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Ozone-induced stomatal sluggishness develops progressively in Siebold's beech (*Fagus crenata*)

Running title: Ozone accelerates stomatal sluggishness in *Fagus crenata*

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Abstract

We investigated the effects of ozone and leaf senescence on steady-state stomatal conductance and stomatal response to light variation. Measurements were carried out in a free-air ozone exposure experiment on a representative deciduous broadleaved tree species in Japan (*Fagus crenata*). Both steady-state and dynamic stomatal response to light variation varied intrinsically with season due to leaf senescence. Ozone induced the decrease in steady-state leaf gas exchange and the sluggish stomatal closure progressively. These findings suggest that ozone reduces the ability of plants to adapt to a fluctuating light environment under natural conditions, and therefore impairs plant growth and ability to control water loss.

Capsule: Ozone progressively induces a sluggishness of stomatal light response.

Key words: tropospheric ozone; stomatal conductance; stomatal sluggishness; photosynthesis; leaf senescence

Introduction

Tropospheric ozone (O_3) is a phytotoxic air pollutant of major concern for forests, and is also recognized as a significant greenhouse gas (Bytnerowicz et al., 2007; Serengil et al., 2011). The tropospheric O_3 level has been increasing continuously since the first direct measurements in 1874, and has now doubled in the northern hemisphere (Volz and Kley, 1988; Akimoto, 2003, Paoletti, 2007). In East Asia, increase of O_3 concentrations are also observed because of rapid increases in emission of the main O_3 precursors, nitrogen oxides and volatile organic compounds (Naja and Akimoto, 2004). In near future, surface O_3 concentrations are expected to be continuously increasing in East Asian countries (Ohara et al., 2007; Yamaji et al., 2008).

Ambient O_3 has a negative impact on tree growth, and therefore on the ability of forest to sequester carbon (Wittig et al., 2009). Entry of O_3 via stomata into a leaf is a crucial factor leading to negative effects on the physiological processes of plants (Omasa et al., 1979; UNECE, 2004, Paoletti and Manning, 2007; Grulke et al., 2007a; Cieslik et al., 2009). Previous studies have reported that O_3 generally induces

stomatal closure (e.g., Wittig et al., 2007), thereby limiting stomatal O₃ uptake (Paoletti and Grulke, 2005). However, O₃ also impairs stomatal response to changes in environmental factors, i.e., stomata respond sluggishly (Paoletti and Grulke, 2005; 2010). Ozone-induced sluggishness of stomata has been reported in their response to photosynthetic photon flux density (PPFD) (Reich and Lassoie, 1984; Reiling and Davison, 1995; Paoletti, 2005; Grulke et al., 2007a; Paoletti and Grulke, 2010), vapor pressure deficit (VPD) (Grulke et al., 2007b; Uddling et al., 2009), CO₂ concentrations (Onandia et al., 2011) and severe water stress imposed by severing a leaf (Paoletti, 2005; Paoletti et al., 2009; Mills et al., 2009; Hoshika et al., 2012).

Ozone is also known to accelerate leaf senescence (Matyssek and Sandermann, 2003). Ozone-induced stomatal sluggishness may be a characteristic of early senescence in O₃-exposed leaves. In fact, leaves also exhibit a sluggish response of their stomata at the end of the growing season, called the “dull leaf” phenomenon (Terashima, 2002). Interactive effects of O₃ and phenology on stomatal response are key to extending our knowledge of elevated O₃ on leaf gas exchange, since both effects may occur simultaneously during the growing season.

Our main objective was to investigate the effects of both O₃ and leaf senescence on steady-state stomatal conductance and stomatal response to light variation. Measurements were carried out in a free-air O₃ exposure experiment on Siebold's beech (*Fagus crenata*), which is a representative deciduous tree species in Japan.

