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Spring leaf flush in aspen (*Populus tremuloides*) clones is altered by long-term growth at elevated carbon dioxide and elevated ozone concentration

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Spring leaf flush is stimulated by elevated [CO_2] and suppressed by elevated [O_3] in aspen (Populus tremuloides).

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ABSTRACT

Early spring leaf out is important to the success of deciduous trees competing for light and space in dense forest plantation canopies. In this study, we investigated spring leaf flush and how long-term growth at elevated carbon dioxide concentration ([CO_2]) and elevated ozone concentration ([O_3]) altered leaf area index development in a closed *Populus tremuloides* (aspen) canopy. This work was done at the Aspen FACE experiment where aspen clones have been grown since 1997 in conditions simulating the [CO_2] and [O_3] predicted for ~2050. The responses of two clones were compared during the first month of spring leaf out when CO_2 fumigation had begun, but O_3 fumigation had not. Trees in elevated [CO_2] plots showed a stimulation of leaf area index (36%), while trees in elevated [O_3] plots had lower leaf area index (−20%). While individual leaf area was not significantly affected by elevated [CO_2], the photosynthetic operating efficiency of aspen leaves was significantly improved (51%). There were no significant differences in the way that the two aspen clones responded to elevated [CO_2]; however, the two clones responded differently to long-term growth at elevated [O_3]. The O_3-sensitive clone, 42E, had reduced individual leaf area when grown at elevated [O_3] (−32%), while the tolerant clone, 216, had larger mature leaf area at elevated [O_3] (46%). These results indicate a clear difference between the two clones in their long-term response to elevated [O_3], which could affect competition between the clones, and result in altered genotypic composition in future atmospheric conditions.

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1. Introduction

Two climate change factors that alter leaf growth and productivity are increasing atmospheric carbon dioxide concentration ([CO_2]) and increasing tropospheric ozone concentration ([O_3]) (Taylor et al., 2001; Ainsworth and Long, 2005; Karnosky et al., 2005; Wittig et al., 2009). Tropospheric [O_3] is expected to increase 20% by 2050 due to increases in precursors to O_3 such as nitrogen oxides (NO_x) and volatile organic compounds (VOCs) from anthropogenic and natural sources (Vingarzan, 2004). Plants show many negative responses to increased [O_3] including decreased photosynthetic assimilation and electron transport (Fiscus et al., 2005; Wittig et al., 2007) and decreased maximum leaf area index (LAI) (Karnosky et al., 2003). At the same time, [CO_2] is expected to increase 50% by 2050 (Solomon et al., 2007). Elevated [CO_2] increases photosynthetic rate in C_3 plants (Ainsworth and Long, 2005; Karnosky et al., 2003), above-ground dry matter production, yield and maximum LAI (Ainsworth and Long, 2005). In combination, elevated [CO_2] provides protection from elevated [O_3] (Karnosky et al., 2003, 2005; Fiscus et al., 2005), although the degree of protection differs among species (Karnosky et al., 2003) and genotypes within a species (Isebrands et al., 2001). Other studies have examined how development of differences in LAI in elevated [CO_2] and elevated [O_3] relates to changes in bud physiology (Riikonen et al., 2008), but development of LAI has not been correlated with leaf growth and physiological properties during the first leaf flush of the season.

Leaves are the primary assimilatory surface of plants and as such are involved in processes that range on many scales from exchange of individual CO_2 and water molecules to global biogeochemical cycling. As the site of carboxylation and light harvesting, leaves are responsible for energy capture in the plant. In *Populus* trees, leaf growth is an important determinant of total tree productivity

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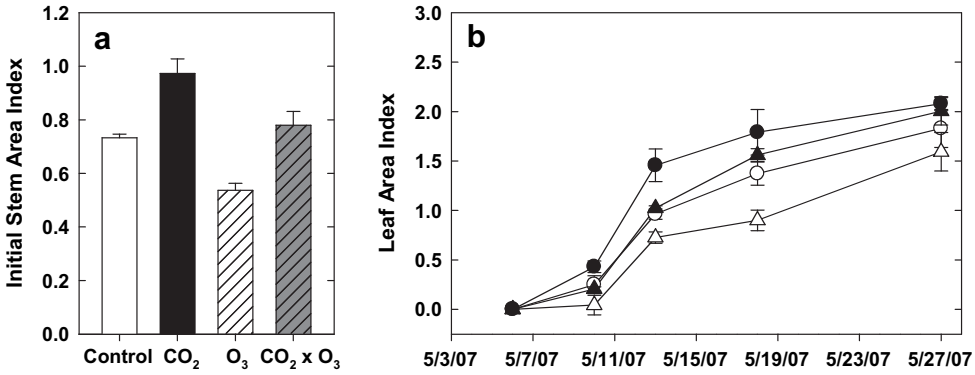

