Combining $\delta^{13}$C and $\delta^{18}$O analyses to unravel competition, CO$_2$ and O$_3$ effects on the physiological performance of different-aged trees

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ABSTRACT

Combined $\delta^{13}$C and $\delta^{18}$O analyses of leaf material were used to infer changes in photosynthetic capacity ($A_{\text{max}}$) and stomatal conductance ($g_l$) in Fagus sylvatica and Picea abies trees growing under natural and controlled conditions. Correlation between $g_l$ and $\delta^{18}$O in leaf cellulose ($\delta^{18}$O$_{\text{cel}}$) allowed us to apply a semi-quantitative model to infer $g_l$ from $\delta^{18}$O$_{\text{cel}}$ and also interpret variation in $\delta^{13}$C as reflecting variation in $A_{\text{max}}$. Extraction of leaf cellulose was necessary, because $\delta^{18}$O from leaf organic matter ($\delta^{18}$O$_{\text{LOM}}$) and $\delta^{13}$C$_{\text{soil}}$ was not reliably correlated.

In juvenile trees, the model predicted elevated carbon dioxide (CO$_2$) to reduce $A_{\text{max}}$ in both species, whereas ozone (O$_3$) only affected beech by reducing CO$_2$ uptake via lowered $g_l$. In adult trees, $A_{\text{max}}$ declined with decreasing light level as $g_l$ was unchanged. O$_3$ did not significantly affect isotopic signatures in leaves of adult trees, reflecting the higher O$_3$ susceptibility of juvenile trees under controlled conditions. The isotopic analysis compared favourably to the performance of leaf gas exchange, underlining that the semi-quantitative model approach provides a robust way to gather time-integrated information on photosynthetic performance of trees under multi-faced ecological scenarios, in particular when information needed for quantitative modelling is only scarcely available.

Key-words: elevated carbon dioxide (CO$_2$); elevated ozone (O$_3$); Fagus sylvatica; Picea abies; cellulose; photosynthetic capacity ($A_{\text{max}}$); semi-quantitative model approach; stable isotope ratios; stomatal conductance for water vapour ($g_l$).

INTRODUCTION

Over two decades of intensive research have shown that the proportion of $^{13}$C to $^{12}$C in plant organic matter (i.e. $\delta^{13}$C$_p$) is often negatively correlated with the long-term mean of leaf internal to external CO$_2$ concentrations ($c_i/c_a$) (Farquhar, O’Leary & Berry 1982) in C$_3$ plants as:

$$\delta^{13}C_p = \delta^{13}C_a - a - (b - a) \frac{c_l}{c_a},$$

where $\delta^{13}$C$_a$ is the carbon isotope ratio of atmospheric CO$_2$ (c. $-8\%o$ under natural conditions), $a$ is the fractionation caused by differential gaseous diffusion of $^{13}$CO$_2$ and $^{12}$CO$_2$ through the stomatal pore (4.4‰) and $b$ is the net fractionation (c. 27‰) caused by the leaf which is dominated by the fractionation of the CO$_2$-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and to a lesser extent by other carboxylases present in the leaves of C$_3$ plants. Equation 1 is a useful but simplified version of the full equation proposed by Farquhar et al. (1982), and neglects smaller fractionation factors such as diffusion through the leaf boundary layer, dissolution and liquid phase diffusion within the leaf as well as fractionation by dark and photorespiration (Farquhar, Ehleringer & Hubick 1989a; Brugnoli & Farquhar 2000).

According to Eqn 1, a lowered leaf internal CO$_2$ concentration ($c_i$) results in an increased $\delta^{13}$C$_p$ by a lower discrimination against $^{13}$CO$_2$ ($\Delta^{13}$C). CO$_2$ diffusion into the leaf depends on stomatal aperture and $c_i$, resulting from the balance between CO$_2$ influx (supply) and carboxylation (CO$_2$ consumption) within the leaf. Hence, the overall activity of the CO$_2$-fixing enzymes, in addition, directly affects $c_i$ and $\delta^{13}$C$_p$ (Farquhar et al. 1989a).

More recently, several authors reported $\delta^{18}$O of leaf organic matter ($\delta^{18}$O$_{\text{LOM}}$) can be negatively correlated with mean stomatal conductance ($g_l$) (Barbour & Farquhar 2000; Barbour et al. 2000; Siegwolf et al. 2001). This is especially true when plants have access to the same source water $\delta^{18}$O ($\delta^{18}$O$_w$) and occupy the same aerial environment so that they are exposed to the same $\delta^{18}$O in water vapour ($\delta^{18}$O$_v$) and to the same leaf external water vapour pressure ($e_v$). Such prerequisites are typically fulfilled when plants are grown in controlled environments (e.g. in glasshouses, phytotrons). In the field, maintaining specific growth conditions is more challenging, although not impossible, to achieve.