Materials and Methods

Experimental site and plant material

The study was carried out in Sapporo Experimental Forest, Hokkaido University, in northern Japan (43°04' N, 141°20' E, 15 m a.s.l., annual mean temperature: 13.5°C, total precipitation: 1254 mm in 2011). Ten-year-old saplings of Siebold's beech (*Fagus crenata*) were exposed to an experimentally enhanced O₃ regime using a free-air O₃ exposure system, as developed by the TUM group in Germany (Nunn et al., 2002). Size of each plot is 5.5 m × 7.2 m. The distance between the O₃ and ambient plot is about 20 m. Soil type is brown forest soil. Ozone generated from pure oxygen is diluted with ambient air in a pressurized tank and fumigated into the canopies by a system of 48 teflon tubes hanging down from a fixed grid above trees. Target O₃ concentration was set to 60 nmol mol⁻¹ during daylight hours (Fig. 1; Table 1). Percentage of fumigation time with elevated O₃ within 20% range of the target value was 69%. An enhanced O₃ regime

(hourly mean $54.4 \pm 11.8 \text{ nmol mol}^{-1}$) during the daytime was applied to ten Siebold's beech saplings, from August 6th, 2011. Comparison was made with Siebold's beech saplings of the same age under ambient conditions (daytime hourly mean O_3 concentration $25.7 \pm 11.4 \text{ nmol mol}^{-1}$). Ozone concentrations at canopy height were continuously recorded by an O_3 monitor (Mod. 202, 2B Technologies, Boulder CO, USA). In 2011 the mean tree height was $3.3 \pm 0.4 \text{ m}$, and the mean stem diameter at breast height was $26.7 \pm 5.9 \text{ mm}$. The soil moisture was measured in the root layer (20 cm depth) by 10HS sensors equipped with an EM5b data logger (Decagon Devices, Pullman WA, USA). On average, soil moisture was $28.8 \pm 4.8\%$ during these measurements. These values were nearly equal to the field capacity (32%).

Measurement of steady-state and dynamic gas exchange

We selected the 4 beech trees for measurement of leaf gas exchange in ambient and elevated O_3 . Fully expanded sun leaves at the top of the canopy were targeted for measurement in ambient and enhanced O_3 treatments. Leaf mass per area (LMA) of healthy leaves in target trees was measured in July (before O_3 exposure) and mid-October (9 weeks after O_3 exposure) in either regime. Gas exchange was measured with a

portable infra-red gas analyzer (Model 6400, Li-Cor instruments, Lincoln, NE, USA) at controlled values of the leaf temperature (25 °C), the leaf-to-air vapour pressure deficit (1.4 kPa) and the CO₂ concentration (380 μmol mol⁻¹). Measurements were carried out on days with clear sky prior to O₃ exposure (at the end of July 2011) and at 3-week intervals (August to October 2011), between 8:00 and 16:00 CET.

After the stomatal conductance (g_s) and net photosynthetic rate (A) had both reached equilibrium at a constant PPFD value of 1500 μmol m⁻² s⁻¹, the PPFD was changed to 100 μmol m⁻² s⁻¹ until a new equilibrium of g_s and A has reached, then back to 1500 μmol m⁻² s⁻¹, similar to the methodology described in Paoletti (2005) (Fig. 2). The data were logged at 30 sec intervals. The steady state was identified by g_s variation < 0.5% per minute. At equilibrium, the values were recorded of steady-state leaf gas exchange under conditions of low light (A_{lo*lt} and g_{lo*lt}) and high light intensity (A_{hi*lt} and g_{hi*lt}), and the instantaneous water use efficiency (defined as A divided by the transpiration rate). The recorded values at equilibrium were used to calculate the following parameters: range of g_s variation,

$\Delta g_s = g_{hi*1t} - g_{lo*1t}$; time to close stomata, $T_{close}(g_s)$; time to open stomata, $T_{open}(g_s)$; rate of decrease in g_s , $\Delta g_s / T_{close}(g_s)$; rate of increase in g_s , $\Delta g_s / T_{open}(g_s)$; range of variation of A, $\Delta A = A_{hi*1t} - A_{lo*1t}$; time to increase in A, $T_{open}(A)$; rate of increase in A, $\Delta A / T_{open}(A)$.

