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Leaf traits and photosynthetic responses of *Betula pendula* saplings to a range of ground-level ozone concentrations at a range of nitrogen loads

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Summary

Ground-level ozone (O_3) concentrations and atmospheric nitrogen (N) deposition rates have increased strongly since the 1950s. Rising ground-level O_3 concentrations and atmospheric N deposition both affect plant physiology and growth, however, impacts have often been studied in isolation rather than in combination. In addition, studies are often limited to a control treatment and one or two elevated levels of ozone and/or nitrogen supply. In the current study, three-year old *Betula pendula* saplings were exposed to seven different O_3 profiles (24 hour mean O_3 concentration of 36 – 68 ppb in 2013, with peaks up to an average of 105 ppb) in precision-controlled hemispherical glasshouses (solardomes) and four different N loads (10, 30, 50 or 70 kg N ha^{-1} y^{-1}) in 2012 and 2013. Here we report on the effects of enhanced O_3 concentrations and N load on leaf traits and gas exchange in leaves of varying age and developmental stage in 2013. The response of leaf traits to O_3 (but not N) vary with leaf developmental stage. For example, elevated O_3 did not affect the chlorophyll content of the youngest fully expanded leaf, but it reduced the chlorophyll content and photosynthetic parameters in aging leaves, relatively more so later than earlier in the growing season. Elevated O_3 enhanced the N content of senesced leaves prior to leaf fall, potentially affecting subsequent N cycling in the soil. Enhanced N generally stimulated the chlorophyll content and photosynthetic capacity. Whilst elevated O_3 reduced the light-saturated rate of photosynthesis (A_{sat}) in aging leaves, it did not affect stomatal conductance (g_s). This suggests that photosynthesis and g_s are not closely coupled at elevated O_3 under-light saturating conditions. We did not observe any interactions between O_3 and N regarding photosynthetic parameters ($V_{c,max}$, J_{max}, A_{sat}), chlorophyll content, g_s, N content in senesced leaves and leaf number. Hence, the sensitivity of these leaf traits to O_3 in young silver birch trees is neither reduced nor enhanced by N load.

Keywords: Air pollution; chlorophyll content; leaf age; nitrogen content; photosynthetic capacity; stomatal conductance.

Abbreviations

A_{sat} = light-saturated rate of photosynthesis at ambient CO_2 (390 ppm)

A/Ci curve = plot of net photosynthesis (A) against leaf internal CO_2 concentration (Ci)

AICc = Akaike Information Criterion, corrected for small sample size

g_s = stomatal conductance

ID = identity

J_{max} = maximum rate of electron transport

LMM = linear mixed model

ppb = parts per billion

$V_{c,max}$ = maximum rate of carboxylation

Introduction

Tropospheric ozone (O_3) concentrations have been increasing from a background of ca. 10-15 ppb in the early 20th century, due to increased emissions of O_3 precursors from anthropogenic sources (Cooper et al., 2014; Royal Society, 2008; Solberg et al. 2005; Volz and Kley, 1988). In Europe, background concentrations have roughly doubled between 1950 – 2000, followed by a decade with no further rise or even a reduction at some sites (Cooper et al., 2014; Parrish et al., 2012). Present day annual average background concentrations are levelling off at ca. 30 – 40 ppb in Europe and North America, (Cooper et al., 2014; Royal Society, 2008), but are still rising significantly in East Asia (Cooper et al., 2014). In addition, atmospheric concentration and deposition of reactive nitrogen (N) has increased strongly since the 1950s with the application of the Haber-Bosch process to produce N fertilizers on an industrial scale (Sutton et al., 2011). Despite the rise in both ground-level O_3 concentrations and atmospheric N deposition since the 1950s, impacts on vegetation have often been studied in isolation rather than in a combination of both factors. The two pollutants are closely interlinked as a major pathway for the formation of tropospheric O_3 is the photochemical reaction with N oxides (Royal Society, 2008).

Generally, O_3 tends to have a negative impact on sensitive plant species, resulting in a decline in photosynthesis, biomass production or crop yield, and accelerated senescence (Ainsworth et al., 2012). A meta-analysis by Wittig et al. (2007) showed that the increase in O_3 since the industrial revolution has been responsible for a reduction in photosynthesis of approximately 11% in trees, which reduced tree productivity by approximately 7% (Wittig et al. 2009). Further reductions were observed with a rise in O_3 concentration above the current ambient level (Wittig et al., 2007, 2009). Partly due to the successful implementation of air pollution abatement policies in Europe, reductions in O_3 precursor emissions have resulted in a decline in peak O_3 concentrations in the summer in recent years at some sites across Europe (Simpson et al., 2014; Torseth et al., 2012). However, mean annual O_3 concentrations have hardly changed in recent decades (EMEP, 2016) due to a rise in the frequency of low-range O_3 concentrations (Simpson et al., 2014). Background O_3 concentrations are currently at levels where they chronically affect sensitive plant species (Mills et al., 2011a).

In contrast, N tends to have a fertilising effect on plant growth and photosynthesis in areas where N is a limiting nutrient. However, above the critical load or level, the impact of N on sensitive species will become negative in most species (Bobbink and Hettelingh, 2011; Cape et al., 2009). For example, forest observations in Switzerland suggested an increase in phosphorus limitation with increasing N deposition, resulting in a decline in growth in *Fagus sylvatica* (Braun et al., 2010). N pollution is now a major threat to terrestrial biodiversity (Bobbink et al., 2010; Dise et al., 2011).

Relatively few studies have investigated the combined impacts of both O_3 and N on vegetation at realistic ozone concentrations (Mills et al., 2016). Evidence suggests that O_3 and N can have both synergistic and antagonistic effects on tree species. Pääkkönen and Holopainen (1995) and Landolt et al. (1997) showed that high N supply ameliorates the negative impact of O_3 in *Betula pendula*. Similar responses were reported for *Larix kaempferi*

(Watanabe et al., 2006), *Populus tremula x Populus tremuloides* (Häikiö et al., 2007) and *Quercus kelloggii* (Handley and Grulke, 2008). However, enhanced N supply increased growth sensitivity to O_3 of *Pinus sylvestris* (Utriainen and Holopainen, 2001b) and *Fagus crenata* (Wanatabe et al., 2012; Yamaguchi et al., 2007) seedlings. The effect of N supply on the sensitivity to O_3 was not significant for *Picea abies* (Thomas et al., 2005; Utiainen and Holopainen, 2001a) or *Quercus robur* seedlings (Marzuoli et al., 2016). Complex interactions were observed between O_3 and N impacts on growth of young trees of *Fagus sylvatica* (Thomas et al., 2006). In a recent review, Mills et al. (2016) showed that the beneficial effect of N on root development was lost at high O_3 exposure whilst the effects of increasing O_3 on root biomass became more pronounced as N supply increased.

Many previous studies investigating the interactive impacts of O_3 and N on trees have been limited to two to three O_3 and N exposure levels. In the current study, three year old silver birch (*Betula pendula*) seedlings were exposed to seven O_3 regimes (24 h mean of 36 – 68 ppb) and four levels of N load (10 – 70 kg N ha^{-1} y^{-1}) for two years (2012 and 2013). Silver birch is known to be sensitive to O_3 (LRTAP Convention, 2015; Mills et al., 2011b) and has a widespread distribution in Europe, from northern Finland to Italy and from Ireland to Kazakhstan. We investigated whether O_3 sensitivity of leaf traits was affected by N supply. We report in detail on the impacts of O_3 and N on chlorophyll content, stomatal conductance (g_s), leaf photosynthetic traits, N concentration in senesced leaves and leaf number during the second year of exposure. Based on the cumulative nature of O_3 impacts on plants (LRTAP Convention, 2015), we hypothesised that negative effects of O_3 on leaf traits will increase with time of exposure, with older leaves being more affected than younger leaves. Based on previously reported results (Landolt et al., 1997; Pääkkönen and Holopainen, 1995) for silver birch, we hypothesised that enhanced N will reduce negative effects of O_3.