Fig. 1. (a) Initial measurement of stem area index measured before leaves flushed in 2007. This initial value for each treatment was subtracted from all subsequent measurements of leaf area index. (b) Leaf area index of trees grown in control plots with ambient [CO_2] and ambient [O_3] (○), elevated [CO_2] and ambient [O_3] (•), ambient [CO_2] and elevated [O_3] (△) and combination plots with elevated [CO_2] and elevated [O_3] (▲) during the 2007 spring leaf flush. Data represent treatment mean values ± 1 standard error.

(Taylor et al., 2001). A study of *Populus tremuloides* (aspen) at the Aspen Free-Air Carbon dioxide Enrichment (FACE) experiment showed that growth at elevated [CO_2] resulted in increased LAI and growth at elevated [O_3] resulted in decreased LAI compared to control (Karnosky et al., 2003). While peak LAI is an important determinant of forest productivity, early spring leaf out is critical to the growth and survival of competing trees in deciduous forests (Augspurger, 2008). Individuals or genotypes that more quickly reach high LAI will more successfully compete with neighbors for light energy and space.

Development of LAI is affected by both individual leaf size and total leaf number. Leaf size is established by the number and size of cells in the meristem determined to become a leaf (leaf starting size), growth rate and growth duration (Granier et al., 2000; Tsukaya, 2006). Each of these growth parameters is in turn controlled by other factors, including carbohydrate supply (Raines and Paul, 2006; Smith and Stitt, 2007), turgor and cell-wall extensibility (Taylor et al., 1994). All of these factors can be altered by growth at elevated [CO_2] or [O_3].

The aim of this experiment was to investigate spring leaf flush in two contrasting aspen clones exposed to elevated [CO_2], elevated [O_3] and the combination of gases for the past decade. Clone 216 was chosen as an O_3-tolerant genotype (Isebrands et al., 2001), while clone 42E was chosen as an O_3-sensitive genotype (Isebrands et al., 2001). We measured physiological and biochemical determinants of leaf growth in both clones during the spring season, when LAI is increasing rapidly. During the first month of leaf growth, trees were fumigated with CO_2, but not with O_3. Therefore, this experiment tested the long-term effects of exposure to elevated [O_3], and the long-term and immediate effects of growth at elevated [CO_2]. Leaf growth, chlorophyll fluorescence and carbohydrate content of expanding leaves were examined. Previous research at Aspen FACE suggested that altered carbon (C) gain in aspen exposed to elevated [CO_2] or elevated [O_3] did not change the amount of C allocated to buds, indicating that altered LAI is not related to changes in initial carbohydrate stores in buds (Riikonen et al., 2008). However, aspen grown in elevated [O_3] had smaller buds, which may contribute to decreased LAI (Riikonen et al., 2008). A previous analysis of trees grown in elevated [CO_2] across four FACE experiments suggested that at low LAI, stimulation of net primary productivity by elevated [CO_2] was attributable to increased light absorption, but as LAI increased, the response to elevated [CO_2] was predominantly caused by increased light-use efficiency (Norby et al., 2005). This study investigated both structural changes in the canopy and functional changes in leaves exposed to elevated [CO_2] and elevated [O_3] during spring leaf growth, and tested the hypothesis that elevated [CO_2] and elevated [O_3] cause changes in initial leaf growth, which contribute to differences in LAI.

2. Materials and methods

2.1. Aspen FACE facility

The Aspen FACE experiment, located in Rhinelander, Wisconsin, was planted with trembling aspen (*P. tremuloides* Michx.), paper birch (*Betula papyrifera* Marsh.) and sugar maple (*Acer saccharum* Marsh.) in 1997. The experiment is a complete-block full-factorial design ($n = 3$), where CO_2 is elevated to 560 ppm and O_3 is elevated to 50% above ambient (Karnosky et al., 2005). Details about the fumigation method and experimental site have been described previously (Dickson et al., 2000; Karnosky et al., 2003). We examined two aspen clones: 216, an O_3-tolerant clone, and 42E, an O_3-sensitive clone.