The relationship between $g_l$ and $\delta^{18}$O$_w$ has a mechanistic basis which differs from that which determines $\delta^{13}$C$_p$ (Farquhar & Lloyd 1993; Barbour et al. 2000). Firstly, the O-isotope composition of leaf water ($\delta^{18}$O$_l$) is determined
by a combination of factors such as the $\delta^{18}O$ at the sites of evaporation (e.g., the water ‘film’ covering the cells just inside the stomatal cavity of the leaf; $\delta^{18}O_e$), the $\delta^{18}O$, and $\delta^{18}O_i$, as well as the evaporative gradient $e_i/e_e$, as originally formulated by Craig & Gordon (1965) and modified by Farquhar & Lloyd (1993) as:

$$\delta^{18}O_i = \delta^{18}O_e + e^* + e_i + (\delta^{18}O_e - \delta^{18}O_i - e_i) \frac{e_i}{e_e},$$

(2)

where $e^*$ is the equilibrium fractionation factor for water exchange between the liquid and vapour phase which depends on leaf temperature (Bottigia & Craig 1969). $e_i$ is the kinetic fractionation occurring during diffusion through the stomata and leaf boundary layer; $e_e$ can be calculated as:

$$e_e = \frac{32g_b + 21g_i}{g_b + g_i},$$

(3)

where $g_b$ and $g_i$ are the boundary and stomatal conductance of water vapour (Cernusak, Farquhar & Pate 2005). The evaporative surface inside the leaf may not always be representative in determining the $\delta^{18}O$ of the bulk leaf water ($\delta^{18}O_l$). In fact, $\delta^{18}O_l$ is linked to $\delta^{18}O_i$ via the Pécelet effect (Farquhar & Lloyd 1993) as follows:

$$\delta^{18}O_l = \delta^{18}O_i + \frac{(\delta^{18}O_l - \delta^{18}O_i)(1 - e^{*-\varphi})}{\varphi}.$$

(4)

where $\varphi$ is a Pécelet number, which is calculated as $EL/(CD)$, with $E$ being the transpiration rate, $L$ is a scaled effective path length, $C$ is the molar concentration of water and $D$ is the diffusivity of $H_{18}O$ in water.

The exchange of oxygen atoms with local water in the meristematic tissues where cellulose is synthesized enhances the impact of $\delta^{18}O_i$ on $\delta^{18}O_{cel}$ as modelled by Barbour & Farquhar (2000):

$$\delta^{18}O_{cel} = \delta^{18}O_i(P_{ex} + P_1) + \delta^{18}O_l(1 - P_{ex} + P_1) + \varepsilon_{nc},$$

(5)

where $\varepsilon_{nc}$ is the equilibrium fractionation between water and carbonoxygen groups (C=O) (Sternberg & Deniro 1983), while $P_{ex}$ and $P_1$ are the proportion of exchangeable oxygen in cellulose and the proportion of xylem water in the meristematic tissue where cellulose is synthesized, respectively. Recently, $P_{ex}$ was estimated to be close to 0.4 (Cernusak et al. 2005), while the degree of variability in $P_{ex}$ is not as clear and creates a weakness in the model (Barbour 2007). Equations 2–5 reflect increasing $g_l$ to be associated with decrease in $\delta^{18}O_{cel}$ via three different effects: (1) decreased $e_i$ associated with increased $g_l$; (2) increased $e_i/e_e$ caused by lowered leaf temperature (resulting from increased transpiration cooling); and (3) an increase of $\varphi$ upon increasing $E$.

Recently, the combined analysis of $\delta^{13}C$ and $\delta^{18}O$ has provided insights into a plant’s long-term photosynthetic performance (given as $A_{max}$) in relation to its water use (given as $g$) (Farquhar et al. 1989b; Sternberg, Mulkey & Wright 1989; Farquhar & Lloyd 1993; Yakir & Israeli 1995; Saurer, Aellen & Siegwolf 1997; Scheidegger et al. 2000; Sullivan & Welker 2007). These studies have embraced the original qualitative nature of the $^{13}C^{18}O$ relationships outlined in a paper by Scheidegger et al. (2000). Here, we present a semi-quantitative modification to this earlier model (Scheidegger et al. 2000) and test it for forest trees of contrasting habit (i.e. Norway spruce versus European beech) and ontogenetic stages (juvenile versus adult trees). We explore the possibility of inferring $g$ directly from $\delta^{18}O$ and subsequently test the advanced model under a variety of ecological scenarios. To induce changes in $A_{max}$ and $g$, and therefore in $\delta^{13}C$ and $\delta^{18}O$ of leaf material, we exposed plants to elevated CO$_2$ and O$_3$ concentrations as well as different light environments. Furthermore, we tested for the impact of intra- and interspecific competition on $\delta^{13}C_l$ and $\delta^{18}O_l$ under controlled chamber conditions. This set of contrasting natural and controlled growth scenarios provided a rewarding way to explore the robustness of the advanced model. Our broader goal from these studies was to provide a generalized approach for using $\delta^{13}C$ and $\delta^{18}O$ in combination to infer physiological performance under a wide range of environmental conditions.

**MATERIALS AND METHODS**

**Phytotron experiment**

In spring 1998, 2- and 3-year-old seedlings of European beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) Karst.), respectively, were planted in containers (0.7 x 0.4 m, and 0.3 m in depth), which had been filled with untreated forest soil (dystric cambisol, Ah-B horizon). Twenty trees (arranged in rows of 4 x 5 individuals) were planted into each of 32 containers such that 16 containers had only one species (eight each of spruce or beech) and 16 others were one-to-one beech/spruce mixtures. To minimize potential edge effects, measurements were only taken on the six central individuals in each container.

