Photosynthetic response of early and late leaves of white birch (*Betula platyphylla* var. *japonica*) grown under free-air ozone exposure

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Running title: Effects of ozone on photosynthesis in heterophyllous white birch

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Abstract

Betula platyphylla var. *japonica* (white birch) has heterophyllous leaves (i.e., early and late leaves) and is a typical pioneer tree species in northern Japan. Seedlings of white birch were exposed to ozone during two growing seasons, and measurements were carried out in the second year. Early leaves did not show an ozone-induced reduction in photosynthesis because of lower stomatal conductance resulting in higher avoidance capacity for ozone-induced stress. Also, an ozone-related increase in leaf nitrogen content may partly contribute to maintain the photosynthetic capacity in early leaves under elevated ozone in autumn. On the other hand, late leaves showed an ozone-induced decline of photosynthesis and early defoliation of leaves occurred. Also, smaller leaf size and higher stomatal density in late leaves were observed under elevated ozone. Differences in stress resistance to ozone may be related to differing functional roles of early and late leaves for birch species.

Key words*:* tropospheric ozone; white birch (*Betula platyphylla* var. *japonica*); heterophyll; photosynthesis

Capsule: Early leaves have higher resistance to ozone stress than late leaves in heterophyllous white birch.

Introduction

Tropospheric ozone (O_3) is a widespread phytotoxic air pollutant, and is also a significant greenhouse gas (Bytnerowicz et al. 2007; Serengil et al., 2011). Ground surface O_3 concentrations are increasing in East Asia because of the rapid increase in emissions of the main O_3 precursors, nitrogen oxides and volatile organic compounds (Naja and Akimoto, 2004). A further increase in O_3 concentrations as a result of continued economic growth in and around East Asia is predicted for the near future (Sitch et al., 2007; Yamaji et al., 2008). Ohara et al. (2001) reported that the increase in O_3 concentration may also be influenced by trans-boundary air pollution. Watanabe et al. (2010a, 2012) suggested that the present O_3 concentration in Japan has a

negative impact on the growth of forest tree species.

The white birch (*Betula platyphylla* var. *japonica*) is a typical pioneer tree species, which rapidly establishes a forest after large disturbances such as wild fire or landslides (Koike and Sakagami, 1985; Koike, 1995). This species is widely used for re-vegetation and is distributed broadly in the cool temperate region of Northeast Asia, including northern Japan (Koike, 1988; Mao et al., 2010; Kawaguchi et al., 2012). White birch has an indeterminate growth pattern, and its shoot development has been classified as heterophyllous (Koike, 1995), i.e., a first flush of early leaves, then expansion of the late leaves.

Kohno et al. (2005) classified the sensitivity of white birch to O_3 as moderate among 18 tree species native to Japan. The AOT40 (accumulated ozone exposure over a threshold of 40 nmol mol^{-1}) value of 16 to 30 μmol mol^{-1} h induced a 10% reduction in growth in their experimental study. Matsumura et al. (2005) reported that exposure to O_3 at 66 nmol mol^{-1} (daytime average) for two growing seasons had adverse effects on growth of white birch, in their open-top chamber (OTC) experiments in central Japan. However, shorter-time exposure

to O_3 during summer did not cause a reduction of growth and a decline of photosynthetic capacity of fully expanded sun leaves in white birch (Hoshika et al., 2012).

Manninen et al. (2009) pointed out that indeterminate growth patterns of birch may have the ability to produce new leaves to compensate for the O_3 damage. Pell et al. (1994) found that aspen (*Populus tremuloides*) underwent continuous formation of new leaves with greater photosynthetic capacity, in compensation for injured older leaves, under elevated O_3. Previous studies reported that newly formed leaves had different leaf morphological traits such as stomatal density under elevated O_3 (Matyssek et al., 1991; Günthardt-Goerg et al., 1993; Pääkkönen et al., 1995b; Frey et al., 1996). The change in leaf morphological traits could be considered a possible compensatory response (Günthardt-Goerg et al., 1993; Pääkkönen et al., 1995a). In fact, reported younger leaves of hybrid poplar (*Populus maximowiczii* × *trichocarpa*) were less responsive to O_3 than older leaves (Pell et al., 1996). Pell et al. (1996) thus suggested that accelerated senescence of older leaves may occur under elevated O_3 as compensatory

mechanisms.

Also, birch has heterophyllous leaves, i.e., early and late leaves (Clausen and Kozlowski, 1965; Kozlowski and Clausen, 1966). Koike (1995) suggested that white birch may first flush its early leaves to avoid damage by late frost, and utilize higher temperatures for expansion of its shoots as long as possible. Previous studies revealed that there are different traits such as leaf gas exchange and leaf longevity (Koike, 1995; Miyazawa and Kikuzawa, 2004). Tabata et al. (2010) reported that maintenance of early leaves was observed rather than of late leaves under water deficit treatment for *Betula ermanii*, although photosynthetic rate was decreased only in early leaves. These imply a different “value” and “vulnerability” of early and late leaves for birch (Matsuki et al., 2004). The different traits of its early and late leaves for white birch may also be related to their difference in susceptibility to O_3-induced stress.