Material and methods

Plant material, experimental site and treatments

On 13th April 2012, three-year old trees of silver birch (*Betula pendula*, UK provenance) were planted in 6.5 litre pots (diameter 21 cm, height 25 cm) filled with John Innes No. 1 compost (J. Arthur Bowers). Mean height of the trees when planted was 68 cm and they had not branched yet. All plants were inoculated with mycorrhiza (obtained from Buckingham Nurseries, UK). The pots were randomly distributed between seven hemispherical glasshouses (solardomes; 3 m diameter, 2.1 m height) at Abergwyngregyn nr. Bangor, North Wales, United Kingdom. After an acclimation period in the solardomes, treatments were started on 30th April 2012. Plants were exposed to O_3 based on an episodic profile recorded at a rural O_3 monitoring site during July 2006 (Aston Hill, Wales, UK, 52°50'N, 3°03'W). The Aston Hill profile was applied as the highest treatment, and for the other treatments the O_3 concentration was reduced by 10 ppb during the peaks and 3 ppb during periods with background O_3 (Figure 1; Hewitt et al., 2016). The profiles cover a range of concentrations found in Europe and this is the only facility that has sufficiently sophisticated control of O_3 to be able to deliver O_3 treatments that increase in small increments. The 24 hour mean concentration was between 36 and 68 ppb in 2013, with peaks up to on average 105 ppb (Figure 1). Plants were exposed to O_3 during the growing season, starting on 30th April and finishing on 12th October in 2012, and starting on 17th May and finishing on 16th September in 2013, when the trees were harvested. At harvest, the roots didn't show any signs of being pot-bound. Between 17th October 2012 and 14th May 2013, the trees were kept outside under ambient conditions. The solardomes were ventilated at a rate of two air changes per minute and charcoal-filtered air was injected with controlled amounts of O_3. O_3 was provided by a G11 O_3 generator (Ozone Industries, UK) equipped with oxygen concentrator (Dryden Aqua, UK). Concentrations were determined by a computer-controlled O_3 injection system (Lab VIEW version 8.6, National Instruments, Texas, US). O_3 was distributed to each solardome via polytetrafluoroethylene (PTFE) tubing, with the concentration inside each solardome measured for 5 min every 30 minutes using two O_3 analyzers (400a, Enviro Technology Services, Stroud, UK) of matched calibration. Four N treatments were applied weekly in each solardome as ammonium nitrate, in 200 ml water containing other macronutrients (P, K, S, Ca, Mg) and various micronutrients (Fe, Mn, Mo, Cu, Zn, B). N addition rates were 10, 30, 50 and 70 kg N ha^{-1} yr^{-1}. N and nutrient additions were started at the same time as the O_3 exposure (see above) and continued for the duration of the experiment, including in the winter. Plants were watered twice a week or as required to maintain soil moisture content near field capacity. Each dome contained 24 pots, i.e. six pots per N supply. The O_3 profiles were assigned randomly to the solardomes. The weekly O_3 profiles for 2012 were described by Hewitt et al. (2014), the weekly O_3 profiles for 2013 were very similar and are shown in Figure 1. A summary of O_3 treatments and climate conditions for 2013 is provided in Table 1. In one solardome, ambient air temperature, photosynthetically active radiation (PAR), temperature and relative humidity were continuously monitored by an automatic weather station (Skye Instruments Ltd, Llandridod Wells, UK) and soil moisture content was continuously monitored using Theta Probes (Delta-T Devices Ltd, Cambridge, UK). Although O_3 treatments were not replicated, numerous previous studies (Hayes et al., 2012; Hewitt et al., 2014, 2016; Mills et al., 2009) have established the statistical validity of conducting un-replicated experiments using the solardome facility, and no solardome effects were detectable in independent measurements of air or leaf temperature (Hewitt et al., 2016).

Chlorophyll content and stomatal conductance (g_s) *measurements*

Chlorophyll content (determined non-destructively using CCM200, ADC, UK, and expressed as an index) and g_s (AP4 Porometer, Delta T, UK) measurements were conducted at about four-weekly intervals on the third leaf (youngest fully expanded leaf) from the top of the canopy throughout the growing season in five O_3 treatments. The maximum g_s for each treatment was determined as the 95-percentile. Silver birch is heterophyllous (Clausen and Kozlowski, 1965), i.e. has early and late leaves which started to flush in April and July respectively in our study. In addition, the chlorophyll content was determined on leaves used for leaf gas exchange measurements (including g_s), i.e. a designated leaf throughout the growing season (see below), and determined near the end of the growing season on the eight leaves on the highest branch of trees (six replicates per treatment).

Leaf gas exchange measurements

Youngest fully expanded leaves were identified on 21 May 2013. Gas exchange measurements were conducted on these designated leaves exposed to the lowest (24 hr mean = 36 ppb) and highest O_3 concentration (24 hr mean = 68 ppb) and supplied with the lowest (10 kg N ha^{-1} yr^{-1}) and highest N rate (70 kg N ha^{-1} yr^{-1}). Measurements on the designated leaves were conducted at 6-weekly intervals during the following periods: 10 - 14 June, 22 - 25 July and 2 - 5 September 2013. To determine photosynthetic capacity of birch leaves, the response curve of the net photosynthetic rate (A) to the intercellular CO_2 concentration (Ci), i.e. the A/Ci curve, was constructed using a portable gas analyser with PLC6 universal leaf cuvette (Ciras-2, PPsystems). The measurements were conducted at light saturation (photosynthetic photon flux density = 1500 μmol m^{-2} s^{-1}; LED light source), a leaf temperature of 22 ^{o}C (determined by built-in infrared temperature sensor), and a leaf-to-air vapour pressure deficit of 0.6 – 1.1 kPa. The A/C_i curve was constructed by measuring A at 11 air CO_2 concentrations between ca. 50 and 1900 μmol mol^{-1}. A_{sat} and associated g_s were determined at ambient CO_2 (390 μmol mol^{-1}). Leaves were inserted into a broad leaf chamber (area = 2.5 cm^2) and allowed to stabilise for 15 min at growth CO_2. At each CO_2 concentration, gas exchange parameters were recorded after readings had stabilised (ca. 2 – 3 min). The maximum rate of carboxylation ($V_{c,max}$) and the maximum rate of electron transport (J_{max}) were calculated from the A/Ci curve according to the methodology described by Sharkey et al. (2007). $V_{c,max}$ and J_{max} were calculated for a leaf temperature of 25 ^oC, using the measurement leaf temperature and atmospheric pressure as input parameters. The ratio between J_{max} and $V_{c,max}$ was also determined. In addition to the A_{sat} measurements from the A/Ci curve, A_{sat} was also measured in late August/early September at all six O_3 concentrations at low and high N supply (4 - 5 replicates per treatment).

Leaf number and N content of leaves

Leaf number was counted on four days during the 2013 growing season: 14 May, 31 July, 2 and 24 September (day before harvest). Throughout September, leaves that were ready to drop off were collected for N analysis. The N content of mature, green leaves was also determined in the middle of July. The leaves were dried at 70 ^oC, ground with a (Cyclotec) and 10 mg was weighed in tin capsules for analysis. Leaves were analysed for N content at the Chemical Analysis Laboratory, Forest Research, UK. The total N content was determined by dry combustion (ISO 13878, using Carbo Erba CN analyser, Flash1112 series).

Statistical analyses

The precise O_3 control system used in the solardomes allowed relatively small changes in O_3 profile to be simulated, facilitating dose-response analyses rather than just comparison of the impacts at low and high ozone exposure. The large number of O_3 treatments allowed for effects of N on the slope of dose-response functions to be determined. We note that the lack of treatment replication may raise concerns, however, we believe that the benefit of using more treatments outweighs this limitation, as published previously (Hayes et al., 2012; Hewitt et al., 2014, 2016; Mills et al., 2009). Air flow rates were matched between solardomes, and recorded climatic conditions did not vary significantly from solardome to solardome (Hewitt et al., 2016). For all linear mixed models (LMMs; normal error) applied below, a model set was created using the package lme4, v1.1-7 (Bates et al., 2015) in R version 3.2.3 (R core team, 2014), sequentially removing interactions and predictor variables from the global model, and model selection was carried out by examining the change in Akaike Information Criterion, corrected for small sample size (AICc). The model with the lowest AICc value is optimal, with models differing in 2 - 7 AICc units from the best model having little empirical support (Burnham and Anderson, 2002). Bayesian Information Criterion (BIC) values, which penalise the number of model parameters more strictly, were also examined. For the optimal model selected, p-values were obtained for each term in the model using the R package lmerTest, v2.0-20 (Kuznetsova et al., 2014). For all models, statistical assumptions (normality and even spread of residuals) were checked using residual plots. Response variables were transformed where necessary (log or square root).

Chlorophyll content and stomatal conductance (g_s)

To investigate factors influencing chlorophyll content of the youngest fully expanded leaf, the LMM included added N, 24 hour mean O_3 concentration (2013, n = 5 levels) and measurement period as fixed, continuous variables and the random effects of dome and plant identity (ID). As there was a suggestion of a quadratic relationship between the chlorophyll content and time, a quadratic time term was included. For eight leaves on the top branch (near the end of the growing season), a further LMM with chlorophyll content as the response variable, the continuous fixed effects of 24 hour mean O_3 (2013, n = 4) and leaf number, N (factor: high and low), and a random effect of plant ID was also run. A quadratic O_3 term was included to allow for the suggested non-linear relationship between chlorophyll content and O_3. The LMM for g_s included added N, 24 hour mean O_3 concentration (2013, n = 5 levels) and measurement period as fixed, continuous variables. A quadratic term for measurement period and random effects of dome and plant ID were also included. Extreme outliers in the g_s data (thought to be due to measurements from damp leaves) were removed using the median absolute deviation (mad) method (using a rejection criteria of median ± 5.2 mad).