2.2. Leaf growth

Digital images of all leaves on two branches from each clone were collected from May 1 to May 29, 2008 in each plot. Each image included a scale mark in order to accurately calculate leaf area and length from image processing software (ImageJ, Rasband, 1997–2008). Leaf length was used to calculate leaf plastochron index (LPI) for each leaf on each shoot using a reference length of 25 mm (Erickson and Michelini, 1957; Larson and Isebrands, 1971). For area measurements, leaves were categorized into different age classes in a manner similar to Noormets et al. (2001): young (leaves that had recently flushed, LPI −1 to 1), rapidly expanding (LPI 3–5), and recently mature (LPI 6–9).

2.3. Leaf area index

LAI was measured using an LAI-2000 (LI-COR, Lincoln, Nebraska). A view cover was used to restrict the sensor's viewing area within a plot. For each observation, one no-canopy measurement was paired with two below-canopy measurements. The below-canopy measurements were collected next to the center scaffold on the perimeter of the plot, with the sensor facing into the plot, but without the scaffold in view. The no-canopy measurement was collected adjacent to the plots in an area with open sky. The sensor was facing in the same direction for no-canopy and below-canopy measurements. It was not possible to separate clones or species, so the data presented are for the entire plot.

Since anything that intercepts light will contribute to the LAI value calculated by the LAI-2000, a measurement was collected before any leaves had emerged (Fig. 1a). This value was used as an estimate of stem mass and subtracted from subsequent LAI values in order to adjust for any differences between plots.

2.4. Chlorophyll fluorescence

Fluorescence parameters, maximal fluorescence from dark-adapted leaves (F_m) and light-adapted leaves (F'_m), minimal fluorescence in dark-adapted (F_o) and light-adapted (F'_o) leaves, were measured between May 20 and May 25, 2008 using a portable chlorophyll fluorometer (PAM-2100, Waltz, Germany). Measurements were taken on all leaves on a branch of each clone, with LPI ranging from 0 to 9. At least six branches per clone per treatment plot were measured. Dark-adapted measurements were taken before dawn with a light-saturating pulse (PAM-2100 setting 9800 ms). Maximum light-adapted fluorescence was measured after the

Table 1
Statistical significance (P) of the analysis of variance of the maximum quantum efficiency of photosystem II (PSII) photochemistry (F_v/F_m), the operating efficiency of PSII (F'_q/F'_m), non-photochemical quenching (NPQ), glucose, fructose, sucrose, total soluble carbohydrate, starch content, and individual leaf area measured on two different aspen clones (216 and 42E) and different leaf plastochron indices (LPI) under control conditions, elevated [CO_2], elevated [O_3], and the combination of elevated [CO_2] and elevated [O_3].

Effect	F_v/F_m	F'_q/F'_m	NPQ	Glucose	Fructose	Sucrose	Total soluble carbohydrate	Starch	Leaf area
Clone	0.90	0.51	0.15	0.69	0.17	0.15	0.28	0.38	0.05
CO_2	0.28	0.03	0.09	0.40	0.49	0.98	0.82	0.08	0.45
Clone*CO_2	0.63	0.19	0.75	0.26	0.33	0.96	0.80	0.59	0.48
O_3	0.77	0.22	0.35	1.00	0.84	0.94	0.91	0.47	0.42
Clone*O_3	0.93	0.78	0.44	0.02	0.02	0.12	0.05	0.44	<0.01
CO_2*O_3	0.65	0.66	0.44	0.56	0.98	0.40	0.40	0.85	0.98
Clone*CO_2*O_3	0.09	0.60	0.50	0.80	0.86	0.93	0.94	0.44	0.75
LPI	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
LPI*Clone	<0.01	0.06	0.10	<0.01	0.01	0.14	0.29	0.88	0.38
LPI*CO_2	0.20	0.31	0.99	0.35	0.52	0.93	0.77	0.30	0.83
LPI*Clone*CO_2	0.98	0.27	0.07	0.97	0.93	0.34	0.42	0.56	0.52
LPI*O_3	0.20	0.90	0.49	0.95	0.91	0.41	0.47	0.75	0.17
LPI*Clone*O_3	0.22	0.79	0.72	0.41	0.01	0.01	0.01	0.28	<0.01
LPI*CO_2*O_3	0.04	0.92	0.17	0.20	0.06	0.82	0.59	0.68	0.98
LPI*Clone*CO_2*O_3	0.41	0.91	0.77	0.65	0.82	0.94	0.95	0.74	0.12