Therefore, the aim of the present study was (1) to test whether new leaf formation of white birch was accelerated while older leaves were shed as suggested by Pell et al. (1996), (2) to examine whether there

was different susceptibility between early- and late-leaves to O_3–induced stress in white birch. To test these predictions, we studied the effects of O_3 on photosynthetic traits of early and late white birch leaves.

Materials and Methods

Experimental site and plant material

The experimental site was located in Sapporo Experimental Forest, Hokkaido University, in northern Japan (43°04' N, 141°20' E, 15 m a.s.l., annual mean temperature: 9.3˚C, total precipitation: 1279 mm in 2012). The snow-free period is usually from early-May to late December. The soil is brown forest soil. Measurements were carried out in a free-air O_3 exposure experiment (for details of the system; see Watanabe et al., 2013). We set up two plots, one for ambient O_3 and another for elevated O_3. Size of each plot is 5.5 m × 7.2 m. The distance between the O_3-enhanced plot and the ambient plot was about 20 m. In June 2011, 3-year-old white birch seedlings were planted in each plot. Exposure to O_3 is based on the system used at Kranzberg

Forest in Germany (Nunn et al., 2002; Werner and Fabian, 2002). The target O_3 concentration was 60 nmol mol^{-1} during daylight hours. This enhanced daytime O_3 treatment was applied to ten white birch seedlings from August to November 2011, and from May to November 2012. Ozone concentrations at canopy height were recorded continuously by an O_3 monitor (Mod. 202, 2B Technologies, Boulder CO, USA). The daytime hourly mean O_3 concentration in ambient and elevated O_3 were 25.7 ± 11.4 nmol mol^{-1} and 56.7 ± 10.5 nmol mol^{-1} during the experimental period in 2011, and were 27.5 ± 11.6 nmol mol^{-1} and 61.5 ± 13.0 nmol mol^{-1} during the experimental period in 2012. In 2012 the mean tree height was 3.0 ± 0.3 m, and the mean stem diameter at breast height was 8.0 ± 2.1 mm. The soil moisture was measured in the root layer (at depth 20 cm) by 10HS sensors equipped with an EM5b data logger (Decagon Devices, Pullman WA, USA). The average soil moisture was 28.1 ± 2.8% during these measurements. These values were nearly equal to the field capacity (32%).

We conducted a survey of the number of leaves at the terminal shoot of white birch at one- to three-week intervals during the growing

season in 2012. Also, the number of shed leaves was assessed by counting the leaf traces at the terminal shoots. We marked early leaves (flushed at the end of April) and late leaves (flushed at the 1st week in June and July).

Measurement of leaf gas exchange

Leaf gas exchange was measured in early leaves (flushed at the end of April) and late leaves (flushed at the 1st week in June and July) using a portable infra-red gas analyzer (Model 6400, Li-Cor instruments, Lincoln, NE, USA) at controlled values of the leaf temperature (25 °C) and of the leaf-to-air vapour pressure deficit (VPD, 1.4 kPa), according to Watanabe et al. (2011). The intercellular CO_2 concentration (C_i) response curve of the net photosynthetic rate (A), i.e., the A/C_i curve, was measured over May 30th to June 9th, August 18th-27th and September 22nd-28th under light saturated conditions (PPFD, Photosynthetic Photon Flux Density: 1500μmol mol^{-1}). The A/C_i curve was obtained by measurement over 12 CO_2 steps (C_a, 50-1700 μmol mol^{-1}). We determined the light-saturated net

photosynthetic rate (A_{sat}) at a CO_2 concentration of 380 µmol mol^{-1} and also the stomatal conductance at this CO_2 concentration (G_s). From the A/C_i curve we calculated the net photosynthetic rate at a CO_2 concentration of 1700 µmol mol^{-1} (A_{max}, indicating maximum rate of ribulose-1,5-bisphosphate regeneration), the maximum rate of carboxylation (V_{cmax}), and the maximum rate of electron transport (J_{max}) (Farquhar et al., 1980; Long and Bernacchi, 2003). Values of the Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) Michaelis constants for CO_2 (K_c) and O_2 (K_o), and the CO_2 compensation point in the absence of dark respiration (Γ^*), used in the analysis of the A/C_i curve, were derived according to the methodology of Bernacchi et al. (2001). All gas exchange measurements relevant to the A/C_i curve were carried out on days with clear sky between 8:00 and 16:00.