After changing the light intensity, g_s underwent a transient increase or decrease in opposite directions, which was promptly reversed, known as the transient wrong way response (WWR) (Reich, 1984; Powles et al., 2006).

Statistical analysis

The effects of O_3 and season on steady-state leaf gas exchange, and on dynamic stomatal responses to change in light intensity, were tested via repeated measures analysis of variance (repeated measures ANOVA). Differences among means were tested by the Holm's test when the significant interaction of O_3 and time was detected. Results were considered significant at $p < 0.05$. All statistical analyses were performed with R software (R Development Core Team, 2005).

Results

Leaf mass per area

LMA of 60 g m^{-2} was recorded in the target trees before O_3 exposure

in July (Table 2). At 9 weeks after O₃ exposure in October, no change in LMA was recorded. There was also no difference in LMA between the O₃ regimes in both July and October.

Steady-state g_s and A

The leaf gas exchange parameters were similar for Siebold's beech trees in ambient and elevated O₃ under static light conditions prior to O₃ exposure (Table 3). Steady-state leaf gas exchange decreased with advancing season, except for g_{10*1t} (Table 3). The value of g_{10*1t} did not decrease significantly over time. Instantaneous water use efficiency (WUE_{hi*1t}) was also reduced in late growing season (Table 3).

Ozone was found to induce a reduction in the steady-state values of g_s and A (Table 3). Ozone-exposed leaves gave lower g_{hi*1t} (-25% and -31% in September and October, respectively) than control leaves. The value of A_{hi*1t} declined more rapidly under conditions of enhanced O₃ (-50% in September and -71% in October, relative to July) than in ambient conditions (-9% and -36%). Under low light conditions (PPFD=100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), O₃ also induced reductions in g_{10*1t} and A_{10*1t} . After 3, 6 and 9 weeks of O₃ exposure, A_{10*1t} in O₃-exposed leaves was

less than half of its value in control leaves.

Response of g_s and A to light intensity variation

After reducing the light intensity from 1500 to 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a gradual decrease in g_s and a sharp decrease in A were observed in both treatments (Fig. 2). When the light intensity was restored to 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, g_s and A gradually increased.

Prior to O_3 exposure, there was no difference between ambient and elevated O_3 in the dynamic response of g_s and A to simulated light variation (Table 4). The times for stomatal responses (closing and opening) and increases in A were about 15 min in controls and 11 min in the O_3 treatment. WWR after changing the light intensity did not differ between control and O_3 treatments.

Owing to phenological effects, all parameters exhibited seasonal dependences (Table 4). When changing light intensity, the amplitude of change in g_s ($\Delta g_s / g_{\text{hi}} * 1t$) was decreased with advancing season. In October, Siebold's beech leaves were slower in the response of stomata and photosynthesis than in July: $T_{\text{close}}(g_s)$ (+92% and +106%, under ambient and enhanced O_3 conditions, respectively), $T_{\text{open}}(g_s)$ (+109%

and +111%), $T_{\text{open}}(\text{A})$ (+117% and +66%). WWR was also longer following changes of the light intensity in October. The leaf gas exchange response was slower in October than July: $\Delta g_s/T_{\text{close}}(g_s)$ (-66% and -87%, under ambient and enhanced O_3 conditions, respectively), $\Delta g_s/T_{\text{open}}(g_s)$ (-78% and -91%), $\Delta A/T_{\text{open}}(\text{A})$ (-66% and -81%).

Siebold's beech leaves exposed to enhanced O_3 exhibited significantly longer $T_{\text{close}}(g_s)$ (+27% and +73%, in August and September, respectively) and slower $\Delta g_s/T_{\text{close}}(g_s)$ (-26% and -64%) than leaves of trees grown in ambient conditions (Fig. 2; Table 4). Although O_3 did not significantly affect $T_{\text{open}}(\text{A})$, it reduced $\Delta A/T_{\text{open}}(\text{A})$ in response to changes in the light intensity from 100 to $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. In the enhanced O_3 treatments, $\Delta A/T_{\text{open}}(\text{A})$ was lower than in ambient conditions (-33% in August and -58% in September).