Being preadapted to ambient and elevated (ambient + 300 µL L$^{-1}$) CO$_2$ concentrations in two climate-controlled greenhouse chambers, the containers were transferred into four walk-in phytotrons (size c. 2.8 x 3.4 m) maintained by the German Aerospace Research Center for Environment and Health in Neuherberg near Munich, Germany. Details on the phytotrons can be found in Payer et al. (1993) and Thiel et al. (1996). During the subsequent two growing seasons in the phytotrons, plants were, in addition to the two CO$_2$ concentrations, exposed in each phytotron either to ambient or twice-ambient O$_3$ concentrations (restricted to <150 nL L$^{-1}$) using Plexiglas subchambers (Röhm GmbH, Darmstadt, Germany) Kozovits et al. (2005a). The result was establishing four CO$_2$/O$_3$ treatments: (1) ambient CO$_2$/ambient O$_3$, hereafter referred to as ‘control’; (2) ambient CO$_2$/elevated O$_3$ = $+O_3'$; (3) elevated CO$_2$/ambient O$_3$ = $+O_3'$; and (4) elevated CO$_2$/elevated O$_3$ = $+CO_2'$/$+O_3'$. The climatic conditions and O$_3$ concentrations were adopted from the study site ‘Kranzberg Forest’ (Germany, 490 m a.s.l.) (Nunn et al. 2002) and reproduced in the phytotrons on an hourly basis throughout the seasonal courses.
(Kozovits et al. 2005a). The external, cumulative O3 exposure under the ambient O3 concentration resulted in an AOT40 (calculated for daylight hours, according to Fuhrer, Skárby & Ashmore 1997) of 10.5 and 9.2 µL L⁻¹ h⁻¹ and in a SUM0 of 89.5 and 82.1 µL L⁻¹ h⁻¹ for the first and second growing season, respectively. In the corresponding twice-ambient O3 treatment, AOT40 and SUM0 were c. 6.5 and 2.0 times higher, respectively (Kozovits et al. 2005a).

Three tensiometers (model T5, UMS, Munich, Germany) per container continuously monitored soil moisture at a depth of 7 cm and were set to trigger irrigation with deionized water whenever soil water tension reached 350 hPa. Irrigation water with a δ¹⁸O of c. -11.3‰ was supplied from a common tank. At any irrigation event, the containers were supplied with 0.5 L (April, May) or 1.0 L (June to September) of deionized water. Liquid fertilizer (1 L of double-concentrated Hoaglands solution) (Hoagland & Arnon 1950) was applied four and six times during the first and second growing season, respectively, to maintain nutrient levels similar to those found in natural soils of Bavarian forests (Kreutzer et al. 1991).

During the winter months of 1998/1999 and 1999/2000, plants were placed in open-top chambers outdoors where corresponding CO2 concentrations were maintained. Leaves were harvested at the end of the second growing season in the phytotrons and dried for 3 d at 65 °C. For details on the phytotron experiment, see Kozovits et al. (2005a,b).

Plants grown under ambient CO2 concentrations were exposed to CO2 from outside air. Mean ambient CO2 concentration was 397.0 ± 0.1 µL L⁻¹ (mean ± SE). The supplementary CO2 added in the elevated CO2 treatment was supplied from a large tank, which was refilled once or twice a year with CO2 of known δ¹³C (in the range of +4.0 to −4.4‰; mean value of −4.2‰). Mean CO2 concentration in the elevated CO2 treatment was 690.0 ± 0.7 µL L⁻¹ and resulted in a mean air δ¹³C (δ¹³Cₐ) of −6.4‰. Because of the different δ¹³C in the two CO2 treatments, δ¹³C of leaf material could not be compared directly. Therefore, comparison was made on the basis of discrimination against ¹³CO₂ (Δ¹³C), calculated as follows (Farquhar et al. 1989a):

\[
\Delta^{13}C = \frac{\delta^{13}C_{a} (\text{‰}) - \delta^{13}C_{cel} (\text{‰})}{1 + \delta^{13}C_{cel} (\text{‰})/1000},
\]

where δ¹³Cₐ is the carbon isotope ratio of atmospheric CO₂ (here: −8‰ and −6.4‰ in the case of ambient and elevated CO₂ concentrations, respectively) and δ¹³C₉ is the carbon isotope ratio of leaf cellulose. We related Δ¹³C or δ¹³C₉ to δ¹³O₉ to evaluate effects of gaseous treatments and types of competition on the trees grown in the phytotrons. To facilitate the comparison between the experiments in phytotrons and in the field, we inverted the orientation of the Δ¹³C-axis (ordinate) in Figs 2 and 4.

Leaf gas exchange was measured with an open-flow, steady-state porometer (CQ130, Walz, Effeltrich, Germany) (Schulze et al. 1982), which was equipped with a differential infrared CO₂/H₂O gas analyser. Measurements

on phenologically representative leaves (n = 12 per atmospheric treatment and type of competition) were performed under growth conditions by approximate monthly intervals throughout the growing seasons of 1999 (1 June, 26 June, 15 July, 27 August, 17 September) and 2000 (7 June, 6 July, 3 August, 24 August). Leaf internal to external CO₂ partial pressure (c/cₑ) and leaf stomatal conductance for water vapour (gₛ) were calculated according to the equations of von Caemmerer & Farquhar (1981), based on one-sided leaf area for beech and projected leaf area for spruce.