Leaf gas exchange measurements

For each parameter (e.g. $V_{c,max}$, J_{max}) of the designated leaf, a LMM including the fixed effects of time, N and O_3 (as factors, low and high), and the random effect of plant ID, was applied. The relationship between A_{sat} and chlorophyll content and A_{sat} and g_s was tested using LMMs, including the fixed effects of O_3 and N and the random effect of plant ID. The A_{sat} measurements taken in late August were analysed using a LMM with a continuous fixed effect for 24 hour mean O_3 (2013, n = 6 levels), N as a factor (high and low), and a random effect of dome. Two further A_{sat} model sets were also run, with chlorophyll content and g_s included as continuous fixed predictors.

N content of leaves and leaf number

The effect of O_3 and added N on the N content of mature (mid July) and senesced leaves (September) was analysed using a general linear model (glm) containing an interaction between the continuous variables of added N and 24 hour mean O_3 concentration (2013, n = 7 levels). As the variance explained by the random effect of dome was zero, a mixed effects model was not used. To investigate changes in leaf number, a generalised linear mixed effect model (GLMM), with leaf count as the response variable and a Poisson error distribution, was run using the glmmPQL function in package MASS (Venables and Ripley, 2002) to deal with over-dispersion in the data. Fixed effects included 24 hour mean O_3 concentration (2012-2013, n = 7 levels) and added N as continuous variables and month as a factor, while dome and plant ID were included as random effects. As the glmmPQL function does not provide AIC values, p-values and estimated standard errors were used to determine which fixed effects were needed in the model.

Results

Chlorophyll content and g_s *of the youngest fully expanded leaf*

The chlorophyll content of the third leaf (youngest fully expanded leaf) declined in the first part of the growing season but then increased again between 23 July and 23 August due to new growth of late leaves ($p < 0.001$ for ‘Time’; Figure 2, Table 2). The third leaf developed from new growth (late leaf) had a similar chlorophyll content as the third leaf near the start of the season (early leaf). Remobilisation of N within the trees is likely to have started as older leaves began to drop off from the middle of July onwards as new leaves developed. Throughout the season, the chlorophyll content was higher at 70 than at 10 kg N ha^{-1} y^{-1} ($p < 0.001$; Figure 2, Table 2), with the difference being lowest at the end of July and highest at the end of August. O_3 concentration did not affect the chlorophyll content of the youngest fully expanded leaf. While visual examination of the raw data suggested that stimulation of the chlorophyll content at high N supply was diminished at the highest O_3 exposure at the end of May, two or three-way interactions were not observed (Table 2). The g_s changed with time and was highest at the end of August (late leaves) compared to earlier in the year (early leaves); g_s was not affected by O_3 or N treatment and there were no significant two or three-way interactions (Figure 3; Table 2). The maximum g_s reached its highest value at 50 kg N ha^{-1} y^{-1}, and was 256, 307, 339 and 337 mmol H_2O m^{-2} s^{-1} at 10, 30, 50 and 70 kg N ha^{-1} y^{-1} respectively.

Chlorophyll content of leaves along the first top branch near the end of the growing season

To assess the response to treatment of the chlorophyll content of leaves of different developmental stages, the chlorophyll content along a branch was measured late in the season (4th September) when a wide range of chlorophyll content was present. The chlorophyll content was higher at 70 kg N ha^{-1} y^{-1} than at 10 kg N ha^{-1} y^{-1} ($p < 0.001$) and decreased with increasing leaf age (i.e. leaf number; $p < 0.001$; Figure 4, Tables 3 and S1). The effect of O_3 was dependent on leaf age, with young leaves showing stimulation in the chlorophyll content (although the highest chlorophyll content was already reached in the youngest leaf at 39 ppb O_3) and medium-aged leaves showing no clear O_3 effect. In the oldest leaves (leaf 8), the chlorophyll content was highest at 36 ppb O_3 and 70 kg N ha^{-1} y^{-1} and lowest at 68 ppb O_3 and 10 kg N ha^{-1} y^{-1}. At 68 ppb O_3 the chlorophyll content declined faster with increasing leaf age than at 36 ppb O_3.

Photosynthetic capacity and other leaf traits of a leaf aging throughout the season

The photosynthetic capacity ($V_{c,max}$ and J_{max}) of an aging leaf was reduced by elevated O_3 and stimulated by high N supply, with no significant interaction being observed between O_3 and N (Figure 5, Table 4 and S2). Whilst elevated O_3 increased the $J_{max}/V_{c,max}$ ratio (ca. 7% on average), enhanced N supply reduced the $J_{max}/V_{c,max}$ ratio (ca. 8% on average; Table S2). Although the photosynthetic capacity declined with time, this was primarily due to a significant decline between June and July, with no further decline being observed between July and September. It should be noted that some of the designated leaves had already dropped off the trees in September, hence their photosynthetic capacity could not be assessed in September. In addition, variation for all measured parameters was highest near the end of the growing season. The negative effect of O_3 on photosynthetic capacity increased with time (Table S2), resulting in an O_3 x time interaction for J_{max} (for $V_{c,max}$ $p = 0.08$ for the interaction term in the model). The response of light-saturated photosynthesis (A_{sat}) to treatment was similar to that of J_{max}, except that the response to N was not significant ($p= 0.12$). The response of the chlorophyll content to treatment was also similar to J_{max} (a measure of the maximum rate of electron transport in the photosystems). The g_s of leaves under ambient CO_2 and light saturation was not affected by time, O_3 exposure or N supply. There was a linear relationship between A_{sat} and chlorophyll content ($p < 0.001$; Figure 6a), indicating that non-destructive measurement of the chlorophyll content provides a good estimate of A_{sat}. This relationship was not affected by O_3 and N treatment. There was also a linear relationship between photosynthetic capacity ($V_{c,max}$ and J_{max}) and chlorophyll content ($p < 0.001$; Figure S1). There was a weak relationship ($p = 0.038$) between A_{sat} and g_s, with a lot of scatter in the data, and the relationship was not affected by O_3 or N treatment (Figures 6b, S2). At the end of August, A_{sat} was also measured at additional O_3 treatments at an N supply of 10 and 70 kg ha^{-1} y^{-1}. These measurements showed a linear decline in A_{sat} with O_3 exposure later in the season ($p < 0.001$; Figure 7). Enhanced N supply stimulated A_{sat} ($p = 0.012$): the average A_{sat} (+/- one SE) was 4.8 (+/- 0.3) and 6.0 (+/- 0.4) $\mu mol\ m^{-2}\ s^{-1}$ at 10 and 70 kg N ha^{-1} y^{-1}, respectively. There was no interaction between O_3 exposure and N supply. Again, there was a strong linear relationship between A_{sat} ($p < 0.001$) and chlorophyll content and a weak linear relationship ($p = 0.033$) between A_{sat} and g_s (Figure S3).

N content of leaves

The N content of green, mature leaves in the middle of July was not affected by treatment; the mean N content of these leaves was 1.2% on average. Near the end of the growing season, the N content of leaves that were ready to drop off had declined to 0.5 – 0.7%. An increase in O_3 exposure resulted in a significant ($p < 0.001$) increase in N content of these leaves (Figure 8). N supply did not significantly affect the N content of senesced leaves and there was no significant O_3 x N interaction.

Leaf number

Independent of time of the season, enhanced N supply stimulated the number of leaves on silver birch ($p < 0.001$; Figure 9). On the other hand, while there was no effect of O_3 on leaf number at the start of the season, leaf number decreased with higher O_3 at the end of the season ($p < 0.001$). There was no evidence of an interaction between O_3 and nitrogen.

