# Interactive effect of leaf age and ozone on mesophyll conductance in Siebold's beech

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Mesophyll conductance ($G_m$) is one of the most important factors determining photosynthesis. Tropospheric ozone ($O_3$) is known to accelerate leaf senescence and causes a decline of photosynthetic activity in leaves. However, the effects of age-related variation of $O_3$ on $G_m$ have not been well investigated, and we, therefore, analysed leaf gas exchange data in a free-air $O_3$ exposure experiment on Siebold's beech with two levels (ambient and elevated $O_3$: 28 and 62 nmol $mol^{-1}$ as daylight average, respectively). In addition, we examined whether $O_3$-induced changes on leaf morphology (leaf mass per area, leaf density and leaf thickness)

may affect $CO_2$ diffusion inside leaves. We found that $O_3$ damaged the photosynthetic biochemistry progressively during the growing season. The $G_m$ was associated with a reduced photosynthesis in $O_3$-fumigated Siebold's beech in August. The $O_3$-induced reduction of $G_m$ was negatively correlated with leaf density, which was increased by elevated $O_3$, suggesting that the reduction of $G_m$ was accompanied by changes in the physical structure of mesophyll cells. On the other hand, in October, the $O_3$-induced decrease of $G_m$ was diminished because $G_m$ decreased due to leaf senescence regardless of $O_3$ treatment. The reduction of photosynthesis in senescent leaves after $O_3$ exposure was mainly due to a decrease of maximum carboxylation rate ($V_{cmax}$) and/or maximum electron transport rate ($J_{max}$) rather than diffusive limitations to $CO_2$ transport such as $G_m$. A leaf age×$O_3$ interaction of photosynthetic response will be a key for modelling photosynthesis in $O_3$-polluted environments.

*Abbreviations*

| | |
|---|---|
| $C_c$ | $CO_2$ concentration inside the chloroplast envelope (μmol $mol^{-1}$) |
| $C_i$ | Sub-stomatal $CO_2$ concentration (μmol $mol^{-1}$) |
| ETR | Rate of electron transport (μmol $m^{-2}$ $s^{-1}$) |
| Φ | Initial slope of photosynthetic light response curve (mol $mol^{-1}$) |
| $\Phi_{CO2}$ | Quantum efficiency of carbon gain (dimensionless) |
| ΦPSII | Quantum yield of PSII in the light (dimensionless) |
| Γ | Light compensation point (μmol $mol^{-1}$) |
| Γ* | $CO_2$ compensation point to photorespiration (μmol $mol^{-1}$) |
| $G_m$ | Mesophyll conductance to $CO_2$ (mol $m^{-2}$ $s^{-1}$ $bar^{-1}$) |
| $G_{sCO2}$ | Stomatal conductance for $CO_2$ (mol $m^{-2}$ $s^{-1}$) |
| $G_{sH2O}$ | Stomatal conductance for water vapour (mol $m^{-2}$ $s^{-1}$) |

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|---|---|---|
| 53 | $G_{totCO2}$ | Total conductance to $CO_2$ (mol $m^{-2}$ $s^{-1}$) |
| 54 | $J_{max}$ | Maximum rate of electron transport required for RuBP regeneration (μmol |
| 55 | $m^{-2}$ $s^{-1}$) | |
| 56 | $l_b$ | Relative biochemical limitation to photosynthesis (%) |
| 57 | LD | Leaf density (g $cm^{-3}$) |
| 58 | $l_b$ | Relative mesophyll conductance limitation to photosynthesis (%) |
| 59 | LMA | Leaf mass per area (g $cm^{-2}$) |
| 60 | $l_b$ | Relative stomatal limitation to photosynthesis (%) |
| 61 | LT | Leaf thickness (mm) |
| 62 | $P_N$ | Net photosynthetic rate (μmol $m^{-2}$ $s^{-1}$) |
| 63 | $P_{Nsat}$ | Light-saturated net photosynthetic rate (μmol $m^{-2}$ $s^{-1}$) |
| 64 | $R_d$ | Respiration in the light (μmol $m^{-2}$ $s^{-1}$) |
| 65 | $R_n$ | Respiration in the dark (μmol $m^{-2}$ $s^{-1}$) |
| 66 | $R_{PR}$ | Photorespiration (μmol $m^{-2}$ $s^{-1}$) |
| 67 | $V_{cmax}$ | Maximum rate of RuBP carboxylation (μmol $m^{-2}$ $s^{-1}$) |
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## Introduction

Anthropogenic emissions of air pollutants such as nitrogen oxides (NOx) and volatile organic compounds have elevated the ground-level concentration of ozone ($O_3$) since pre-industrial times (Mills et al. 2018). Ozone is phytotoxic to plants, adversely affecting physiological and biochemical processes contributing to a decline in plant growth (Matyssek et al. 2013, Grulke and Heath 2020).

In cool-temperate forests in Japan, deciduous broadleaved Siebold's beech (*Fagus crenata*) is the dominant tree species (Koike et al. 1998). Previous experimental studies have shown that Siebold's beech is highly sensitive to $O_3$ (e.g. Yamaguchi et al. 2011, Izuta 2017). Watanabe et al. (2012) estimated that the current concentration of $O_3$ in Japan may reduce the growth of Siebold's beech from 3.2% to a maximum of 9.7%. This $O_3$ induced reduction in tree growth is considered to be related to lower photosynthetic activity (Watanabe et al. 2014a). The exact mechanisms underlying the effects of $O_3$ on photosynthesis are still unclear. However, previous studies have suggested that $O_3$ causes biochemical limitations associated with reduced ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity in leaves (Fiscus et al. 2005, Hoshika et al. 2013, Watanabe et al. 2014a, 2014b, Bagard et al. 2015). The loss of Rubisco activity is commonly inferred from decreases in the maximum rate of carboxylation ($V_{cmax}$), as indicated by a decrease in the initial slope of a $P_N/C_i$ curve (net photosynthetic rate versus estimated sub-stomatal $CO_2$ concentration; e.g. Matyssek et al. 2012, Watanabe et al. 2013). Any loss in the biochemical efficiency of $CO_2$ assimilation induced by $O_3$ exposure may also be evident in a decline in the maximum rate of electron transport required for ribulose-1,5-bisphosphate (RuBP) regeneration ($J_{max}$; Hoshika et al. 2013). The traditional calculation of photosynthetic parameters from $P_N/C_i$ curves assumes an infinite mesophyll

conductance to $CO_2$ ($G_m$; e.g. Long and Bernacchi 2003). However, $G_m$ may exert similar limits to $P_N$ as stomatal resistance to $CO_2$ transport (Terashima et al. 1995, Lauteri et al. 2014, Killi and Haworth 2017, Gago et al. 2019, Veromann-Jürgenson et al. 2020). This indicates that previous observations of the $O_3$-induced reduction of $V_{cmax}$ and $J_{max}$ parameters calculated from $P_N/C_i$ curves assuming infinite $G_m$ incorporate not only biochemical limitations but also the effect of $G_m$ (Warren et al. 2007).

The generation of reactive oxygen species during $O_3$ exposure could disrupt the physical integrity and biochemistry of mesophyll cells (Kangasjärvi et al. 2005). This would suggest that the exposure to $O_3$ would impair both the physical and biochemical transport of $CO_2$ from the sub-stomatal leaf air-space to chloroplasts. In fact, a reduction in $G_m$ was observed in $O_3$-sensitive snapbean (*Phaseolus vulgaris*) exposed to 60 nmol $mol^{-1}$ $O_3$, while no effect was observed on the $G_m$ values of resistant varieties (Flowers et al. 2007). Xu et al. (2019) found that the $O_3$-induced reduction of photosynthesis was mainly due to a decreased $G_m$ in a hybrid poplar clone. In addition, Watanabe et al. (2018a) reported that $O_3$ exposure reduced $G_m$ in Siebold's beech without any effect on $C_c$-based $V_{cmax}$. However, European beech (*F. sylvatica*) grown in an $O_3$ FACE system showed no effect of a doubling of ambient $O_3$ concentration on $G_m$ (Warren et al. 2007). Likewise, fumigation of localized areas of Holm oak (*Quercus ilex*) and pubescent oak (*Q. pubescens*) leaves with $O_3$ between 50 to 350 nmol $mol^{-1}$ did not reduce $G_m$ (Velikova et al. 2005).

Mesophyll conductance is generally lower in leaves with thicker cell walls and greater leaf density (Adachi et al. 2013, Ivanova et al. 2018) that are conditions limiting the diffusion of $CO_2$ in the leaf liquid phase (Tomás et al. 2013, Onoda et al. 2017). Ozone may affect leaf morphology, which is likely to modify the $CO_2$

131 diffusion inside leaves (Heath 1994, Moura et al. 2018). The white poplar (*Populus*
132 *alba*) grown in an $O_3$-enriched atmosphere (150 nmol $mol^{-1}$) developed thicker and
133 denser leaves than that grown in clean air (Fares et al. 2006). Likewise, leaf density
134 (LD) was increased by $O_3$ in European silver birch (*Betula pendula*; Oksanen 2005)
135 and European beech (Bussotti et al. 2005). In contrast, the leaves of Pima cotton
136 (*Gossypium barbadense*) formed in $O_3$-enriched air showed lower mesophyll
137 density (Grantz and Shrestha 2006), probably due to damage and disruption of the
138 mesophyll cells (Günthardt-Goerg et al. 1993). Watanabe et al. (2018a) did not find
139 any effect of $O_3$ on leaf morphology in Siebold's beech, although plants were
140 exposed to relatively low $O_3$ concentrations (29 to 47 nmol $mol^{-1}$ as daylight
141 average). The density of cells in the mesophyll layer is closely related to leaf mass
142 per area (LMA; Haworth and Raschi 2014). Meta-analysis suggests that the effect
143 of $O_3$ on LMA is not pronounced or uni-directional (Poorter et al. 2009), raising
144 the likelihood that $O_3$ effects on leaf structure depend on species or varieties
145 (Pääkkönen et al. 1995).

146 Previous works have shown that $G_m$ is variable with leaf age (Loreto et al. 1994,
147 Niinemets et al. 2009, Tosens et al. 2012, Marino et al. 2020). After the onset of
148 leaf senescence, $G_m$ reduced due to age-dependent variation of leaf structure and
149 physiological activity (Niinemets et al. 2009). It is known that leaf senescence is
150 accelerated by $O_3$ (Grulke and Heath 2020). As a matter of fact, $O_3$-induced
151 structural changes in chloroplasts appear to be similar to those during autumn
152 senescence (e.g. Vollenweider et al. 2019), which may be associated with a reduced
153 mesophyll $CO_2$ diffusion. The question was therefore raised if $O_3$ and leaf age
154 could interactively affect $G_m$, but the age-related change of $O_3$ effect on $G_m$ has
155 not been addressed in previous studies.