Field experiment

In the field, leaf material was sampled from 55- to 60-year-old Norway spruce and European beech trees grown in a forest stand in southern Bavaria, Germany (‘Kranzberg Forest’, for details see Pretzsch, Kahn & Grote 1998). In May 2000, a ‘free-air’ O₃ fumigation was set into operation in the canopy, and five entire crowns of beech and spruce each were exposed to twice-ambient O₃ concentrations (2x O₃, being restricted to < 150 nL L⁻¹) (Nunn et al. 2002; Werner & Fabian 2002). Leaves of trees grown under ambient O₃ (1x O₃ = control) and 2x O₃ were accessible via scaffolding and canopy crane and were sampled at four dates throughout the year 2001 (10 June, 5 July, 2 August and 30 September). For spruce, leaf sampling was concentrated on 1-year-old needles. We sampled at three different crown positions: sun, intermediate and shade crown corresponding to specific leaf area (SLA) of 11.9 ± 0.7, 21.8 ± 2.0 and 40.0 ± 1.4 m² kg⁻¹ for beech and 3.1 ± 0.4, 4.9 ± 0.5 and 6.0 ± 0.7 m² kg⁻¹ for spruce, respectively (means ± SE). SLA significantly differed between the three crown positions (P < 0.001 and P = 0.003 for beech and spruce, respectively).

Because of several nearby canopy gaps, the stand was well ventilated, and CO₂ gradients in concentration and δ¹³C were unlikely to be large (Werner, Ecoclimatology, Technische Universität München, Germany, personal communication).

Cellulose extraction

Cellulose was extracted from homogenized leaf material using a modified method first published by Brendel, Iannetta & Stewart (2000) that adds an extraction step using 17% w/v NaOH and subsequent rinsing steps with water (three times) which enhanced the purity of the extracted cellulose used for isotope analysis. The modified Brendel method produces reliable cellulose extracts for ¹³C and ¹⁸O analyses (for full details on these modifications and comparison to other methods of cellulose extraction, see Gaudinski et al. 2005).

Hot-water extraction of water-soluble carbohydrates

During 2 days of high O₃ levels in the field experiment, on 26 July and 1 August 2001, leaves were sampled for the
analysis of hot-water extractable carbohydrates. The 24 h means of O₃ concentrations at 1× O₃ and 2× O₃ were 41.8 and 82.6 nL L⁻¹, respectively, on 26 July, and 60.6 and 120.7 nL L⁻¹ on 1 August, respectively. Leaves were sampled during afternoon hours (1415–1745 h), killed immediately in a microwave oven (Popp et al. 1996) and subsequently dried for 3 d at 65 °C. Hot-water extracts (1 h at 95 °C) were prepared from milled plant material. Non-soluble material was centrifuged (5 min at 10 000 g), and the supernatant was used for carbon isotope analysis (δ¹³C) after drying in an oven overnight at 65 °C.

**Analysis for stable isotopes ¹³C and ¹⁸O**

Analysis of carbon isotope ratio (δ¹³C) was performed with a PDZ Europa 2020 isotope ratio mass spectrometer (Manchester, UK), while oxygen isotope analysis was conducted in a Finnigan MAT Delta PlusXL (Finnigan MAT, Bremen, Germany) following the method of Farquhar, Henry & Styles (1997) housed at the Center for Isotope Biogeochemistry, U.C. Berkeley, USA. All isotope ratios are expressed in δ notation using PeeDee Belemnite (PDB) as the standard for carbon and Vienna-standard mean ocean water (V-SMOW) as the standard for oxygen (see Dawson et al. 2002). Long-term (3+ year) external precisions for carbon and oxygen isotope analyses are 0.17‰ and 0.23‰, respectively.

**Statistics**

Statistical analysis was performed using SPSS 12.0 for Windows (SPSS, GmbH, Munich, Germany). In the phytotron experiment, main effects of O₂, CO₂ and type of competition (intra- versus interspecific) were analysed using a three-way analysis of variance (ANOVA). In the following, we refer to main effects of the applied gaseous treatments using the terms ‘elevated CO₂’ and ‘elevated O₂’, while effects of individual treatments are denoted using the aforementioned terms ‘control’, ‘+O₂’, ‘+CO₂’ and ‘+O₂/+CO₂’. Data from the field site (Kranzberg Forest) were analysed in a two-way ANOVA with the independent variables ‘O₃ treatment’ and ‘irradiance level’ (i.e. crown position). Because in the field experiment, crown positions of sampled leaves were located within the O₃ treatments, a nested design (crown position nested into O₃ treatment) was employed. Replicated measures in time were taken into account when present (Fig. 5 and Table 1).

**RESULTS**

The correlations between δ¹³C of leaf organic matter (δ¹³CLOM) and leaf cellulose (δ¹³CCEL) were highly significant in both species, irrespective of their growing environment in the phytotron (Fig. 1a) or field (Fig. 1b). In all cases, δ¹³CCEL was higher (more enriched in ¹³C) than in δ¹³CLOM. These differences were smaller in juvenile spruce (0.5–1.5‰) than in juvenile beech trees (1.5–3.5‰). The correlation between δ¹⁸OLOM and δ¹⁸OCEL was only significant in needles from adult spruce trees (small symbols in Fig. 1d) although the variance was high (e.g., r² = 0.28). In all cases, leaf cellulose was enriched in ¹⁸O relative to LOM (i.e. δ¹⁸OCEL > δ¹⁸OLOM). This offset was highest in adult trees growing in the forest (c. 5.5‰ on average, Fig. 1d) compared with juvenile trees of beech (1 to 4‰) and spruce (2–4‰) grown in the phytotrons (Fig. 1c). Given the lack of a reliable correlation between δ¹⁸OLOM and δ¹⁸OCEL, subsequent analyses were based on δ¹⁸O from purified leaf cellulose.

Discrimination against ¹³C (Δ¹³C) by juvenile trees growing in the phytotrons was calculated from δ¹³CCEL and δ¹³CLOM (see Eqn 6). Δ¹³C was significantly correlated with c/cₑ in both years 1999 and 2000; this most likely occurred because more than 85% of total needle biomass was constructed during these 2 years (Fig. 2b). Correspondingly, in the deciduous beech trees, we elected to use the leaves for 2000 in the correlation shown (Fig. 2a).

