Variations in $^{13}$C discrimination during CO$_2$ exchange by Picea sitchensis branches in the field

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ABSTRACT

We report diurnal variations in $^{13}$C discrimination ($^{13}$A) of Picea sitchensis (Bong.) Carr. branches measured in the field using a branch chamber technique. The observations were compared to predicted $^{13}$A based on concurrent measurements of branch gas exchange. Observed $^{13}$A values were described well by the classical model of $^{13}$A including isotope effects during photorespiration, day respiration and CO$_2$ transfer through a series of resistances to the sites of carboxylation. A simplified linear of model $^{13}$A did not capture the observed diurnal variability. At dawn and dusk, we measured very high $^{13}$A values that were not predicted by either of the said models. Exploring the sensitivity of $^{13}$A to possible respiratory isotope effects, we conclude that isotopic disequilibria between the gross fluxes of photosynthesis and day respiration can explain the high observed $^{13}$A values during net photosynthetic gas exchange. Based on the classical model, a revised formulation incorporating an isotopically distinct substrate for day respiration was able to account well for the high observed dawn and dusk $^{13}$A values.

Key-words: branch chambers; carbon isotopes; day respiration; photosynthetic discrimination.

INTRODUCTION

Nearly 30% of the entire atmospheric pool of CO$_2$ (750 Gt) is exchanged across leaf surfaces each year. The $^{8}$C enrichment that accompanies the summer drawdown of CO$_2$ in the atmosphere can be ascribed to photosynthetic discrimination against $^{13}$CO$_2$ by the terrestrial biosphere. Globally, photosynthetic uptake of CO$_2$ discriminates by 15 to 18‰ against $^{12}$C (weighted mean of C$_3$ and C$_4$ vegetation, Lloyd & Farquhar 1994). For plants with the C$_3$ photosynthetic pathway, the photosynthetic discrimination against $^{12}$C ($^{13}$A) has been related to the lower reactivity of $^{13}$C during fixation by photosynthetic enzymes, as well as to differential diffusivities of $^{13}$CO$_2$ and $^{12}$CO$_2$ in air (Farquhar, O’Leary & Berry 1982; O’Leary 1984). The magnitude of photosynthetic $^{13}$C discrimination is sensitive to environmental variables such as vapour pressure deficit, photon flux density and air temperature. The $^{8}$C composition of CO$_2$ thus provides a natural tracer for component processes and their regulation by environmental conditions.

The $^{8}$C signature of photosynthetic fluxes often responds differently to changes in environmental conditions than that of concurrent plant and soil respiratory fluxes, creating transient isotopic disequilibria (Lloyd et al. 1996). This is employed at the ecosystem and global scales to interpret field data and to simulate isotopic gas exchange between the terrestrial biosphere and the atmosphere with numerical models. For example, time series of the $^{8}$C of atmospheric CO$_2$ are tools in inversion studies for tracking changes in CO$_2$ fluxes of the terrestrial biosphere and ocean in response to climate variability (Tans, Berry & Keeling 1993; Francey et al. 1995). Plant physiology models incorporating carbon isotope ratios are important for predicting the influence of different biomes on the global patterns of $^{8}$C in atmospheric CO$_2$, providing a tool for constraining large-scale fluxes (Lloyd & Farquhar 1994; Randerson et al. 2002). At the ecosystem scale, partitioning may be possible at sites measuring eddy flux and CO$_2$ concentration profiles with observations of the $^{8}$C composition of CO$_2$ (Lloyd et al. 1996; Yakir & Wang 1996; Bowling, Tans & Monson 2001; Ogée et al. 2003, 2004).

But field measurements of photosynthetic $^{13}$C discrimination have only been reported for a few ecosystems (Harwood 1997; Harwood et al. 1998). Thus, a gap exists between studies of photosynthetic $^{13}$C discrimination at the leaf scale (generally conducted under steady-state conditions in the laboratory) on the one hand, and the interpretation of field data and modelling at the ecosystem and global scale on the other. We try to link these scales by exploring the natural variability of $^{13}$C discrimination at the largest scale possible for direct measurements: whole branches enclosed in chambers. Through this choice of scale and method of observation, we have a useful platform for making observations under field conditions relevant for the

Here, we present daily variations in environmental conditions, CO₂ and water fluxes of branches in a Picea sitchensis (Bong.) Carr. plantation in central Scotland alongside concurrent measurements of discrimination against ¹³C (Δ₁₃) in the field. This paper demonstrates how trace gas measurements during 5 min closure intervals of branch chambers can be used to: (1) obtain diurnal data sets of Δ₁₃ from flask observations in the field; (2) derive estimates of Δ¹³C discrimination from gas-exchange and microclimate data that are directly comparable to the flask observations; and (3) derive Δ¹³Δ estimates that correspond to those expected in the absence of chambers. We also explore the sensitivity of net Δ¹³C discrimination to isotope effects during internal CO₂ transfer, photorespiration and day respiration.

MATERIALS AND METHODS

Study site and gas-exchange measurements

The study was made in Griffin Forest, an even-aged plantation of Queen Charlotte Islands provenance P. sitchensis (Bong.) Carr. located near Aberfeldy, Perthshire, UK (56°37′N, 3°48′W). A description of the site, a long-term monitoring station within the CARBOEUROPE-IP network can be found at http://www.carboeurope.org.

Three branch chambers constructed from extruded acrylic and polypropylene film (34 μm thick, ICI Propafilm, Dumfries, UK) were installed on two trees of 13.6 and 9.5 m height within a 0.01 ha plot from 18 May 2001 to 22 July 2001. Bud burst was partially occurring in the canopy in May, while in July shoot expansion was complete. Branches were chosen as representative of sun-lit branches visible from the tower. Two branches were used in the upper canopy at 10.5 m (chamber 1) and 9.4 m (chamber 3) and one in the middle canopy at 8.1 m (chamber 4) in height. During the July campaign, chamber 4 was sealed without a branch inside and designated as a control. Chambers were positioned to the south of the tower to minimize shading effects. They were held in position by metal booms projecting from the canopy access tower and nylon cord, allowing the chambers to move with the branches. The chambers are fully described by Rayment & Jarvis (1999) and Wingate (2003). The experimental set-up has been used to investigate branch O₂/CO₂ exchange and ¹⁸O discrimination (Seibt et al. 2004, 2006). The latter also employed the same simulation approach as the present study.

During the day, the bags were closed for 5 min, after which they were opened and ventilated for 15 min, i.e. measurements were made on each branch every 20 min. At night, they were closed for 10 min and ventilated for 30 min, i.e. the measurement cycle changed to every 40 min. During closure periods, relative humidity (h) and air temperature (Tₐ) were monitored within each bag [Vaisala HMP 35A; Vaisala (UK) Ltd, Cambridge, UK]. Needle temperature (Tₙ) was measured in three locations per chamber (0.2 mm in diameter Cu-Con thermo-junction, referenced to air temperature). Photosynthetic photon flux density (Q) incident upon each branch was measured using a photosynthetic photon flux density sensor (SD101QV; Macam Ltd, Livingston, UK) attached vertically on the branch midway along its length. At the same time, the CO₂ mole fraction of chamber air was monitored with an infrared gas analyser (IRGA) (LI-6262; Li-Cor Inc., Lincoln, NE, USA). Outputs were recorded with a logger (CR10; Campbell Scientific Ltd, Shepshed, UK) that also served to initiate the measurement sequence. The CO₂ concentration in reference bottles of air was calibrated against gas mixtures prepared by mixing pure CO₂ with CO₂-free air using three precision gas mixing pumps (G27/3F, SA18/3F and SA17/3F; H.Wösthoff GmbH, Bochum, Germany). H₂O vapour span was set using a dew point generator (LI-610; Li-Cor Inc.). The chambers were kept open in a continuously ventilated state when not in use.

Rates of net CO₂ assimilation (A, μmol m⁻² s⁻¹), transpiration (E, mol m⁻² s⁻¹) and branch conductances to water vapour and CO₂ (gₑ, gₛ, mol m⁻² s⁻¹) were calculated and integrated over the full chamber closure periods (see Appendix I). To calculate the leaf surface CO₂ mole fraction (Cᵣ), the leaf boundary layer conductance for CO₂ inside the chamber (gₛ) was estimated as 1.5 ± 0.5 mol m⁻² s⁻¹ from energy balance considerations. The intercellular CO₂ mole fraction incorporating ternary effects (Jarman 1974), C₁₈ (μmol mol⁻¹), was calculated following the notation of von Caemmerer & Farquhar (1981) as

\[ C_{18} = \left( \frac{g_e - \frac{E}{2}}{g_s + \frac{E}{2}} \right) \left( C_r - A \right) \tag{1} \]

The subscript g denotes that for a heterogeneous system such an estimate is weighted according to the conductances and not that of A (Farquhar 1989) (see Appendix III).