156 To examine a leaf age×$O_3$ interaction on $G_m$, we measured leaf gas exchange

three times (June, August, October) in a free-air $O_3$ exposure experiment on Siebold's beech. The aim of the present study was to: (1) characterise the effect of $O_3$ fumigation on diffusive resistances to $P_N$, in particular the impact on $G_m$ values derived from the variable J method (Harley et al. 1992, Loreto et al. 1992), while comparing with those derived from the $P_N/C_i$ curve fitting approach (Ethier and Livingston 2004); (2) assess the impact of $O_3$ exposure on the efficiency of photosynthetic light capture; and (3) analyse the interaction between $O_3$ fumigation and leaf morphology (LMA, LD and leaf thickness), and the impact upon photosynthetic physiology throughout the growing season.

## Materials and Methods

### Experimental site and plant material

Measurements were conducted at a free-air $O_3$ exposure experiment facility located in the Sapporo Experimental Forest, Hokkaido University, in northern Japan (43°04' N, 141°20' E, 15 m a.s.l., mean annual temperature: 8.9˚C, mean total annual precipitation: 1107 mm, the snow-free period was late April to mid-November). The details of the system are described in Watanabe et al. (2013). The soil is a brown forest soil (Dystric Cambisols). Two plots were established, one for ambient $O_3$ (AA) and the other for elevated $O_3$ ($eO_3$). The size of each plot was 5.5 m × 7.2 m. The distance between the $O_3$-enhanced plot and the ambient plot was about 20 m, which was enough distance for the $O_3$-enriched air not to affect the AA plot (Watanabe et al. 2013). Ten seedlings of two-year-old Siebold's beech (*Fagus crenata*) were obtained from a nearby nursery and planted in each plot in May 2003 and then grown under ambient conditions from 2003 to 2010. The plants were ten years old and well-established when fumigation with $O_3$ began in 2011. The 11-year-old Siebold's beech saplings analysed in this study were around 3.4 m in height in 2012. We confirmed that photosynthetic parameters, plant height and diameter were not different between treatments before $O_3$ exposure (in July 2011, data not shown). The target $O_3$ concentration was 60 nmol $mol^{-1}$ during daylight hours. The enhanced $O_3$ treatment was employed during daylight hours to

187 ten Siebold's beech saplings from August to November in 2011, and from May to
188 November in 2012. Ozone concentrations at canopy height were recorded
189 continuously by an $O_3$ monitor (Mod. 202, 2B Technologies). The daytime hourly
190 mean of $O_3$ concentration in AA and $eO_3$ were 28 nmol $mol^{-1}$ and 62 nmol $mol^{-1}$,
191 respectively, during the 2012 experimental period. The level of $O_3$ concentration
192 in $eO_3$ was equal to the environmental standard value in Japan (= 60 nmol $mol^{-1}$),
193 and has been frequently observed in many Asian regions including forested areas
194 (Izuta 2017). The soil moisture was measured in the root layer (at depth 20 cm) by
195 10HS sensors equipped with an EM5b data logger (Decagon Devices). The average
196 soil moisture (volumetric soil water content) was 28.1 ± 2.8% during these
197 measurements. These values were equivalent to the field capacity of the soil.
198 Previous studies have established that no reduction in $P_N$ due to soil water content
199 occurred during the growing season for Siebold's beech in this experiment
200 (Hoshika et al. 2012, 2013, Watanabe et al. 2014a).

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202 **Measurement of leaf gas exchange**

203 Leaf gas exchange was measured in fully expanded leaves exposed to full sun at
204 the top of the canopy (n = 6 trees in each $O_3$ treatment) using a Li6400 portable
205 infra-red gas analyzer (Li-Cor instruments). Only first flush leaves (flushed in
206 May) were used for these measurements, because the number of second flush leaves
207 was very small (0.16% of the total leaf number taken over all saplings).
208 Measurements were undertaken on 15-16 June, 26-31 August and 9-16 October on
209 days with clear sky between 8:00 and 16:00 h. Conditions within the leaf cuvette
210 were set to maintain a leaf temperature of 25°C and the leaf-to-air vapour pressure
211 deficit (VPD) within a range of 1.0-1.8 kPa. The Li6400 was connected to a 6400-
212 40 leaf chamber fluorimeter cuvette with red and blue LEDs when conducting the
213 $P_N$ – photosynthetic photon flux density (PPFD) response curves. The response of
214 $P_N$ to PPFD was measured at 11 light intensities (1500, 1100, 800, 500, 400, 300,
215 200, 100, 75, 50, 0 μmol $m^{-2}$ $s^{-1}$). At each stage of the $P_N$/PPFD curve, $P_N$, stomatal
216 conductance ($G_{sH2O}$), and the quantum yield of PSII in the light (ΦPSII) were
217 recorded. The $P_N$/PPFD response curves were utilised to calculate the light
218 saturated rate of $P_N$ ($P_{Nsat}$), initial slope of photosynthetic light response curve (Φ),
219 light compensation point (Γ) and the quantum efficiency of carbon gain ($\Phi_{CO2}$)
220 following Kaipiainen et al. (2009). The Kok (1948) method was used to estimate

respiration in the light ($R_d$). The effect of variation in the sub-stomatal concentration of [$CO_2$] ($C_i$) on $R_d$ was corrected using the iterative method of Kirschbaum and Farquhar (1987). Respiration in the dark ($R_n$) was measured by switching off the LED light unit after the Kok protocol, shading the area of the plant being measured and recording the rate of $CO_2$ efflux from the leaf, after values had remained stable for 5-10 min. Mesophyll conductance was calculated at $P_{Nsat}$ using the variable J method as described by Loreto et al. (1992):

$$G_m = \frac{P_{Nsat}}{C_i - \frac{\Gamma^* \cdot [ETR + 8 \cdot (P_N + R_d)]}{ETR - 4 \cdot (P_N + R_d)}} \quad (1),$$

where the electron transport rate (ETR) is calculated from fluorescence (Genty et al. 1989) as following:

$$ETR = \text{PPFD} \cdot \varphi\text{PSII} \cdot \alpha \cdot \beta \quad (2),$$

where the partitioning factor ($\beta$) between photosystems I and II was considered to be 0.5 and leaf absorbance (α= 0.85; Laisk and Loreto 1996). The $CO_2$ compensation point to photorespiration (Γ*) was calculated using the Rubisco specificity factor of Galmes et al. (2005). Total conductance to $CO_2$ ($G_{totCO2}$) was calculated as:

$$G_{totCO2} = [G_{sCO2} \cdot G_m]/[G_{sCO2} + G_m] \quad (3).$$

where $G_{sCO2}$ is the stomatal conductance for $CO_2$. The concentration of [$CO_2$] inside the chloroplast envelope ($C_c$) was calculated using the value of $G_m$ produced by the variable J method as:

$$C_c = C_i - \frac{P_{Nsat}}{G_m} \quad (4).$$

Photorespiration ($R_{PR}$) was determined following Sharkey (1988):

$$R_{PR} = \frac{P_{Nsat} + R_d}{\frac{C_c}{\Gamma^*} - 1} \quad (5).$$

Hoshika et al. (2013) previously reported the $P_N/C_i$ curves of the Siebold's beech exposed to $O_3$-FACE, based on the approach by Long and Bernacchi (2003) that assumed infinite $G_m$. Here the $P_N/C_i$ data were re-analysed using the protocol of Ethier and Livingston (2004) to consider $G_m$ values using the curve-fitting approach. The $P_N/C_i$ curves were obtained by measuring $P_N$ over 12 [$CO_2$] steps ($C_a$, 380, 300, 220, 140, 60, 300, 380, 500, 800, 1100, 1400, 1700 μmol mol$^{-1}$). The response of $P_N$ to $C_i$ was then used to calculate the carboxylation capacity of ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco; $V_{cmax}$) and the maximum rate of electron transport required for ribulose-1,5-bisphosphate (RuBP) regeneration ($J_{max}$), assuming that $G_m$ were constant at all [$CO_2$] steps. All gas exchange data were corrected for leaks from gaskets using an empty cuvette and the entire chamber head was enclosed in a plastic bag to minimise the effect of leak (Flexas et al. 2007).

According to Grassi and Magnani (2005), the relative limitations in $P_{Nsat}$ (stomatal limitation, $l_s$; mesophyll conductance limitation, $l_m$; biochemical limitation, $l_b$) were determined as follows:

$$l_s = \frac{G_{totCO2}/G_{sCO2}\cdot\delta P_{Nsat}/\delta C_c}{G_{totCO2}+\delta P_{Nsat}/\delta C_c} \quad (6),$$

$$l_m = \frac{G_{totCO2}/G_m\cdot\delta P_{Nsat}/\delta C_c}{G_{totCO2}+\delta P_{Nsat}/\delta C_c} \quad (7),$$

$$l_b = \frac{G_{totCO2}}{G_{totCO2}+\delta P_{Nsat}/\delta C_c} \quad (8),$$

where $\delta P_{Nsat}/\delta C_c$ is an initial slope of $P_N/C_c$ curves ($C_c$: over a range of 0–150 μmol mol$^{-1}$).

**Measurements of leaf traits**

After the measurement of gas exchange rate, leaves were collected for the determination of the leaf mass per unit area (LMA), leaf density (LD) and leaf thickness (LT). LT was measured avoiding the mid-vein by using a hand-held micrometer (Digimatic micrometer, Mitsutoyo). The projected area (one side of the leaf) was determined with image-processing software (LIA 32, Nagoya University,

Japan). Leaves were then dried in an oven at 70˚C for 1 week and were then weighed. The LMA was calculated as the ratio of the dry mass to the area of the leaves. The LD was calculated as the leaf dry mass per unit leaf volume.

**Data analysis**

Statistical analyses were performed using R 3.5.1 (R Core Team 2018). To test the effects of $O_3$ fumigation treatment and measurement months, a two-way ANOVA was used to assess differences in variance between samples. Linear regression was used to investigate possible relationships between $P_N$ and diffusive limitations to $CO_2$ transport and leaf traits. For these analyses, we used $G_m$ values derived from variable J method. Principle component analysis (PCA) was used to investigate patterns in the photosynthetic capacity, light harvesting and diffusive components of photosynthesis in Siebold's beech after fumigation with $O_3$. Results were considered significant at $P<0.05$.