In juvenile beech, atmospheric treatments and types of competition (intra- versus interspecific) resulted in c/cₑ varying between 0.74 and 0.86 as averaged across four sampling dates throughout the different growing seasons (Fig. 2a). Variation in c/cₑ within a treatment (e.g. mixed culture under +CO₂ open squares: 0.78 ± 0.06), was the result of seasonal changes. Mean c/cₑ correlated linearly with Δ¹³C (solid line, r² = 0.86); however, in the modelled relationship (see Eqn 1, dotted lines) variation in c/cₑ only

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Table 1. δ¹³C of hot water extractable carbohydrates (δ¹³C_HWC) from leaves of adult beech and spruce trees grown in Kranzberg Forest under ambient (1× O₃) and twice-ambient O₃ (2× O₃) concentrations

<table>
<thead>
<tr>
<th>Tree Type</th>
<th>Date</th>
<th>O₃ Treatment</th>
<th>δ¹³C_HWC (‰) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beech</td>
<td>26 July</td>
<td>1× O₃</td>
<td>-28.2 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2× O₃</td>
<td>-27.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>1 August</td>
<td>1× O₃</td>
<td>-27.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2× O₃</td>
<td>-27.5 ± 0.3</td>
</tr>
<tr>
<td>Spruce</td>
<td>1 August</td>
<td>1× O₃</td>
<td>-28.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2× O₃</td>
<td>-28.6 ± 1.0</td>
</tr>
</tbody>
</table>

Leaves from sun, intermediate (half-shade) and shade crown positions were sampled during 2 days with high O₃ concentrations (26 July and 1 August 2001). Ozone concentrations in 1× O₃ and 2× O₃-plots were 41.8 and 82.6 nL L⁻¹ on 26 July and 60.6 and 120.7 nL L⁻¹ on 1 August, respectively (above canopy, 24 h means). Data are means ± SE (n = 3–5).
accounts for a 3‰ shift as opposed to the observed 6‰ shift in $\Delta^{13}C$. The discrepancy between the modelled and observed variation in $\Delta^{13}C$ could be accounted for via the reduction in the parameter, $b$, the net fractionation caused by the leaf, at lowered $c_i/c_a$ (Fig. 2a). A value of 21 for $b$ was needed to reproduce the low $\Delta^{13}C$ of c. 17‰ at the $c_i/c_a$ shown. In spruce, the data were also linearly correlated and in accordance with the model at standard parameterization ($b = 27$, Fig. 2b).

In juvenile beech, $g_l$ correlated negatively with $\delta^{18}O_{cel}$ (Fig. 3a) but not with $\delta^{18}O_{LOM}$ (data not shown). Beech saplings grown in mixture with spruce, and particularly under enhanced CO$_2$ or O$_3$ concentrations, had reduced $g_l$ and, thus, their $\delta^{18}O_{cel}$ increased. The highest $g_l$ in beech was measured under the gaseous control in monoculture. The model output (Eqn 5) fits well with the observed values if one assumes that leaf temperature increased above air temperature by 2 and 4K at high and low $g_l$ (i.e. 120 and 40 mmol m$^{-2}$ s$^{-1}$), respectively. In general, $g_l$ and $\delta^{18}O_{cel}$ obtained from juvenile spruce trees were less affected by the gaseous treatments and types of competition than in beech (Fig. 3b). As $g_l$ of spruce varied between 85 and 130 mmol m$^{-2}$ s$^{-1}$, the $\delta^{18}O_{cel}$ fell between 30.0 and 31.5‰. In spruce, a significant negative correlation was found only when one outlier (monoculture under +O$_3$: closed triangle) was excluded from the analysis (Fig. 3b, $r^2 = 0.67$, $P = 0.024$).

At an increased leaf temperature of c. 3K above air temperature, the model output (Eqn 5) fits well with the observed values.

Discrimination against $^{13}C$ ($\Delta^{13}C$) was then related to $\delta^{18}O_{cel}$ so as to evaluate the effects of the gaseous treatments and types of competition on tree performance in the phytotron experiment (Fig. 4). In beech trees grown under elevated O$_3$, $\Delta^{13}C$ was significantly reduced by up to 2.5‰ (main effect, $P < 0.003$, Fig. 4a), while $\delta^{18}O_{cel}$ increased (in particular, under +O$_3$). Plant response to elevated O$_3$ can be visualized in the uppermost right-hand insert ‘O$_3$’ in Fig. 4a with the arrow pointing up and to the right reflecting the O$_3$-induced shift in $\Delta^{13}C$ and $\delta^{18}O_{cel}$. Conversely, elevated CO$_2$ led to an increase in $\Delta^{13}C$ by up to 4.0‰ (main effect, $P < 0.001$), while $\delta^{18}O_{cel}$ remained largely unchanged (see right-hand ‘CO$_2$’ insert with downward arrow). When concentrations of both gases were increased (+CO$_2$/+O$_3$), no significant change in the isotope ratios compared with the gaseous control treatment were found in beech leaves. $\Delta^{13}C$ and $\delta^{18}O_{cel}$ were most strongly affected by the different types of competition. In mixed culture, beech showed a decrease in $\Delta^{13}C$ by up to 4.5‰, while $\delta^{18}O_{cel}$ was increased by up to 2.0‰ compared with beech grown in the corresponding gaseous treatments in monoculture ($P < 0.001$ for both isotopes, visualized in the uppermost right-hand insert ‘Comp’ by the arrow pointing upward-right). In the less responsive species,
spruce, only elevated CO₂ affected the isotope ratios of C and O, increasing both $\Delta^{13}C$ and $\delta^{18}O_{cel}$ ($P<0.001$ and $P<0.01$, respectively), as visualized by the arrow pointing downward-right in the ‘CO₂’ insert of Fig. 4b.