Collection and analysis of organic material

During the May field campaign, needle and non-green twig samples were collected from the same or adjacent Sitka spruce trees. Samples were taken at the same heights and close to the chamber locations. All samples were stored in glass sample vials, transferred to the laboratory as rapidly as possible and stored at -20 °C until analysis. Samples were dried and ground with a ball mill to a fine homogeneous powder. Subsamples were weighed and their carbon isotope ratios determined on an elemental analyser (NA 1100 CN; CE Instruments, Rodano, Italy), coupled via open split interface to a mass spectrometer (ConFlo III and Delta PlusXL; both Finnigan MAT, Bremen, Germany). SD for dry matter δ¹³C was 0.05‰. The samples were analysed at the Max Planck Institute for Biogeochemistry, Jena, Germany.

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Collection and analysis of flask samples

Pairs of air samples from branch chambers were collected at intervals of approximately 3 h over 24 h in spring (18/19 May) and summer (20 July) of 2001. Air was circulated from the branch chambers through sampling lines into a flask sampling system (separate from that used for gas-exchange measurements), and back into the chamber. Within the sampling system, the air stream was passed through a magnesium perchlorate cylinder to remove water vapour, and pumped through two flasks in series. We used 1 dm$^3$ glass flasks with a valve (Glass Expansion, Melbourne, Australia) on each end, sealed with Teflon perfluorooalkoxy (PFA) O-rings (DuPont Fluoro products, Wilmington, DE, USA), and 1.3 dm$^3$ flasks with two valves (Louwers, Hapert, the Netherlands) on the same end. Flask samples were taken at two points in the sequence of opening and closing of branch chambers. The first flask was collected 3 to 4 min before closure (‘open sample’) and reflects the CO$_2$ mole fraction and isotopic signature of ambient canopy air. The second flask (‘closed sample’) was collected shortly before reopening of the chamber.

The CO$_2$ mole fractions in the air samples were determined using a gas chromatograph (HP 6890; Hewlett Packard, Palo Alto, CA, USA) linked to a methanizer and flame ionization detector. Subsamples of CO$_2$ were then extracted cryogenically from the dry air samples (‘BGC-AirTrap’, Werner, Rothe & Brand 2001), and their isotope fractionation was measured. Additional uncertainties arising from the sampling procedure, applying to samples collected at the end of closure periods, were estimated from control measurements in an empty chamber. They were 1.3 μmol mol$^{-1}$ for CO$_2$ mole fraction and 0.2‰ for $\delta^{13}$C.

Calculations of $\delta^{13}$C signatures of CO$_2$ exchange from flask data

The observed values of $^{13}$C discrimination during photosynthesis, $^{13}\Delta_{obs}$ (‰) of foliage in the closed branch chamber were determined following Guy et al. (1989):

$$^{13}\Delta_{obs} = \frac{\ln \frac{R_{\delta}}{R_{\alpha}} + 1 + \ln \frac{R_{\delta}}{R_{\alpha}}}{\ln C_o/C_e}.$$

where $C_o$ and $C_e$ are the mole fractions (μmol mol$^{-1}$) and $R_{\delta}$ and $R_{\alpha}$ the $^{13}$C/$^{12}$C ratios of CO$_2$ at the beginning and end of closure periods, respectively. These correspond to the samples collected from the open and closed chambers for flask measurements, and to starting time values and those integrated over the closure periods for gas-exchange data (see Appendix I). We use Eqn 2 for both flask observations and $^{13}\Delta$ predictions from gas-exchange data (although not strictly valid as $^{13}\Delta$ is changing during the closure period) because it allows us to directly compare the two approaches. Estimated uncertainties for $^{13}\Delta_{obs}$ were calculated using Gaussian error propagation. They were inversely related to net flux rates, usually < 1‰, but > 5‰ at dawn and dusk. Discrimination values were also largest at these times.

All calculations were performed in interactive data language (IDL) (Version 6.1; Research Systems Inc., Boulder, CO, USA). Correlation parameters between predicted and observed $^{13}\Delta$ were obtained from least absolute deviation regression using the flask observations as independent variable. Four data points were excluded due to lack of reliable gas-exchange measurements (18 May, 0710 h IRGA tubing detached, 0740 h relative humidity constant at 50%, 1740 h sensor artefacts from direct sunlight, 20 July, 0340 h air saturated).

SIMULATIONS

Predictions of $^{13}\Delta$ during foliage gas exchange

We calculated $^{13}\Delta$ values from chamber measurements of environmental conditions, air composition and fluxes (see Appendix I) based on the three equations described next. Summary of symbols and definitions are given in Table 1. The most comprehensive (‘classical’) model of net $^{13}$C discrimination ($^{13}\Delta_{classical}$, ‰) for C$_3$ plants describes our present understanding of fractionation steps during net assimilation of CO$_2$ (Farquhar et al. 1982) as

$$^{13}\Delta_{classical} = a_o C_o - C_e + a C_e - C_i + a_m (C_i - C_o) + b C_i/C_e,$$

