have signs of severe  $O_3$  damage (Fig. 3a). Fully expanded, matured leaves were the ones that suffered most severely as they were killed (100% necrosis) and shed within 72–96 h. Older or lower canopy leaves had few necrotic spots (about 30% necrosis) on them but did not abscise. Interestingly, maple leaves immediately adjacent to dead aspen leaves did not show any visible symptoms of acute  $O_3$  damage (Fig. 3b).

Instantaneous gas exchange rates were measured in the upper and mid canopy before the occurrence of acute  $O_3$  damage (Table 1). There were no significant differences ( $P > 0.05$ ) in either photosynthesis or stomatal conductance rates of aspen upper canopy leaves under control treatment compared to those under  $+O_3$ . We observed a significant difference between upper- and mid-canopy leaves in both photosynthesis ( $P < 0.01$ ) and stomatal conductance ( $P = 0.02$ ) rates under  $+O_3$  treatment. The mature, mid-canopy aspen leaves that became damaged by  $O_3$  had nearly three times ( $P < 0.01$ ) the gas exchange rates of adjacent, mature maple leaves. Moreover, gas exchange rates of upper canopy leaves (measured on DOY 202), indicate that instantaneous photosynthesis and conductance rates of expanding aspen leaves with no visible damage were not significantly different ( $P > 0.05$ ) from those of mature, undamaged maple leaves (mid canopy leaves). This is in contrast to the mid-canopy aspen leaves that had three times higher stomatal conductance rates ( $P < 0.01$ ). This implies that stomatal  $O_3$  uptake was three times higher in mature aspen leaves than in maple leaves or young expanding aspen leaves.

Stomatal aperture was significantly larger ( $P < 0.01$ ), but stomatal density lower ( $P > 0.05$ ) in mature aspen than maple leaves (Table 1). Despite lower stomatal density, stomata apertures occupied a larger proportion of the leaf surface in aspen ( $8\% \pm 1\%$ ) compared to maple ( $3\% \pm 0.3\%$ ).

Specific leaf area did not differ significantly between species ( $0.0076 \pm 0.0002 \text{ cm}^2 \text{ g}^{-1}$  and  $0.0078 \pm 0.0003 \text{ cm}^2 \text{ g}^{-1}$  for aspen and maple respectively) indicating that the photosynthetic tissues in aspen leaves were serviced by 2.7 times the stomatal pore space as that of maple.

We observed equally severe acute  $O_3$  damage under elevated  $CO_2 + O_3$  as in  $O_3$  alone. The images in Fig. 3 were taken from the elevated  $CO_2 + O_3$  treatment. Even though photosynthesis rates were significantly higher in both species under elevated  $CO_2 + O_3$  relative to control ( $P < 0.001$  for both species), stomatal conductance did not differ significantly between treatments in both species. Consequently, stomatal uptake of  $+O_3$  did not differ between treatments for each species (Table 2).



**Fig. 3** (a) Visible acute  $O_3$  damage is more severe on mature, actively photosynthesizing leaves (mid-canopy leaves) compared to young expanding leaves (upper-canopy leaves) and older leaves (lower-canopy leaves). Hence, damage is not uniform in the canopy profile or with leaf age. (b) Although aspen and maple were equidistance from the fumigation vents, there were no visible symptoms on maple leaves but aspen leaves were severely damaged (100% necrotic).

## Discussion

### Acute $O_3$ damage was related to variation in leaf age and physiology throughout the canopy

In our study, we found that leaf damage due to  $O_3$  exposure varied throughout the aspen-maple canopy due to variation in both atmospheric  $O_3$  concentration and physiological factors related to leaf age. Ozone damage decreased along a decreasing

**Table 1** A comparison of physiological and morphological characteristics between lower, mid and upper canopy (young expanding, recently matured and old matured shaded, respectively) leaves of aspen and maple under ambient and elevated O<sub>3</sub> concentrations. The lower photosynthetic rates (P<sub>n</sub> rates in μmol m<sup>-2</sup>s<sup>-1</sup>), stomatal conductance (G<sub>s</sub> in mol m<sup>-2</sup>s<sup>-1</sup>), stomatal aperture (Sa in μm<sup>2</sup>), Stomatal density (Sd) and larger Leaf area (La in cm<sup>2</sup>) contributed to maples higher tolerance to acute O<sub>3</sub>. At the time measurements were taken, the subject leaves were LPI 1–10–Upper canopy, LPI 11–21 ± 3 mid canopy and LPI >21 for lower-canopy leaves<sup>a</sup>