Discussion

Steady-state leaf gas exchange and dynamic light stomatal response changed as time passes due to leaf senescence (Reich, 1984; Atkinson

et al., 1989). In October, Siebold's beech leaves underwent a decrease in steady-state leaf gas exchange (except for g_{10*1t}), slower stomatal response and longer WWR after changes in the light intensity, in controls and enhanced O_3 regime (Tables 3, 4).

Chronic O_3 exposure caused a decrease in the steady-state values of g_s and A (Table 3), in agreement with the meta-analysis by Wittig et al. (2007). At a moderate level of chronic O_3 exposure, O_3 -induced stomatal closure is a reaction to the increased internal CO_2 concentration resulting from the inhibition of carbon assimilation (Paoletti and Grulke, 2005). However, g_{10*1t} did not decrease by the end of the growing season, although O_3 -exposed leaves showed a significant reduction in A_{10*1t} (Table 3). This observation suggests that stomata were unable to close efficiently in the O_3 treatments.

Measurement of dynamic stomatal response found that $\Delta g_s / T_{close}(g_s)$ was slower for leaves exposed to O_3 (Fig. 2; Table 4). The $T_{close}(g_s)$ value developed progressively over time (Table 4). The effects could translate into a weaker ability to close stomata, i.e., sluggish stomatal control, in the enhanced O_3 treatments. Similar findings were reported in O_3 -exposed leaves of Mediterranean evergreen broadleaf trees

(*Arbutus unedo*) (Paoletti, 2005).

Plants could not use water as efficiently when the O₃-induced sluggish stomatal closure is occurred. Previous studies have found progressive loss of stomatal control over transpiration by a stand sap flux approach in the Aspen FACE experiment (Onandia et al., 2011) and in Appalachian foothills of the USA (McLaughlin et al., 2007).

Leaf senescence is often accelerated by O₃ (e.g., Reich and Lassoie, 1984). The effects we observed of O₃ on leaf gas exchange were similar to the effects of leaf senescence, i.e., significant reductions in steady-state leaf gas exchange and loss of stomatal response to change in light intensity, and were observed earlier (August and September; see Tables 3, 4). Ozone particularly caused sluggishness of closing response of stomata to decreasing light intensity (Table 4). The mechanisms of this sluggish stomatal response as a result of O₃ exposure are not yet fully determined. The observed impairment in stomatal function, i.e., sluggish closure, could be the result of physiological and/or structural damage caused by O₃. Previous studies reported that stomatal response was affected by O₃-induced changes such as a slight increase in the permeability of epidermal cell

membranes and changes in the osmotic pressure, modulating a balance in turgidity between the guard and subsidiary cells (Omasa, 1990; Günthardt-Goerg et al., 1993). Mills et al. (2009) reported that O₃ reduced the sensitivity of stomata to abscisic acid (ABA). The loss of stomatal response to ABA may be related to O₃-induced ethylene emissions (Wilkinson and Davies, 2010). Vahisalu et al. (2010) showed that O₃ could temporarily desensitize stomata by blocking the K⁺ channels.

In conclusion, we have found evidence that O₃-induced sluggish closure of stomata and reduction in the net photosynthetic rate developed progressively, in combination with ageing processes. This process results in reduced plant adaptability to fluctuating environmental factors under natural conditions. A fast gas exchange response to environmental stimuli is key to successful plant acclimation and competition (Tinoco-Ojanguren and Pearcy, 1993). The present results suggest that O₃ reduces the ability of plants to adjust to changing light levels, and thereby impair plant function including growth and water economy.