saturating light pulse was superimposed on the prevailing environmental light levels. These parameters were used to calculate the maximum quantum efficiency of photosystem II (PSII) photochemistry (F_v/F_m), the operating efficiency of PSII (F'_q/F'_m) and non-photochemical quenching, the apparent rate constant for non-radiative decay (heat loss) from PSII (Baker and Rosenqvist, 2004).

2.5. Leaf carbohydrate content

Tissue samples were collected from two plants from three LPI (0, 4 and 8) from each clone in each plot for a total of 12 samples per plot from 22–25 May 2007. Tissue was sampled with a cork borer (81 mm^2), placed in aluminum foil and immediately frozen in liquid nitrogen. Soluble carbohydrates were extracted in 80% (v/v) buffered ethanol (2 mM HEPES, pH 7.8) at 80 °C. Glucose, fructose and sucrose content were determined using the methods of Jones et al. (1977). Starch was extracted in 0.1 M NaOH at 95 °C. The extract was then brought to pH 4.9 and starch content was determined by digesting the sample to glucose and determining glucose content (Hendriks et al., 2003).

2.6. Statistics

Data were analyzed using a mixed model analysis of variance using the Kenwood Rogers option to calculate degrees of freedom (SAS Institute, Cary, NC). CO_2, O_3, and clone were treated as fixed effects in the model. Date and LPI were treated as repeated effects where appropriate. Block and block interactions with fixed effects were treated as random effects in the model. For all variables, statistics were performed on the plot means. Effects were considered significant at a p-value of less than 0.1.

3. Results

Elevated [CO_2] increased LAI immediately at the onset of leaf growth ($F = 20.13$, $P < 0.01$; Fig. 1b), while growth at elevated [O_3] resulted in lower LAI ($F = 11.04$, $P < 0.05$; Fig. 1b). In the combination treatment, effects of elevated [CO_2] and [O_3] were additive; thus, there was no significant interaction of elevated [CO_2] and elevated [O_3] ($F = 0.06$, $P = 0.82$), and control was not significantly different from the combination treatment.

The maximum quantum efficiency of PSII photochemistry (F_v/F_m) was not significantly altered by growth at elevated [CO_2], elevated [O_3] or the combination of treatments in either clone (Table 1). F_v/F_m was between 0.70 and 0.74 for leaves with LPI of 0, and between 0.72 and 0.80 for older leaves (data not shown). The operating efficiency of PSII (F'_q/F'_m) was significantly higher (51.6%) for leaves grown at elevated [CO_2] in both the O_3-tolerant clone, 216, and the O_3-sensitive clone, 42E (Fig. 2, Table 1). There was no significant effect of O_3 or a $CO_2 \times O_3$ interaction effect on F'_q/F'_m, but there was a trend towards a decrease in F'_q/F'_m (−18.6%) in leaves grown at elevated [O_3] (Fig. 2). There was a significant effect of LPI on non-photochemical quenching (Fig. 3, Table 1), and a significant decrease in NPQ (−42%) in leaves grown at elevated [CO_2] (Fig. 3, Table 1). However, there was no effect of O_3 and no $CO_2 \times O_3$ interaction on NPQ.

Pools of glucose, fructose and sucrose (soluble carbohydrates) and pools of starch were measured in leaves at three different LPI, 0, 4 and 8 (Figs. 4 and 5). There was a significant $O_3 \times$ clone interaction on the soluble carbohydrate pool (Table 1), with soluble carbohydrates lower on average (−15.6%) in Clone 42E at elevated