Measurement of leaf traits

After measurement of the gas exchange rate, three leaf discs (8 mm diameter) were collected for determination of the leaf mass per unit

area (LMA) and the nitrogen (N) content of leaves. These leaf discs were dried in an oven at 70˚C for 1 week and were then weighted. The LMA was calculated as the ratio of the dry mass to the area of the leaves. The N content of the leaves per unit mass (N_{mass}) was determined by gas chromatography (GC-8A, Shimadzu, Kyoto, Japan) after combustion with circulating O_2 using an NC analyzer (Sumigraph NC-900, Sumika Chemical Analysis Service, Osaka, Japan). A calibration curve was generated using acetanilide (N=10.36%, C=71.09%, Wako, Osaka, Japan). We calculated the area-based N content (N_{area}) as the product of N_{mass} and LMA, and the photosynthetic nitrogen use efficiency (PNUE) as A_{sat} divided by N_{area}.

In August, one further leaf disc was collected to determine the chlorophyll content. The leaf disc was frozen in liquid N immediately after collection and was stored in a deep freezer at -80˚C prior to analysis. Chlorophyll was extracted with dimethyl sulfoxide as according to Barnes et al. (1992) and Shinano et al. (1996), and was determined using a spectrophotometer (Gene spec III, Hitachi, Tokyo, Japan).

Additional leaf samples were collected to assess mean leaf size, leaf air space and stomatal density in August. Leaf air space was calculated as according to Koike (1988). The amount of leaf air space was given by the following formula;

$$V_a = V_f - (w_f - w_d)/\rho_w - w_d/\rho_d$$

where V_a denotes the amount of air space in a leaf (cm^3), V_f is the volume of fresh leaves (cm^3) which is estimated as the value of single leaf area multiplied by leaf thickness of fresh leaves, w_f is the fresh weight of leaves (g), w_d is the dry weight of leaves (g), and ρ_w is the density of water (g cm^{-3}); thus ρ_w=1; ρ_d is the density of the dry matter of leaves (g cm^{-3}), and assuming to be 1.45 (Yokoi and Kishida, 1985). The volume of air space in a leaf is given by V_a/V_f (%).

The stomatal density was determined by the SUMP method (Koike et al., 1998), which involves making a replica of the abaxial leaf surface using a celluloid sheet (Universal Micro-printing, SUMP, Tokyo, Japan). Stomata were counted at 5-7 locations, chosen randomly from

intercostals fields, of area 0.4 mm^2, under a light-microscope.

Statistical analysis

The effects of O_3 and leaf age on LMA, on leaf N content and on leaf gas exchange, were tested via repeated measures analysis of variance (ANOVA). The Student's t-test was used to test the effects of O_3 on leaf traits, and on the number of leaf at the terminal shoot in white birch. Data were checked for normal distribution (Kolmogorov-Smirnov D test). Results were considered significant at $P<0.05$. All statistical analyses were performed with SPSS software (20.0, SPSS, Chicago, USA).

Results

Number of leaves at the terminal shoot

The number of attached leaves at the terminal shoot was initially enhanced under elevated O_3 (Fig. 1A). The number of shed leaves was enhanced under elevated O_3 in August (Fig. 1B). Also, an early decline in the number of leaves was observed under elevated O_3 (Fig.1).

However, early leaves were maintained until mid-October in both treatments (Fig.1B).

Leaf traits

There was no difference in LMA of either early or late leaves between ambient and elevated O_3 (Table 1). Ozone did not affect leaf N content of late leaves, although for early leaves N_{mass} and N_{area} were greater under elevated O_3 than under ambient in September (t-test, P = 0.008 for N_{mass}, P = 0.039 for N_{area}).

Fig. 2 shows leaf size, stomatal density and leaf air space of early and late leaves in August. For early leaves there was no difference in leaf size, stomatal density and leaf air space between ambient and elevated O_3. For late leaves, especially flushed in July, there was a difference between ambient and elevated O_3 in several parameters of leaf traits. Ozone led to smaller leaf size (-42% in late leaves flushed in July) and higher stomatal density (+24% in late leaves flushed in June, +18% in late leaves flushed in July). Chlorophyll content was not different between the O_3 treatments in both early and late leaves

(data not shown).

Gas exchange traits of leaves

Ozone exposure did not reduce A_{sat}, V_{cmax}, J_{max} and A_{max} in early leaves of white birch throughout the measurement period (Table 2). The value of G_s for early leaves decreased under elevated O_3. Ozone induced a reduction of photosynthetic parameters in late leaves (Table 2). In late leaves flushed in June, O_3 caused reductions in A_{sat} (-32% in August; -32% in September), V_{cmax} (-26% in August; -26% in September), and J_{max} (-21% in August; -16% in September). Ozone reduced A_{sat} (-28%), V_{cmax} (-31%) and J_{max} (-19%) of younger late leaves (flushed in July) in September. The photosynthetic nitrogen use efficiency (PNUE) was also decreased in both early and late leaves found under elevated O_3.