Surface O₃ concentrations are increasing continuously (Paoletti,

2007). Modeling efforts have assisted in establishing the risk of O₃ and in estimating the future effect of O₃ on plant carbon acquisition (Emberson et al., 2000; UNECE; 2004; Karlsson et al., 2007; Sitch et al., 2007). Our understanding of stomatal response to O₃ is still limited and uncertainties remain in the estimation of stomatal O₃ uptake by current models (Gulke et al., 2007a). Our results contribute to assist in determining the role of O₃ pollution on leaf gas exchange and providing more accurate estimates of forest responses to O₃.

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Table 1 Mean values (\pm SD) of environmental factors during the experimental period (August 6th to October 15th, 2011) at the experimental site in Sapporo.

O ₃ concentration during daylight hours (nmol mol ⁻¹)		*AOT40 (μ mol mol ⁻¹ h)		Air temperature (°C)	VPD (kPa)	** Precipitation (mm)	Volumetric soil water content (%)
Ambient	Elevated O ₃	Ambient	Elevated O ₃				
25.7 (11.4)	54.4 (11.8)	0.3	11.9	18.4 (6.2)	0.4 (0.5)	512.5	28.8 (4.8)

* Accumulated concentrations of O₃ exceeding 40 ppb during daylight hours (i.e. solar radiation > 50 W m⁻² (Mills et al., 2010)).

** Total precipitation during August-October.

Table 2 Mean values (\pm SD) (N = 4 plants) and levels of significance (P value) of repeated measures ANOVA for the effects of O₃ exposure and time on LMA

Treatment		Exposure duration (weeks)	LMA (g m ⁻²)
Ambient	July	0	60.2 (6.8)
	October	9	68.9 (3.4)
O ₃	July	0	58.7(6.3)
	October	9	63.1(12.0)
<i>ANOVA results</i>			
Time			0.135
Ozone			0.387
Time × Ozone			0.583

Table 3 Mean values (\pm SD) (N = 4 plants) and levels of significance (P value) of repeated measures ANOVA for the effects of O₃ exposure and time on steady-state leaf gas exchange

Treatment	Date	Exposure					WUE _{hi*lt}	WUE _{lo*lt}
		duration (weeks)	g _{hi*lt} (mmol m ⁻² s ⁻¹)	g _{lo*lt} (mmol m ⁻² s ⁻¹)	A _{hi*lt} (μ mol m ⁻² s ⁻¹)	A _{lo*lt} (μ mol m ⁻² s ⁻¹)	(μ mol CO ₂ mmol ⁻¹ H ₂ O)	(μ mol CO ₂ mmol ⁻¹ H ₂ O)
Ambient	7/21,22,27	0	267.5 (21.9)	128.3 (40.7)	11.8 (1.2)a	4.4 (1.6)	3.2 (0.4)	2.9 (1.9)
	8/24,26,27,28	3	253.1 (41.0)	109.1 (25.3)	11.3 (2.1)ab	3.8 (1.5)	3.4 (1.1)	2.7 (1.4)
	9/14,19,20,21	6	238.3 (40.0)	112.3 (30.0)	10.7 (1.2)ab	4.3 (1.6)	3.3 (0.4)	3.0 (1.6)
	10/11,12,13,14	9	215.1 (31.2)	141.8 (35.1)	7.5 (1.5)c	2.8 (0.7)	2.6 (0.7)	1.4 (0.2)
O ₃	7/21,22,27	0	228.9 (66.2)	88.7 (17.8)	10.8 (1.4)ab	3.4 (0.8)	3.5 (0.6)	2.8 (0.7)
	8/24,26,27,28	3	212.9 (33.6)	71.6 (19.2)	8.2 (0.6)bc	1.2 (0.5)	2.9 (0.6)	1.3 (0.6)
	9/14,19,20,21	6	177.8 (11.4)	108.1 (18.5)	5.4 (1.4)cd	1.8 (1.4)	2.2 (0.4)	1.2 (0.9)
	10/11,12,13,14	9	147.8 (47.8)	126.0 (48.5)	3.1 (0.3)d	0.9 (0.8)	1.6 (0.4)	0.8 (1.1)
ANOVA results								
Time			0.023	0.162	<0.001	0.072	0.003	0.062
Ozone			0.022	0.022	0.002	<0.001	0.122	0.081
Time \times Ozone			0.755	0.572	0.014	0.522	0.068	0.430