	Aspen Upper Canopy		Aspen Mid Canopy		Aspen Lower Canopy		Maple Mid Canopy	
	Control	+O <sub>3</sub>	Control	+O <sub>3</sub>	Control	+O <sub>3</sub>	Control	+O <sub>3</sub>
P <sub>n</sub> rate	10.6 ± 3b	10.1 ± 2.4b	16.4 ± 0.5a	16.4 ± 0.4a	13.4 ± 0.5b	08.7 ± 1.0b	09.7 ± 0.40b	07.3 ± 0.60b
G <sub>s</sub>	0.16 ± 0.02c	0.15 ± 0.03c	0.45 ± 0.03a	0.42 ± 0.01a	0.38 ± 0.02ab	0.33 ± 0.02b	0.16 ± 0.01c	0.13 ± 0.01c
Sa			170 ± 09a	119 ± 13b			89 ± 04c	80 ± 02c
Sd			26 ± 03c	14 ± 02d			66 ± 03a	47 ± 03b
La			65 ± 04b	52 ± 03c			104 ± 06a	91 ± 11a

<sup>a</sup> Grouping by Tukey's post-hoc multiple comparison test are indicated by different letters a, b, c and d (alpha = 0.05).

O<sub>3</sub> gradient from the emitters to the plot center [Mid canopy leaves of plants close to the emitters had 100% necrosis, while those 8 m away (midway between vents and ring center) had 50% and those 14 m away had hardly any (<5%) necrotic spots on their mid-canopy leaves], which corresponded to decreasing visible O<sub>3</sub> damage on aspen leaves. This is in agreement with the localized ozone fumigation experiment conducted by Velikova *et al.*,<sup>18</sup> in which leaves close to the fumigation vents received about 300 nmol mol<sup>-1</sup> while other leaves on a different branch 30 cm away received about 200 nmol mol<sup>-1</sup>. In that experiment, high variance in O<sub>3</sub> concentration within the crown of one tree affected the O<sub>3</sub> dose received by each leaf. Thus, leaf-level visual and physiological indicators of O<sub>3</sub> stress varied substantially across our large scale experiment.

Fig. 3 clearly illustrates that acute O<sub>3</sub> damage was not uniform across leaves of different ages: acute and severe O<sub>3</sub> damage (100% necrosis) occurred only on mature, fully expanded, and actively photosynthesizing leaves. Plant response to atmospheric O<sub>3</sub> depends upon the amount of O<sub>3</sub> that enters the tissue and causes damage inside the leaves by disrupting physiological and biochemical processes.<sup>50</sup> Leaf O<sub>3</sub> uptake is thought to be exclusively through stomata because the cuticle is highly resistant to O<sub>3</sub> conductance.<sup>29</sup> Thus, O<sub>3</sub> damage depends upon three factors: external O<sub>3</sub> which drives O<sub>3</sub> diffusion (O<sub>3</sub> flux) through stomata and into leaves, the rate of stomatal conductance which allows gas diffusion to occur, and internal resistance to oxidation, primarily through production of antioxidant compounds.<sup>29,46,51,52</sup>

In our study, the new expanding leaves (upper canopy leaves) of aspen had up to about 50% (significantly) lower stomatal conductance than the mature, sun-lit leaves at mid canopy, and they had no visible O<sub>3</sub> injury, which is consistent with other

studies.<sup>16,18</sup> Likewise, mid-canopy maple leaves had no visible symptoms of acute O<sub>3</sub> damage, and had similar stomatal conductance rates as the newly expanding aspen leaves. The high rates of O<sub>3</sub> flux into mature aspen leaves resulted in a mid-canopy zone of 100% leaf necrosis for trees near the dispersal vents, followed by shedding of those leaves 2–3 days later. Older (lower canopy), shaded aspen leaves, with decreased stomatal conductance, had much less visible damage compared to the mid canopy leaves. This is in contrast to reports that O<sub>3</sub> damage to leaves is greater in lower canopy leaves and at low light levels despite the lower stomatal conductance and O<sub>3</sub> uptake relative to upper canopy leaves.<sup>31,53</sup> This contradiction could possibly be due to differences in plant response to chronic compared to acute O<sub>3</sub> dose. During the short, acute exposure time in our study, there was no decrease in conductance of the most active leaves, which is often not the case for plants exposed to long-term but lower O<sub>3</sub>.<sup>32</sup>