Discussion

Formation of new leaves may help to offset reduced carbon gain in injured older leaves under elevated O_3 (Pell et al., 1994, 1996; Watanabe et al., 2010b). In fact, O_3 stimulated leaf production of white

birch from May to July (Fig. 1), and no difference in photosynthetic capacity of younger leaves (i.e., late leaves flushed in July) was found between the treatments in August (Table 2). Also, the enhancement of number of shed leaves was observed under elevated O_3 in August (Fig. 1B). This is supported by the previous observations (Pell et al., 1994, *Populus tremuloides* Michx.; Pell et al., 1996, *Populus maximowiczii* × *trichocarpa*; Pääkkönen et al., 1996, *Betula pendula*). However, the present study also shows that early leaves, which were the oldest leaves, were maintained until mid-October, similarly in both O_3 treatments (Fig. 1B). This suggests that white birch shed late leaves rather than early leaves under elevated O_3.

In fact, O_3-induced decline of A_{sat} was observed only in late leaves (Table 2). Early leaves did not show any significant reduction in photosynthetic rate during the growing season under elevated O_3. Fig. 3 shows a comparison of the relative decrease of light saturated net photosynthetic rate (A_{sat}) in early and late leaves of white birch against AOT40 found in the present study. The decrease of A_{sat} was greater in late leaves than early leaves. This observation indicates that early

leaves may have less susceptibility to O_3–induced stress than late leaves, regardless of actual leaf age.

Early leaves had lower G_s than late leaves (Table 2), which may result in lower stomatal O_3 flux. Moreover, O_3 induced stomatal closure in early leaves while no reduction in photosynthetic capacity was observed (Table 2). This may lead to a limitation of stomatal O_3 flux for avoidance of further O_3-induced stress (Kitao et al., 2009; Hoshika et al., 2013). Also, early leaves, which generally lived longer than late leaves, might have higher tolerant capacity such as detoxification and/or repair of O_3-induced stress than late leaves. Long-lived leaves are generally known to invest higher cost for maintenance and/or defense capacity to the biotic and abiotic stresses than short-lived leaves (Chapin et al., 1980; Coley, 1988; Matsuki and Koike, 2006; Bussotti, 2008). However, Tabata et al. (2010) reported that the content of soluble ascorbate peroxidase and glutathione reductase, which affect detoxification, were similar in early and late leaves of *Betula ermanii*.

Pell et al. (1994) suggested that plants may have the opportunity to

utilize N by re-translocation from older leaves to new leaves if O_3 accelerates the senescence of old leaves under elevated O_3. However, early leaves, which were the oldest leaves, had greater N_{mass} and N_{area} under elevated O_3 in September. This result may be partly related to the maintenance of the photosynthetic capacity in early leaves even under elevated O_3. In fact, O_3 caused a reduction of PNUE in not only late leaves but also early leaves. Therefore, the O_3-induced greater N content in early leaves might be an acclimation process to elevated O_3 in early leaves. Also, previous studies suggested that nitrogen may be allocated to maximize carbon gain (Field, 1983; Hirose and Wegner, 1987). The retention of N in early leaves might have a benefit for carbon gain in white birch rather than re-translocation of leaf N content from early leaves to newly formed late leaves.

The present study also shows that newly formed leaves had different leaf morphological traits (Fig. 2). Especially, the stomatal density in late leaves was higher under elevated O_3 (Fig. 2). This finding is consistent with previous studies for European birch, *Betula pendula* (Matyssek et al., 1991; Günthardt-Goerg et al., 1993; Pääkkönen et al.,

1995b; Frey et al., 1996). Also, late leaves showed smaller leaf size under elevated O_3 in the present study. Frey et al. (1996) suggested that O_3-induced increase in stomatal density may be related to a reduced leaf size. In spite of their higher stomatal density, no difference in G_s was observed in late leaves under elevated O_3 (Table 2). This suggests that the increased stomatal density was apparently overridden by narrowing stomatal apertures (Matyssek et al., 1991). These structural changes of leaves might contribute to reduce the O_3 damage (Günthardt-Goerg et al., 1993; Pääkkönen et al., 1995a). However, late leaves eventually showed a decline of photosynthesis (Table 2), and apparently younger leaves were not less responsive to O_3 (Fig. 3). In the present study, these structural changes of leaves were not enough to reduce O_3 damage.

In conclusion, early leaves of white birch (*Betula platyphylla* var. *japonica*) did not show an O_3-induced reduction in photosynthesis, and the O_3-induced early defoliation of leaves was occurred in late leaves only. This result indicates less susceptibility in early leaves to chronic O_3 stress than late leaves in white birch, because early leaves may

have a higher avoidance capacity for O_3-induced stress due to lower G_s. Also, early leaves had greater leaf N content under elevated O_3 in September. This may partly contribute to maintain the photosynthetic capacity in early leaves as an acclimation process to elevated O_3. The early leaves play an essential role in the growth and development of the new shoot (Kozlowski and Clausen, 1966). Also, a difference in the importance of early and late leaves for birch may depend on their contribution to subsequent growth (Matsuki et al., 2004). Differences in stress resistance to O_3 between early and late leaves may be related to differing functional roles of early and late leaves for birch species.