Discussion

In the current study, conducted at a range of O_3 and N exposure levels, no significant interactions between the impacts of O_3 and N on photosynthesis and other leaf traits were observed, either for young leaves or aging leaves. This suggests that the sensitivity of these traits to O_3 in young silver birch trees is neither ameliorated (as shown for some trees species, e.g. Häikiö et al., 2007; Handley and Grulke, 2008; Watanabe et al., 2006) nor enhanced by N fertilization (as shown for some other tree species, e.g. Utriainen and Holopainen, 2001b; Wanatabe et al., 2012; Yamaguchi et al., 2007). Although Pääkkönen and Holopainen (1995) suggested that sufficient N supply might reduce O_3-sensitivity in silver birch, interactions between O_3 and N supply were not consistent between experiments and only observed consistently for certain leaf parameters (e.g. leaf area and leaf dry mass per area). They had conducted experiments at only two O_3 exposure levels (ambient and ca. 1.6 x ambient) and up to three N loads (37, 74 and 150 kg N ha^{-1} y^{-1}). Our results, however, confirmed that the response of leaf traits to O_3 is dependent on the developmental stage and age of the leaf studied, in agreement with various other studies (Bagard et al., 2008; Hoshika et al., 2013; Mäenpää et al., 2011; Oksanen et al., 2003). As the magnitude of the impact of O_3 is related to the accumulated O_3 flux (Phytotoxic Ozone Dose) entering the stomata (Mills et al., 2011b), relatively little impact was expected early in the growing season, with impacts increasing with leaf age and longer-term exposure. Indeed, O_3 x time interactions were observed for most photosynthetic parameters and the chlorophyll content of aging leaves. In contrast, the g_s of aging leaves was not affected by O_3 and no O_3 x time interactions were found.

Traits of a young leaf throughout the growing season

Although the leaf primordia for early leaves were developed during the previous year, there did not seem to be a carry-over effect of ozone on the number of leaves developed in 2013. Enhanced N, however, seems to have stimulated the number of leaves present at the start of 2013, before O_3 and N treatments were started again (Figure 8). The late leaves started to develop in July and the first older leaves started to drop off near the end of July. The heterophyllous character of silver birch explains why the chlorophyll content index of the third leaf (youngest fully expanded leaf) declined first between the end of May and 23rd July, and subsequently rose again by the 23rd of August to a level slightly higher than at the end of May (Figure 2). It is likely that remobilisation of N occurred during this period from the older to the young, newly grown leaves. As to be expected, the chlorophyll content of the young leaves increased significantly with increasing N supply, and this increase was independent of the Julian day. O_3, however, did not affect the chlorophyll content of the young leaves, which might be due to the relatively short time of exposure to O_3 (as discussed below, elevated O_3 reduced the chlorophyll content of aging leaves). Linear relationships between chlorophyll content and $V_{c,max}$, J_{max} and A_{sat} (see Figures 6, S1 and S2) suggest that a similar response to

O_3 and N can be expected for these photosynthetic traits for the third leaf when developing throughout the season. Indeed, Riikonen et al. (2009) did not find an effect of O_3 on $V_{c,max}$, J_{max} and A_{sat} in *Betula pendula* saplings on the youngest fully expanded leaf, however, the level of O_3 exposure was very low in their study (mean O_3 concentration of 32 – 34 ppb). On the other hand, g_s was not affected by O_3 exposure or N supply in our study. Although many studies have reported a decline in g_s in trees with increasing O_3 (Wittig et al., 2007, and references therein), including for *Betula pendula* (Riikonen et al., 2009), other studies have reported no change or even an increase in g_s in response to O_3 exposure (Mills et al., 2016, and references therein). The varying responses might well be explained by inherent differences in responses of species/genotypes to O_3, variation in exposure facilities, magnitude and duration of O_3 exposure. For example, Wittig et al. (2007) observed no change at low to medium O_3 concentrations (30 – 59 ppb), a decline at medium to high O_3 concentration (60 – 119 ppb) and no change at very high O_3 concentrations (>120 ppb).

Traits of an aging leaf throughout the growing season

Leaf photosynthetic capacity ($V_{c,max}$, J_{max}) and A_{sat} declined between 10 – 14th June and 22 – 25th July (Figure 5), but did not decline any further between 22 – 25th July and 2 – 5th September, apart from at 10 kg N ha^{-1} y^{-1} and 68 ppb O_3, where the decline continued. The halt in decline later in the season may be influenced by the fact that older, senesced leaves started to drop off with some new growth and remobilisation of N occurring (see above). High O_3 exposure reduced the photosynthetic capacity and A_{sat} of leaves, and more so later than early in season. A significant interaction between O_3 and time (although not significant for $V_{c,max}$) confirms that effects of O_3 are dependent on the accumulated phytotoxic O_3 dose (LRTAP Convention, 2015; Mills et al., 2011b). Noormets et al. (2010) also found that elevated O_3 decreased photosynthetic parameters and leaf chlorophyll content, particularly in the lower canopy, and that the negative impact of O_3 increased with time. A similar response to O_3 was found for A_{sat} in *Fagus crenata* seedlings, with a significant interaction between O_3 and N only observed in July (Yamaguchi et al., 2007). However, it is not clear for which leaves A_{sat} was determined in *Fagus crenata*. Whilst Marzuoli et al. (2016) reported a significant decline in $V_{c,max}$ due to elevated O_3 in *Quercus robur* seedlings in the first year, no significant effect of elevated O_3 on $V_{c,max}$ was observed in the second year of O_3 exposure. In contrast to Yamaguchi et al. (2007), we did not find any evidence that N impacts dominate early in the season and that O_3 impacts dominate later in the season. Although elevated N supply stimulated photosynthetic capacity ($V_{c,max}$ and J_{max}) as to be expected, N supply did not significantly affect A_{sat}. Whilst high N supply seems to have alleviated to some extent the impact of O_3 on photosynthetic capacity observed at low N supply later in the growing season, there was no significant interaction between O_3 and N treatment, not even when the data for September were analysed separately. The latter might be due to the low number of replication (four instead of six as some leaves had already dropped off the tree) and the higher variability later in the season. The higher decline in A_{sat} compared to photosynthetic capacity in September at high O_3 exposure and high N supply can be explained by a decline in g_s (Figure 5). Nevertheless, O_3 exposure, N supply and time did not affect g_s in the aging leaf in our study when measured at light saturation. In contrast, Yamaguchi et al. (2007) reported a significant increase in g_s in September due to high O_3 exposure in *Fagus crenata*, with no O_3 effect observed earlier in the year, however, they did not define clearly which leaf was measured. We observed a significant and strong linear relationship between A_{sat} and the chlorophyll content of leaves. Although significant, the linear relationship between A_{sat} and g_s was weak ($p = 0.038$), with the data showing a lot of scatter. This suggests that impacts of treatment on A_{sat} were mainly driven by impacts on photosynthetic capacity rather than changes in g_s. Indeed, previous studies have shown that O_3-induced reductions in A_{sat} are mainly driven by reductions in photosynthetic capacity in the chloroplasts (Farage et al., 1991; Hoshika et al., 2013; Niu et al., 2014; Yamaguchi et al., 2007). In agreement with Noormets et al. (2010) and Bagard (2008), the biochemical component of photosynthesis ($V_{c,max}$) was damaged more by O_3 than electron transport (J_{max}), as the ratio of J_{max} to $V_{c,max}$ was enhanced at elevated O_3 exposure. Decreases of both Rubisco content and activation state have been reported for trees and crops (Ainsworth et al., 2012), often associated with down regulation of photosynthetic genes and upregulation of genes involved in programmed cell death and/or tissue senescence (Bohler et al., 2007; Ernst, 2013; Heath, 2008; Pell et al., 1997). In general, terrestrial plants allocate as much as 50% of their leaf N content to Rubisco because of inefficient catalytic activity of the enzyme (Spreitzer and Salvucci, 2002). If A_{sat} is mainly determined by the amount of Rubisco in birch leaves, then the O_3-induced reduction in A_{sat} might well be due to a decrease in allocation of leaf N to Rubisco (Yamaguchi et al., 2007). Because photosynthetic measurements are often made on the most recently fully expanded upper canopy leaves, it is often difficult to demonstrate differences in net photosynthesis due to O_3 until near or after the time when production of new upper canopy leaves has ceased. The growth of new leaves observed in July might have enabled birch to compensate for some of the O_3 damage to the photosynthetic apparatus occurring in older leaves later in the growing season.