Many studies have discussed the role of stomatal conductance and O<sub>3</sub> dose on O<sub>3</sub> damage,<sup>18,32,54</sup> but few studies consider the role that stomatal size and density play in O<sub>3</sub> sensitivity. We found that total stomatal pore space per unit leaf area was 3.3 times higher in aspen compared to maple. This is a species-specific (although somewhat plastic) morphological characteristic that contributes to differing rates of stomatal conductance per unit leaf area, and consequently differences among species in O<sub>3</sub> dose.

A comparison of the O<sub>3</sub> concentration measured in the middle of the rings from 1998 through 2001 (21–65 nmol mol<sup>-1</sup>)<sup>9</sup> is similar to what was recorded in July of 2010 (26–67 nmol mol<sup>-1</sup>). Because O<sub>3</sub> did not differ at plot center between years, it is critical to mention the importance of wind speed in diffusing the highly concentrated O<sub>3</sub> at the emitters for efficient mixing with ambient air. The average wind speed at a height of 2 m during the study

**Table 2** Elevated CO<sub>2</sub> + O<sub>3</sub> did not ameliorate the adverse effect of O<sub>3</sub> on stomatal conductance and O<sub>3</sub> flux since CO<sub>2</sub> significantly increased photosynthetic rate (μmol m<sup>-2</sup> s<sup>-1</sup>) in both species, but CO<sub>2</sub> had no effect on stomatal conductance (mol m<sup>-2</sup> s<sup>-1</sup>) in both species, and had no effect on O<sub>3</sub> (mol m<sup>-2</sup> s<sup>-1</sup>) uptake on both species<sup>a</sup>

	Control	Aspen + O <sub>3</sub>	+CO <sub>2</sub> + O <sub>3</sub>	Control	Maple + O <sub>3</sub>	+CO <sub>2</sub> + O <sub>3</sub>
P <sub>n</sub>	17.9 ± 0.8b	14.9 ± 0.8c	26.4 ± 0.7a	10.7 ± 0.4d	08.5 ± 0.7e	15.0 ± 0.7c
G <sub>s</sub>	0.42 ± 0.02a	0.40 ± 0.02a	0.37 ± 0.03a	0.16 ± 0.01b	0.13 ± 0.02b	0.14 ± 0.01b
O <sub>3</sub> flux	15.3 ± 0.4b	52.3 ± 2.2a	48.3 ± 2.8a	05.7 ± 0.3c	17.0 ± 0.9b	18.3 ± 1.5b

<sup>a</sup> Groupings by Tukey's post-hoc multiple comparison test are indicated by different letters (alpha = 0.05).

period was 0.25 m s<sup>-1</sup> compared with 2.4 m s<sup>-1</sup> recorded in 1998 when the original planted trees were about the same age and size. But there was no acute O<sub>3</sub> damage because the wind helped to diffuse the O<sub>3</sub> quickly. Under the 3-day low wind conditions in 2010, the fumigation of between 110 and 490 nmol mol<sup>-1</sup> over the study period and an occasional brief peaks reaching 1990 nmol mol<sup>-1</sup>, exposed plants near the emitters to very high O<sub>3</sub> concentration. It should be noted, however, that although we observed little visual evidence of O<sub>3</sub> damage in the interior of the treatment rings, those same lower O<sub>3</sub> concentrations decreased stand volume growth by 29%<sup>55</sup> after 7 years, which was a function of both smaller tree size and increased mortality.<sup>56</sup>