Acknowledgements

We are grateful for financial support by the Environment Research & Technology Development Fund of Japan (B-1105) and for a Grant-in-aid from the Japanese Society for Promotion of Science (Type B 23380078, Young Scientists B 24710027 and ditto B 24780239, and Young Scientists for research abroad).

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Table 1 Leaf mass per unit area (LMA) and leaf nitrogen content of white birch seedlings grown under ambient and elevated O_3 measured in the second year of treatment (the year 2012)

	June		August		September		ANOVA results		
	Ambient	Elevated O_3	Ambient	Elevated O_3	Ambient	Elevated O_3	Leaf age	O_3	Leaf age x O_3
Early leaves									
LMA (g m^{-2})	50.8 (5.6)	50.7 (7.5)	62.5 (9.0)	60.1 (6.3)	58.7 (5.8)	59.0 (3.4)	<0.001	0.677	0.812
N_{mass} (%)	2.9 (0.3)	3.0 (0.3)	2.4 (0.2)	2.5 (0.1)	1.9 (0.2)	2.2 (0.2)	<0.001	0.066	0.427
N_{area} (g m^{-2})	1.5 (0.3)	1.5 (0.2)	1.4 (0.2)	1.5 (0.1)	1.1 (0.1)	1.3 (0.1)	0.025	0.427	0.365
Late leaves (flush in June)									
LMA (g m^{-2})			51.2 (4.9)	52.2 (7.9)	53.8 (9.8)	55.4 (6.8)	0.495	0.972	0.793
N_{mass} (%)			2.8 (0.2)	2.9 (0.3)	2.6 (0.2)	2.6 (0.3)	0.022	0.742	0.585
N_{area} (g m^{-2})			1.4 (0.2)	1.4 (0.2)	1.4 (0.3)	1.3 (0.1)	0.239	0.781	0.625
Late leaves (flush in July)									
LMA (g m^{-2})			58.9 (8.2)	57.2 (4.5)	61.3 (9.3)	60.8 (4.8)	0.368	0.662	0.859
N_{mass} (%)			3.0 (0.4)	3.2 (0.1)	2.8 (0.3)	2.8 (0.2)	0.025	0.314	0.239
N_{area} (g m^{-2})			1.7 (0.4)	1.9 (0.2)	1.7 (0.5)	1.7 (0.2)	0.795	0.527	0.489

Each value denotes the mean (±SD) of 4 - 6 replicated trees and levels of significance (*P* value) of repeated measures ANOVA.

Table 2 Photosynthetic traits of leaves of white birch seedlings grown under ambient and elevated O_3 measured in the second year of treatment (the year 2012)

	June		August		September		ANOVA results		
	Ambient	Elevated O_3	Ambient	Elevated O_3	Ambient	Elevated O_3	Leaf age	O_3	Leaf age x O_3
Early leaves									
A_{sat} (μmol m^{-2} s^{-1})	15.1 (1.7)	15.8 (2.4)	8.0 (2.1)	6.1 (3.1)	3.9 (2.2)	3.4 (1.5)	<0.001	0.522	0.633
G_s (mol m^{-2} s^{-1})	0.27 (0.04)	0.28 (0.05)	0.14 (0.03)	0.09 (0.04)	0.13 (0.05)	0.07 (0.03)	<0.001	0.036	0.177
V_{cmax} (μmol m^{-2} s^{-1})	78.8 (13.2)	78.1 (10.8)	50.8 (12.1)	40.9 (20.0)	25.7 (14.6)	24.5 (8.3)	<0.001	0.547	0797
J_{max} (μmol m^{-2} s^{-1})	148.8 (32.2)	145.6 (16.8)	106.1 (13.5)	90.3 (36.0)	64.9 (35.0)	73.0 (23.7)	<0.001	0.628	0.669
A_{max} (μmol m^{-2} s^{-1})	27.5 (5.7)	26.0 (5.0)	20.7 (4.1)	16.5 (6.0)	10.6 (5.2)	12.8 (5.0)	<0.001	0.421	0.447
PNUE (μmol mol^{-1} s^{-1})	162.3 (23.8)	148.6 (37.2)	78.8 (15.9)	58.1 (30.4)	49.7 (27.4)	35.1 (12.6)	<0.001	0.084	0.858
Late leaves (flush in June)									
A_{sat} (μmol m^{-2} s^{-1})			10.5 (2.3)	7.1 (2.7)	6.5 (1.2)	4.4 (1.6)	0.003	0.010	0.312
G_s (mol m^{-2} s^{-1})			0.30 (0.11)	0.23 (0.09)	0.16 (0.08)	0.12 (0.06)	0.001	0.118	0.409
V_{cmax} (μmol m^{-2} s^{-1})			46.5 (7.3)	34.3 (12.0)	38.8 (3.0)	28.6 (6.8)	0.125	0.009	0.553
J_{max} (μmol m^{-2} s^{-1})			106.7 (11.6)	84.4 (25.2)	90.0 (5.2)	75.4 (10.5)	0.099	0.057	0.484
A_{max} (μmol m^{-2} s^{-1})			22.9 (2.6)	20.1 (4.8)	16.4 (2.2)	14.5 (3.0)	0.008	0.204	0.831
PNUE (μmol mol^{-1} s^{-1})			109.5 (24.9)	73.1 (34.4)	69.0 (24.5)	41.5 (11.4)	0.031	0.006	0.435
Late leaves (flush in July)									
A_{sat} (μmol m^{-2} s^{-1})			14.9 (1.5)	14.5 (1.9)	10.4 (2.6)	7.4 (1.0)	<0.001	0.027	0.120
G_s (mol m^{-2} s^{-1})			0.40 (0.10)	0.44 (0.21)	0.25 (0.09)	0.21 (0.10)	0.011	0.953	0.537
V_{cmax} (μmol m^{-2} s^{-1})			68.6 (8.1)	59.4 (9.9)	58.9 (15.6)	40.7 (9.0)	0.013	0.010	0.358
J_{max} (μmol m^{-2} s^{-1})			141.1 (16.0)	130.9 (22.2)	130.6 (25.5)	106.0 (21.9)	0.120	0.031	0.504
A_{max} (μmol m^{-2} s^{-1})			32.8 (2.9)	32.4 (3.1)	23.2 (4.0)	20.4 (3.8)	<0.001	0.245	0.466
PNUE (μmol mol^{-1} s^{-1})			135.7 (24.7)	113.4 (22.5)	84.9 (16.1)	60.4 (10.3)	<0.001	0.037	0.755