Crown position had a marked effect (main effect, $P<0.001$) on $\delta^{13}C_{LOM}$ from adult beech trees growing in Kranzberg Forest at the four sampling dates during the growing season of 2001 (Fig. 5a). The highest $\delta^{13}C_{LOM}$ occurred in the sunlit leaves (approximately $-28.0\%$) and lowest in the lowermost and shaded crown parts (approximately $-32.2\%$, Fig. 5a). At all crown positions, $\delta^{13}C_{LOM}$ decreased slightly throughout the growing season. No significant effect of elevated O₃ concentrations was found. Needles of adult spruce trees had changes in $\delta^{13}C_{LOM}$ of similar spatio-temporal pattern as encountered in beech (Fig. 5b). However, the effect by crown position was only marginally significant ($P=0.051$). The elevated O₃ treatment tended to increase $\delta^{13}C_{LOM}$ in spruce needles ($P=0.085$), in particular, early in the season.

Cellulose was extracted from leaves sampled at the end of September, analysed for $\delta^{18}O$ and plotted in relation to $\delta^{13}C_{LOM}$ (Fig. 6). In addition to the mentioned decline of $\delta^{13}C_{LOM}$ with decreasing light level (see also insert in Fig. 6a), $\delta^{18}O_{cel}$ in beech leaves was also significantly affected by crown position ($P<0.001$, Fig. 6a). However, this response was complex, because $\delta^{18}O_{cel}$ increased from shade to half-shade crown position, but decreased from half-shade to sun-lit positions. In contrast, $\delta^{18}O_{cel}$ of spruce needles was not affected by canopy position. Overall, no significant O₃ effect was found on $\delta^{13}C_{LOM}$ and $\delta^{18}O_{cel}$ of leaves from adult beech and spruce trees at the end of the growing season.
$\delta^{13}$C of hot-water extractable carbohydrates ($\delta^{13}$CHWC) were analysed in leaves from adult trees sampled on 26 July and 1 August, 2001, two days with high O3 concentrations, to investigate short-term O3 effects on $\delta^{13}$CHWC (Table 1).

Crown position significantly influenced the $\delta^{13}$CHWC for both species ($P < 0.001$ and $P = 0.027$ in beech and spruce, respectively). No significant ozone effect was found however in $\delta^{13}$CHWC of either species. In general, findings in $\delta^{13}$CHWC were similar to $\delta^{13}$CLOM (cf. Fig. 5).

**DISCUSSION**

Stable isotopes are well-established integrators of physiological plant responses to abiotic and biotic factors (Dawson et al. 2002). In this regard, the proportion of $^{13}$C to $^{12}$C in plant organic matter (i.e. $\delta^{13}$Cp) is known to be negatively correlated with the leaf internal to external CO2 concentration ($c_i/c_a$) (Farquhar et al., 1982) and has been used in numerous investigations of crop and wild plant species (e.g. Farquhar et al. 1989a; Dawson et al. 2002). More recently, several authors reported that $\delta^{18}$OLOM can serve as a time-integrated estimate of relative humidity (Yakir 1992; Roden & Ehleringer 1999) or $g_l$ (Barbour & Farquhar 2000; Barbour et al. 2000; Siegwolf et al. 2001) although the nature of the latter correlation is currently under debate (Sheshshayee et al. 2005; Farquhar, Cernusak & Barnes 2007). In the data presented here, variation in transpiration is under control of stomatal aperture and not driven by variation in evaporative demand. In such a case, theory predicts reductions in $g_l$ to be associated with increasing $\Delta^{18}$O (Farquhar et al. 2007); this prediction is supported by our data (Fig. 3).

Changes in $c_i$ (and therefore in $c_i/c_a$) typically result from variation in stomatal aperture and thereby impact CO2 diffusion into the leaf, changes to the CO2 demand by chloroplasts the leaf mesophyll (i.e. photosynthetic capacity, $A_{max}$) or some proportion of both. The combination of C and O isotope analysis can indicate if observed changes in $\delta^{13}$CLOM were caused by modified stomatal aperture and/or $A_{max}$ (Farquhar et al. 1989b; Sternberg et al. 1989; Yakir & Israeli 1995; Saurer et al. 1997). To this end, Scheidegger et al. (2000) introduced a conceptual model to infer photosynthetic performance of herbaceous plants from $\delta^{13}$CLOM and $\delta^{18}$OLOM. They estimated changes in relative humidity from...
Figure 6. Correlations between $\delta^{13}C$ of LOM ($\delta^{13}C_{LOM}$) and $\delta^{18}O$ in cellulose ($\delta^{18}O_{cel}$) of leaves harvested in late September from adult beech (a) and spruce (b) trees at Kranzberg Forest. Open symbols represent sunlit, gray intermediate (half-shaded) and black shaded leaves. Circles and triangles denote ambient and twice-ambient O3 concentrations, respectively. The insert indicates a significant main effect by decreasing light level. Data are means ± SE ($n = 5$).