## Results

### Leaf density is modulated by ozone and season

No significant effects of $O_3$ or measurement month on LMA and LT were found (Table 1). On the other hand, LD was significantly increased by $O_3$. In addition, LD increased with season as it was relatively smaller in June (0.45 g $cm^{-3}$ in AA and 0.44 g $cm^{-3}$ in $eO_3$) compared to in October (0.48 g $cm^{-3}$ in AA and 0.54 g $cm^{-3}$ in $eO_3$).

### Photosynthetic parameters are progressively decreased by ozone

Most photosynthetic parameters of Siebold's beech were significantly affected by $O_3$ exposure (Fig. 1, Table 2). Ozone decreased $P_{Nsat}$ of Siebold beech trees in

August (−28%) and October (−46%), although $P_{Nsat}$ values in both $O_3$ treatments were similar in June (−7% in $eO_3$ compared to in AA; Fig. 1A). Significant increases of $R_n$ were induced by $O_3$ (+70% in August and +88% in October; Fig. 1B). There were no significant changes in $R_{PR}$ associated with elevated $O_3$ in either June or August (Fig. 1C). However, $O_3$ decreased $R_{PR}$ by 56% relative to AA plants in October. Ozone decreased $G_{sH2O}$, $G_m$ and $G_{totCO2}$, although significant differences were not observed between the treatments in October (Fig. 1D-F). In addition, the measurements by fluorimeter indicated that the PSII electron transport rate (ETR) and actual quantum efficiencies of PSII (ΦPSII) were significantly decreased by $O_3$ (Table 2, Fig. 2H,L). The $eO_3$ treatment significantly reduced $P_{Nsat}$ and values of $V_{cmax}$ and $J_{max}$ from the $P_N/C_c$ curves (Table 2, Fig. S1). A significant increase in the $J_{max}/V_{cmax}$ ratio was found in October due to a larger reduction in $V_{cmax}$ than $J_{max}$ after $O_3$ exposure. Estimated values of $G_m$ derived from the $P_N/C_i$ response curves approach were matched with those calculated by the variable J method (Fig. S2).

Rates of $P_{Nsat}$ were positively correlated to the measures of $CO_2$ conductance under AA conditions (Fig. 3A-C). However, in $eO_3$, $P_{Nsat}$ was not associated with $G_{sH2O}$ (Fig. 3A). In addition, no significant relationship between $G_m$ and $G_{sH2O}$ was found in $eO_3$ (Fig. 3D).

The light-saturated net photosynthetic rate ($P_{Nsat}$) and diffusive conductance parameters ($G_{sH2O}$, $G_m$ and $G_{totCO2}$) were not correlated with LD under the AA condition (Fig. 4A-D). On the other hand, in $eO_3$, $P_{Nsat}$, $G_m$ and $G_{totCO2}$ showed negative relationships with LD (Fig. 4A,C,D), while there was no significant correlation between LD and $G_{sH2O}$ (Fig. 4B). LMA and LT did not correlate to those diffusive conductance parameters in both $O_3$ treatments (Figs S3 and S4).

**Relative limitations to photosynthesis are affected by ozone and season**

The relative limitations to photosynthesis ($l_s$, $l_m$ and $l_b$) are shown in Table 3. Relative limitation to photosynthesis by stomata ($l_s$) decreased with season. In particular, in $eO_3$, the reduction in $l_s$ was remarkable in the end of the growing season ($l_s$: −39% in August, −54% in October compared to June). On the other hand, the $l_b$ increased over the season, and this was highlighted in $eO_3$ according to the post-hoc Tukey test. A relative limitation to photosynthesis by mesophyll conductance ($l_m$) was rather constant among the sampling campaigns (approximately 30 to 40% over the season).

**Principal component analysis revealed the interaction of leaf age and ozone on photosynthetic response**

Principal component analysis of parameters derived from analyses of the photosynthetic capacity ($P_N/C_c$ curves), light harvesting ($P_N$/PPFD curves) and diffusive limitations to $CO_2$ transport ($G_{sH2O}$, $G_m$ and $G_{totCO2}$) of Siebold's beech indicated a high degree of overlap in the multi-variate space occupied by AA- and $eO_3$-fumigated plants when measured in June (Fig. 5; student's t-test of PCA scores: component 1, P=0.215; component 2, P=0.646). However, the measurement month affected variations in component 1 and component 2, while $O_3$ was a significant factor affecting only component 1 (two-way ANOVA of PCA scores: component 1, $O_3$: P<0.001, Month: P<0.001, $O_3$×Month: P=0.002; component 2, $O_3$: P=0.164, Month: P=0.029, $O_3$×Month: P=0.059). In fact, $eO_3$-treated plants did not overlap those treated by AA in August and October. In addition, Siebold's beech measured during October occupied distinct multivariate space in comparison to previous analyses in June and August. In this analysis, component 1 accounts for 49.2% of the variance within the dataset and component 2 accounts for 14.8%

365 of variance.
366
367 **Discussion**

368 **Ozone effects on mesophyll conductance and other photosynthetic parameters**

369 We analysed leaf gas exchange data in Siebold's beech exposed to elevated $O_3$ to
370 assess whether $O_3$ affected $G_m$. We found that $O_3$ caused a significant reduction of
371 $G_m$ in August accompanied by a reduction of $P_{Nsat}$ (Fig. 3B). On the other hand, no
372 significant relationship between $G_{sH2O}$ and $P_{Nsat}$ was found in $eO_3$ (Fig. 3A). As a
373 result, $G_m$ could better explain the observed reduction of photosynthetic rate rather
374 than $G_{sH2O}$ in $eO_3$ (Table 3). In addition, the present study indicates that $eO_3$ caused
375 a decoupling of $G_{sH2O}$ from $G_m$ (Fig. 3D), although a significant relationship has
376 been previously found between $G_{sH2O}$ and $G_m$ (Lloyd et al. 1992, Lauteri et al.
377 2014). Although stomata generally regulate $CO_2$ uptake, several studies have
378 shown evidence that chronic $O_3$ exposure impairs efficient stomatal control of gas
379 exchange (Paoletti 2005, Hoshika et al. 2019, 2020).

380 In the previous report by Hoshika et al. (2013), $O_3$ was found to decrease $C_i$-
381 based $V_{cmax}$ of Siebold's beech leaves in August and October based on the
382 calculation from $P_N/C_i$ curves assuming an infinite mesophyll conductance to $CO_2$
383 (Long and Bernacchi 2003). Such a decrease of $C_i$-based $V_{cmax}$ is, however,
384 attributed to a decreased $G_m$ or degradation of Rubisco activity (Loreto et al. 1994).
385 In the present study, $O_3$ decreased $G_m$ in August while $C_c$-based $V_{cmax}$ was not
386 significantly altered (Fig. 1E, Table 2), which is supported by previous findings in
387 Siebold's beech (Watanabe et al. 2018a). In addition, we found that $O_3$ exposure
388 decreased $C_c$-based $V_{cmax}$ and $J_{max}$ in October while no reduction was found in $G_{sH2O}$,
389 $G_m$ and $G_{totCO2}$. The results suggest that a reduction in $G_m$ may be a primary
390 response in $O_3$-induced decrease of photosynthesis of Siebold's beech.

391

392 **Leaf age effects on mesophyll conductance and other photosynthetic**
393 **parameters**

394 Mesophyll conductance ($G_m$) decreased with leaf ageing, in association with a
395 reduction of the leaf photosynthetic rate (Fig. 1A, E). Leaf morphological traits
396 were not significantly correlated with $G_m$ during the ageing process in control
397 condition (AA). This is probably because the change in leaf morphology was small
398 during leaf ageing in AA, although LD statistically significantly increased with
399 season. Tosens et al. (2012) similarly reported the slow increase in LD and LMA
400 with leaf ageing after leaf maturity in *Populus tremula*, while LT remained constant.
401 The observed reduction of $G_m$ in senescent leaves may be attributed to age-
402 dependent alterations such as changes of chloroplasts number and size, the location
403 of chloroplasts, photosynthetic enzyme content, and aquaporin status (Evans and
404 Vellen 1996, Niinemets et al. 2009). In addition to $G_m$, most of photosynthetic
405 parameters decreased with leaf ageing (Fig. 1, Table 2). Interestingly, the relative
406 contribution to photosynthesis by mesophyll conductance ($l_m$) was rather constant
407 over the season (Table 3). Additionally, the stomatal limitation to photosynthesis
408 ($l_s$) decreased with leaf ageing, while the relative biochemical limitation ($l_b$)
409 increased in the end of the growing season. It is known that stomata in senescent
410 leaves remain open even in low light conditions, called the “dull leaf” phenomenon.
411 They are, therefore, unable to regulate leaf gas exchange efficiently (Terashima,
412 2002). On the other hand, the low biochemical photosynthetic capacity in senescent
413 leaves was due to the decline of leaf nitrogen (Watanabe et al., 2018b), which may
414 further limit photosynthesis in autumn. These results suggest that photosynthesis
415 was primarily determined by diffusive limitations of $CO_2$ transport during summer
416 in Siebold’s beech, while biochemical limitations were relatively important in

417 senescent leaves in autumn. This seasonal change is consistent with the PCA
418 analysis for photosynthetic characteristics (Fig. 5).
419
420 **Interactive effects of ozone and leaf age on leaf gas exchange**
421 Leaf senescence is often accelerated by $O_3$ exposure (Grulke and Heath 2020). The
422 effects of $O_3$ on leaf gas exchange were similar to those of leaf senescence, i.e.
423 significant reductions in the photosynthetic capacity, light harvesting and diffusive
424 components of photosynthesis, but the reduction of photosynthetic capacity was
425 observed earlier in $eO_3$ than in AA (Fig. 1A-F, Table 2). Mesophyll conductance
426 ($G_m$) showed a sharp decrease after $O_3$ exposure in August and no further change
427 in October. Meanwhile, LD increased with leaf age especially at $eO_3$ (Table 1).
428 Previous studies similarly reported that $O_3$ altered leaf cell ultrastructure in
429 Siebold's beech (Yonekura et al. 2001) and increased LD in European beech
430 (Bussotti et al. 2005). A larger LD may decrease $CO_2$ diffusion in the liquid phase
431 and, thus, $G_m$ (Niinemets 2015). As a result, a significant relationship between $G_m$
432 and LD over the season was found at $eO_3$ (Fig. 4C), suggesting that the reduction
433 of $G_m$ was accompanied by changes in the physical structure of mesophyll cells. It
434 is known that some leaf structural changes are highlighted only when senescence
435 is accelerated by environmental stress (Vollenweider et al. 2019). In fact, as a leaf
436 age×$O_3$ interaction, inner cell wall thickening has been observed in aged leaves
437 after $O_3$ exposure due to progressing cell injury in $eO_3$ (Günthardt-Goerg and
438 Vollenweider 2007). However, we should note that the correlation between LD and
439 $G_m$ was not strong ($R^2$ = 0.24). This suggests that there may also be other factors
440 causing a reduced $G_m$ after $O_3$ exposure. For example, aquaporins are involved in
441 the regulation of $G_m$ (e.g. Hanba et al. 2004) and water across the bundle sheath
442 (Shatil-Cohen et al. 2011). Ozone and the resultant reactive oxygen species (ROS)

443 reach the cell membrane (Vainonen and Kangasjärvi 2015), which may affect $G_m$
444 through direct effects on aquaporins. In fact, $O_3$ may decrease leaf hydraulic
445 conductance (Zhang et al. 2018), which is related to vein architecture in
446 association with aquaporins (Scoffoni and Sack 2017). This hypothesis needs to be
447 tested in further studies.