where $a_o$ and $a$ are the fractionations during diffusion of CO$_2$ in air through the leaf boundary layer and the stomata (≈ 2.9 and 4.4‰, Craig 1953), and $b$ is the combined fractionation during carboxylation by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phospho-enol-pyruvate carboxylase (PEPc) (assumed to be 29‰, Roeske & O’Leary 1984; Guy et al. 1989; Guy, Fogel & Berry 1993). $a_m$ is the sum of the fractionation factors during internal CO$_2$ transfer, combining an equilibrium fractionation of CO$_2$ entering solution ($e_s = 1.1$‰ at 25 °C, Mook, Bommerston & Staverman 1974) and a diffusional fractionation of dissolved CO$_2$ in water ($a_d = 0.7$‰, O’Leary 1984). The respiratory fractionation factors are $f$ for photorespiration (8 ± 1‰, Rooney 1988; Gillon & Griffiths 1997) and $e$ for day respiration. $R_k$, $C_i$ is the CO$_2$ mole fraction at the sites of carboxylation, $k$ is the carboxylation efficiency and $R_k$ is the CO$_2$ compensation point in the absence of day respiration.
**Table 1. List of symbols used in the text**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>Measured net rate of assimilation</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$A_{\text{max}}$</td>
<td>Fitted maximum assimilation rate</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$a$</td>
<td>$^{13}$C fractionation during diffusion through the stomata</td>
<td>$4.4%$</td>
</tr>
<tr>
<td>$a_b$</td>
<td>$^{13}$C fractionation during diffusion through the boundary layer</td>
<td>$2.9%$</td>
</tr>
<tr>
<td>$a_{\text{d}}$</td>
<td>$^{13}$C fractionation during diffusion through water</td>
<td>$0.7%$</td>
</tr>
<tr>
<td>$a_m$</td>
<td>$^{13}$C fractionation during internal CO$_2$ transfer ($e + a_d$)</td>
<td>$1.8%$</td>
</tr>
<tr>
<td>$b$</td>
<td>Net $^{13}$C fractionation during carboxylation by ribulose 1,5-bisphosphate carboxylase/oxygenase</td>
<td>$29%$</td>
</tr>
<tr>
<td>$b'$</td>
<td>Estimated net $^{13}$C fractionation during carboxylation, internal CO$_2$ transfer and decarboxylation</td>
<td>$27%$</td>
</tr>
<tr>
<td>$C_a$</td>
<td>CO$_2$ mole fraction in ambient air</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_c$</td>
<td>CO$_2$ mole fraction at the sites of carboxylation</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_e$</td>
<td>CO$_2$ mole fraction of chamber air at the end of closure period</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_i$</td>
<td>CO$_2$ mole fraction in the intercellular spaces</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_{i,A}$</td>
<td>Assimilation weighted intercellular CO$_2$ mole fraction</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_{i,g}$</td>
<td>Conductance weighted intercellular CO$_2$ mole fraction</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_o$</td>
<td>CO$_2$ mole fraction of chamber air at the beginning of closure period</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$C_s$</td>
<td>CO$_2$ mole fraction at the leaf surface</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$^{13}A$</td>
<td>Net discrimination against $^{13}$C during photosynthesis</td>
<td></td>
</tr>
<tr>
<td>$A_{\text{obs}}$</td>
<td>Observed net $^{13}$C discrimination (Eqn 2)</td>
<td></td>
</tr>
<tr>
<td>$A_{\text{classical}}$</td>
<td>Predicted net $^{13}$C discrimination (Eqn 3, or eqn B24 of Farquhar et al. 1982)</td>
<td></td>
</tr>
<tr>
<td>$A_{\text{pred}}$</td>
<td>Predicted net $^{13}$C discrimination</td>
<td></td>
</tr>
<tr>
<td>$A_{\text{revised}}$</td>
<td>Predicted net $^{13}$C discrimination (Eqn 4, or eqn 10 of Farquhar et al. 1982)</td>
<td></td>
</tr>
<tr>
<td>$A_{\text{calc}}$</td>
<td>Carbon isotope signature of the respiratory flux (Eqn A2.10) defined as net respiratory discrimination in analogy to photosynthetic gas exchange</td>
<td></td>
</tr>
<tr>
<td>$\delta^{13}C$</td>
<td>Carbon isotope composition ($\delta_{\text{sample}} / \delta_{\text{standard}} - 1$) $\times 1000$</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta^{13}C_a$</td>
<td>Carbon isotope composition of ambient air</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta^{13}C_{\text{assimilates}}$</td>
<td>Carbon isotope composition of assimilates</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta^{13}C_e$</td>
<td>Carbon isotope composition of chamber air at the end of closure period</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta^{13}C_{\text{needle}}$</td>
<td>Carbon isotope composition of bulk needle material</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta^{13}C_{\text{plant}}$</td>
<td>Carbon isotope composition of chamber air at the beginning of closure period</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta^{13}C_{\text{R}}$</td>
<td>Carbon isotope composition of respired CO$_2$</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta^{13}C_{\text{substrate}}$</td>
<td>Carbon isotope composition of substrate for dark respiration</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta^{13}C_{\text{wood}}$</td>
<td>Carbon isotope composition of bulk twig material</td>
<td>$%$</td>
</tr>
<tr>
<td>$D_s$</td>
<td>Vapour mole fraction deficit</td>
<td>mmol mol$^{-1}$</td>
</tr>
<tr>
<td>$E$</td>
<td>Transpiration rate</td>
<td>mmol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$e$</td>
<td>$^{13}$C fractionation during decarboxylation</td>
<td>$0$ or $-6%$</td>
</tr>
<tr>
<td>$e_*$</td>
<td>Apparent discrimination against $^{13}$C during decarboxylation ($= \delta_{\text{substrate}} / \delta_{\text{assimilates}} - 1$)</td>
<td>$%$</td>
</tr>
<tr>
<td>$f$</td>
<td>Discrimination against $^{13}$CO$_2$ during photosynthesis</td>
<td>$8%$</td>
</tr>
<tr>
<td>$g$</td>
<td>Total branch conductance to water vapour</td>
<td>mmol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$g_b$</td>
<td>Branch boundary layer conductance for CO2</td>
<td>$1.1$ mmol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$g_i$</td>
<td>Branch conductance to CO$_2$</td>
<td>mmol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$g_o$</td>
<td>Internal transfer conductance to CO$_2$</td>
<td>$0.16$ mmol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$g_s$</td>
<td>Branch conductance to water vapour</td>
<td>mmol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$T_p$</td>
<td>Temperature dependent CO$_2$ compensation point (Brooks &amp; Farquhar 1985)</td>
<td>$\mu$mol mol$^{-1}$</td>
</tr>
<tr>
<td>$h$</td>
<td>Relative humidity</td>
<td>$%$</td>
</tr>
<tr>
<td>$K$</td>
<td>Carboxylation efficiency</td>
<td>mmol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$L_A$</td>
<td>Projected needle area</td>
<td>m$^2$</td>
</tr>
<tr>
<td>$P$</td>
<td>Atmospheric pressure</td>
<td>Pa</td>
</tr>
<tr>
<td>$Q$</td>
<td>Photosynthetic photon flux density</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$R_d$</td>
<td>Measured rate of dark respiration</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$R_{d,s}$</td>
<td>Estimated rate of day respiration, $T$ dependent</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$R_{d,i}$</td>
<td>Estimated rate of day respiration, $T$ and $Q$ dependent</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$R_{20}$</td>
<td>Fitted rate of dark respiration at 20 °C</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$\delta_{\text{ambient}}$</td>
<td>Isotope ($^{13}$C) ratio of ambient CO$_2$</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta_{\text{assimilates}}$</td>
<td>Isotope ($^{13}$C) ratio of assimilates</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta_{\text{chamber}}$</td>
<td>Isotope ($^{13}$C) ratio of chamber air at end of closure period</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta_{\text{plant}}$</td>
<td>Isotope ($^{13}$C) ratio of chamber air at beginning of closure period</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta_{\text{standard}}$</td>
<td>Isotope ($^{13}$C) ratio of V–PDB–CO$_2$ standard</td>
<td>$%$</td>
</tr>
<tr>
<td>$\delta_{\text{substrate}}$</td>
<td>Isotope ($^{13}$C) ratio of substrate used for dark respiration</td>
<td>$%$</td>
</tr>
<tr>
<td>$T_a$</td>
<td>Air temperature</td>
<td>°C</td>
</tr>
<tr>
<td>$W_a$</td>
<td>Air vapour mole fraction</td>
<td>mmol mol$^{-1}$</td>
</tr>
<tr>
<td>$W_{a0}$</td>
<td>Initial air vapour mole fraction in chamber at the starting time $t_0$</td>
<td>mmol mol$^{-1}$</td>
</tr>
<tr>
<td>$W_{a(corr)}$</td>
<td>Air vapour mole fraction calculated from relative humidity corrected for the return flow of dry air during flask sampling</td>
<td>mmol mol$^{-1}$</td>
</tr>
<tr>
<td>$W_a$</td>
<td>Leaf vapour mole fraction</td>
<td>mmol mol$^{-1}$</td>
</tr>
<tr>
<td>$V_a$</td>
<td>Molar volume of air enclosed in the chamber</td>
<td>mol</td>
</tr>
</tbody>
</table>
The most often applied (‘simple’) model of $^{13}$C discrimination ($^{13}$Δ$_{\text{simple}}$) neglects any effects of photo- and day respiration on net discrimination, but implicitly accounts for transfer conductance by using a lower value for $b$ (Farquhar et al. 1982; Farquhar & Richards 1984):

$$13\Delta_{\text{simple}} = a + (b - a) \frac{C_i}{C_a},$$

(4)

where $b$ is the weighted fractionation during internal transfer of CO$_2$ and fixation by Ru$_2$P and PEP carboxylases (27‰), applied to $C_i$ (instead of $C_a$ as in Eqn 3).

Equation 3 is based on the assumption that recent assimilates form the substrates of photorespiration and day respiration. This is likely to be the case for photorespiration as it recycles freshly assimilated carbon (Fig. 3). However, day respiration may also utilize other substrates, such as older carbohydrates (Schnyder et al. 2003; Noguès et al. 2004). If the $\delta^{13}$C composition of these substrates is not the same as that of freshly assimilated carbon, the respiratory $\delta^{13}$C signature would differ from instantaneous $\Delta^{13}$A by more than the fractionation factor ($e$). To take this effect into account, we incorporate an apparent fractionation for day respiration, $e^*$, defined as $e^* = R_{\text{assimulate}}/R_{\text{substrate}} - 1$, expressing the difference between the isotopic composition of the respiratory substrate ($\delta^{13}$C$_{\text{substrate}}$) and photosynthetic assimilates ($\delta^{13}$C$_{\text{assimilate}}$ $\approx$ $\delta^{13}$C$_a$) at a given time, i.e. $e^* = \delta^{13}$C$_a - \delta^{13}$C$_{\text{substrate}}$. This leads to the ‘revised’ model, a modified version of the classical model (Eqn 3, omitting boundary layer effects for clarity):

$$13\Delta_{\text{revised}} = \frac{a + (a_n - d) \frac{C_i}{C_a} + (b - a_n) \frac{C_i}{C_a} - f \frac{\Gamma_n}{C_i} - \frac{(e + \delta^{13}C_a - \delta^{13}C_{\text{substrate}}) R_3}{A + R_3} C_1 - \frac{\Gamma_n}{C_i}}{1 - \frac{R_3}{A + R_3} C_i}$$

(5)

Alternatively, Eqn 6 retains the apparent factor $e^*$ so that it can be easily compared to Eqn 5:

$$13\Delta_{\text{revised}} = a_d \frac{C_a - C_e}{C_a} + \frac{C_i - C_a}{C_a} + a_m \frac{C_i - C_a}{C_a} + b \frac{C_i}{C_a} - f \frac{\Gamma_n}{C_i} - \frac{(e + e^*) R_3}{kC_i}$$

(6)

The derivation of Eqns 5 and 6 are given in Appendix II (note that the $\Delta^{13}$ term of $e^*$ is included in the left hand side of Eqn 5). When $\delta^{13}$C$_{\text{substrate}}$ $\approx$ $\delta^{13}$C$_{\text{assimilate}}$ (i.e. $e^* = 0$), Eqn 5 is again the classical Farquhar et al. (1982) description (Eqn 3).