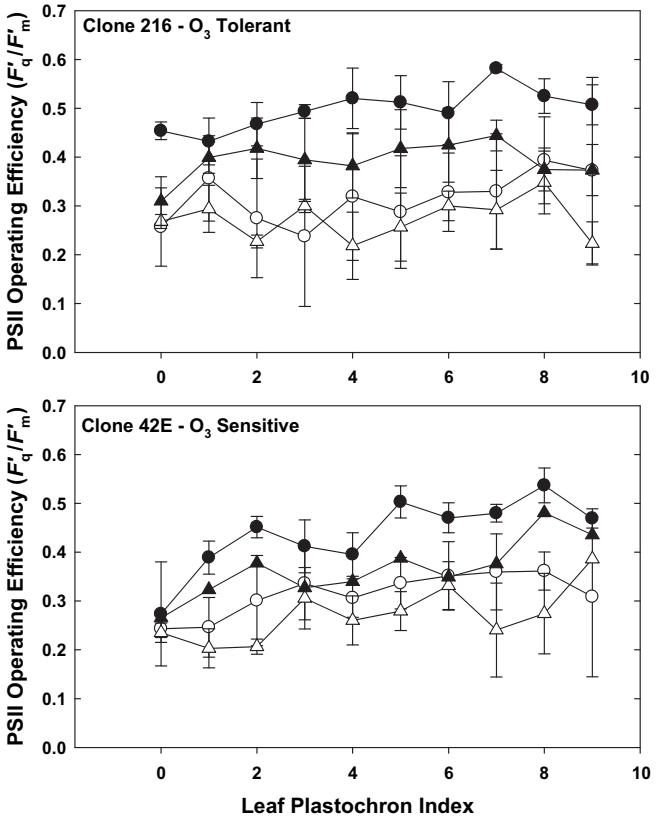

Fig. 2. The operating efficiency of photosystem II (F'_q/F'_m) of leaves of different leaf plastochron indices (LPI), ranging from young expanding leaves (LPI 0) to fully expanded leaves (LPI 8 and 9). The upper panel shows the O_3-tolerant clone, 216, while the lower panel shows the O_3-sensitive clone, 42E. Symbols are described in the legend of Fig. 1.

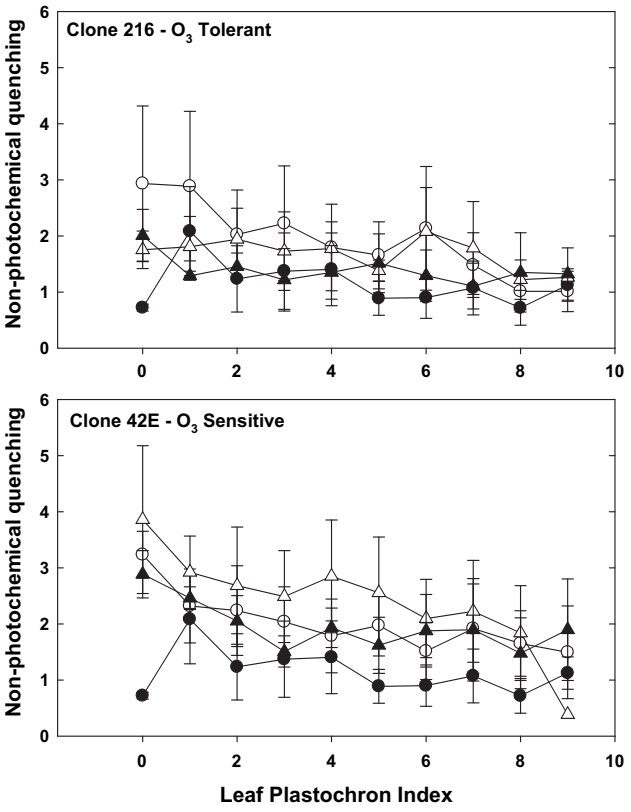

Fig. 3. Non-photochemical quenching of leaves of different leaf plastochron indices (LPI), ranging from young expanding leaves (LPI 0) to fully expanded leaves (LPI 8 and 9). The upper panel shows the O_3-tolerant clone, 216, while the lower panel shows the O_3-sensitive clone, 42E. Symbols are described in the legend of Fig. 1.