$\delta^{18}O_{LOM}$ and used this information to predict changes that would have likely occurred in $g_l$. In the expansion of the original Scheidegger-model presented here (see Fig. 7), we infer $g_l$ directly from $\delta^{18}O_{cel}$ bypassing the previous used relative humidity/$\delta^{18}O_{LOM}$ relationship. The applied correlation between $g_l$ and $\delta^{18}O$ of leaf material has been presented previously by Barbour et al. (2000) as well as others cited above and was confirmed in our study as shown in Fig. 3. A prerequisite for using this relationship is establishing similar $\delta^{18}O$ among the study plants and their exposure to similar $\delta^{18}O$. This can be accomplished very well in phytotrons or even in the field if the trees form joined canopies or live in close proximity. Otherwise, $\delta^{18}O$, and $\delta^{14}O$, may vary between plants, and thus, would need to be corrected for in applying the model (e.g. by determination of $\delta^{18}O$, in xylem water of individual plants). In addition to changes in $g_l$, a potential change in $c_i/c_a$ is derived from $\Delta^{13}C$ or $\delta^{13}C_{LOM}$ (Fig. 2). Combining information on $g_l$ and $c_i/c_a$ therefore allows one to draw conclusions about $A_{max}$ as shown in Fig. 7. For example, in scenario ‘A’ in Fig. 7, $\delta^{18}O$ and thus $g_l$ remains unchanged (see ‘o’ in the $g_l$ row), and therefore a reduction in $c_i/c_a$ (indicated by ‘–’ in the $c_i/c_a$ row) results from increasing $A_{max}$ (indicated by ‘+’ in the $A_{max}$ row). The resulting model output is an upward arrow, indicating a higher $A_{max}$, while $g_l$ remains unchanged. Following this rationale, all possible $\delta^{18}C/\delta^{18}O$ combinations (scenarios shown in boxes A to H in the top row of the figure) are exemplified and converted to changes in $A_{max}$ versus $g_l$ (very bottom row of Fig. 7).

To predict $c_i/c_a$, we relied on $\Delta^{13}C$ calculated from $\delta^{18}C_{cel}$ (Fig. 2) or on $\delta^{13}C_{LOM}$ on the basis that a satisfactory correlation between $\delta^{13}C_{LOM}$ and $\delta^{13}C_{cel}$ could be established (Fig. 1a,b). However, a reliable correlation between $\delta^{18}O_{LOM}$ and $\delta^{18}O_{cel}$ was not found (Fig. 1c,d). Although earlier studies had presented such correlations for herbaceous and other woody plants (Barbour et al. 2000;

\[ \delta^{18}O_{LOM} \] and $\delta^{18}O_{cel}$ combinations are given in the boxes at the top of the figure as scenarios A to H (model input). Arrows represent changes between two treatments (e.g. increase of CO2 from ambient to elevated concentrations). Stomatal conductance ($g_l$) is derived directly from $\delta^{18}O_{cel}$ (see Fig. 3) and $c_i/c_a$ from $\delta^{13}C_{cel}$ (see Fig. 2); ‘+’, ‘o’ and ‘–’ represent increase, no response or decrease in levels, respectively, of $g_l$, $c_i/c_a$ and $A_{max}$. Information derived from $\delta^{18}O_{LOM}$ and $\delta^{13}C_{LOM}$ about $g_l$ and $c_i/c_a$ lead to subsequent interpretation of photosynthetic capacity ($A_{max}$). The model output in the bottom line of boxes gives relative changes of $A_{max}$ versus $g_l$ caused by treatments (e.g., increase of CO2 from ambient to the elevated concentrations). The cases B, D and E are highlighted, as they represent plant responses to treatments applied in this study (cf. Figs 4 & 6).
barbour, Andrews & Farquhar 2001; Cernusak, Pate & Farquhar 2004), our data from central European trees suggest that caution is needed when using $\delta^{18}O_{LOM}$ to infer physiological performance. In contrast to the recent findings shown by Sullivan & Welker (2007), our study was unable to find a reliable correlation between mean seasonal $g_l$ and $\delta^{18}O_{LOM}$. We did, however, find a correlation between $g_l$ and $\delta^{13}C_{CEL}$. This correlation is based on changes in leaf temperature and transpiration-driven Pécelet effect (Cernusak et al. 2003; Farquhar et al. 2007), which are the direct consequences of variation in $g_l$. These findings are substantiated because irrigation water and climatic conditions were identical for all plants (Kozovits et al. 2005a,b). However, a variety of leaf secondary metabolites such as lignin and fatty acids have a lower $\delta^{13}C$ compared with that of cellulose (Gray & Thompson 1977; Bricout 1979; Schmidt, Werner & Rossmann 2001) and may be responsible for the observed differences between the $\delta^{13}C_{CEL}$ and $\delta^{18}O_{LOM}$. On average, $\delta^{18}O_{LOM}$ was lowered by c. 5.5‰ compared with $\delta^{18}O_{CEL}$ (Fig. 1; cf. Barbour & Farquhar 2000; Barbour et al. 2000). In addition, secondary metabolites such as lignin and phenolics may vary in concentration with light levels and plant exposure to elevated $O_3$ (Lange, Lapierre & Sandermann 1995; Sandermann 1996; Zinser, Ernst & Sandermann 1998). Such abiotic interferences may also have contributed to the poor correlation between $\delta^{18}O_{LOM}$ and $\delta^{18}O_{CEL}$ (Fig. 1cd). For these reasons, we relied on using $\delta^{18}O_{CEL}$ instead of $\delta^{18}O_{LOM}$.