Each value is the mean (±SD) of 4 - 6 trees and levels of significance (*P* value) of repeated measures ANOVA.

A_{sat}, net photosynthetic rate at growing CO_2 concentration (380 μmol mol^{-1}); G_s, stomatal conductance to water vapour;

V_{cmax}, maximum rate of carboxylation; J_{max}, maximum rate of electron trasport; A_{max}, light-saturated net photosynthetic rate at CO_2 saturation;

PNUE, photosynthetic nitrogen use efficiency.

Figure legends

Fig. 1 The number of attached leaves (A) and shed leaves (B) at terminal shoots of white birch grown under ambient and elevated O_3 measured in the second year of treatment (the year 2012). Data are represented as mean ± SE (n=6). t-test: **P*<0.05, ***P*<0.01, ****P*<0.001. Black arrow denotes the timing of shed of early leaves in white birch.

Fig. 2 Individual leaf size (A), stomatal density (B) and leaf air space (C) of white birch seedlings grown under ambient and elevated O_3 as measured in August 2012 (in the second year of treatment). Data are represented as mean ± SD (n=5-6). t-test: n.s. no significant, **P*<0.05, ***P*<0.01.

Fig. 3 A comparison of the relative decrease of light saturated net photosynthetic rate (A_{sat}) in early and late leaves of white birch against AOT40. Solid line denotes the regression line for early leaves (R^2=0.67), dashed line denotes the regression line for late leaves (R^2=0.86).