In the following, we use the time series from the July campaign as an example to illustrate the different model predictions. Using Eqns 3, 4 and 5, $\Delta$ values were calculated and integrated over the chamber closure periods as described in Appendix I. Parameters required for the models are $\delta^{13}$C$_a$ from flask observations (i.e. $\delta^{13}$C$_o$), $g_a$, $g_c$ and $C_a$ from chamber measurements (see Methods), fractionation factors ($a_b$, $a_a$, $a_m$, $b$, $f$, $e$), and $g_e$, $\Gamma_n$, $R_3$ and $\delta^{13}$C$_{\text{substrate}}$. The internal transfer conductance to CO$_2$ ($g_e$) was estimated from a linear regression of the difference between predicted $\Delta^{13}$ (Eqn 5 with infinite $g_i$) and $\Delta^{13}$ against $A/C_i$ (von Caemmerer & Evans 1991) using data from both months (not shown). The value obtained ($g_i$ = 0.16 mol m$^{-2}$ s$^{-1}$) was consistent with the relationship $g_i$ = 0.019 $\cdot$ $\Delta_{\text{max}}$ (Warren et al. 2003) and $\Delta_{\text{max}}$ of 8.0 ± 1.5 µmol m$^{-2}$ s$^{-1}$ (obtained by fitting a non-rectangular hyperbola to the light response of branches).

The CO$_2$ compensation point in the absence of dark respiration, $\Gamma_n$ (µmol mol$^{-1}$), was calculated from needle temperature (Farquhar & von Caemmerer 1982; Brooks & Farquhar 1985). Mitochondrial respiration associated with the tricarboxylic acid (TCA) cycle continues in the light, although it does not necessarily proceed at the same rate as that observed in the dark (Villar, Held & Merino 1994; Atkin et al. 2000; Tcherkez et al. 2005). To account for the potential range of day respiration rates, we calculated temperature-dependent rates ($R_d$) using $R_d$ derived from nocturnal measurements (see Results section) and observed needle temperature, and based on these, light inhibited rates ($R_d$) as described by Lloyd et al. (1995). Day respiration was estimated to release 15% of gross photosynthetic CO$_2$ uptake during the day (only 4% for light-inhibited rate). During mornings and evenings, $R_a$ and $R_d$ contributions were much larger (30 to 60%) while photorespiration was small (5 to 7%). Values of $\delta^{13}$C$_R$ measured for nocturnal respiration (Table 2) were used as estimates of $\delta^{13}$C$_{\text{substrate}}$ of day respiration in Eqn 5. We assumed that either 60 or 100% of day respiration was fed from this older substrate pool, with the remainder from newly assimilated carbon (Schnyder et al. 2003; Noguès et al. 2004). We used values of 0 and −6% for fractionation during day respiration ($e$) in Eqns 3 and 5 (see Discussion for a more detailed sensitivity analysis).

**RESULTS**

**Diurnal patterns of branch microclimate and gas exchange**

Daily time courses for environmental variables within branch chambers (at the beginning of chamber closure periods) are shown in Fig. 1. During May, direct beam irradiance dominated in the upper canopy, but in July, diffuse irradiance was persistent as indicated by low $Q$ in the upper canopy (Fig. 1a,g). Marked diurnal patterns in air temperature ($T_a$) and vapour mole fraction deficit ($D_v$) were observed during both field campaigns (Fig. 1b,c,h,i). Temperatures were similar for both upper canopy chambers (1 and 3) during May and July, reaching 15 to 25 °C over the midday period (1000–1400 h), while $T_a$ was lower in the middle canopy chamber (4) in May, ranging between 10 to 15 °C. Air vapour mole fraction deficits in May were higher than the values observed in the same chambers during July.
et al. 13C of organic material collected in May 2001 at Griffin Forest, alongside the observed 13CR signature of nocturnal branch respiration derived from mass balance, the

Table 2. Assimilation-weighted composition of photoassimilates for the simple (Eqn 4), classical (Eqn 3) and revised models (Eqn 5) including the best fit parameter set for all branch chambers on 19 May and 20 July 2001.

<table>
<thead>
<tr>
<th>Sampling campaign</th>
<th>Canopy Location</th>
<th>Chamber</th>
<th>Assimilation-weighted composition of photoassimilates (‰)</th>
<th>Estimated uncertainties for δ13C, conductance-weighted and δ13A2 (‰)</th>
<th>δ13Cassimilates and δ13A2 (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 May, Upper 1</td>
<td>Midday</td>
<td>1</td>
<td>30.1 ± 0.3 (4)</td>
<td>2.7 ± 0.6 (4)</td>
<td>27 ± 4.7 (9)</td>
</tr>
<tr>
<td>19 May, Middle</td>
<td>Noon</td>
<td>4</td>
<td>30.4 ± 0.3 (4)</td>
<td>2.7 ± 0.6 (4)</td>
<td>27 ± 4.7 (9)</td>
</tr>
<tr>
<td>19 May, Lower</td>
<td>Sunrise</td>
<td>1</td>
<td>29.0 ± 0.1 (4)</td>
<td>2.7 ± 0.6 (4)</td>
<td>27 ± 4.7 (9)</td>
</tr>
<tr>
<td>19 May, Dusk</td>
<td>Sunset</td>
<td>3</td>
<td>29.0 ± 0.2 (4)</td>
<td>2.7 ± 0.6 (4)</td>
<td>27 ± 4.7 (9)</td>
</tr>
</tbody>
</table>

Daily time courses of gas exchange (beginning of closure periods) are also shown in Fig. 1. During May, assimilation rates (A) were highest before midday when Q was high and D was low, and were greater than in July (11 and 8 µmol m⁻² s⁻¹, respectively, Fig. 1d,j). From 0900 to 1040 h and 1230 to 1500 h on 18 May 2001, technical difficulties in chambers 1 and 3 prevented determination of A, although all other measurements remained unaffected.

Values of branch conductance to water vapour (g) ranged between 100 and 250 mmol m⁻² s⁻¹ in the middle canopy and 30 to 170 mmol m⁻² s⁻¹ in the upper canopy during May (Fig. 1e), but values were much lower in July, between 50 and 150 mmol m⁻² s⁻¹ (Fig. 1k). Maximum g values were observed in the morning, subsequently declining over the day. The ratio of intercellular to ambient CO₂ mole fraction (Ci/Ca, conductance-weighted) during May remained between 0.4 to 0.8 for the upper canopy and 0.7 to 0.9 for the middle canopy (Fig. 1f). During July, Ci/Ca was stable over most of the day and ranged between 0.5 and 0.8 for both upper branch chambers (Fig. 1l). However, as Q approached 0 during dawn and dusk, Ci/Ca was typically 0.8 to 1. At dawn, the large Ci/Ca occurred when values of A were low but g, high. At dusk, high Ci/Ca occurred primarily because of low A.

Nocturnal branch gas-exchange data for each chamber (Fig. 1d,j) were fitted with an Arrhenius type ‘activation energy’ response to needle temperature (e.g. Lloyd & Taylor 1994). The derived parameter, Rs, (efflux rate at 20 °C) ranged from 2.5 to 3.3 µmol m⁻² s⁻¹ in May, and 1.3 to 1.4 µmol m⁻² s⁻¹ in July. Nocturnal branch respiration rates during May 2001 were larger than those observed in July 2001, even though temperatures were similar during the night.

Diurnal 13C discrimination

We determined 22 values of 13C discrimination during photosynthesis, 13Δobs, from flask sample pairs (Eqn 2). During both field campaigns, we found a pronounced systematic diurnal variability in 13Δobs (Fig. 2). Highest 13Δobs occurred at dawn and dusk, ranging between 30 to 35‰. Around midday, 13Δobs were lowest at around 15‰. The middle canopy showed systematically higher 13Δobs than the upper canopy at similar times of the day. The highest daytime value (41‰) was measured in the mid canopy at noon in May. This and the high dusk and dawn values coincided with low net carbon and water vapour fluxes (Fig. 1). We also determined seven values for isotopic signatures of nocturnal foliage respiration, δ13CR, from mass balances using flask samples pairs [δ13CR = (δ13CIEC - δ13CCE) / (CE - CA)]. Values of δ13CR in the upper canopy were more negative than in the middle canopy (Fig. 2). The latter was associated with a large uncertainty. For the same branch, δ13CR differed by nearly 2‰ between May and July. A relatively enriched δ13CR value of ~19‰ was measured shortly before dawn during the July field campaign (Fig. 2b).
Predictions of $^{13}\Delta$ during foliage gas exchange