### Elevated CO<sub>2</sub> did not ameliorate the adverse effects of acute O<sub>3</sub> exposure

There have been many reports that elevated CO<sub>2</sub> ameliorates adverse effects of elevated O<sub>3</sub>;<sup>35</sup> however, this is not always the case.<sup>20,38,57,58</sup> In our study, although there was ameliorative effect of elevated CO<sub>2</sub> on O<sub>3</sub> effects with respect to photosynthetic rate, there was no change in stomatal conductance indicating that the CO<sub>2</sub> effect on photosynthesis was largely due to a stronger CO<sub>2</sub> diffusion gradient. These findings are in agreement with many reports<sup>19,20,34,42,57,59–61</sup> but not all.<sup>40,62</sup> Because the stomatal conductance of leaves under the elevated O<sub>3</sub> alone did not differ from leaves under elevated CO<sub>2</sub> + O<sub>3</sub>, their O<sub>3</sub> uptake rates were similar. Interestingly, we observed greater incidence of leaf necrosis in the CO<sub>2</sub> + O<sub>3</sub> treatment compared to O<sub>3</sub> alone. This is consistent with Gupta *et al.*<sup>39</sup> who reported that elevated CO<sub>2</sub> + O<sub>3</sub> at times exacerbated the adverse effect of O<sub>3</sub> on gene expression of aspen. For example, elevated CO<sub>2</sub> exacerbated the adverse effect of O<sub>3</sub> on senescence-associated genes (SAGs) and genes involved in the flavanoid pathway. The many contrasting reports of CO<sub>2</sub> effects on conductance and O<sub>3</sub> injury might be explained by variation in responses among taxa and climatic conditions. Darbah *et al.*<sup>63</sup> reported that elevated CO<sub>2</sub> significantly decreased stomatal conductance in one aspen clone but not another. In that same experiment, Darbah *et al.*<sup>20</sup> found that climatic variation among different years contributed to stomatal responses to elevated CO<sub>2</sub> and O<sub>3</sub>. This shows that caution should be used when making predictions about possible ameliorative effects of elevated CO<sub>2</sub> on tropospheric O<sub>3</sub> pollution, especially where acute exposures are possible.

### Conclusions

We found that severity of acute O<sub>3</sub> damage is not uniform in leaves of different physiological ages even on the same plant and that this was related to stomatal conductance differences among the leaves of various ages. Leaf morphology and physiology both contributed to variation in sensitivity to acute O<sub>3</sub> dose among different leaf ages and species. Elevated CO<sub>2</sub> did not ameliorate the adverse effect of acute O<sub>3</sub> damage on leaves as it did not significantly decrease stomatal conductance rates. Finally, within the same experiment, different plants and different leaves on the same plant received different O<sub>3</sub> doses depending on their relative position to the source of the O<sub>3</sub> and that determined how much damage was caused to the leaf tissue and hence the photosynthetic machinery.

### Acknowledgements

The Northern Forest Ecosystem Experiment was funded by the USDA Forest Service Northern Research Station. The authors gratefully acknowledge the contributions of Scott Jacobson in operating and maintaining the experimental infrastructure and Daniel Baumann who provided the wind data and produced the wind rose shown in Fig. 1. The authors are thankful to Brian McCarthy, Martha Bishop and Connie Pollard of the Plant Biology Department (Ohio University) for helping with the microscopy work.