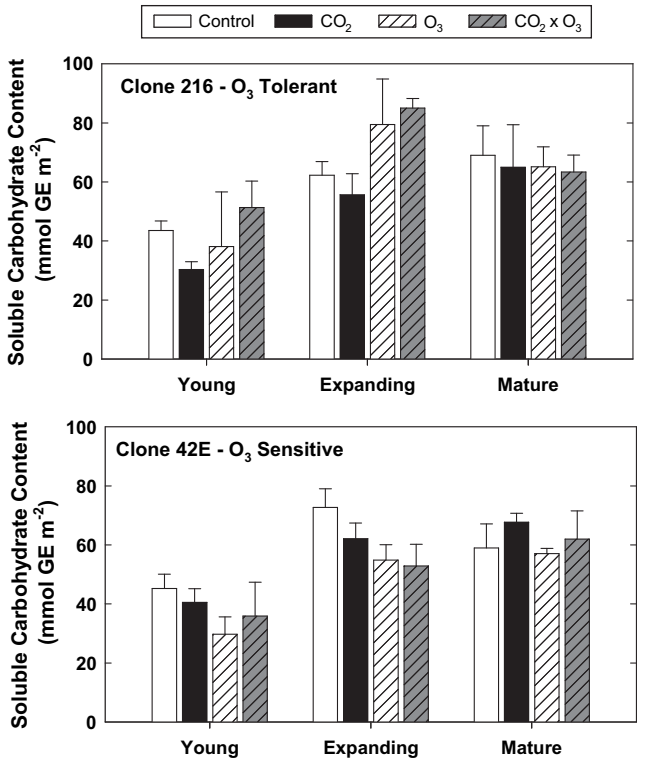

Fig. 4. Total ethanol soluble carbohydrate content measured in glucose equivalents (GE) for the O_3-tolerant clone, 216 (upper panel), and the O_3-sensitive clone, 42E (lower panel). Measurements were taken on leaves of three different leaf plastochron stages: young (−1 to 1), expanding (3–5) and mature (6–9). Bars represent treatment mean values ± 1 standard error.

O_3 (Fig. 4), and not significantly different from control in Clone 216 (Fig. 5). There was a trend towards an increase in starch content (69.5%) at elevated [CO_2] (Table 1; Figs. 4 and 5). There was also a significant effect of LPI on both soluble carbohydrate content and starch content. Soluble carbohydrate contents were lower in both clones at LPI 0 compared to LPI 4 and 8, while starch content was higher in LPI 8 compared to LPI 0 and 4 (Figs. 4 and 5).

Individual leaf area for leaves of different LPI was measured the week before samples were taken for fluorescence and carbohydrate analysis. There was a significant effect of clone and LPI on individual leaf area, as well as a significant three way interaction of LPI, clone and O_3 (Table 1). In elevated [O_3], leaf area of the O_3-sensitive clone, 42E, tended to be smaller (−32.2%), whereas leaf area of the O_3-tolerant clone, 216, was larger at LPI 8 (45.6%; Fig. 6, Table 1).

4. Discussion

Growth of aspen clones in elevated [CO_2] resulted in an immediate stimulation of LAI upon the initial leaf flush. The increase in LAI at elevated [CO_2] in a Florida scrub oak ecosystem was also greatest during the spring coinciding, with the time of maximum leaf area expansion (Hymus et al., 2002). Significant differences in initial stem material provided more nodes for leaf growth, and therefore faster LAI development in elevated [CO_2]. An increase in stem size and node number also contributed to larger LAI in three Poplar species exposed to elevated [CO_2] at the PopFACE experiment (Liberloo et al., 2006). However, in the current experiment, individual leaf area was not significantly larger in trees grown at elevated [CO_2], which contrasts with a previous study of *Populus x euramericana* where elevated [CO_2] increased individual leaf area by 30% in fully expanded leaves (Taylor et al., 2003), and a previous study at this field site that found increases in some leaf ages, at all canopy positions, but most prominently in the upper canopy (Noormets et al., 2001). The lack of effect may be due to differences in light environment. A previous study found elevated [CO_2] increased photosynthetic rate in upper but not lower canopy leaves, likely because light, not CO_2, is limiting in the lower canopy (Takeuchi et al., 2001). In this study the canopy was fully closed and leaves were sampled randomly throughout the canopy, thus any CO_2 effect may have been masked.

At low LAI in forests, structural changes in elevated [CO_2], which increase absorbed photosynthetically active radiation, have been considered the major determinant of enhanced net primary productivity (Norby et al., 2005). Our results in part agree with this interpretation; however, we also showed that there were significant functional differences in leaves grown at elevated [CO_2]. The operating efficiency of PSII was improved at elevated [CO_2] across all LPI, while non-photochemical quenching was reduced. Thus, in aspen clones, it appears that both structural and functional differences at elevated [CO_2] contribute to changes in productivity at the beginning of the growing season. Previous work at Aspen FACE suggested that carbohydrate content of the buds was not related to changes in leaf growth at elevated [CO_2] (Riikonen et al., 2008), and our work supports that finding in that there were no statistically significant differences in soluble carbohydrate content in young leaves (LPI 0 and 4) grown at elevated [CO_2].