Developmental stage of the leaves affects the response to treatments

The results discussed above indicate that the response of leaf traits to treatments is affected by the developmental stage of leaves, with different responses being observed in young leaves compared to aging leaves (Bagard et al., 2008; Hoshika et al., 2013; Mäenpää et al., 2011; Oksanen et al., 2003). This was further confirmed when the chlorophyll content of different leaves along a branch was determined. Whereas the chlorophyll content declined with O_3 concentration in older leaves, in younger leaves the highest chlorophyll content was found at intermediate O_3 concentrations at high N supply (Table 2, Figure 4). For medium-aged leaves, there was no effect of O_3 on the chlorophyll content. Hoshika et al. (2013) reported differences in stress resistance to O_3 between early and late leaves in white birch (*Betula platyphylla* var. *japonica*), with early leaves not showing an O_3-induced reduction in photosynthesis, and an O_3-induced decline in photosynthesis and early defoliation occurring in late leaves. Hence, late leaves were more sensitive to O_3 than early leaves in white birch. However, our results indicate that differences in response to O_3 are primarily determined by the age/developmental stage of leaves and exposure time to O_3 rather than the type of leaf. Responses to treatments are further confounded when nearer the end of the growing season N is remobilised from old leaves to young leaves, maintaining the photosynthetic capacity of the young leaves. The various responses to O_3, and the interaction between O_3 and N, reported in the literature might well be due to measurements on leaves at different developmental stages, which confounds comparisons between experiments. In agreement with Uddling et al. (2005), we observed that enhanced O_3 impairs the resorption of N in leaves of silver birch near the end of the growing season. Similar results were also reported for *Fagus crenata* (Yamaguchi et al., 2007). An increase in leaf N, partly due to the release of NH_4^+ from both protein degradation and enhanced activity of the shikimate pathway, is known to occur at high ozone dose (early senescence) and a part of this N could be transiently re-assimilated via an increased activity of the cytosolic glutamine synthetase (Bohler et al., 2007; Dizengremel et al., 2012; Galant et al., 2012) or stored in gamma-aminobutyric acid (Dizengremel et al., 2012). The enhanced N content in litter at elevated O_3 is likely to affect the subsequent cycling of N in the soil.

Conclusions

At the leaf level, we did not observe any interactions between O_3 and N regarding photosynthetic parameters, chlorophyll content, N content in senesced leaves and leaf number. This suggests that the sensitivity of these leaf traits to O_3 in young silver birch trees was neither reduced nor enhanced by N fertilization under our experimental conditions. Our results suggest that the response of leaf traits to O_3 is dependent on the developmental stage/age of the leaf studied. Whilst O_3 reduced the net rate of photosynthesis in aging birch leaves later in the season, O_3 did not affect the g_s of those leaves. This suggests that there was only a weak coupling between photosynthesis and g_s in aging silver birch leaves under light-saturating conditions. When studying the impacts of O_3 on leaf traits, it is important to define the developmental stage of the leaves, as this will affect the measured response to O_3.

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Table 1. Summary of ozone treatments and climate conditions in 2013.

Ozone treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**
24 hr mean (ppb)	35.7	39.3	42.3	47.8	53.1	59.1	67.9
Daylight mean (ppb)	36.1	40.0	43.6	50.0	54.7	63.9	71.5
AOT40 (ppm h^{-1})	2.4	4.2	7.1	12.1	16.1	25.1	32.5
Season max. (ppb)	62.4	71.3	81.3	100.4	111.3	113.5	126.3

Air temperature (°C)			**VPD (kPa)**			**PAR (umol m^{-2} s^{-1})**	
24 h mean	*Daylight mean*	*Daily max. mean*	*24 h mean*	*Daylight mean*	*Daily max. mean*	*Daylight mean*	*Daily max. mean*
19.8	24.0	25.6	0.67	1.24	1.55	515	808

Table 2. Summary statistics of effects of treatments and time[1] on the chlorophyll content and stomatal conductance (G_S) of the third leaf (youngest fully expanded) measured throughout the growing season.

Variable	**Chl. content**	**G_s**
O_3	n.e.	n.e.
N	$p<0.001$	n.e.
Time	$p<0.001$	$p<0.01$
Time^2	$p<0.001$	$p<0.01$

[1] No two or three-way interactions were found.

n.e. = no significant effect at p = 0.05.

Time^2 = time squared.

Table 3. Summary statistics of effects of treatments and leaf number on the chlorophyll content of silver birch leaves of different age along the first top branch near the end of the growing season (on 4 September 2013).

Variable	**Chl. content**
O_3	n.e.
N	$p<0.001$
Leaf number	$p<0.001$
O_3 * N	n.e.
O_3 * Leaf number	$p<0.001$
N * Leaf number	n.e.
O_3 * N * Leaf number	n.e.

n.e. = no significant effect at p = 0.05.

Table 4. Summary statistics of effects of treatments and time[1] on photosynthetic parameters, stomatal conductance (G_s) and chlorophyll (Chl.) content (index) of the same birch leaves measured throughout the season, i.e. aging with time.

Variable	**$V_{c,max}$**	**J_{max}**	**$J_{max}/V_{c,max}$**	**A_{sat}**	**G_s (at A_{sat})**	**Chl. content**
O_3	$p<0.01$	$p<0.01$	$p=0.05$	$p<0.01$	n.e.[2]	($p=0.055$)
N	$p<0.01$	$p=0.02$	$p=0.01$	n.e.	n.e.	$p=0.03$
Time	$p<0.001$	$p<0.001$	($p=0.09$)	$p<0.001$	n.e.	$p<0.001$
O_3 * Time	($p=0.08$)	$p<0.01$	n.e.	$p=0.001$	n.e.	$p=0.04$

[1] No O_3 x N, N x Time or O_3 x N x Time interactions were found.

[2] n.e. = no significant effect at $p = 0.05$.

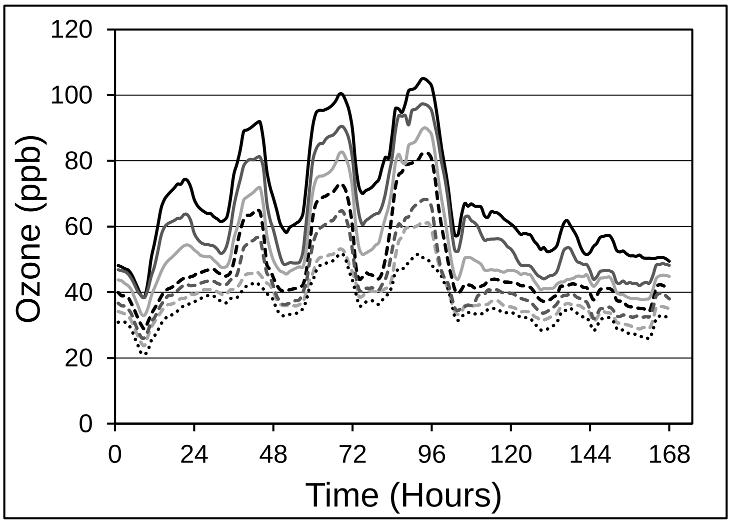

Figure 1. Weekly ozone profile in seven solardomes at Abergwyngregyn, nr. Bangor, North Wales in 2013.

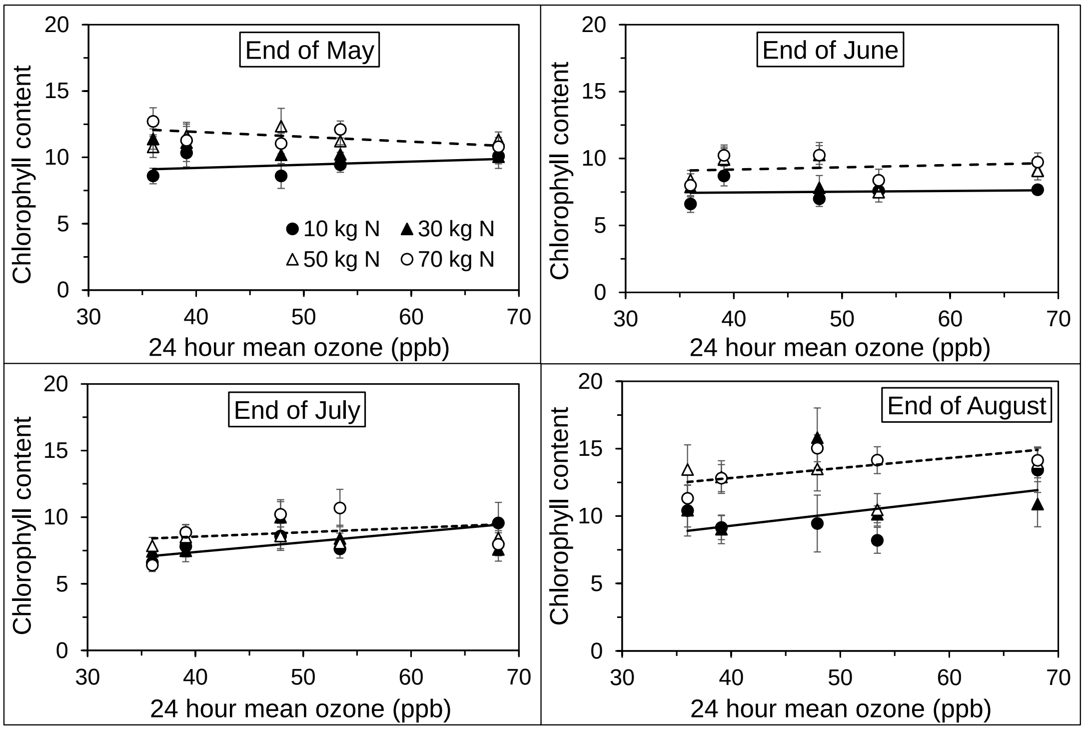

Figure 2. Effect of ozone exposure and nitrogen supply on the chlorophyll content (index) of silver birch leaves (third leaf on main stem, fully expanded) from late May to late August 2013. Values are means ± one SE (n = 5 – 6). Solid line: 10 kg N ha^{-1} y^{-1}, broken line: 70 kg N ha^{-1} y^{-1}.