### References

- 1 G. Torsethaugen, E. J. Pell and S. M. Assmann, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**(No. 23), 13577.
- 2 U.S. Environmental Protection Agency, in *Air Quality Criteria for Ozone and Related Photochemical Oxidants (Office of Research and Development, National Center for Environmental Assessment, Research Triangle Park, NC)*, EPA, 1996, Report no. EPA-600/P-93/004cF.
- 3 T. E. E. Grams, S. Anegg, K.-H. Haberle, C. Langebartels and R. Matyssek, *New Phytol.*, 1999, **144**, 95–107.
- 4 R. Matyssek and H. Sandermann Jr., 2003. In: *Progress in Botany*, 2003 Volume 64. Springer-Verlag, Heidelberg, Germany, pp. 349–404.
- 5 J. T. Houghton, Y. Ding, D. J. Griggs, M. Noguer, P. J. Van der Linden, X. Da, K. Maskell, C. A. Johnson (ed.), *Climate Change 2001: The Scientific Basics*. Cambridge University Press, Cambridge.
- 6 IPCC, Climate Change 2007. Working Group I Report: The Physical Basis of Climate Change, 2007, <http://ipcc-wg1.ucar.edu/wg1/wg1-report.html>.
- 7 S. Stich, P. M. Cox, W. J. Collins and C. Huntingford, *Nature*, 2007, **448**, 784.
- 8 The Royal Society 2008, *Science Policy Report 15/08*, October 2008, <http://royalsociety.org/displaypagedoc.asp?id=31506>.
- 9 D. F. Karnosky, D. R. Zak and K. S. Pregitzer, *et al.*, *Funct. Ecol.*, 2003, **17**, 289.
- 10 B. Felzer, D. Kicklighter, J. Melillo, C. Wang, Q. Zhuang and R. Prinn, *Tellus, Ser. B*, 2004, **56**, 230.
- 11 A. Chappelka, J. Skelly, G. Somers, R. Renfro and E. Hildebrand, *Water, Air, Soil Pollut.*, 1999, **116**(1–2), 261.
- 12 M. Schaub, J. M. Skelly and J. W. Zhang, *et al.*, *Environ. Pollut.*, 2005, **133**, 553.
- 13 H. Xu, S. B. Chen, D. K. Biswas, Y. G. Li and G. M. Jiang, *Photosynthetica*, 2009, **47**(2), 247–254.
- 14 E. J. Pell, C. D. Schlagnhauser and R. N. Arteca, *Physiol. Plant.*, 1997, **100**, 264.
- 15 D. F. Karnosky, Z. E. Gagnon, R. E. Dickson, M. D. Coleman, E. H. Lee and J. G. Isebrands, *Can. J. Forest Res.*, 1996, **26**, 23.
- 16 C. Nali, L. Guidi, F. Filippi, G. F. Soldatini and G. Lorenzini, *Trees*, 1998, **12**, 196.
- 17 A. Noormets, A. Sober and E. J. Pell, *et al.*, *Plant, Cell Environ.*, 2001, **24**, 327.
- 18 V. Velikova, T. Tsonev, P. Pinelli, G. A. Alessio and F. Loreto, *Tree Physiology*, 2005, **25**, 1523.
- 19 J. N. T. Darbah, *PhD Thesis*, 2007, Michigan Technological University, USA, pp. 1–183.
- 20 J. N. T. Darbah, M. E. Kubiske, N. Nelson, K. Kets, J. Riikonene, A. Sober, L. Rouse and D. Karnosky, *Environ. Pollut.*, 2010, **158**, 983.
- 21 K. Reiling and A. W. Davison, *New Phytol.*, 1995, **12**, 587.
- 22 J. Fuhrer, L. Skarby and M. R. Ashmore, *Environ. Pollut.*, 1997, **97**, 91–106.
- 23 P. B. Reich, *Tree Physiol.*, 1986, **3**, 63.
- 24 S. Pasqualini, M. Antonielli, L. Ederli, C. Piccioni and F. Loreto, *Plant Physiol. Biochem.*, 2002, **40**, 599.
- 25 R. Matyssek, G. Wieser and A. J. Nunn, *et al.*, *Atmos. Environ.*, 2004, **38**, 2271.
- 26 L. Zhang, R. Vet, J. R. Brook and A. H. Legge, *Sci. Total Environ.*, 2006, **370**(1), 117.
- 27 F. Loreto and S. Fares, *Plant Physiol.*, 2007, **143**, 1096.