Fig.1

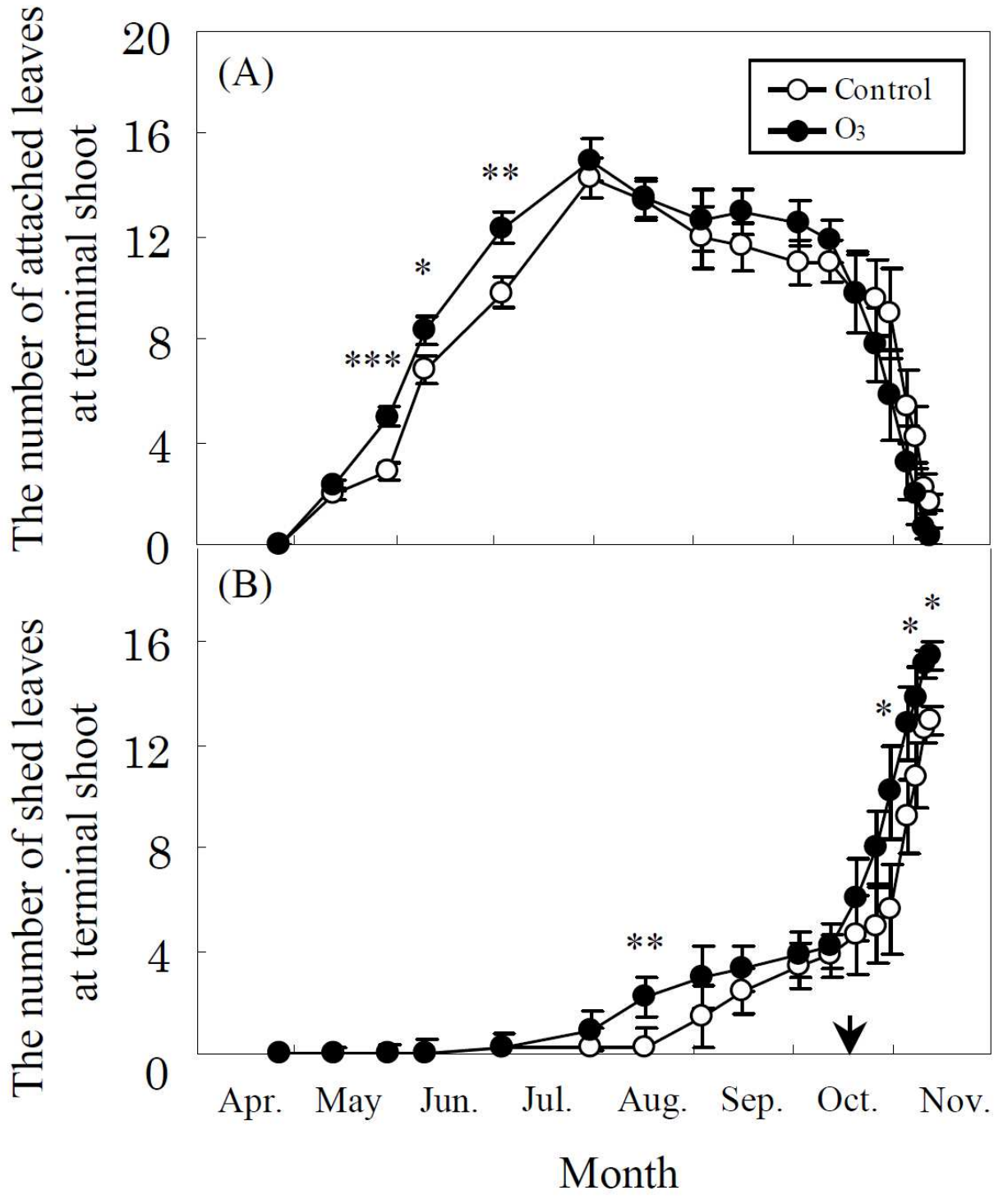

(A)
Control
O3
The number of attached leaves at terminal shoot
20
16
12
8
4
0
***
*
**
(B)
The number of shed leaves at terminal shoot
16
12
8
4
0
**
*
*
*
Apr.
May
Jun.
Jul.
Aug.
Sep.
Oct.
Nov.
Month