448 Limiting factors for the reduction of $P_N$ after $O_3$ exposure may vary with leaf
449 age. In fact, diffusive resistances to $CO_2$ transport were dominant factors for
450 photosynthesis at $eO_3$ in August, while the reduction of $P_N$ after $O_3$ exposure in
451 October was mainly due to the co-limitation of $V_{cmax}$ and $J_{max}$ (Table 3). At the
452 same time, $O_3$ progressively increased the $J_{max}/V_{cmax}$ ratio with leaf age (Table 2).
453 This increase in $J_{max}/V_{cmax}$ ratio suggests that the photosynthetic rate was limited
454 more by Rubisco carbon fixation than RuBP regeneration by electron transport.
455 Such a selective degradation of Rubisco quantity or activity was also observed in
456 a poplar clone exposed to $O_3$ (e.g. Bagard et al. 2008).

457 Rubisco activity is closely related to the capacity of photorespiration ($R_{PR}$)
458 (Farquhar et al. 1980). In fact, $R_{PR}$ in Siebold's beech was decreased by enhanced
459 $O_3$ treatments with leaf ageing (Fig. 1C). The reduced $R_{PR}$ is equivalent to reduced
460 photoprotective function that, alongside a lower energy usage for photochemistry,
461 would produce ROS, damaging photosynthetic biochemistry (Kangasjärvi et al.,
462 2005). In addition, the plant photorespiratory system requires the glycine
463 decarboxylase (GDC) reaction cycle in the mitochondria, which requires the
464 oxidized form of nicotinamide adenine dinucleotide (NAD+) and generates NADH
465 (the reduced form of NAD; Obata et al. 2016). This decrease in $R_{PR}$ may result in
466 a reduced NADH/NAD+ ratio stimulating mitochondrial respiration (Dizengremel
467 et al. 2012) as confirmed here in the $O_3$-induced increase of dark respiration rate
468 (Fig. 1B).

## Conclusion

Our results indicate that $O_3$ damaged photosynthetic mechanisms in Siebold's beech leaves. Mesophyll conductance was associated with a reduced photosynthesis in $O_3$-fumigated Siebold's beech in August. This $O_3$-induced reduction of $G_m$ was negatively correlated with leaf density, which was increased by $O_3$ exposure. This suggests that the reduction of $G_m$ in August was accompanied by changes in the physical structure of mesophyll cells. However, the response of photosynthetic parameters to $O_3$ was different in the later growing season. In October, the $O_3$-induced reduction of photosynthesis was mainly due to the co-limitation of $V_{cmax}$ and $J_{max}$ rather than diffusive resistances to $CO_2$ transport. Mesophyll conductance ($G_m$) decreased in October due to leaf senescence regardless of $O_3$ treatments. In consequence, the $O_3$-induced decrease of $G_m$ was diminished. These results suggest that seasonal changes of the photosynthetic response to $O_3$ will be a key to modelling photosynthesis in an $O_3$-polluted environment.

## Author contributions

Y.H. conceived the study and methodology, and analysed leaf gas exchange data from the ozone FACE experiment performed by Y.H., M.W. and T.K.; M.H. co-conceived the study and contributed to the analysis of light-response curve. All authors were involved in writing the paper, although Y.H. took a lead role.

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**Data availability statement**

Data sharing is not applicable to this article as all new created data is already contained within this article.

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744 **Supporting information**
745 Additional Supporting Information may be found in the online version of this
746 article:
747 **Fig. S1.** Responses of net photosynthetic rate ($P_N$) to the sub-stomatal
748 concentration of $CO_2$ ($C_i$) or the concentration of $CO_2$ inside the chloroplast
749 envelope ($C_c$).
750 **Fig. S2.** Relationship between mesophyll conductance ($G_m$) values derived from
751 the variable J method and those derived from the $P_N/C_i$ curve fitting method.
752 **Fig. S3.** Relationships between leaf gas exchange parameters and leaf mass per

753 area (LMA) of Siebold's beech saplings.
754 **Fig. S4.** Relationships between leaf gas exchange parameters and leaf thickness
755 (LT) of Siebold's beech saplings.
756
757
758 **Figure legends**
759 **Figure 1.** Photosynthetic parameters of Siebold's beech grown under ambient (AA)
760 and elevated $O_3$ ($eO_3$). (A) Light-saturated net photosynthetic rate [$P_{Nsat}$], (B) dark
761 respiration rate [$R_N$], (C) photorespiration [$R_{PR}$], (D) stomatal conductance to water
762 vapor [$G_{sH2O}$], (E) mesophyll conductance to $CO_2$ [$G_m$], (F) total conductance to
763 $CO_2$ [$G_{totCO2}$]. Each value is the mean ± standard error (n=6). Two-way ANOVA: *
764 P<0.05, ** P<0.01, *** P<0.001, ns denotes not significant. Different letters show
765 significant differences among treatments (P<0.05, Tukey test).
766 **Figure 2.** Responses of net photosynthetic rate ($P_N$), stomatal conductance ($G_{sH2O}$),
767 mesophyll conductance ($G_m$) and electron transport rate (ETR) to photosynthetic
768 photon flux density (PPFD). The $P_N$/PPFD curves were obtained by measuring $P_N$
769 over 11 PPFD steps (1500, 1100, 800, 500, 400, 300, 200, 100, 75, 50, 0 μmol $m^{-2}$
770 $s^{-1}$) in Siebold's beech exposed to different $O_3$ concentrations (ambient [AA] or
771 elevated $O_3$ [$eO_3$]). Each value is the mean ± standard error (n=6).
772 **Figure 3.** Relationships between (A) light-saturated net photosynthetic rate [$P_{Nsat}$]
773 and stomatal conductance for water vapor [$G_{sH2O}$], (B) $P_N$ and mesophyll
774 conductance to $CO_2$ [$G_m$], (C) $P_N$ and total conductance to $CO_2$ [$G_{totCO2}$], (D) $G_m$
775 and $G_{sH2O}$, in Siebold's beech. Linear regression analysis was used to assess the
776 significance of the relationship in ambient (AA) and elevated $O_3$ ($eO_3$). * P<0.05,
777 *** P<0.001, ns denotes not significant.
778 **Figure 4.** Relationships between leaf gas exchange parameters (light-saturated net
779 photosynthetic rate [$P_{Nsat}$], stomatal conductance [$G_{sH2O}$], mesophyll conductance

780 [$G_m$] and total conductance to $CO_2$ [$G_{totCO2}$]) and leaf density (LD) of Siebold's
781 beech saplings under different $O_3$ concentrations (ambient [AA] or elevated $O_3$
782 [$eO_3$]). Simple linear regression analysis was applied to assess the relationships. *
783 $P<0.05$, ns denotes not significant.

784 **Figure 5.** Principal component analysis of parameters derived from analyses of the
785 photosynthetic capacity ($P_N/C_c$ curves), light harvesting ($P_N$/PPFD curves) and
786 diffusive limitations to $CO_2$ transport ($G_{sCO2}$, $G_m$ and $G_{totCO2}$) of Siebold's beech
787 grown under different $O_3$ concentrations (ambient [AA] or elevated $O_3$ [$eO_3$]).
788 Ellipses represent 95% confidence intervals for measurements conducted in June,
789 August and October. The correlation circle is also shown. Measurements of AA-
790 treated plants in June and August and $eO_3$-treated plants in June occupy the same
791 position in multi-variate space. On the other hand, $eO_3$-treated plants analysed in
792 October occupy statistically distinct multi-variate space. Component 1 accounts
793 for 49.2% of the variance within the dataset and component 2 accounts for 14.8%
794 of variance.