Photosynthetic $^{13}$C discrimination calculated using the different models (Eqns 3–5) is presented in Fig. 4c together with the observations. As $^{13}\Delta_{\text{simple}}$ is linearly dependent on $C_i/C_a$ and constrained by $a$ and $b$ (Eqn 4), the largest values predicted at dusk and dawn were 27‰, with most values between 16 and 22‰ for the rest of the day. In the afternoon, $^{13}\Delta_{\text{simple}}$ was close to $^{13}\Delta_{\text{obs}}$, indicating that during this period the actual drop between $C_i$ and $C_c$ may have corresponded to that implicitly included in $b$ of Eqn 4. But at dusk and dawn, the observations exceeded the $^{13}\Delta_{\text{simple}}$ predictions consistently by up to 7‰ in both May (not shown) and July (Fig. 4c). Including additional effects in $^{13}\Delta_{\text{classical}}$ (Eqn 3), in particular those of internal CO$_2$ transfer when $A$ was large during the day, improved the fit between predictions and observations (Fig. 4c). The contribution of photorespiration to net $^{13}\Delta$ values was generally less than 1‰, smaller than the effect including the internal conductance term, $g_i$ ($\approx$ 2‰). Using the largest negative fractionation reported for dark respiration so far (–6‰, Duranceau et al. 1999), the resulting contribution of
day respiration increased $^{13}\Delta_{\text{classical}}$ considerably but did not fully reconcile offsets between $^{13}\Delta_{\text{classical}}$ and $^{13}\Delta_{\text{obs}}$ at dusk and dawn. To illustrate the isotopic effect of day respiration on net $^{13}\Delta$ from the revised model (Eqn 5), the apparent fractionation factor ($e^*$) is shown in Fig. 4b. During the day, the calculated $\delta^3$C signature of assimilates (e.g. $\delta^3C_{\text{assimilates}} = -25\%$, was close to that of the respiratory substrate ($\delta^3C_{\text{substrate}} = -26\%$, estimated from measurements of dark respiration), yielding small positive $e^*$ values ($\approx 1\%$). Accordingly, day respiration had only minor effects on net $^{13}\Delta_{\text{obs}}$ during most of the day (Fig. 4c). On the other hand, assimilates were substantially depleted ($\approx -36\%$) compared to the respiratory substrate ($-26\%$) at dusk and dawn, yielding large negative $e^*$ values ($\approx -10\%$) at these times. The negative $e^*$ together with high contributions of day respiration resulted in high $^{13}\Delta_{\text{obs}}$ at dusk and dawn comparable to the observations (Fig. 4c).

Values of $^{13}\Delta$ obtained from chamber integrations for the three modelling approaches ($^{13}\Delta_{\text{simple}}$) were compared with flask observations ($^{13}\Delta_{\text{obs}}$). The classical and revised models both improved the correlation between predicted and observed $^{13}\Delta$ values (Table 3). During the day, the classical and revised models predicted almost identical $^{13}\Delta$ values due to the generally small contribution of day respiration. But only the revised formulation showed reasonable agreement with the high $^{13}\Delta_{\text{obs}}$ at dusk and dawn (Fig. 5). Assuming that day respiration was fully supplied by ‘old’ substrates with a $\delta^3$C signature equal to that measured at night, together with a fractionation factor ($e$) of $-6\%$, provided the highest correlation for our data set (Table 3).

### Daily foliage isofluxes and the $\delta^3$C of organic matter

The aforementioned models of $^{13}$C discrimination were applied to diurnal gas-exchange data (20 min time step) for the different branch chambers to yield diurnal variations of $^{13}\Delta$ expected in the absence of chambers. The $\delta^3C_{\text{assimilates}}$ values, used in Eqn 5 and to compute $\delta^3C_{\text{substrate}}$, were derived from a linear regression fitted to open branch chamber flask observations: May: $\delta^3C_{\text{assimilates}} = 8194/C_{\text{assimilates}} - 30.1\%$ ($r^2 = 0.91$, $n = 11$, $P < 0.0001$) and July: $\delta^3C_{\text{assimilates}} = 6846/C_{\text{assimilates}} - 26.5\%$ ($r^2 = 0.98$, $n = 9$, $P < 0.0001$), with daytime $\delta^3C_{\text{assimilates}}$ variations of less than 1‰ for both field campaigns. Assimilation weighted diurnal average $^{13}\Delta$ and the corresponding $\delta^3C_{\text{assimilates}}$ values were then calculated for each
branch chamber and month (Table 2). The simple model predicted a 0.7–2.1‰ higher average $\Delta$ than the classical and revised models, and a correspondingly depleted $\delta^{13}C$ of assimilates of daily assimilated plant material. For all models, $\delta^{13}C$ assimilates were enriched relative to $\delta^{13}C$ needle for chambers 1 and 4, but similar for chamber 3. There were no consistent offsets between $\delta^{13}C$ assimilates and $\delta^{13}C$ signatures of nocturnal respiration. For chamber 1, $\delta^{13}C$ assimilates were enriched relative to $\delta^{13}C$ R, but depleted for chambers 3 and 4.

The $\delta^{13}C$ values of foliage ($\delta^{13}C$ needle) and woody ($\delta^{13}C$ wood) material in the upper canopy were typically enriched by 2 to 3‰ relative to lower canopy values (Table 2). This translates to a vertical gradient of 0.5‰ decrease in $\delta^{13}C$ needle for every meter down the canopy profile ($r^2 = 0.9, n = 17, P < 0.0001$). We found that $\delta^{13}C$ R was similar to $\delta^{13}C$ wood but enriched relative to $\delta^{13}C$ needle (2 to 4‰). This is consistent with many other observations (see Badeck et al. 2005 for review), however, we emphasize that the organic material was not collected from the branches measured for gas exchange, and that large uncertainties were associated with measurements at low flux rates (Table 2).

**DISCUSSION**

Our study aimed to characterize the diurnal patterns of $\Delta_{\text{obs}}$ during photosynthesis of *P. sitchensis* branches in the field (Fig. 2), and to explore how important internal CO2 transfer, photorespiration and day respiration (see Fig. 3) were in shaping these patterns (Fig. 4). We measured $\Delta_{\text{obs}}$ over several diurnal periods on whole branches of *P. sitchensis*. We found a range of 15 to 41‰, comparable to that reported for a leaf scale study on *Piper aduncum* in a tropical forest of Trinidad (Harwood et al. 1998). Despite the differences in ecosystem and plant type, a similar diurnal pattern was observed in both studies: high $\Delta_{\text{obs}}$ values in the morning and low values around midday. Harwood et al. (1998) also documented large $\Delta_{\text{obs}}$ values at dawn. The observations reported in our and previous studies (Gillon et al. 1997; Harwood et al. 1998) were not captured by the commonly used simple Farquhar et al. (1982) model because it expresses $\Delta$ as a linear function of $C_i/C_a$ alone (Eqn 4) and does not account explicitly for the various processes concurrently contributing to the net photosynthetic flux. In contrast, $\Delta_{\text{obs}}$ values were described well during most of the day by the classical Farquhar et al. (1982) model (Eqn 3) that incorporates photorespiration, day respiration and the diffusion of CO2 to the site of carboxylation through a series of resistances. But the surprisingly large $\Delta_{\text{obs}}$ values at dawn and dusk systematically observed in our study (and by Harwood et al. 1998) could not be reconciled using Eqn 3.

It seems unlikely that the difference between predicted $\Delta$ and $\Delta_{\text{obs}}$ was due to erroneous assumptions for the parameters $f$ and $g_i$. Both tend to decrease $\Delta$, and neither has large effects at dawn and dusk. Second, any alteration

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**Table 3.** Regression output parameters between predicted and observed discrimination using the simple (Eqn 4), classical (Eqn 3) and revised models (Eqn 5) for all branch chambers during May and July 2001

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameters and values assigned</th>
<th>Slope</th>
<th>Int</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple ($\Delta_{\text{simple}}$)</td>
<td>$b = 27$, $f = 0$, $g = 0$, $R_d = 0$</td>
<td>0.28</td>
<td>16.3</td>
<td>6.1</td>
</tr>
<tr>
<td>Classical ($\Delta_{\text{classical}}$)</td>
<td>$b = 29$, $f = 8$, $g = 0.16$, $R_d = 0.16$</td>
<td>0.54</td>
<td>8.2</td>
<td>5.7</td>
</tr>
<tr>
<td>Light inhibited</td>
<td>$b = 29$, $f = 8$, $g = 0.16$, $R_d = 0.16$</td>
<td>0.54</td>
<td>8.2</td>
<td>5.7</td>
</tr>
<tr>
<td>Revised ($\Delta_{\text{revised}}$)</td>
<td>$b = 29$, $f = 8$, $g = 0.16$, $R_d = 0.16$, $R_{d,n} = 0.95$</td>
<td>0.76</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Best Fit</td>
<td>$b = 29$, $f = 8$, $g = 0.16$, $R_d = 100$, $R_{d,n} = 0.95$</td>
<td>0.62</td>
<td>7.1</td>
<td>5.2</td>
</tr>
</tbody>
</table>
in either of these would also affect all other $^{13}\Delta$ predictions, and even increasing $^{13}\Delta$ to the limits of $b$ by adopting an infinite $g$, cannot explain all $^{13}\Delta_{\text{obs}}$ values. To attribute the large dusk and dawn $^{13}\Delta_{\text{obs}}$ primarily to fractionation by Rubisco, we would require a $b$ value of at least 34%. The value determined for Rubisco fractionation in vitro is $\approx 30%$ (Roese & O’Leary 1984; Guy et al. 1987), with the additional contributions of PEP carboxylase typically decreasing the overall fractionation during carboxylation by 0.7 to 1.4%. Apparent values of $b$ were found between 27% for rice and radish and 32% for wheat (von Caemmerer & Evans 1991), hence, it is unknown how realistic a $b$ of 34% is. On the other hand, changing $b$ would increase $^{13}\Delta$ predictions over the whole diurnal cycle and thus require a concurrent decrease in our estimate of $g$, by one third (to 0.1 mol m$^{-2}$ s$^{-1}$) to recover the midday $^{13}\Delta_{\text{obs}}$ values. Transfer conductances of 0.1 to 0.2 mol m$^{-2}$ s$^{-1}$ have been observed for woody species (von Caemmerer & Evans 1991; Lloyd et al. 1992; Loreto et al. 1992; Epron et al. 1995; Warren et al. 2003). Temperature-related fluctuations in $g$, (Bernacchi et al. 2002) were probably negligible because of the conservative temperature range during flask sampling. However, recalculations of the classical model incorporating a $b$ of 34% and $g$, of 0.1 mol m$^{-2}$ s$^{-1}$ still underestimated the dusk and dawn values by 3 to 5%.