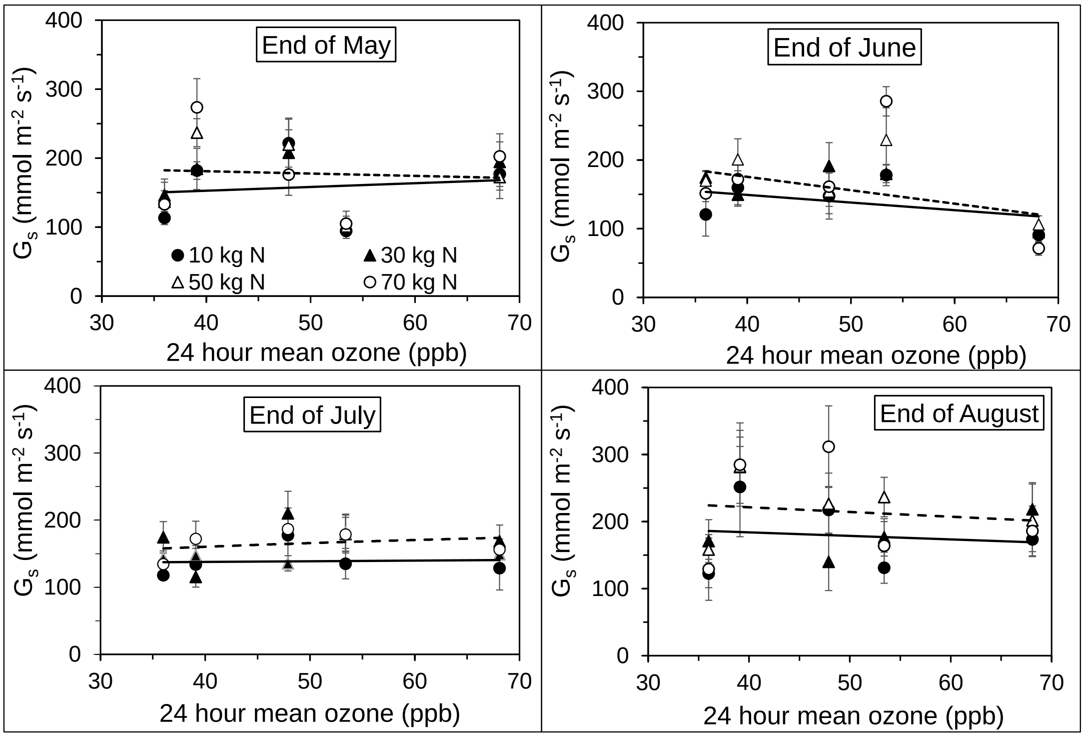

Figure 3. Effect of ozone exposure and nitrogen supply on the stomatal conductance (G_s) of silver birch leaves (third leaf on main stem, fully expanded) from late May to late August 2013. Values are means ± one SE (n = 5 – 6). Solid line: 10 kg N ha^{-1} y^{-1}, broken line: 70 kg N ha^{-1} y^{-1}.

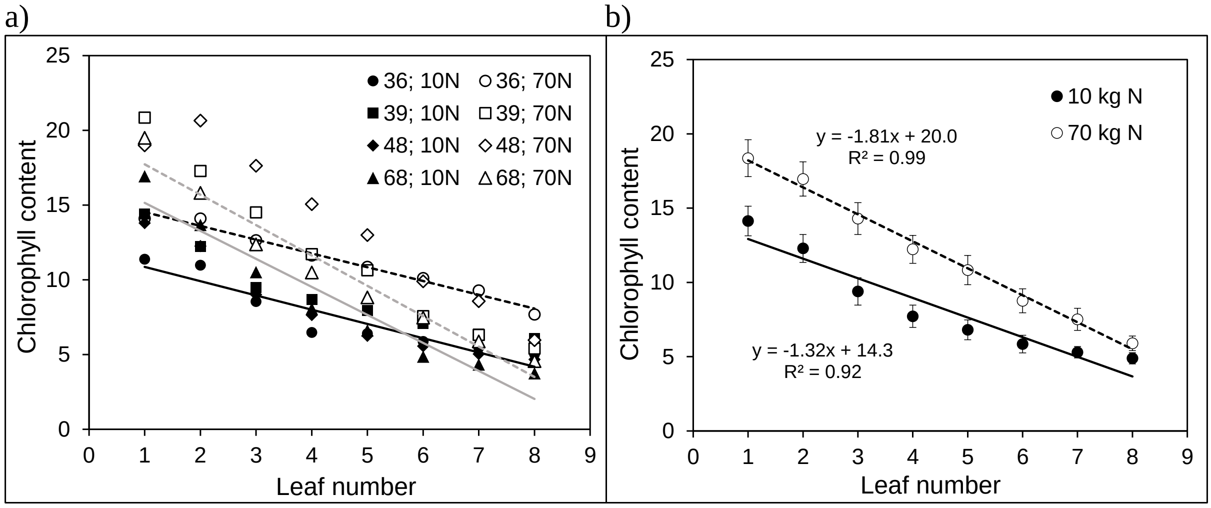

Figure 4. Effect of ozone exposure and nitrogen supply on the chlorophyll content (index) of silver birch leaves of different age along the first top branch near the end of the growing season (4 September 2013). a) Data are means (n = 6); the first number in the legend refers to the 24 hr mean ozone concentration (ppb) and the second number to the nitrogen supply (kg N ha^{-1} y^{-1}); trend lines are shown for the lowest (black lines) and highest (grey lines) ozone exposure for low (solid lines) and high (broken lines) nitrogen supply. See Table S1 for values and standard errors. b) Data are means ± one SE (n = 24), with ozone exposure data averaged for low (filled circles, solid line) and high (open circles, broken line) nitrogen supply. Leaf number one is the youngest leaf near the top of the branch, whereas leaf number eight is the oldest measured leaf situated near the origin of the branch.