While LAI was higher at elevated [CO_2], it was significantly lower in elevated [O_3], supporting previous measurements at the Aspen FACE experiment (Karnosky et al., 2003, 2005; Riikonen et al., 2008). Lower LAI was in part caused by differences in initial stem material and in part by differences in individual leaf area of

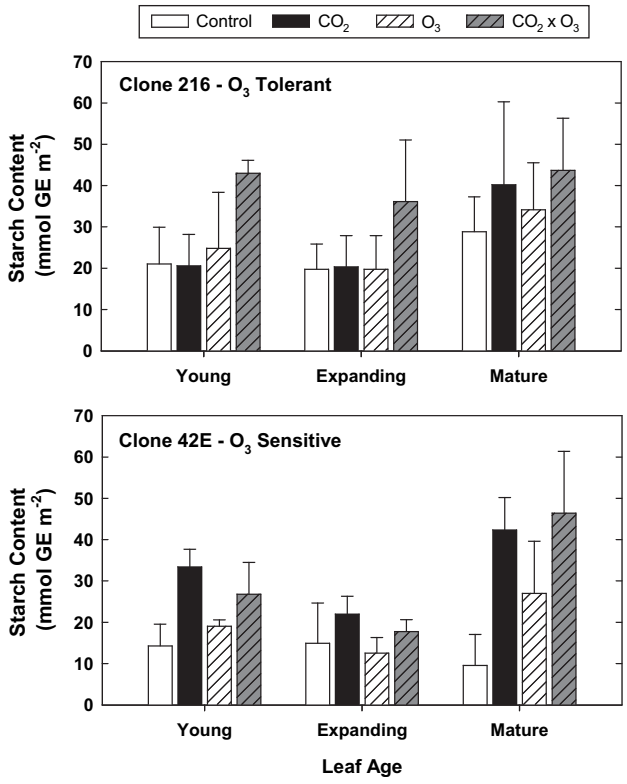

Fig. 5. Total starch content measured in glucose equivalents (GE) for the O_3-tolerant clone, 216 (upper panel), and the O_3-sensitive clone, 42E (lower panel). Measurements were taken on leaves of three different leaf plastochron stages: young (−1 to 1), expanding (3–5) and mature (6–9). Bars represent treatment mean values ± 1 standard error.

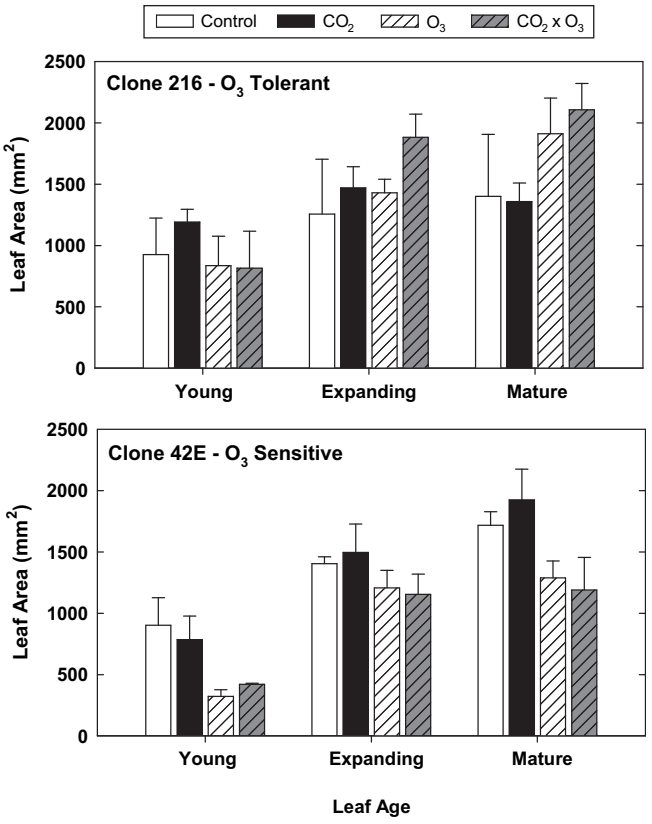

Fig. 6. Individual leaf area for different leaf plastochron stages, young (−1 to 1), expanding (3–5) and mature (6–9), for the O_3-tolerant clone, 216 (upper panel), and the O_3-sensitive clone, 42E (lower panel), measured from 15 to 17 May 2007. Bars represent treatment mean values ± 1 standard error.