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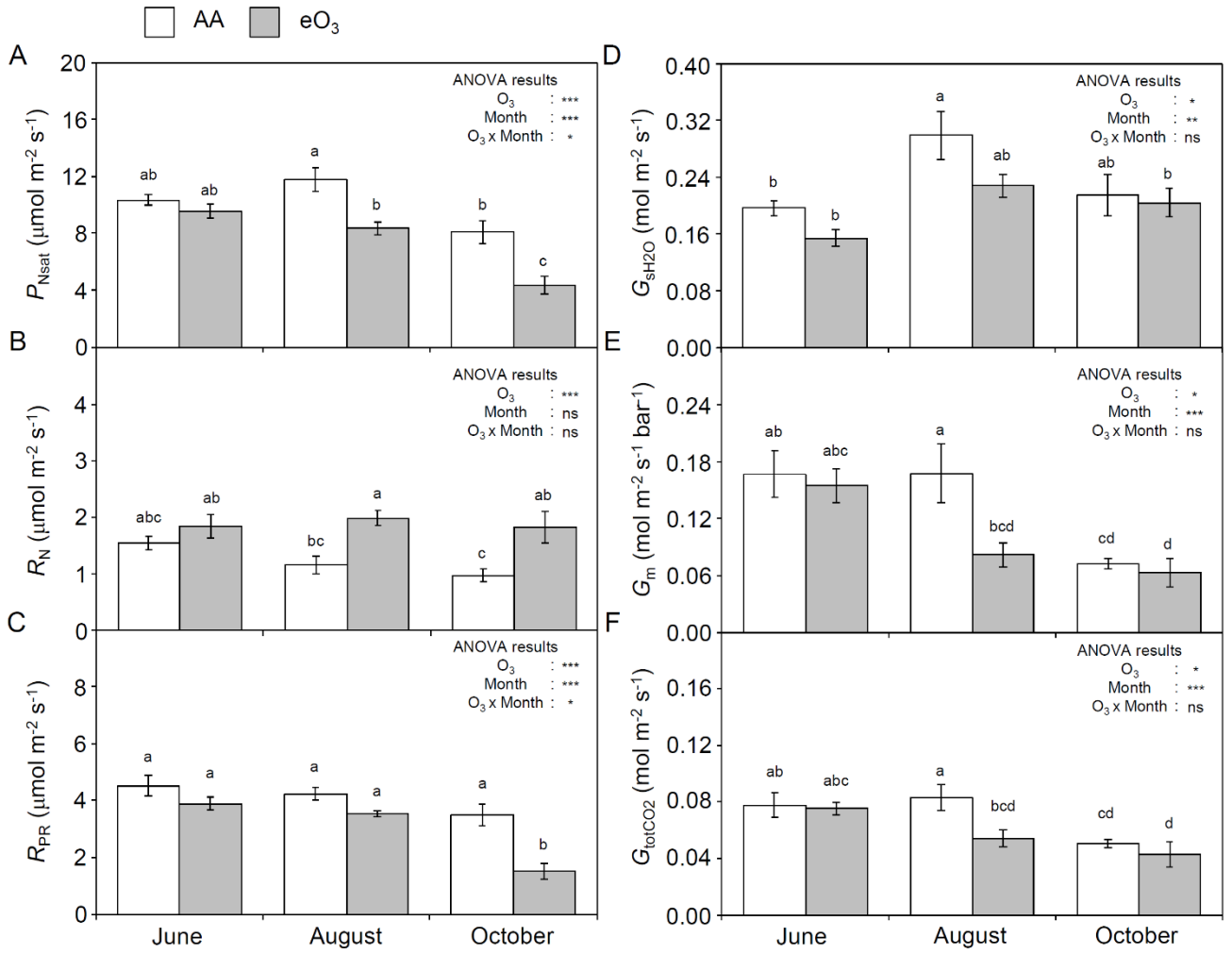


**Figure 1.** Photosynthetic parameters of Siebold's beech grown under ambient (AA) and elevated $O_3$ ($eO_3$): (A) Light-saturated net photosynthetic rate [$P_{Nsat}$], (B) dark respiration rate [$R_N$], (C) photorespiration [$R_{PR}$], (D) stomatal conductance to water vapor [$G_{sH2O}$], (E) mesophyll conductance to $CO_2$ [$G_m$], (F) total conductance to $CO_2$ [$G_{totCO2}$]. Each value is the mean ± standard error (n = 6). Two-way ANOVA: * $p$<0.05, ** $p$<0.01, *** $p$<0.001, ns denotes not significant. Different letters show significant differences among treatments ($p$<0.05, Tukey test).

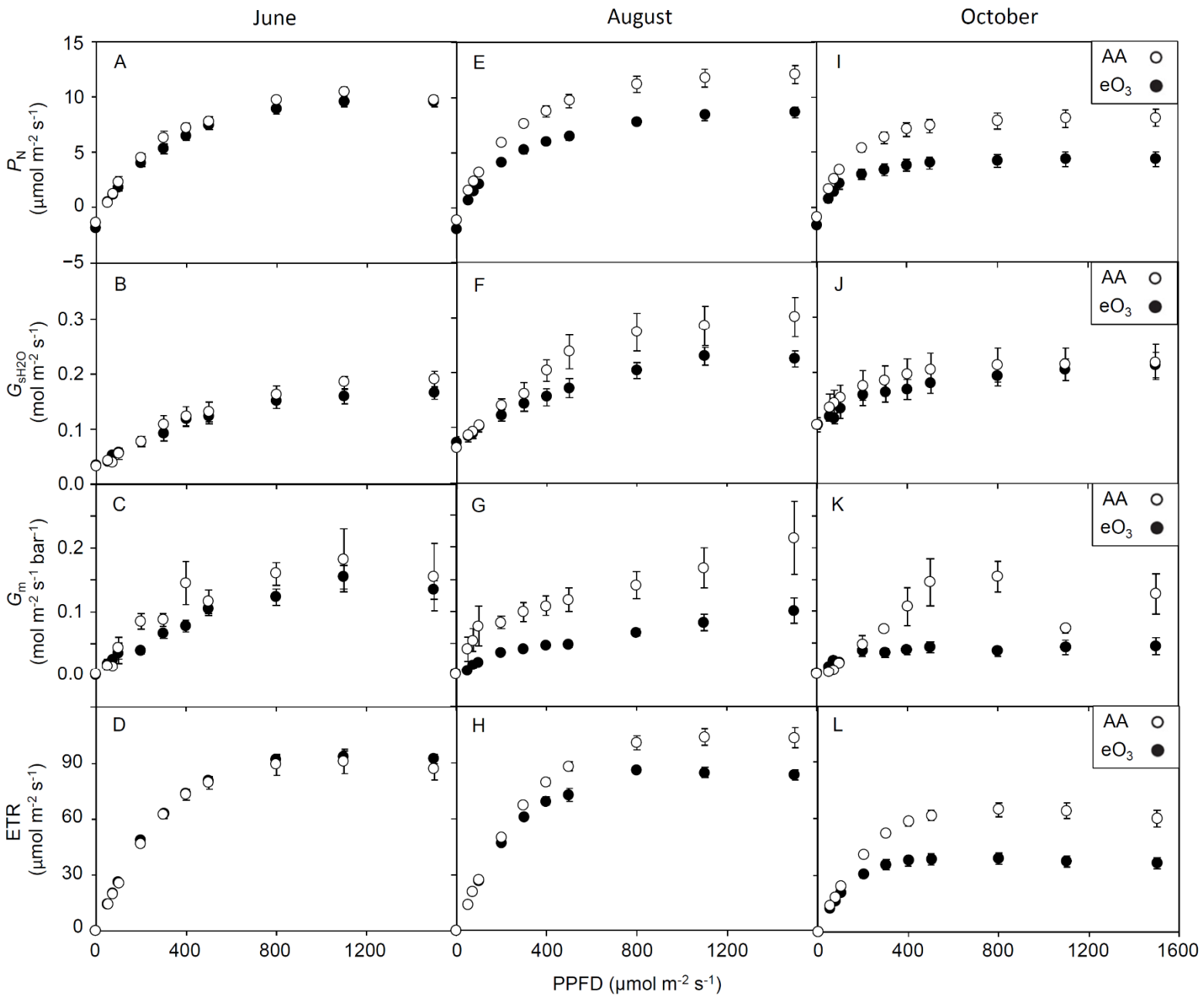


**Figure 2.** Responses of net photosynthetic rate ($P_N$), stomatal conductance ($G_{sH2O}$), mesophyll conductance ($G_m$) and electron transport rate (ETR) to

photosynthetic photon flux density (PPFD). The $P_N$/PPFD curves were obtained by measuring $P_N$ over 11 PPFD steps (1500, 1100, 800, 500, 400, 300, 200, 100, 75, 50, 0 μmol $m^{-2}$ $s^{-1}$) in Siebold's beech exposed to different $O_3$ concentrations (ambient [AA] or elevated $O_3$ [e$O_3$]). Each value is the mean ± standard error (n = 6).

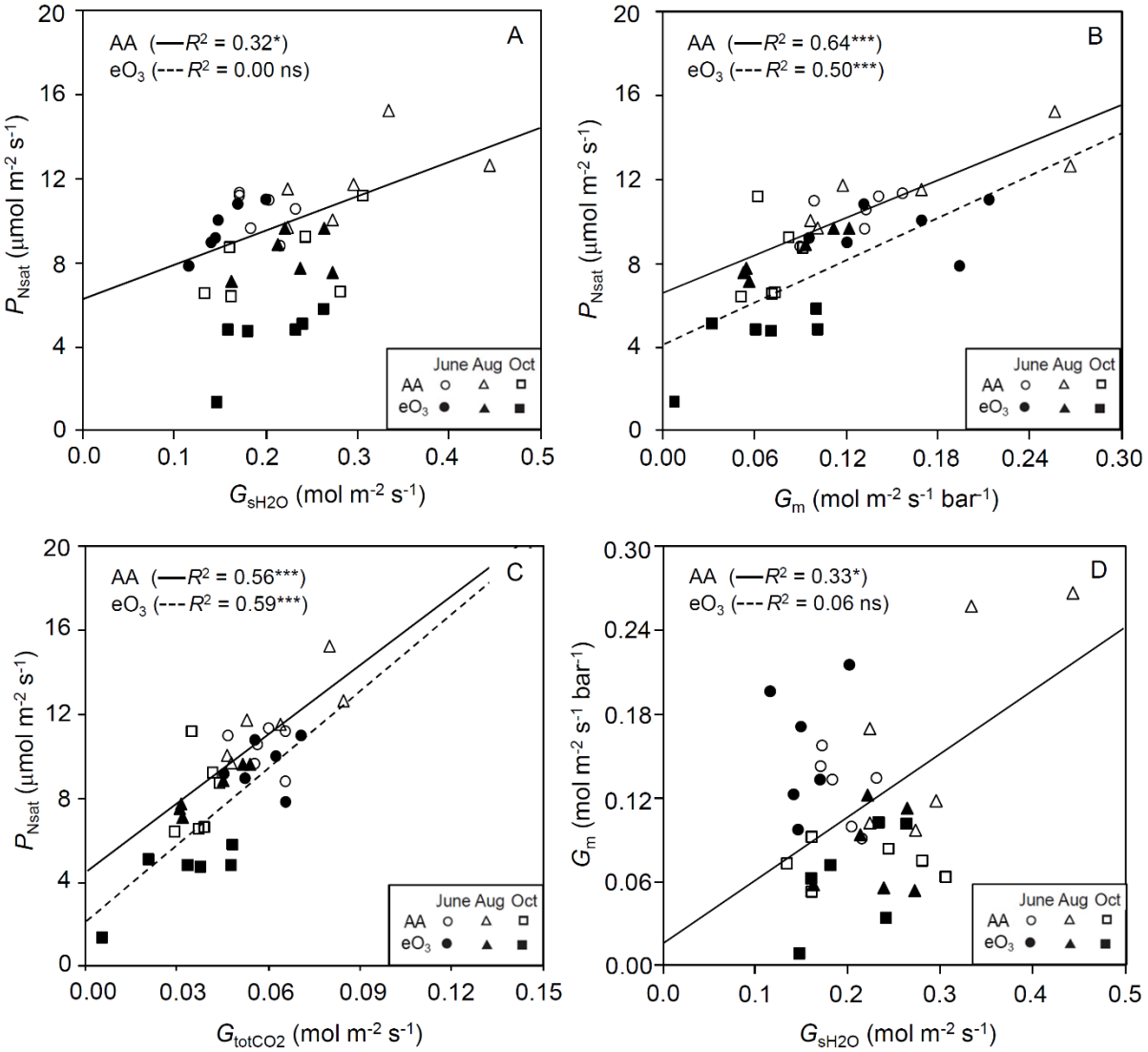


**Figure 3.** Relationships between (A) light-saturated net photosynthetic rate [$P_{Nsat}$] and stomatal conductance for water vapor [$G_{sH2O}$], (B) $P_N$ and mesophyll conductance to $CO_2$ [$G_m$], (C) $P_N$ and total conductance to $CO_2$ [$G_{totCO2}$], (D) $G_m$ and $G_{sH2O}$, in Siebold's beech. Linear regression analysis was used to assess

the significance of the relationship in ambient (AA) and elevated $O_3$ ($eO_3$).  * $p<0.05$, *** $p<0.001$, ns denotes not significant.