The assumption incorporated in the classical model (Eqn 3) is that day respiration utilizes recently synthesized carbohydrates, with a $\delta^{13}C$ signature indistinguishable from that of the concurrent net assimilation flux. Hence, day respiration affects $^{13}\Delta_{\text{classical}}$ (Eqn 3) only if there is fractionation ($e$) during this process. Estimates of fractionation during dark respiration range from 6 to $-6\%$ (Duranceau et al. 1999; Ghashghaie et al. 2003), but these might not be valid for day respiration (Tcherkez et al. 2004). However, we would need unrealistic values of $e$ (up to $-20\%$) compared to the effects of much lower $e$ values shown in Fig. 6a to explain $^{13}\Delta_{\text{obs}}$ purely based on fractionation during day respiration. Thus, recently synthesized carbohydrates may not constitute the only substrate for day respiration of P. sitchensis branches.

For dark respiration, older carbon pools may provide as much as 60% of the substrate (Schnyder et al. 2003; Nogués et al. 2004). Mitochondrial respiration is likely to utilize different substrates over time (Gleixner & Schmidt 1996; Schnyder et al. 2003; Tcherkez et al. 2003; Nogués et al. 2004; Prater, Mortazavi & Chanton 2006). Substrate shifts could occur between day and night in response to light-activated changes in metabolic pathways (Atkin et al. 2000; Tcherkez et al. 2005). In addition, these pathways may be affected by the non-statistical distribution of $^{13}C$ in carbohydrates (Gleixner & Schmidt 1996) or differ in isotope fractionation during the reactions themselves (Ghashghaie et al. 2003; Tcherkez et al. 2003). If day respiration utilizes carbon pools other than recently synthesized carbohydrates, its isotopic signature could differ from that of the concurrent assimilation flux (even for $e = 0$). This would create ‘isotopic singularities’, with extremely enriched or depleted $^{13}\Delta$ and $\delta^{13}C_R$ values as the net flux approaches zero. Such extreme

![Figure 6. Sensitivity of $^{13}\Delta_{\text{revised}}$ (Eqn 5) to parameter assumptions relating to day respiration assuming (a) different values for $e$ and using only fresh assimilates ($^{13}\Delta_{\text{classical}}$), (b) different values for $e$ and using 50% fresh assimilates and 50% old substrate, and (c) different values for $\delta^{13}C_{\text{substrate}}$, using only old substrate and an $e$ of 0%. The example chosen here was the high dusk value in July 2001, with $^{13}\Delta_{\text{obs}} = 34.4\%$. $Q = 36 \mu$mol m$^{-2}$ s$^{-1}$, $g_s = 0.1 \mu$mol m$^{-2}$ s$^{-1}$, $A = 0.8 \mu$mol m$^{-2}$ s$^{-1}$, $R_d = 0.5 \mu$mol m$^{-2}$ s$^{-1}$, $C_s = 360 \mu$mol mol$^{-1}$, $C_c = 341 \mu$mol mol$^{-1}$, $\delta^{13}C_a = -7.5\%$, $\delta^{13}C_{\text{substrate}} = -23\%$.](image)

values are the result of interpreting two opposing fluxes of similar magnitude with different isotopic signatures as a single net flux with a combined ‘apparent’ isotopic signature. Similar analyses of apparent isotopic signatures of net
fluctuations have been presented at large scales for $^{13}$C (Miller & Tans 2003; Helliker et al. 2005), and at the leaf scale for $^{13}$C (Seibt 2003; Wingate 2003) and $^{18}$O (Cernusak et al. 2005). Here, we incorporated the opposing flux of day respiration into the equation for net $^{13}$C discrimination (Eqn 5) to account for the mixing and recycling of respired CO$_2$ within the intercellular spaces in the same way as for photorespiration (see Fig. 3 & Appendix II). For example, using the fluxes associated with the large dusk $^{13}$D$_{obs}$ in July 2001, $34.4\%_o$, measured net $A$ of $0.8 \mu$mol m$^{-2}$ s$^{-1}$, estimated $R_d = 0.5 \mu$mol m$^{-2}$ s$^{-1}$, correspondingly higher gross photosynthetic rate), calculations without recycling (eqn 7 of Miller & Tans 2003) predicted $1\%_o$ larger net $^{13}$D than Eqn 5 for identical $\delta^{13}C$ signatures of the two gross fluxes. Incorporating an isotopically different substrate for day respiration, Eqn 5 thus can explain the large net $^{13}$D$_{obs}$ values at dawn and dusk that could not be resolved by either of the other models (Eqns 3 & 4). Alternatively, the elevated $^{13}$D$_{obs}$ can be thought of as reflecting a residual isoflux composed of the rate of day respiration, the $\delta^{13}C$ signature of the respiratory substrate and the fractionation. Very large absolute values of $e$ are required if the substrate is assumed identical to newly assimilated carbohydrates. Smaller $e$ values are needed the more the respiratory substrate differs from recent assimilates, and the larger the relative contribution of this substrate is (see Fig. 6). The respiratory isoflux thus contains too many unknowns to be decomposed without independent knowledge of its components. Ultimately, this can only be addressed through controlled laboratory and field measurements.

For the low $^{13}$D values usually coinciding with high $A$, $Q$ and $T$, there was little difference between the classical and revised formulations. The slightly higher values of the classical model fitted the observed data better than our revised description. As the day respiration term is the only difference between the two models, this might indicate that the revised version overestimates the day respiratory contribution at these times. This could be resolved easily by assuming stronger inhibition of day respiration at high light and/or temperature levels (Atkin et al. 2000). Such a variable day respiration rate, contributing more at dawn and dusk and less during the rest of the day, would explain the observed diurnal pattern of $^{13}$D including the high dusk and dawn values. This scenario does not rule out, but also does not depend on, additional shifts in respiratory substrates, such as two similar but opposite substrate switches, one at dawn, the other at dusk, to explain the high $^{13}$D values observed at both times.

Lastly, we note that in our gas-exchange calculations, the foliage in the chambers was treated as a big leaf. However, enclosing whole branches meant that chamber measurements included the gas exchange of different aged needle cohorts, as well as contributions from woody material. Flask measurements provide assimilation-weighted $^{13}$D values, whereas gas-exchange measurements give conductance-weighted values for different age classes. Potential age class-related weighting effects on $^{13}$D (see Appendix III) are illustrated in Table 4. In addition, we calculated rates of woody respiration based on temperature measurements (Bosc, de Grandcourt & Loustau 2003). Woody respiration was estimated to contribute about 10% to day respiration rates at low light, i.e. less than 5% of photosynthetic foliage fluxes (even less during the rest of the day), due to the small ratio of stem/twig to foliage area. Therefore, the contributions of woody tissue fluxes to net CO$_2$ exchange and $\delta^{13}C$ fractionation observed in this study were considered negligible and ignored in the calculations detailed in Appendix I.