In juvenile beech trees, exposure to the enhanced $O_3$ concentrations resulted in an increase in both $\delta^{18}O_{CEL}$ and $\delta^{13}C_{CEL}$ (Fig. 4a), which represents scenario ‘B’ in the extended model proposed in Fig. 7. In this case, the model output predicts a reduction of $g_l$, while $A_{max}$ remains unchanged. This interpretation has been confirmed by assessments of $A_{max}$ using $A/c_i$-curves (Winkler, GSF-National Research Center for Environment and Health, Germany, personal communication) as well as the analysis of $CO_2$ assimilation shown by Kozovits et al. (2005a). However, applying the model described by Eqn 1, one realizes that only about half of the increase in $\delta^{18}O_{CEL}$ is explained by the variation in $c_i/c_a$ (Fig. 2a) with discrepancies being most pronounced under treatments resulting in low $g_l$ (e.g. +$O_3$). With regard to understanding what may have led to $O_3$-induced increase of $\delta^{13}C_{CEL}$, previous studies have reported an up-regulation of the $CO_2$-fixing enzyme PEP-C under enhanced $O_3$ concentrations (cf. Saurer et al. 1995; Landolt et al. 1997), which reduces total net fractionation by the leaf (parameter $b$ in Eqn 1) because of the lower $\Delta^{13}C$ of PEP-C (Farquhar et al. 1989a). Hence, increased PEP-C activity might explain, at least partially, the low $\Delta^{13}C$ at narrowed stomatal aperture (e.g. under +$O_3$, Fig. 2a). In addition, other factors such as a lowered leaf internal conductance ($g_i$) might have contributed to the low net fractionation by the leaf (value of 21 for the parameter $b$, cf. Brugnoli & Farquhar 2000). However, a recent study showed $g_l$ to be unaffected by enhanced $O_3$ concentrations (Warren et al. 2007), and therefore it appears unlikely that changes in $g_l$ are involved. Overall, the application of Eqn 1 to the data shown in Fig. 2a demonstrates the additional potential of a quantitative modelling approach compared with a semi-quantitative assessment to provide further mechanistic insight. Likewise, in Fig. 3, variability in leaf temperature was indicated by the application of the model Eqn 5. On the other hand, those modelling approaches require additional data, such as relative air humidity, air temperature, isotopic signatures of air and water, etc. which might not always be readily accessible. In those cases, the application of the semi-quantitative approach of Fig. 7 appears most rewarding.

Both species responded to enhanced $CO_2$ concentrations in a similar way with decreasing $\delta^{13}C_{CEL}$ and increase in spruce trees in combination with increasing $\delta^{18}O_{CEL}$. This corresponds to scenarios ‘D’ and ‘E’ in our model for spruce and beech, respectively (Fig. 7). In both cases, the model output predicts a reduction in the photosynthetic capacity ($A_{max}$), a common response for trees when exposed to enhanced $CO_2$ concentrations (Curtis 1996), and is confirmed by previous findings for both juvenile beech and spruce trees grown under similar conditions (Lippert et al. 1997; Grams et al. 1999). Interestingly, the strongest effect we observed was caused by interspecific competition. Both $\delta^{13}C_{CEL}$ and $\delta^{18}O_{CEL}$ of beech were increased in mixed culture, whereas these isotope ratios remained unchanged in spruce grown in mixed culture compared with monoculture. The response of beech trees to competition with spruce represents case ‘B’ in the model (Fig. 7), which results in a reduced $g_l$ (cf. Fig. 3). Such a competitive effect we interpret as being caused by water limitation in beech brought about by the interspecific resource competition with spruce, which is currently under investigation. In addition, this result reflects the higher overall responsiveness of juvenile beech compared with juvenile spruce to the competition treatments used (Grams et al. 2002; Grams and Andersen 2007; Luedemann et al. 2005). In the field, the model reflected the well-documented natural gradient in $c_i/c_a$ with canopy height and light level in both species (Waring & Silvester 1994; Buchmann, Brooks & Ehleringer 2002). Effects of $O_3$ on adult trees were less clear, which is in accordance with recent reports on lower $O_3$ susceptibility of adult trees relative to juvenile beech and spruce trees grown in phytotrons (Wieser et al. 2002; Nunn et al. 2005).

In conclusion, the correlation between average $g_l$ and $\delta^{18}O$ from leaf cellulose ($\delta^{18}O_{CEL}$) as presented here (Fig. 3) and by others (Barbour & Farquhar 2000; Barbour et al. 2000; Siegwolf et al. 2001) permits us to extend the model originally proposed by Scheidegger et al. (2000) and directly estimate changes in $g_l$ from $\delta^{18}O_{CEL}$. The $\delta^{18}O_{CEL}$ data, when coupled with $\delta^{13}C_{CEL}$, can be used further to infer how $A_{max}$ in turn has responded in trees of different sizes and in relation to competition, $O_3$ and $CO_2$ treatments. In view of the analyses presented here for trees grown under controlled chamber and field scenarios, the extended semi-quantitative model proposed here offers a straightforward approach for gathering time-integrated information on the photosynthetic and stomatal performance for plants experiencing a host of different growing conditions. The application of the
semi-quantitative model proposed here (Fig. 7) appears to be most revealing if data needed for a fully quantitative modelling approach are not available. The combined analysis of δ13C and δ18O in leaf material provides a robust way to investigate the impacts of elevated CO2 and O3 concentrations, light limitation and competition on trees, and advocates its use also in other multi-faceted ecophysiological investigations.

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