Fig.2

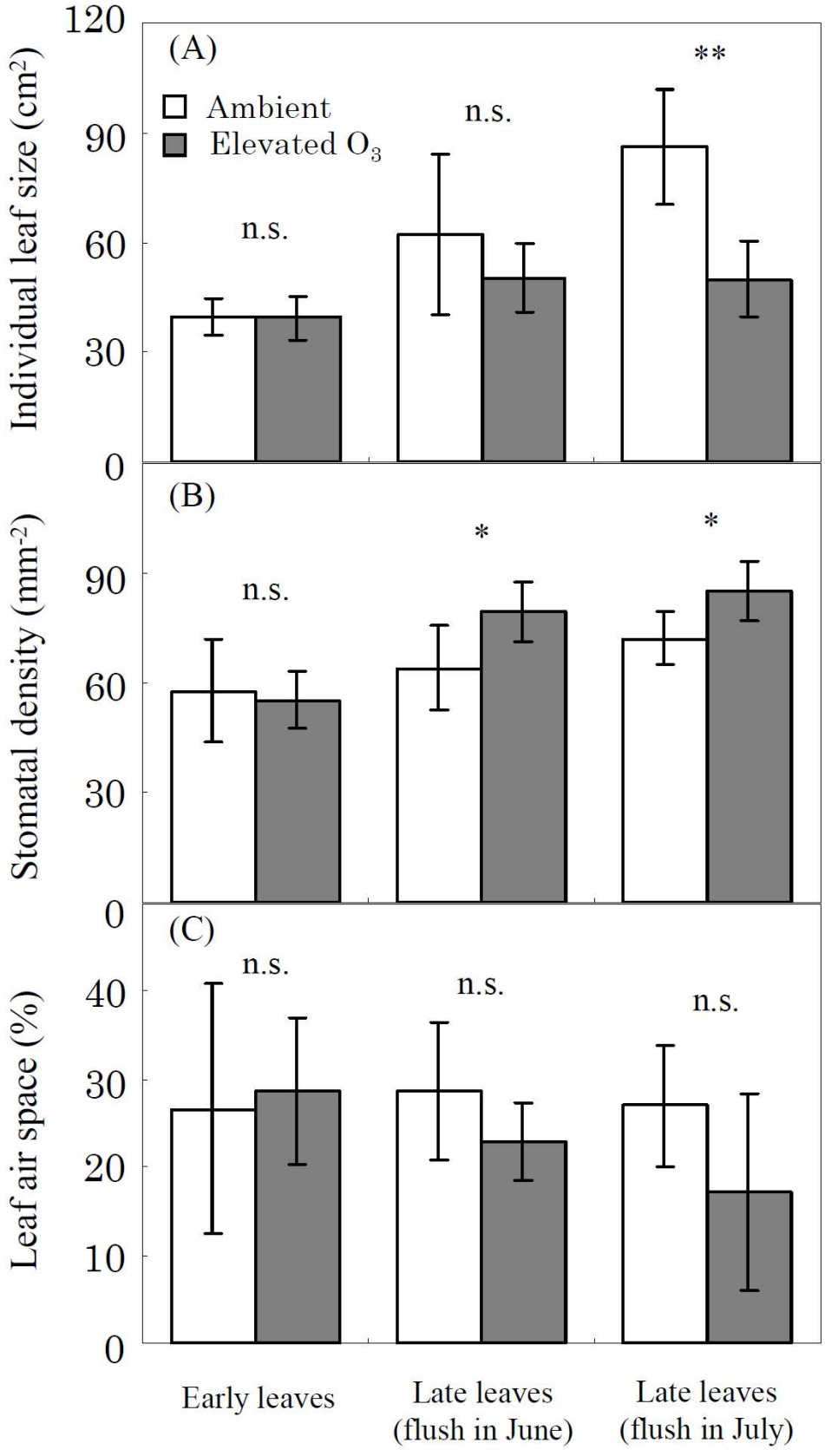
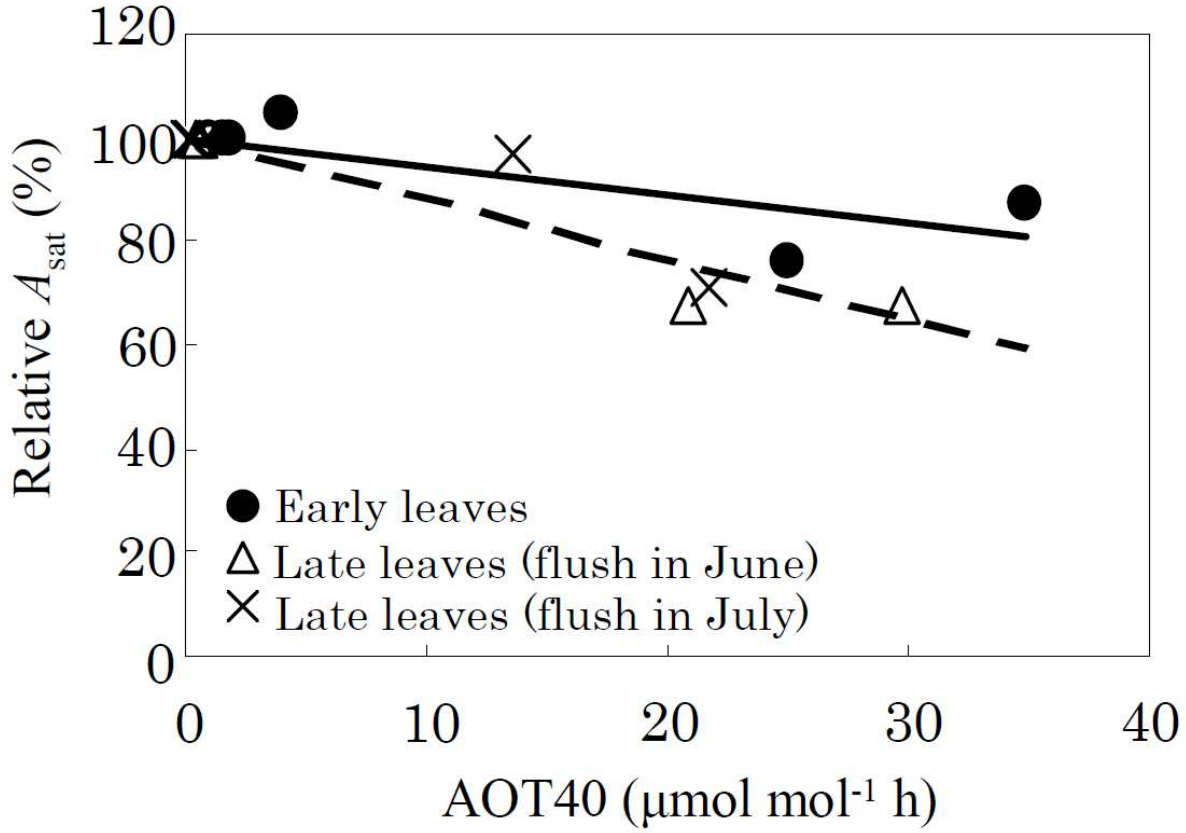

(A)
Ambient
Elevated O_3
n.s.
n.s.
**
Individual leaf size (cm^2)
120
90
60
30
0
(B)
n.s.
*
*
Stomatal density (mm^{-2})
90
60
30
0
(C)
n.s.
n.s.
n.s.
Leaf air space (%)
40
30
20
10
0
Early leaves
Late leaves (flush in June)
Late leaves (flush in July)

Fig.3


120
100
80
60
40
20
0
Relative A_{sat} (%)
Early leaves
Late leaves (flush in June)
Late leaves (flush in July)
0
10
20
30
40
AOT40 (μmol mol-1 h)