At larger spatial and temporal scales, the isotopic effects of day respiration can probably be neglected as the net flux of CO$_2$ during dusk and dawn periods represents such a small contribution to the daily flux weighted $^{13}$D signal. However, considering the increasing temporal resolution of $\delta^{13}C$ measurements (Bowling et al. 2001; Ogée et al. 2003, 2004; Knohl & Buchmann 2005), in particular, the high-resolution isotopic time series constructed using tunable diode laser absorption spectrometry (TDLAS) technology (Bowling et al. 2003; Griffis et al. 2005), we are increasingly likely to directly encounter the isotopic contributions from day respiration during leaf and ecosystem

| Table 4. Effects of averaging over different needle age classes on the value predicted for $^{13}$D |
|---|---|---|---|---|---|---|
| Age class | $L_A$ (% of total) | $A$ (% of current) | $g_s$ (% of current) | Predicted $^{13}$D (%) | $A$ weighted $^{13}$D (%) | Difference $g_s - A$ weighted $^{13}$D (%) |
| Current | 66 | 100 | 100 | 16.7 | 15.7 | 0.3 |
| Current + 1 | 30 | 56 | 39 | 12.2 |
| Current + 2 | 4 | 54 | 40 | 13.1 |

Relative needle area ($L_A$), assimilation rate ($A$) and stomatal conductance ($g_s$) for three different needle age classes, and their total values for the whole branch, with $^{13}$D values predicted for each age class separately and for the whole branch. The conductance-weighted age class average $^{13}$D corresponds to that calculated from branch total values, i.e. from gas-exchange measurements. The assimilation-weighted age class average $^{13}$D corresponds to that expected from isotope measurements.
scale studies. We also hypothesize that these respiratory signals could be more pronounced during periods of high meristematic activity, for instance, during shoot elongation [see, e.g. the increased $R_a$ values in May (Table 2) associated with bud swelling]. During tissue growth in temperate species, storage carbohydrates such as starch are re-mobilized to provide metabolic energy at times of high demand. In the case of evergreen species such as *P. sitchensis*, these starch reserves accumulate during favourable environmental conditions over the winter (Bradbury & Malcolm 1978), whereas for deciduous species, they are accumulated towards the end of the previous year’s growing season. These reserves could then contribute their distinct isotopic signatures to day respiration and, hence, net $\delta^{13}C$ discrimination. The revised model (Eqn 5) presented here, together with detailed studies of metabolic cycling and storage in plants, will enable us to explain the resulting field observations of net $\delta^{13}C$. At the same time, high-resolution field data on $\delta^{13}C$ signatures of CO$_2$ fluxes during the light and dark will enable us to address some of the hypotheses and uncertainties highlighted in our study, and to make use of the potentially valuable information on C metabolism contained in photosynthetic isotope signals.

CONCLUSIONS

Our study highlights isotopic disequilibria during net $\delta^{13}C$ discrimination leading to pronounced diurnal variability of $\Delta_{\text{day}}$ in the field. The results of our study also emphasize the need for independent observations of transfer conductance to CO$_2$, as well as studies on the potential variability of $b$ among and within species. With better constraints on the most critical parameters ($g_i$ and $b$), observations of $\Delta_{\text{day}}$ and metabolite composition under controlled experimental conditions could allow us to indirectly probe respiratory signals during the light using stable carbon isotopes as tracers.

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REFERENCES


Epron D., Godard D., Cornic G. & Genty B. (1995) Limitation of net CO$_2$ assimilation rate by internal resistances to CO$_2$ transfer

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in the leaves of two tree species (Fagus sylvatica L. and Castanea sativa Mill.), Plant, Cell & Environment 18, 43–51.


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APPENDIX I: ANALYSIS OF CLOSED CHAMBER MEASUREMENTS

Gas exchange in closed chambers will lead to transient changes in micro-environmental conditions, air composition and fluxes during the closure period. Flask samples collected at the start and end of chamber closure periods integrate over such changes. Furthermore, the air flushed through the flasks and back into the chambers was dried, weakening the increase in chamber water vapour content. Here, we briefly describe how we take both into account when integrating chamber gas-exchange data (5 s time step) to obtain 13C predictions that are directly comparable to flask observations (see also appendix II of Seibt et al. 2006). The data processing described in the following (for chamber 1 on 20 July 2001, 1440 to 1445 h) was implemented in an IDL program.

During the 5 min chamber closure, photon flux density was 127 ± 3 μmol m−2 s−1, and air and leaf temperatures were 14 ± 0.7 °C. We assumed that to a good approximation, stomatal conductance remained constant within this period (Ludlow & Jarvis 1971). Relative humidity data (h) were used to calculate the vapour mole fraction of the enclosed air [wair(CO2)], Fig. 7a). During flask sampling periods, the air vapour mole fraction was corrected for the flow of dry air (3 dm3 min−1) returned from the flask sampling system [wair(CO2)]. Assuming saturated air at leaf temperature yielded a leaf vapour mole fraction (wL) of 16.4 mmol mol−1. The total conductance to water vapour (g, 0.051 mmol m−2 s−1 for this period) was obtained from the increase in air vapour mole fraction [wair(CO2)− wair(CO2)] of 9 to 14 mmol mol−1, h: 56 to 78% by fitting

\[ w_L = w_i + (w_{air} - w_i) e^{-\frac{L}{v_a} (1-h)} \]  

(A1.1)

to leaf and air vapour mole fraction data at time t during the closure periods, where wair is the initial air vapour mole fraction at the starting time t0. LA and Vb are the leaf area and air molar volume enclosed in the chamber and g represents the total conductance to water vapour combining boundary layer and stomatal components. The transpiration rate decreased from 0.4 to 0.1 mmol m−2 s−1 during the closure period. The CO2 mole fraction of chamber air (C∞, Fig. 7b) decreased from 350 to 275 μmol mol−1. The C∞ data were fitted with a quadratic equation, its derivative yielding a linear approximation of A (most R2 > 0.99). Because C∞ and A varied concurrently, the change inC∞ (Eqn 1, Fig. 7b) was weaker than that of C∞ leading to a small increase in C∞/C∞ over the closure period (Fig. 7c).

Using the δ13C values observed in the open chambers at starting point values, we then calculated 13C (Eqns 3–5) and a new δ13C (Eqn 2) for each 5 s time step. These 13C and δ13C values increased from 17 to 18‰ (Fig. 7c) and –7
to −2‰ (Fig. 7d) during the closure periods, respectively. Weighted averages of $\delta^{13}C_a$ and $C_i$ were calculated from the time steps to reflect the air mixture collected in the ‘closed’ flask sample. Integrated $\delta^{13}C_a$ values were then obtained from $\delta^{13}C_a$ and $C_i$ at the start and their weighted averages at the end of chamber closure periods using Eqn 2. This approach is equivalent to the calculations using flask data, making both methods directly comparable. For the example used here, the integrated $\Delta$ value of 16.9‰ was closer to the flask observed values of 17.0‰ than the starting point estimate of 16.6‰.

APPENDIX II: THE EFFECT OF DAY RESPIRATION ON NET DISCRIMINATION

Currently, the most complete, ‘classical’ model for net $^{13}C$ discrimination, $\Delta$, during photosynthesis of C₃ plants (Farquhar et al., 1982) accounts for fractionation (e) during day respiration ($R_d$) but assumes that current assimilates form its substrate (Eqn 3). In the following, we use a simplified version of Eqn 3 (see eqn B24 of Farquhar et al., 1982) omitting boundary layer and transfer conductances for clarity (the actual calculations always included all terms):

$$ \Delta_{\text{classical}} = a + (b - a) \frac{C_i}{C_a} \Gamma_s \frac{f}{C_a} - \frac{kP}{C_a} \Gamma_s - \frac{R_d}{kC_a} $$  (A2.1)

Here, we derive an equation for net photosynthetic $^{13}C$ discrimination that accounts for an isotopically distinct substrate used for day respiration. No specific assumption is made about the substrate and fractionation (e), which may come from various pools (sucrose, starch, lipids, etc.) with different metabolic pathways (Gleixner & Schmidt, 1996; Schnider et al., 2003; Tcherkez et al., 2003, 2004; Nogués et al., 2004). In the following, we use the notation of Farquhar et al. (1982), with B indicating equations from their appendix II. Using eqns B12, B12′, B18, B18′, B19 and B19′, the $\Delta$ of net assimilation (including photorespiration and daytime dark respiration) is given by

$$ \frac{\Delta'}{A} = (1 - \Delta) \frac{R_s}{g'} - \frac{gP}{g'} \frac{k'(C_i - \Gamma_s) - R_d'}{k(C_i - \Gamma_s) - R_d} $$  (A2.2)

where $R_s = C_i/C_a$ denotes the isotope ratio of atmospheric CO₂, and $P$ is atmospheric pressure. The fractionations

**Figure 7.** Changes in branch chamber 1 during the 5 min closure period at 1440 h on 20 July 2001: (a) observed relative humidity ($h$) and calculated air vapour mole fraction, uncorrected [$w_a(h)$] and corrected [$w_a(\text{corr})$] for the recirculation of dried air by the sampling system; (b) CO₂ mole fraction observed by the infrared gas analyser (IRGA) [$C_a(\text{IRGA})$] and flask sampling method [$C_a(\text{FLASK})$]; and calculated intercellular CO₂ mole fraction ($C_i$) and net assimilation rate ($A$); (c) calculated ratio of ambient to intercellular CO₂ mole fraction ($C_i/g/C_a$) and photosynthetic $^{13}C$ discrimination ($\Delta$), and (d) flask observed ($\delta^{13}C_a$) and calculated [$\delta^{13}C_a(\text{pred})$] isotopic signature of chamber air.
associated with diffusion \((a, \text{ eqn B22})\), carboxylation \((b, \text{ eqn B16})\) and photorespiration \((f, \text{ eqn B5})\) are defined as in Farquhar et al. (1982). But the isotope ratio of recent assimilates \(A'/A\) in eqn B8 \([R_d'/R_d = (1-e)(A'/A)]\) is replaced by the isotope ratio of the substrate used for day respiration, \(R_{\text{substrate}}\):

\[
\frac{R'_d}{R_d} = (1-e)R_{\text{substrate}} \tag{A2.3}
\]