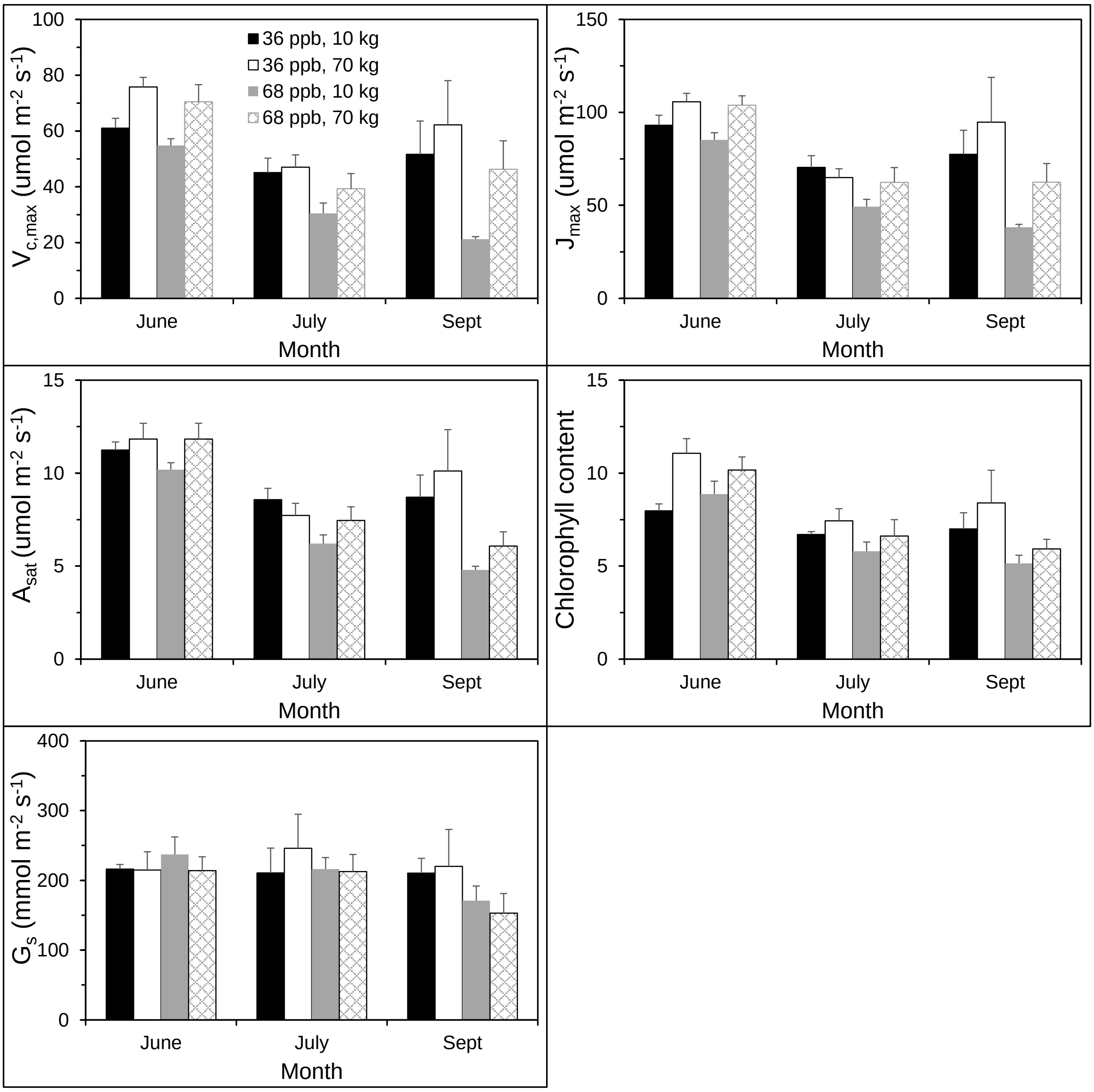

Figure 5. Photosynthetic capacity ($V_{c,max}$ and J_{max}), light-saturated rate of photosynthesis (A_{sat}), chlorophyll content (index), and stomatal conductance (G_s; at ambient CO_2) of aging silver birch leaves, i.e. the same leaves were measured throughout the growing season. Trees were exposed to a 24 hr mean ozone concentration of 36 or 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013. Data are means ± one SE (n = 4 - 6).

a) b)

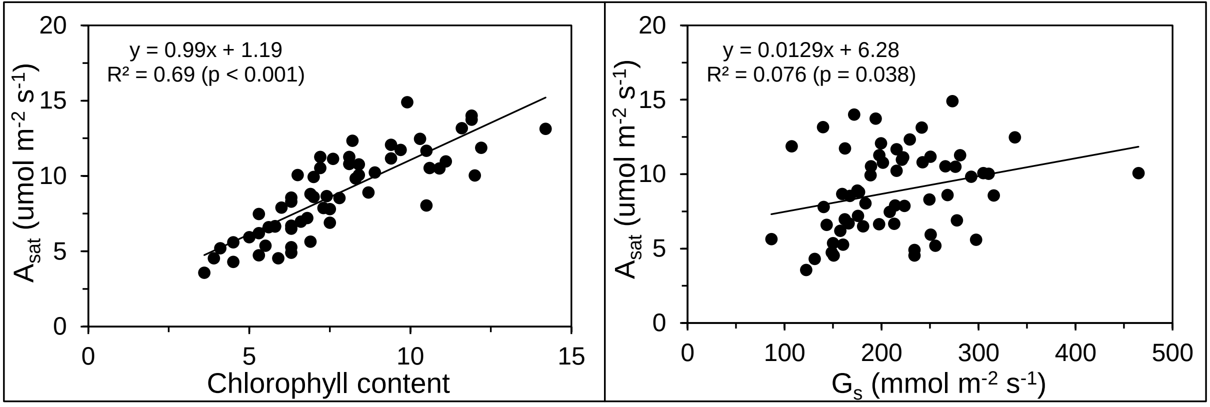

Figure 6. Relationship between light-saturated rate of photosynthesis (A_{sat}) and a) chlorophyll content (index) and b) stomatal conductance (G_s) of aging silver birch leaves (see Figure 5). Trees were exposed to a 24 hr mean ozone concentration of 36 or 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013.

a) b)

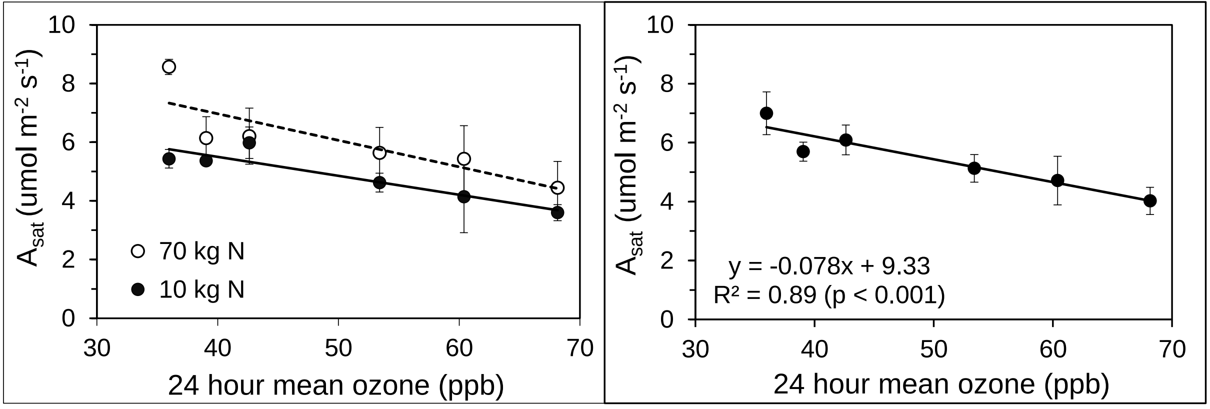

Figure 7. Light-saturated rate of photosynthesis (A_{sat}) of aged silver birch leaves near the end of the growing season (late August, early September). Trees were exposed to a 24 hr mean ozone concentration of 36 - 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013. In a) mean values (± one SE; n = 4 – 5) are shown for different nitrogen supply rates, whereas in b) mean values (± one SE; n = 8 – 10) for different nitrogen supply rates were pooled.

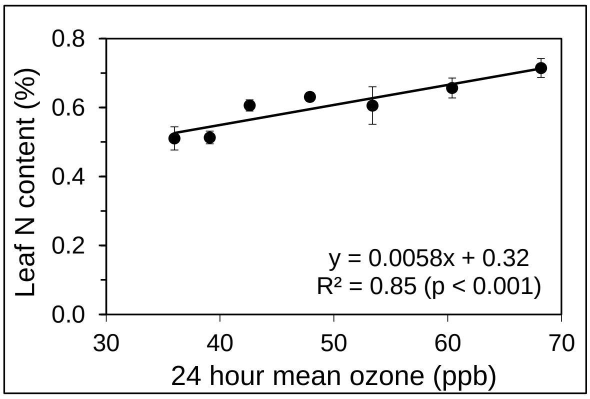

Figure 8. Impacts of ozone exposure on the leaf nitrogen concentration of silver birch leaves just before they fell of the tree. Values are the mean of four different nitrogen supply rates (± one SE).

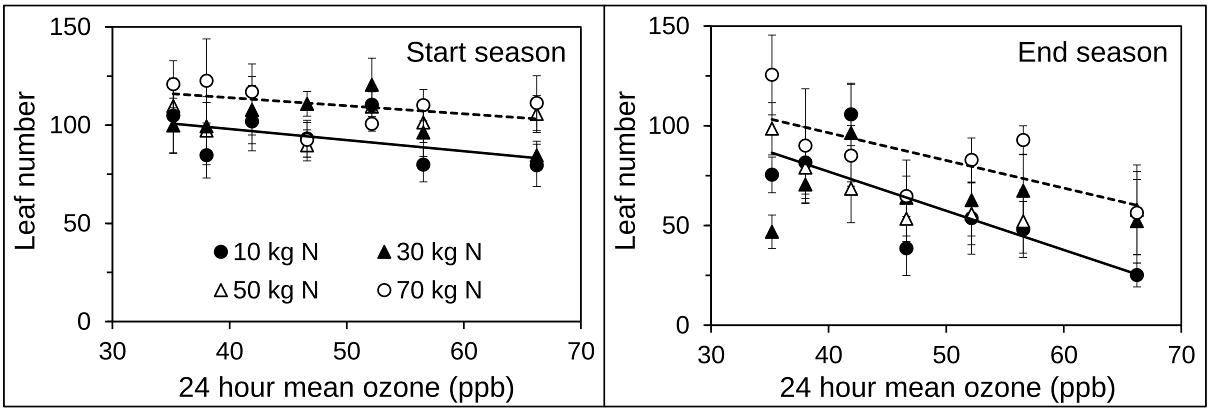

Figure 9. Impact of ozone and nitrogen on leaf number near the start (5th May) and end of the 2013 growing season (24th September). Data are means ± one SE (n = 5 - 6). Any effect early in the season reflect a carry-over effect from the previous growing season. Trend lines are shown for the lowest (solid line) and highest (broken line) nitrogen supply.