- 28 N. E. Grulke, E. Paoletti and R. L. Heath, *Environ. Pollut.*, 2007, **2007** (146), 640.
- 29 S. Fares, F. Loreto, E. Kleistand and J. Wildt, *Plant Biol.*, 2008, **10**, 44.
- 30 S. Fares, M. McKay, R. Holzinger and A. H. Goldstein, *Agric. Forest Meteorol.*, 2010, **150**, 420.
- 31 S. Fares, E. Oksanen, M. Lännenpää, R. ulkunen-Tiitto and F. Loreto, *Photosynth. Res.*, 2010, **104**, 61.
- 32 F. Loreto and V. Velikova, *Plant Physiol.*, 2001, **127**, 1781.
- 33 E. Paoletti, *Environ. Pollut.*, 2005, **134**, 439.
- 34 J. C. Volin and P. B. Reich, *Physiol. Plant.*, 1996, **96**, 674.
- 35 J. C. Volin, P. B. Reich and T. J. Givnish, *New Phytol.*, 1998, **138**, 315.
- 36 O. Kull, A. Sober, M. D. Coleman, R. E. Dickson, J. G. Isebrands, Z. Gagnon and D. F. Karnosky, *Can. J. Forest Res.*, 1996, **26**, 639.
- 37 E. Oksanen, J. Sober and D. F. Karnosky, *Environ. Pollut.*, 2001, **115**, 437.
- 38 P. Sharma, A. Sober and J. Sober, *et al.*, *Ecologia-Bratislava*, 2003, **22** (suppl., 1), 304.
- 39 P. Gupta, S. Duplessis, H. White, D. F. Karnosky, F. Martin and G. K. Podila, *New Phytol.*, 2005, **167**, 129.
- 40 F. Li, S. Shaozhong Kang and Jianhua J. Zhang, *Agric. Water Manage.*, 2004, **67**(3), 221.
- 41 H. Harmens, G. Mills, L. D. Emberson and M. R. Ashmore, *Environ. Pollut.*, 2007, **146**, 763.
- 42 J. Uddling, A. J. Hogg, R. M. Teclaw, M. A. Carroll and D. S. Ellsworth, *Environ. Pollut.*, 2010, **158**, 2023.
- 43 R. E. Dickson, K. F. Lewin, J. G. Isebrands, M. D. Coleman, W. E. Heilman, D. E. Reimenschneider, J. Sober, G. E. Host, G. R. Hendrey, K. S. Pregitzer, D. F. Karnosky, USDA Forest Service North Central Research Station, *General Technical Report*, 2000, NC-214, 68 pp.
- 44 D. F. Karnosky, B. Mankovska and K. Percy, *et al.*, *Water, Air, Soil Pollut.*, 1999, **116**, 311.
- 45 G. R. Hendrey, D. S. Ellsworth, K. F. Lewin and J. Nagy, *Global Change Biol.*, 1999, **1999**(5), 293.
- 46 G. Wieser and W. M. Havranek, *Trees*, 1993, **7**, 227.
- 47 W. M. Havranek and G. Wieser, in *Environmental research with plants in closed chambers. Air pollution research report 26*, ed. H. D. Payer, T. Pfirmann and P. Mathy, Commission of the European Communities, Brussels, 1990, pp. 148–152.
- 48 D. M. Olszyk and D. T. Tingey, *PlantPhysiol.*, 1985, **79**, 949.
- 49 P. R. Larson and J. G. Isebrands, *Can. J. Forest Res.*, 1971, **1**, 1–11.
- 50 D. T. Tingey, G. E. Taylor, in *Effects on gaseous air pollution in agriculture and horticulture*, ed. H. M. Unsworth, D. D. Ormond, 1982, Butterworth, London, pp. 113–138.
- 51 R. L. Heath, *Annu. Rev. Plant Physiol.*, 1980, **31**, 395.
- 52 R. Guderian, D. T. Tingey, R. Rabe, in *Air pollution by photochemical oxidants. Ecological studies*, ed. R. Guderian, Springer, Berlin Heidelberg, NY, 1985, vol. 52, pp. 129–333.
- 53 A. Noormets, O. Kull, A. Söber, M. E. Kubiske and D. F. Karnosky, *Environ. Pollut.*, 2010, **158**, 992.
- 54 E. Paoletti and N. E. Grulke, *Environ. Pollut.*, 2005, **137**, 483.
- 55 M. E. Kubiske, V. S. Quinn, W. E. Heilman, E. P. McDonald, P. E. Marquardt, R. M. Teclaw, A. L. Friend and D. F. Karnosky, *Global Change Biol.*, 2006, **12**, 1054.
- 56 M. E. Kubiske, V. S. Quinn, P. E. Marquardt and D. F. Karnosky, *Plant Biol.*, 2007, **9**, 342.
- 57 J. Uddling, R. M. Teclaw, K. S. Pregitzer and D. S. Ellsworth, *Tree Physiol.*, 2009, **29**, 1367.
- 58 B. A. Wustman, E. Oksanen and D. F. Karnosky, *et al.*, *Environ. Pollut.*, 2001, **115**, 473.
- 59 D. S. Ellsworth, *Plant, Cell Environ.*, 1999, **22**, 461.
- 60 C. J. Bernacchi, C. Calfapietra, P. A. Davey, V. E. Wittig, G. E. Scarascia-Mugnozza, C. A. Raines and S. P. Long, *New Phytol.*, 2003, **159**, 609.
- 61 S. G. Keel, S. Pepin, S. Leuzinger and C. Kö rner, *Trees–Structure & Function*, 2007, **21**, 151.
- 62 C. A. Gunderson, J. D. Sholtis, S. D. Wullschleger, D. T. Tissue, P. Hanson and R. J. Norby, *Plant, Cell Environ.*, 2002, **25**, 379.
- 63 J. N. T. Darbah, T. D. Sharkey, C. Calfapietra and D. F. Karnosky, *Environ. Pollut.*, 2010, **158**, 1008.