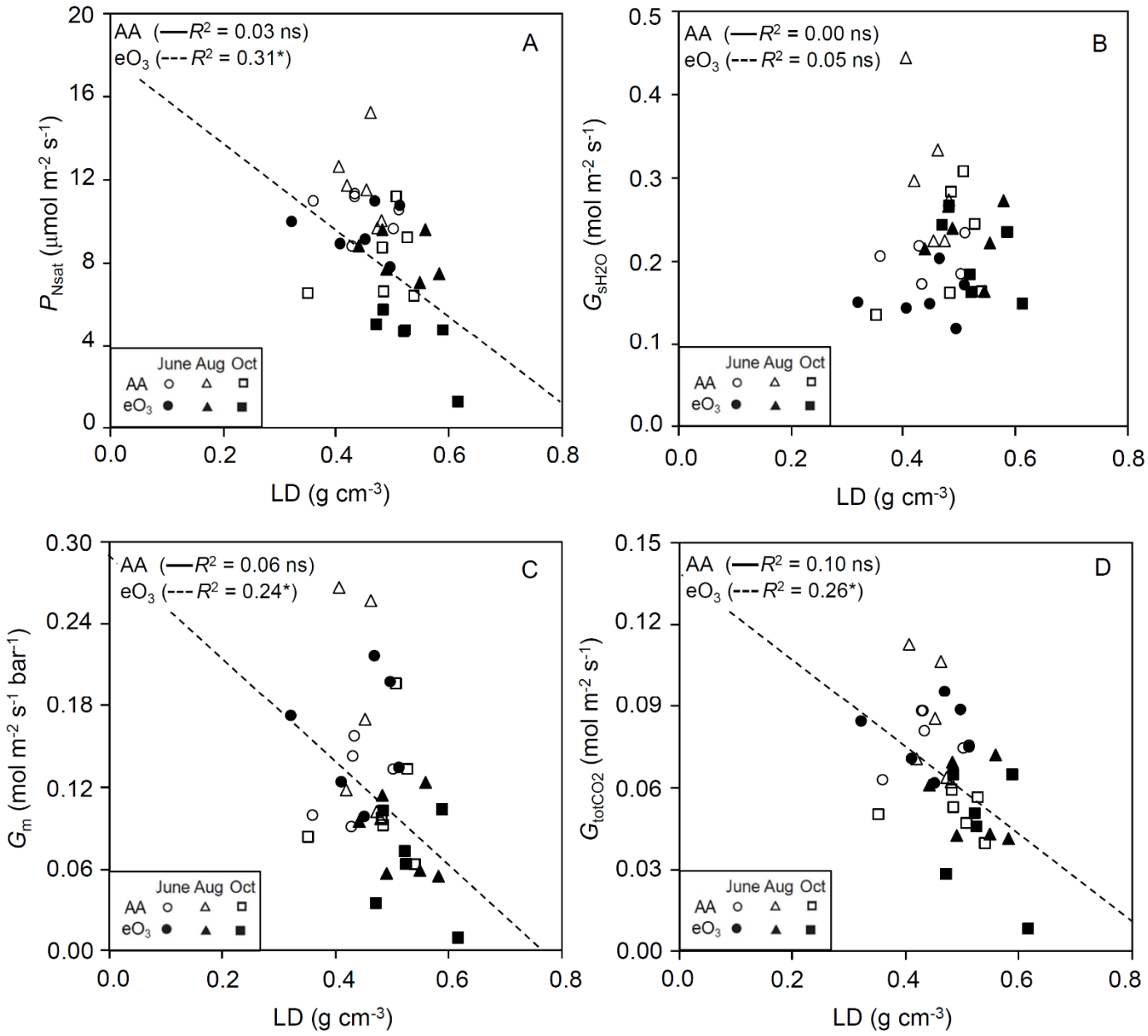


**Figure 4.** Relationships between leaf gas exchange parameters (A: light-saturated net photosynthetic rate [$P_{Nsat}$], B: stomatal conductance [$G_{sH2O}$], C: mesophyll

conductance [$G_m$] and D: total conductance to $CO_2$ [$G_{totCO2}$]) and leaf density (LD) of Siebold's beech saplings under different $O_3$ concentrations (ambient [AA] or elevated $O_3$ [$eO_3$]). Simple linear regression analysis was applied to assess the relationships. * $p<0.05$, ns denotes not significant.

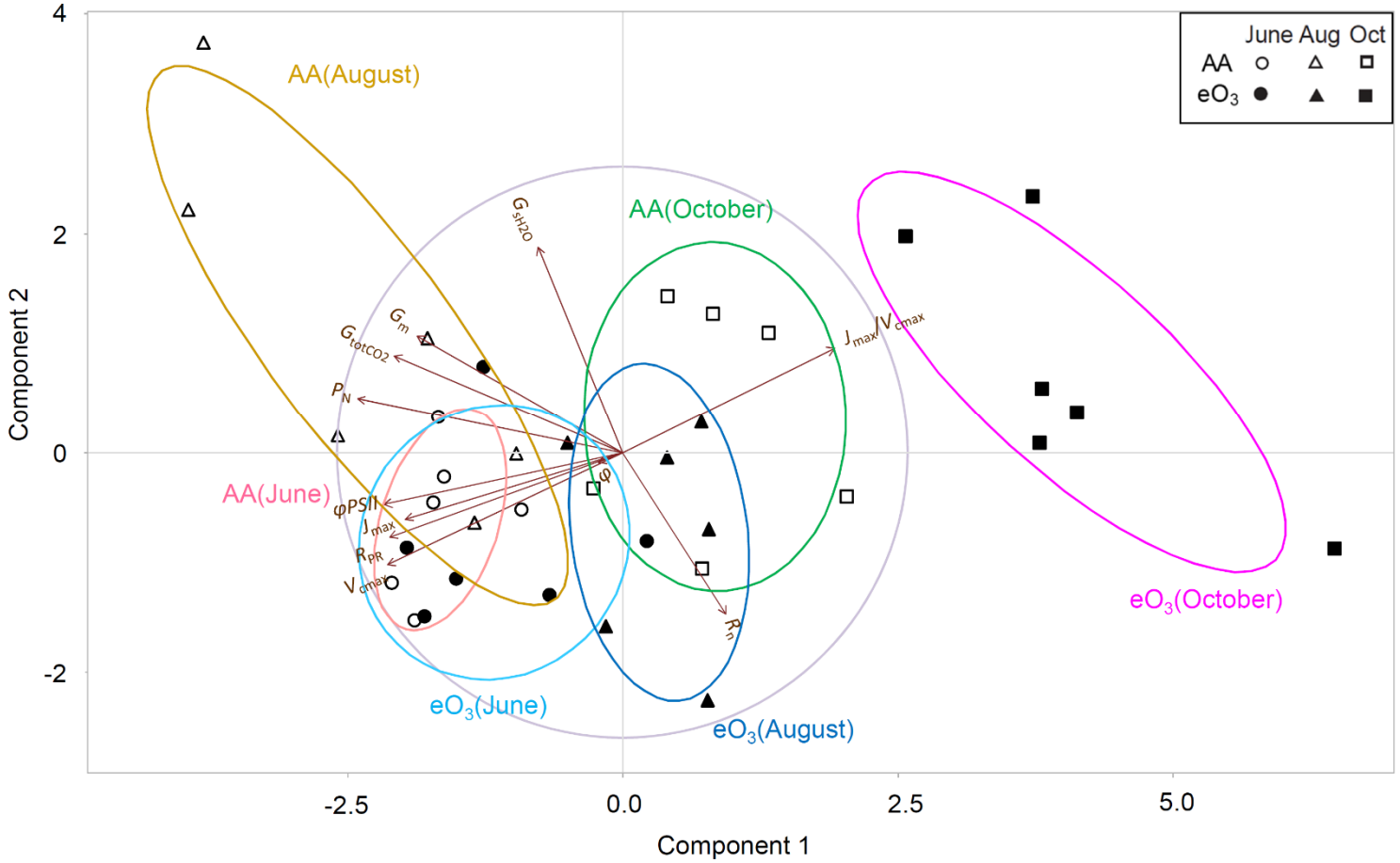


**Figure 5.** Principal component analysis of parameters derived from analyses of the photosynthetic capacity ($P_N/C_c$ curves), light harvesting ($P_N$/PPFD curves) and diffusive limitations to $CO_2$ transport ($G_{sCO2}$, $G_m$ and $G_{totCO2}$) of Siebold's beech grown under different $O_3$ concentrations (ambient [AA] or elevated $O_3$ [$eO_3$]). Ellipses represent 95% confidence intervals for measurements conducted in June, August and October. The correlation circle is also shown. Measurements of AA-treated plants in June and August and $eO_3$-treated plants in June occupy the same position in multi-variate space. On the other hand, $eO_3$-treated plants analysed in October occupy statistically distinct multi-variate space. Component 1 accounts for 49.2% of the variance within the dataset and

component 2 accounts for 14.8% of variance.