Supplement

Table S1. Effect of ozone exposure and nitrogen supply on the chlorophyll content of silver birch leaves of different age along the first top branch near the end of the growing season (4 September 2013). Leaf number one is the youngest leaf near the top of the branch.

			Chlorophyll content	
Ozone (ppb)	N (kg ha^{-1} y^{-1})	Leaf no.	Mean	Standard error
35.7	10	1	11.4	2.2
		2	11.0	2.1
		3	8.6	1.2
		4	6.5	1.0
		5	6.4	0.8
		6	5.9	0.9
		7	5.5	0.8
		8	5.0	0.8
	70	1	14.1	2.9
		2	14.1	2.7
		3	12.7	1.9
		4	11.6	1.6
		5	10.9	1.6
		6	10.1	1.7
		7	9.3	1.8
		8	7.7	1.0
39.3	10	1	14.4	1.1
		2	12.2	1.3
		3	9.5	1.8
		4	8.7	1.8
		5	8.0	1.8
		6	7.1	1.7
		7	6.4	1.1
		8	6.1	1.0
	70	1	20.9	2.0
		2	17.3	1.2
		3	14.5	1.0
		4	11.7	1.0
		5	10.6	1.7
		6	7.6	0.8
		7	6.3	0.8
		8	5.4	0.9
47.8	10	1	13.8	2.3
		2	12.3	2.9
		3	9.0	2.5
		4	7.7	1.6
		5	6.3	1.4
		6	5.6	1.2
		7	5.0	0.2
		8	4.7	0.4
	70	1	19.0	2.4
		2	20.7	2.6
		3	17.6	2.5
		4	15.1	2.3
		5	13.0	2.5
		6	9.9	1.9
		7	8.6	1.8
		8	6.0	0.7
67.9	10	1	16.9	2.0
		2	13.7	1.6
		3	10.5	2.0
		4	8.1	1.7
		5	6.6	1.5
		6	4.9	0.9
		7	4.3	0.5
		8	3.8	0.4
	70	1	19.5	2.3
		2	15.8	2.1
		3	12.4	2.6
		4	10.5	2.3
		5	8.8	2.1
		6	7.5	1.9
		7	5.9	1.1
		8	4.6	1.1

Table S2. Relative difference (%) in photosynthetic parameters, chlorophyll (Chl.) content and stomatal conductance (g_s) at high (24 hr mean of 68 ppb) compared to low ozone (24 hr mean of 36 ppb) at different times during the growing season. Replace Chlorophyll index with Chlorophyll content in table.

Month	N (kg ha^{-1} y^{-1})	A_{sat}	$V_{c,max}$	J_{max}	$J_{max}/V_{c,max}$	Chl. index	g_s
June	10	9.4	10.2	8.5	-2.0	-11.2	-9.8
	70	-0.1	7.1	1.8	-7.0	8.1	0.3
July	10	27.4	32.4	30.0	-5.5	13.4	-2.5
	70	3.6	16.5	4.0	-14.8	11.0	13.6
September	10	45.0	59.0	50.7	-14.9	26.4	18.8
	70	39.9	25.7	34.0	7.7	29.5	30.4
June	Combined[1]	5.0	10.4	6.1	-5.3	3.2	-4.8
July	Combined[1]	15.3	24.6	16.8	-11.2	13.0	7.6
September	Combined[1]	42.2	40.8	41.5	-3.6	28.1	24.8

[1] Data for low and high N were combined as there was no significant O_3 x N interaction.

Negative values indicate a higher value of the variable at higher compared to low O_3 exposure.

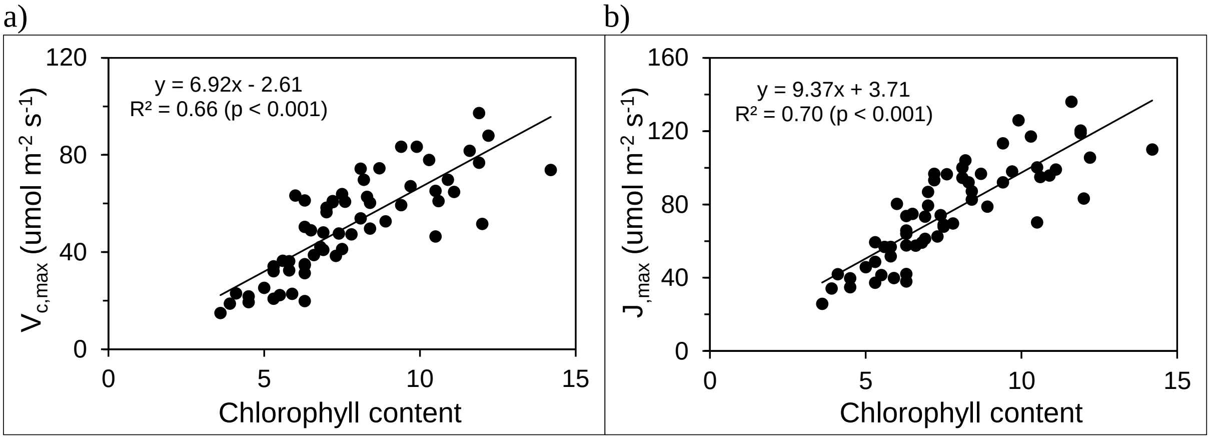

Figure S1. Relationship between a) $V_{c,max}$ and b) J_{max} and chlorophyll content of aging silver birch leaves (see Figure 5). Trees were exposed to a 24 hr mean ozone concentration of 36 or 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013. See also Figure 5.

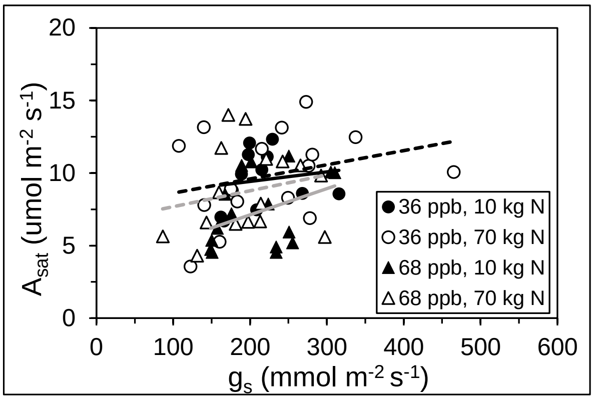

Figure S2. Relationship between light-saturated rate of photosynthesis (A_{sat}) stomatal conductance of aging silver birch leaves (see Figure 6b). Trees were exposed to a 24 hr mean ozone concentration of 36 (black lines) or 68 ppb (grey lines) and supplied with 10 (solid lines) or 70 kg N ha^{-1} yr^{-1} (broken lines) in 2013.

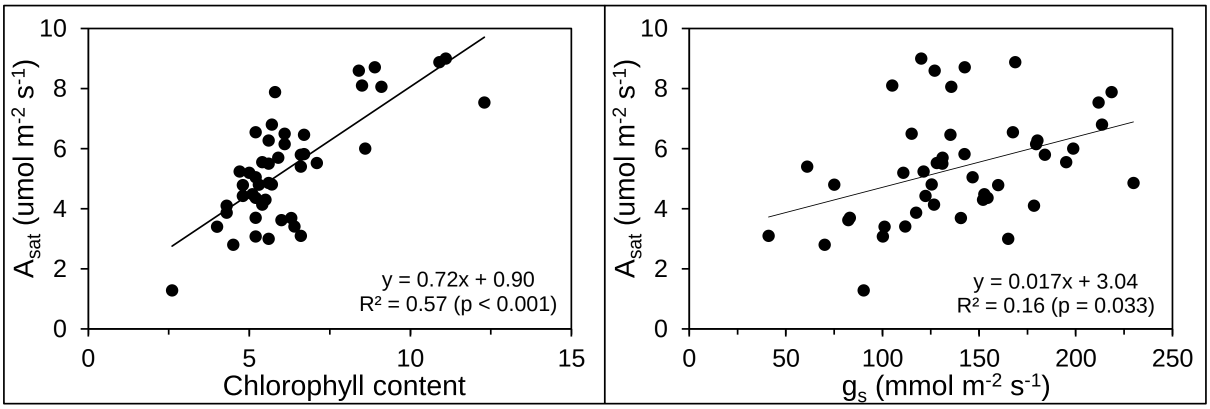

Figure S3. Relationship between light-saturated rate of photosynthesis (A_{sat}) and and a) chlorophyll content and b) stomatal conductance of aged silver birch leaves near the end of the growing season (late August, early September). Trees were exposed to a 24 hr mean ozone concentration of 36 - 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013. See also Figure 7.