g_{hi*lt}, stomatal conductance at saturating-light condition (PPFD=1500 μ mol m⁻² s⁻¹); g_{lo*lt}, stomatal conductance at low light intensity (PPFD=100 μ mol m⁻² s⁻¹); A_{hi*lt}, net photosynthetic rate at light saturation; A_{lo*lt}, net photosynthetic rate at low light intensity; WUE_{hi*lt}; instantaneous water use efficiency (defined as net photosynthetic rate/transpiration rate) at light saturation; WUE_{lo*lt}: instantaneous water use efficiency at low light intensity. When the significant interaction of O₃ and time was detected, Holm's test was performed to identify significant differences between values within each column. Values with different letters are significantly different at p<0.05.

Table 4 Mean values (\pm SD)(N = 4 plants) and levels of significance (P value) of repeated measures ANOVA for the effects of O₃ exposure and time on dynamic response of leaf gas exchange to changes of light intensity

Treatment	Date	Exposure		$\Delta g_s/g_{hi*1t}$ (%)	T _{close} (g _s) (sec)	T _{open} (g _s) (sec)	$\Delta g_s/T_{close}(g_s)$	$\Delta g_s/T_{open}(g_s)$	WWR (sec)	T _{open} (A) (sec)	$\Delta A/T_{open}(A)$
		duration (weeks)									
Ambient	7/21,22,27	0		52.7(12.1)	983(313) a	870(315)	0.166 (0.067)	0.174 (0.097)	0(0)	630 (122)	0.012 (0.002)
	8/24,26,27,28	3		55.7(13.0)	1054(255) a	1084(286)	0.143 (0.018)	0.129 (0.054)	0(0)	874 (178)	0.009 (0.004)
	9/14.19.20.21	6		52.7(11.7)	1095(301) a	1309(341)	0.107 (0.015)	0.108 (0.019)	51(55)	971 (214)	0.007 (0.004)
	10/11,12,13,14	9		34.4(11.5)	1886(100) b	1819(232)	0.057 (0.008)	0.039 (0.010)	120(49)	1369 (368)	0.004 (0.001)
O ₃	7/21,22,27	0		60.6(0.3)	934(225) a	851(103)	0.160 (0.045)	0.158 (0.032)	0(0)	720 (107)	0.011 (0.003)
	8/24,26,27,28	3		66.5(0.7)	1339(181) ab	1328(237)	0.106 (0.023)	0.115 (0.051)	6(7)	1125 (250)	0.006 (0.001)
	9/14.19.20.21	6		38.9(11.5)	1890(184) b	1669(161)	0.038 (0.011)	0.040 (0.020)	109(126)	1166 (220)	0.003 (0.001)
	10/11,12,13,14	9		17.0(18.3)	1927(427) b	1793(407)	0.020 (0.015)	0.014 (0.012)	75(114)	1196 (231)	0.002 (0.001)
ANOVA results											
Time				0.002	<0.001	<0.001	0.001	0.007	0.072	0.003	<0.001
Ozone				0.298	0.048	0.281	0.009	0.062	0.780	0.182	0.016
Time × Ozone				0.090	0.015	0.347	0.314	0.515	0.459	0.329	0.617

$\Delta g_s/g_{hi*1t}$, amplitude of g_s when changing light intensity; T_{close}(g_s), time to close stomata; T_{open}(g_s), time to open stomata; $\Delta g_s/T_{close}(g_s)$ and $\Delta g_s/T_{open}(g_s)$, rate of change of stomatal conductance in opening and closing stomata; WWR, duration of the transient wrong way response; T_{open}(A), time to increase photosynthesis; $\Delta A/T_{open}(A)$, rate of increase of photosynthesis. When the significant interaction of O₃ and time was detected, Holm's test was performed to identify significant differences between values within each column. Values with different letters are significantly different at p<0.05.