the sensitive clone, 42E. There was no significant effect of elevated [O_3] on the operating efficiency of PSII or non-photochemical quenching, which is consistent with previous studies of photochemistry in *Betula pendula* (Riikonen et al., 2005). Therefore, differences in absorbed photosynthetically active radiation due to structural differences in the canopy likely contribute the most to changes in productivity at the beginning of the growing season in elevated [O_3].

The individual effects of elevated [CO_2] and elevated [O_3] reveal differences in mechanistic responses, but aspen responses to both elevated [CO_2] and elevated [O_3] is also of interest because that treatment better represents anticipated changes in the future atmosphere (Vingarzan, 2004; Gardner et al., 2005; Solomon et al., 2007). There was no significant interaction between CO_2 and O_3 in any of the parameters measured. If elevated [CO_2] provided additional protection against rising [O_3], synergistic interactions would have been expected. However, the lack of significant $CO_2 \times O_3$ interactions suggests that rising [O_3] will offset the benefit of rising [CO_2] (Karnosky et al., 2003). It is important to note that all of these measurements were collected at the beginning of the growing season before ambient O_3 concentrations were high enough to warrant fumigation. Therefore, although plots were exposed to elevated or control [CO_2], all plots were exposed to ambient [O_3]. Any treatment effects of elevated [O_3] must, therefore, be due to whole-plant, lifetime-accumulated effects. The mechanism of CO_2 protection from elevated [O_3] is generally considered to be caused by a decrease in stomatal conductance, which reduces O_3 uptake into the leaf (Fiscus et al., 2005). Our findings reveal that the O_3 damage was not caused directly by O_3 uptake through the leaf. This implicates a whole-plant response to O_3 that results from prior exposure, which is independent of [CO_2], and may explain the lack of $CO_2 \times O_3$ interactions on leaf-level processes. Possible signals for the response to prior O_3 exposure include decreased soluble carbohydrate content in young leaves of sensitive clones reported in this study, decreases in bud size and altered bud carbon metabolism (Riikonen et al., 2008).

This whole-plant response to elevated [O_3] affected both leaf structure and leaf physiology, and there was significant variation between the two clones. Leaf area of the O_3-sensitive clone, 42E, was smaller at all leaf ages in elevated [O_3], and peak leaf area was reached earlier in elevated [O_3] (~LPI 4) compared to ambient [O_3] or elevated [CO_2] (LPI ~8). In contrast, leaf area of the O_3-tolerant clone, 216, when grown in elevated [O_3] was not different at the youngest age, and continued to increase after leaves in control and elevated [CO_2] stopped growing. Similarly, 42E had decreased soluble carbohydrate concentrations in elevated [O_3], but 216 did not.

LAI was decreased in the whole stand in elevated [O_3] (Karnosky et al., 2003, 2005; Riikonen et al., 2008). Clone 216 is considered relatively tolerant to elevated [O_3], so it is possible that the other clones respond more similarly to clone 42E, and have smaller individual leaf area in elevated [O_3]. It is also likely that decreased leaf number contributed to decreased LAI, as indicated by the lower stem mass measured at the beginning of this study, and fewer leaves per branch as previously reported (Wustman et al., 2001).

5. Conclusions

Smaller LAI in elevated [O_3] was due to decreases in leaf area and leaf number, whereas evidence for increase in leaf number was

found to contribute to increased LAI in elevated [CO_2]. There was no evidence that an increase in individual leaf area in either clone 216 or 42E contributed to greater LAI in elevated [CO_2]. Clone 42E was shown to be sensitive to long-term growth at elevated [O_3] in this study. It had lower soluble carbohydrates and decreased leaf area, whereas clone 216 was largely tolerant to elevated [O_3]. This difference between the two clones indicates variability in O_3 tolerance within the *P. tremuloides* species, and suggests that early season competition for light energy and space will be altered under future environmental conditions. Furthermore, O_3 can cause damage that is independent of CO_2 effects.

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