**Table 1** Leaf mass per area (LMA), leaf density (LD) and leaf thickness (LT) of Siebold's beech saplings grown under ambient (AA) and elevated $O_3$ ($eO_3$) measured in 2012. Each value is the mean ± standard error (n = 6). Two-way ANOVA: * $p<0.05$, ns denotes not significant. Different letters show significant differences among treatments ($p<0.05$, Tukey test).

| | LMA (g $m^{-2}$) | | | | LD (g $cm^{-3}$) | | | | LT (mm) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AA | | $eO_3$ | | AA | | $eO_3$ | | AA | | $eO_3$ | |
| June | 90.9 ± 7.3 | a | 83.0 ± 6.0 | a | 0.45 ± 0.02 | ab | 0.44 ± 0.03 | a | 0.20 ± 0.01 | a | 0.19 ± 0.01 | a |
| August | 81.3 ± 3.0 | a | 92.0 ± 2.6 | a | 0.45 ± 0.01 | ab | 0.52 ± 0.02 | bc | 0.18 ± 0.01 | a | 0.18 ± 0.00 | a |
| October | 90.8 ± 8.0 | a | 96.6 ± 4.4 | a | 0.48 ± 0.03 | abc | 0.54 ± 0.02 | c | 0.19 ± 0.01 | a | 0.18 ± 0.00 | a |
| ***ANOVA results*** | | | | | | | | | | | | |
| $O_3$ | | ns | | | | * | | | | ns | | |
| Month | | ns | | | | * | | | | ns | | |
| $O_3$ × Month | | ns | | | | ns | | | | ns | | |

**Table 2** Parameters derived from analyses of light harvesting ($P_N$/PPFD curves: initial slope of photosynthetic light response curve [Φ], quantum yield of photosystem II photochemistry [φPSII], electron transport rate [ETR]) and photosynthetic capacity ($P_N$/Cc curve: maximum rate of carboxylation [$V_{cmax}$], maximum rate of electron transport for RuBP regeneration [$J_{max}$], the ratio of $J_{max}$ to $V_{cmax}$ [$J_{max}/V_{cmax}$ ratio]) of Siebold's beech grown under ambient (AA) and elevated $O_3$ ($eO_3$). Each value is the mean ± standard error (n = 6). Two-way ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns denotes not significant. Different letters show significant differences among treatments ($p<0.05$, Tukey test).

| | Φ (mol mol$^{-1}$) | | | | φPSII | | | | ETR (μmol m$^{-2}$ s$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AA | | $eO_3$ | | AA | | $eO_3$ | | AA | | $eO_3$ | |
| June | 0.042 ± 0.003 | a | 0.037 ± 0.002 | a | 0.20 ± 0.01 | ab | 0.20 ± 0.01 | ab | 91.0 ± 6.0 | ab | 93.4 ± 3.2 | ab |
| August | 0.043 ± 0.002 | a | 0.041 ± 0.001 | a | 0.22 ± 0.01 | a | 0.18 ± 0.01 | b | 103.8 ± 4.6 | a | 84.8 ± 2.7 | b |
| October | 0.044 ± 0.001 | a | 0.038 ± 0.004 | a | 0.14 ± 0.01 | c | 0.08 ± 0.01 | d | 64.3 ± 4.0 | c | 37.6 ± 2.9 | d |
| ***ANOVA results*** | | | | | | | | | | | | |
| $O_3$ | | ns | | | | *** | | | | *** | | |
| Month | | ns | | | | *** | | | | *** | | |
| $O_3$ × Month | | ns | | | | ** | | | | ** | | |

| | $V_{cmax}$ (μmol m$^{-2}$ s$^{-1}$) | | | | $J_{max}$ (μmol m$^{-2}$ s$^{-1}$) | | | | $J_{max}/V_{cmax}$ ratio | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AA | | $eO_3$ | | AA | | $eO_3$ | | AA | | $eO_3$ | |
| June | 66.8 ± 6.9 | a | 62.7 ± 6.1 | a | 118.0 ± 7.8 | ab | 114.9 ± 10.1 | ab | 1.8 ± 0.1 | a | 1.9 ± 0.1 | a |
| August | 63.2 ± 2.5 | a | 54.5 ± 6.0 | a | 113.5 ± 1.8 | ab | 87.9 ± 6.4 | bc | 1.8 ± 0.1 | a | 1.7 ± 0.2 | a |
| October | 47.2 ± 4.9 | a | 24.8 ± 2.4 | b | 95.2 ± 3.4 | abc | 70.1 ± 6.1 | c | 2.1 ± 0.2 | a | 2.9 ± 0.1 | b |
| ***ANOVA results*** | | | | | | | | | | | | |
| $O_3$ | | ** | | | | ** | | | | * | | |
| Month | | *** | | | | *** | | | | *** | | |
| $O_3$ × Month | | ns | | | | ns | | | | ** | | |

**Table 3** Relative contribution of stomatal, mesophyll conductance and biochemical limitations ($l_s$, $l_m$ and $l_b$, respectively) to light-saturated net photosynthesis ($P_{Nsat}$) of Siebold's beech grown under ambient (AA) and elevated $O_3$ ($eO_3$). Each value is the mean ± standard error (n = 6). Two-way ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns denotes not significant. Different letters show significant differences among treatments ($p<0.05$, Tukey test).

| | $l_s$ (%) | | | | $l_m$ (%) | | | | $l_b$ (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AA | | $eO_3$ | | AA | | $eO_3$ | | AA | | $eO_3$ | |
| June | 27.9 ± 0.8 | ab | 35.1 ± 2.1 | a | 35.2 ± 4.7 | a | 29.6 ± 4.4 | a | 36.9 ± 5.1 | ab | 35.3 ± 2.9 | a |
| August | 23.9 ± 1.4 | b | 21.3 ± 1.4 | bc | 27.2 ± 4.1 | a | 34.1 ± 4.0 | a | 48.9 ± 4.9 | ab | 44.6 ± 4.9 | ab |
| October | 22.2 ± 2.7 | bc | 16.0 ± 1.8 | c | 36.3 ± 3.2 | a | 30.3 ± 4.8 | a | 41.5 ± 2.3 | ab | 53.7 ± 4.9 | b |
| ***ANOVA results*** | | | | | | | | | | | | |
| $O_3$ | | ns | | | | ns | | | | ns | | |
| Month | | *** | | | | ns | | | | * | | |
| $O_3$ × Month | | ** | | | | ns | | | | ns | | |

**Supplementary materials**

# Interactive effect of leaf age and ozone on mesophyll conductance in Siebold's beech

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**Supplementary figures**

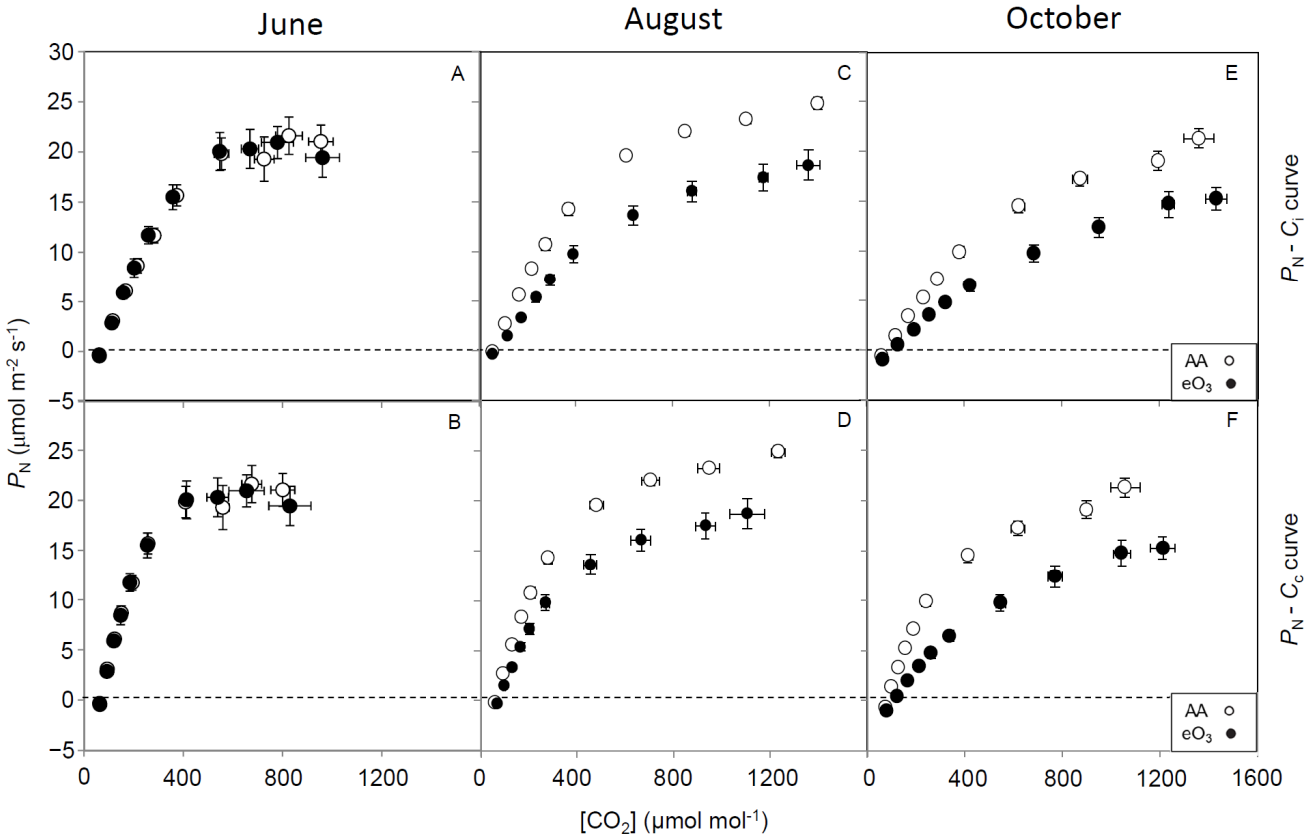


Figure S1. Responses of net photosynthetic rate ($P_N$) to the sub-stomatal concentration of $CO_2$ ($C_i$) (upper figures) or the concentration of $CO_2$ inside the chloroplast envelope ($C_c$) (bottom figures) at different ages. The $P_N/C_i$ curves were obtained by measuring $P_N$ over 12 ambient $CO_2$ concentration steps ($C_a$, 380, 300, 220, 140, 60, 300, 380, 500, 800, 1100, 1400, 1700 µmol mol$^{-1}$). Values of $G_m$ derived from the curve fitting method (Ethier and Livingston, 2004) were used to calculate the $P_N/C_c$ curves. Each value is the mean ± standard error (n = 6).