Using the fractionation factors and \(\Gamma_d/\Gamma_a = (1 + b - 13\Delta - f) \cdot R_a\) (eqn B17) in Eqn A2.2 gives

\[
(1-13\Delta) \cdot R_a = (1-a) \cdot \frac{g + kP}{(1-a) + kP(1-b)} \cdot \frac{g + kP}{k(1-b)(C_a - \Gamma_a) - R_d} \cdot \frac{k(1-b)(C_a - \Gamma_a) - R_d}{R_a(1-e)R_{\text{substrate}}} \tag{A2.4}
\]

Rearranging and neglecting second-order terms lead to the following expression:

\[
(1-13\Delta) \cdot R_a = (1-a) \cdot \frac{g + kP}{g + kP - (ag + bkP)} \cdot \frac{k(1-b)(C_a - \Gamma_a) - R_d}{k(C_a - \Gamma_a) - R_d} \cdot \frac{k(1-b)(C_a - \Gamma_a) - R_d}{R_a(1-e)R_{\text{substrate}}}
\]

\[
= (1-a) \cdot \frac{1}{g + kP} \cdot \left\{ R_a - \frac{k(bC_a - (13\Delta + f))\Gamma_a}{k(C_a - \Gamma_a) - R_d} \right\}
\]

\[
= (1-a) \frac{ag + bkP}{g + kP} \cdot \left[ 1 - \frac{b}{k(C_a - \Gamma_a) - R_d} \right] \cdot \frac{k\Gamma_a}{k(C_a - \Gamma_a) - R_d} + e \frac{k(C_a - \Gamma_a) - R_d}{R_a(1-e)} \cdot R_{\text{substrate}}
\]

\[
= \frac{R_d}{k(C_a - \Gamma_a) - R_d} \left( \frac{R_{\text{substrate}}}{R_a} - 1 \right) \cdot R_a.
\]

Substituting \(\frac{kP}{g + kP} = \frac{kC_a}{k(C_a - \Gamma_a) - R_d} \) (eqns B12, B18 and B19), we get

\[
13\Delta = \frac{kC_a}{k(C_a - \Gamma_a) - R_d} \left[ a + (b-a) \frac{C_i}{C_a} - (13\Delta + f) \right] \tag{A2.6}
\]

\[
\frac{\Gamma_a}{C_a} = e \frac{R_d}{kC_a} \frac{R_{\text{substrate}}}{R_a} + \frac{R_d}{kC_a} \left( \frac{R_{\text{substrate}}}{R_a} - 1 \right)
\]

Because \(13\Delta\) appears on both sides of Eqn A2.6, we rearrange (neglecting second-order terms) to get the net \(^{13}\)C discrimination for any dark respiratory substrate:

\[
13\Delta_{\text{revised}} = \frac{kC_a}{k(C_a - \Gamma_a) - R_d} \left[ a + (b-a) \frac{C_i}{C_a} - f \frac{\Gamma_a}{C_a} \right]
\tag{A2.7}
\]

\[
\left( e + \delta^{13}\text{C}_{\text{atm}} - \delta^{13}\text{C}_{\text{substrate}} \right) \frac{R_d}{kC_a}
\]

If the substrate effect of dark respiration on net \(^{13}\)C discrimination is expressed as apparent fractionation, \(e^a = \delta^{13}\text{C}_{\text{atm}} - \delta^{13}\text{C}_{\text{substrate}} - 13\Delta\), i.e. the isotopic difference between dark respiration and photosynthesis, we can substitute \(R_{\text{substrate}} = (1-e^a) \cdot (A'/A)\) in Eqn A2.3 to give \(R'_d/R_d = [1-(1-e^a)(A'/A)]\) in Eqn A2.3. Using this apparent factor \(e^a\), Eqn A2.7 can be directly compared to Eqn A2.1:

\[
13\Delta_{\text{revised}} = a + (b-a) \frac{C_i}{C_a} - f \frac{\Gamma_a}{C_a} - (e + \delta^{13}\text{C}_{\text{atm}} - \delta^{13}\text{C}_{\text{substrate}}) \frac{R_d}{kC_a}
\tag{A2.8}
\]

If \(\delta^{13}\text{C}_{\text{substrate}} = \delta^{13}\text{C}_{\text{atm}} - 13\Delta\) (\(e^a = 0\)), the revised equation (A2.7, A2.8 & A2.9) is again the classical model of Farquhar et al. (1982) as in Eqn A2.1.

Alternatively, the carboxylation efficiency \(k\) can be substituted in Eqn A2.7 using \(k = (A + R_d)/(C_i - \Gamma_a)\) from appendix II, (Farquhar et al. 1982) to yield Eqn 5 of the main text, a useful version because it contains only variables that were measured in our study:

\[
a + (b-a) \frac{C_i}{C_a} - f \frac{\Gamma_a}{C_a} - (e + \delta^{13}\text{C}_{\text{atm}} - \delta^{13}\text{C}_{\text{substrate}}) \frac{R_d}{A + R_d} \frac{C_i - \Gamma^a}{C_a}
\tag{A2.9}
\]

Lastly, in the absence of light, \(k = 0\) and Eqn A2.7 reduces to

\[
13\Delta_R = e - \delta^{13}\text{C}_{\text{substrate}} + \delta^{13}\text{C}_{\text{atm}},
\tag{A2.10}
\]

demonstrating that in the absence of carboxylation, the \(^{13}\)C signature of the respiratory flux \((13\Delta_R)\) with respect to the atmospheric composition is given by the respiratory substrate minus the fractionation factor (keeping the notation of Eqn A2.7, but \(e\) referring to dark respiration here). We therefore recommend the use of Eqn A2.7 instead of Eqn A2.1 because it is valid at any time of the day, even for dark respiration (J. Ogée, personal communication).
APPENDIX III: WEIGHTING OF GAS-EXCHANGE AND ISOTOPE MEASUREMENTS

The calculations of gas exchange in this study have effectively assumed the branch can be represented as a ‘big leaf’, despite the inclusion of multiple needle cohorts where uniform assimilation, stomatal and internal conductances are unlikely. The implications of such heterogeneity on the weighting of gas-exchange and isotope measurements have already been shown for individual leaves (Farquhar 1989; Lloyd et al. 1992) and plant canopies (Lloyd et al. 1996). Indeed, there are systematic differences in the gas-exchange characteristics of differing needle age classes for P. sitchensis (Ludlow & Jarvis 1971; Watts, Neilson & Jarvis 1976; Leverenz et al. 1982; Barton 1997). The presence of different needle age classes on the same branch thus represents a similar situation to that of stomatal heterogeneity for single leaves. Branches in our study contained a maximum of three age classes, with each age class transpiring and assimilating at different rates. Carbon isotope discrimination is carried by the net flux of CO₂, i.e. different age classes contribute to the average ¹³Δ according to their respective assimilation rates. Deriving Cᵢ/Cₐ from flask ¹³Δ measurements thus provides an assimilation-weighted value of Cᵢ (Cᵢ,A). In contrast, different age classes contribute to the average Cᵢ/Cₐ derived from IRGA gas-exchange data according to their respective stomatal conductances, i.e. gas-exchange measurements give a conductance-weighted value of Cᵢ (Cᵢ,g see Eqn 1) (Farquhar 1989).

Potential age class-related weighting effects on ¹³Δ are illustrated in Table 4. Values of ¹³Δ were determined for each needle age class separately, based on variations of A and gₛ observed across age classes for P. sitchensis (Ludlow & Jarvis 1971). Assimilation and conductance weighted ¹³Δ averages for the whole branch were then calculated according to the contributions of the different age classes to the total needle area measured on our branches. If differences between ¹³Δ predicted from gas exchange and ¹³Δₐₒₛ determined from flask measurements were attributed to differences between Cᵢ,g and Cᵢ,A via stomatal heterogeneity, we would expect similar offsets between conductance-weighted ¹³Δ and flask ¹³Δₐₒₛ values as between conductance-weighted and assimilation-weighted ¹³Δ values. But calculated offsets had opposite signs indicating that the differences between predicted and observed ¹³Δ are unlikely to result from foliage heterogeneity. Moreover, the shift expected from weighting was small (0.24‰) compared to the actual difference between predicted and observed ¹³Δ values. Although estimating such shifts is limited by the fact that diverse (and even contrasting) patterns of changes in A and gₛ across needle age classes have been observed in different studies (Ludlow & Jarvis 1971; Wang 1988), none of these patterns would seem sufficient to induce shifts of the observed direction and magnitude. Again, the more likely explanation is the contribution of further isotopic effects during net uptake such as day respiration, photorespiration and internal CO₂ transfer (see text).