Figure legends

Fig. 1 Example of diurnal variation in hourly mean O₃ concentrations under ambient (gray line and open circle) and enhanced O₃ condition (black line and closed circle) in 2011.

Fig. 2 Example of dynamic response of (A) stomatal conductance and (B) net photosynthesis to changes in light intensity (white horizontal bars: PPFD=1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$; black horizontal bars: PPFD=100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) observed under enhanced O₃ (closed circle, measured on Sep. 21th, 2011) and ambient conditions (open circle, measured on Sep. 21th, 2011).

Figures

Fig.1

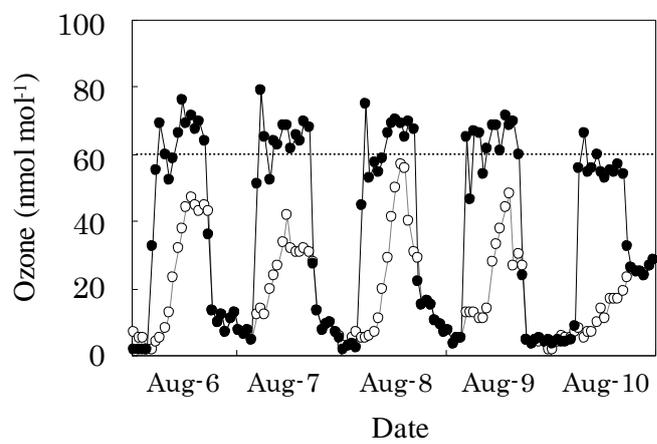
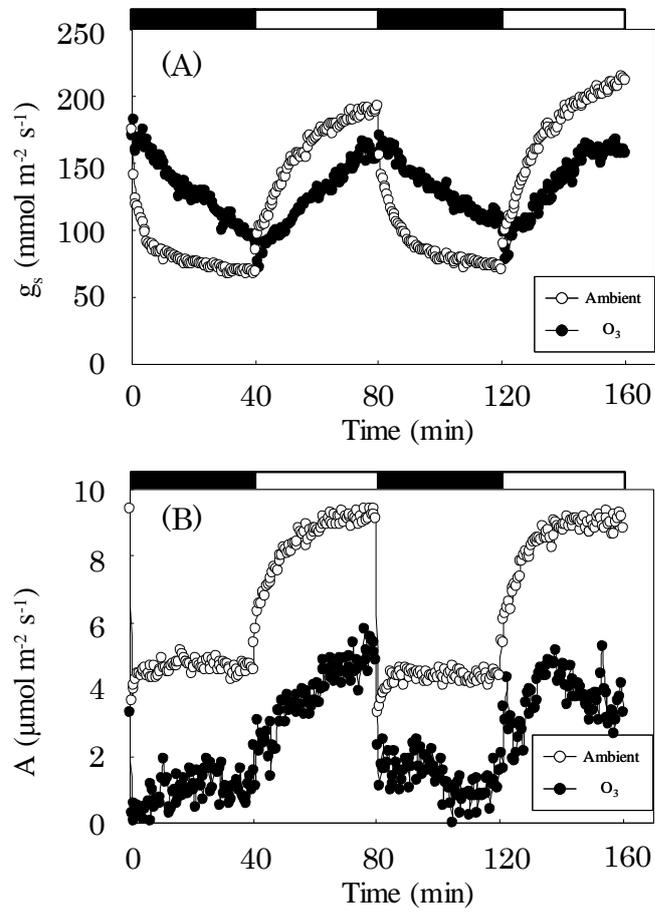


Fig.2



Research Highlights

We investigated the effects of ozone and leaf senescence on stomatal response to light variation.

Measurements were carried out in a free-air ozone exposure experiment on Siebold beech.

Stomatal response to light variation varied intrinsically with season due to leaf senescence.

Steady-state stomatal conductance was reduced under elevated ozone.

Ozone increased time for stomatal closing and reduced light-saturated photosynthesis progressively.