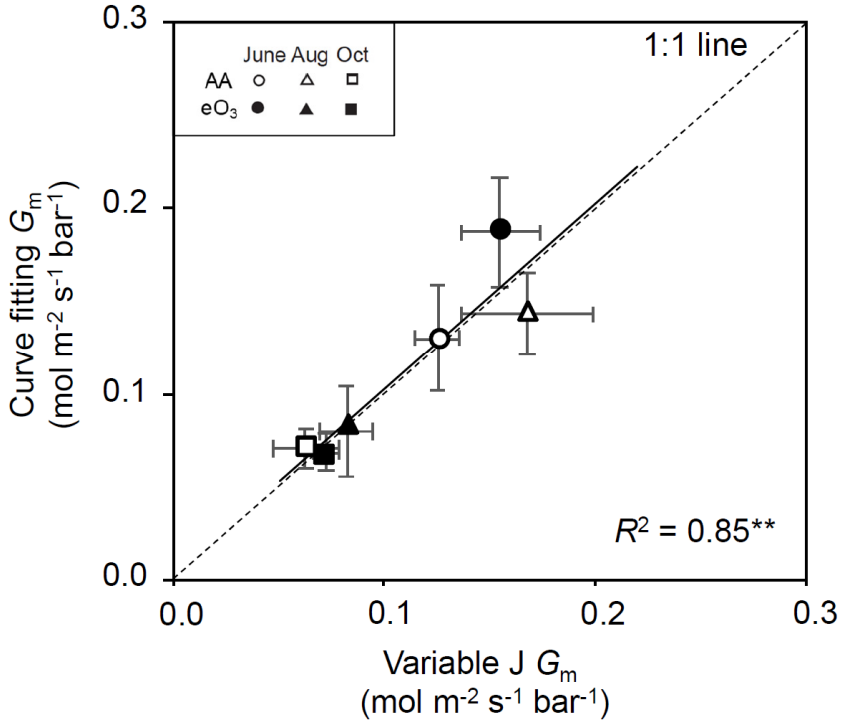


Figure S2. Relationship between mesophyll conductance ($G_m$) values derived from the variable J method and those derived from the $P_N/C_i$ curve fitting method in Siebold's beech exposed to different $O_3$ concentrations (ambient [AA] or elevated $O_3$ [$eO_3$]). Each value is the mean ± standard error (n = 6). A linear regression analysis: ** $p$<0.01.

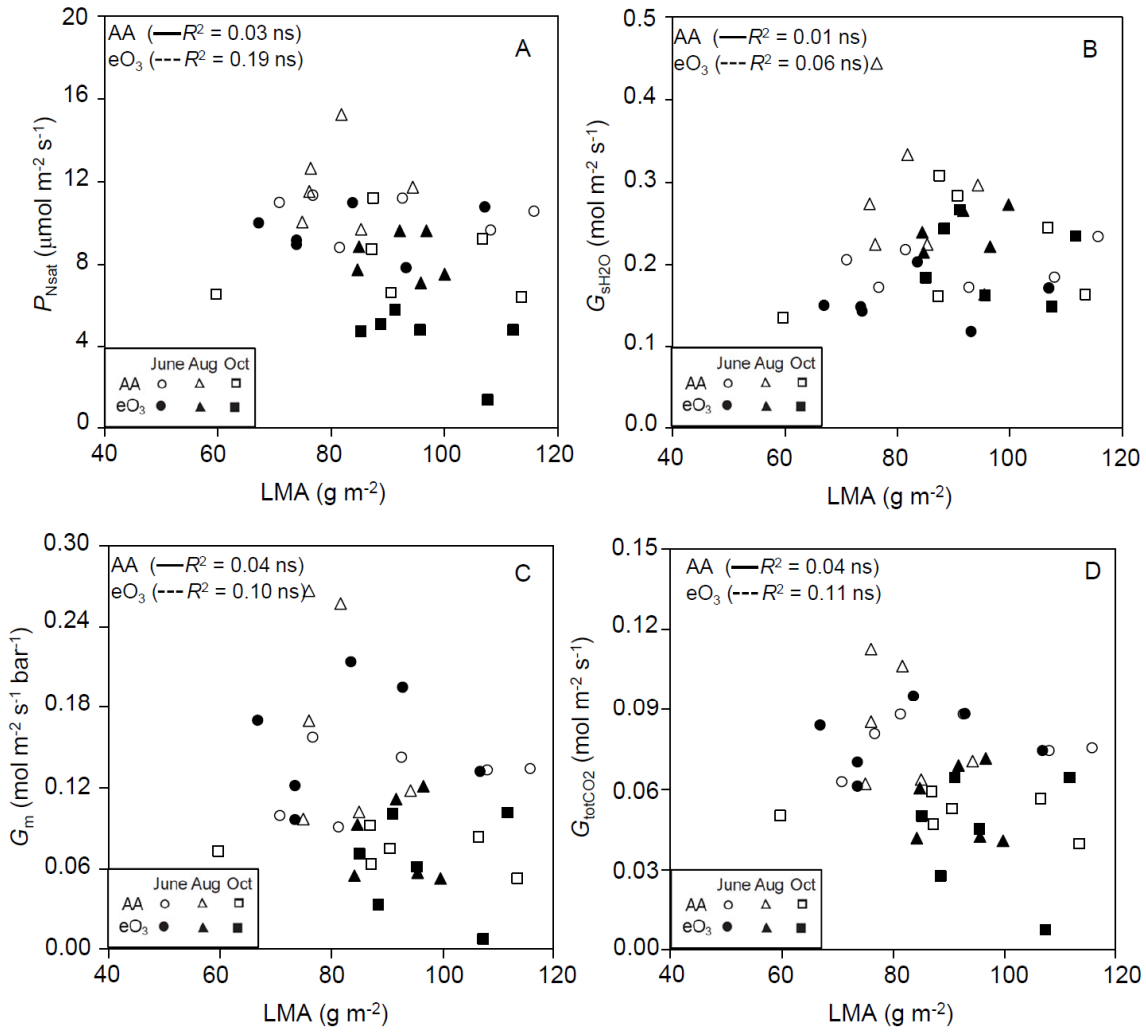


Figure S3. Relationships between leaf gas exchange parameters (A: light-saturated net photosynthetic rate [$P_{Nsat}$], B: stomatal conductance [$G_{sH2O}$], C: mesophyll conductance [$G_m$] and D: total conductance to $CO_2$ [$G_{totCO2}$]) and leaf mass per area (LMA) of Siebold's beech saplings under different $O_3$ concentrations (ambient [AA] or elevated $O_3$ [$eO_3$]). Simple linear regression analysis was applied to assess the relationships. ns denotes not significant.

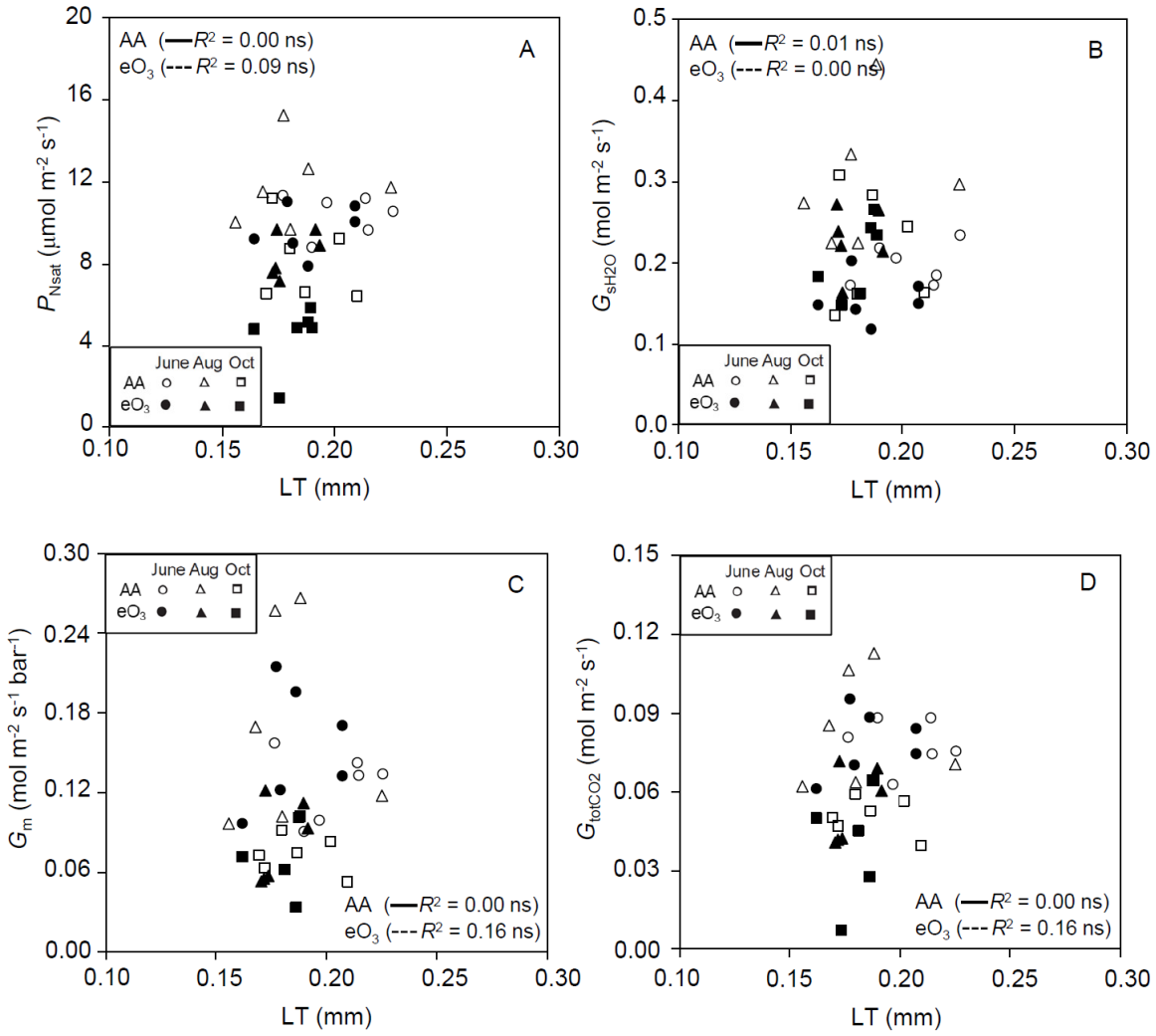


Figure S4. Relationships between leaf gas exchange parameters (A: light-saturated net photosynthetic rate [$P_{Nsat}$], B: stomatal conductance [$G_{sH2O}$], C: mesophyll conductance [$G_m$] and D: total conductance to $CO_2$ [$G_{totCO2}$]) and leaf thickness (LT) of Siebold's beech saplings under different $O_3$ concentrations (ambient [AA] or elevated $O_3$ [eO$_3$]). Simple linear regression analysis was applied to assess the relationships. ns denotes not significant.