

Diurnal variation of the $\delta^{13}\text{C}$ of pine needle respired CO_2 evolved in darkness

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ABSTRACT

The $\delta^{13}\text{C}$ of pine needle CO_2 evolved in darkness ($\delta^{13}\text{C}_r$) for slash pine trees (*Pinus elliottii*) was determined by placing recently collected pine needles in darkness and collecting respired CO_2 over a short time period (<15 min). $\delta^{13}\text{C}_r$ measurements were made over several 24 h periods to test the hypothesis that significant variation in $\delta^{13}\text{C}_r$ would be observed during a diurnal cycle. The $\delta^{13}\text{C}_r$ measurements from the 24 h time series trials showed a consistent midday ^{13}C -enrichment (5–10‰) relative to bulk biomass. The $\delta^{13}\text{C}_r$ values became more ^{13}C -depleted at night and following shading, and approached bulk-biomass $\delta^{13}\text{C}$ values by dawn. The effect of night-time respired ^{13}C -enriched CO_2 on the $\delta^{13}\text{C}$ value of the remaining assimilate is shown to be minimal (^{13}C depleted by 0.22‰) under field conditions for *P. elliottii* needles.

Key-words: carbon isotopes; dark respiration; photosynthetic uptake, slash pine.

INTRODUCTION

As C_3 plants fix carbon through photosynthesis, the $\delta^{13}\text{C}$ of CO_2 is modified by isotopic fractionations caused by diffusion and by enzymatic reactions converting CO_2 to biomass. Fractionation occurs during the diffusion of CO_2 through stomata because of the faster transfer of the lighter isotope (the fractionation for diffusion through the stomata, a , is about 4.4‰), whereas the isotopic effects of diffusion and dissolution into the liquid phase and transfer to the chloroplast, where photosynthesis takes place, has been considered to be minimal (O'Leary 1981; Farquhar, O'Leary & Berry 1982). As enzymes associated with the C_3 photosynthetic pathway act on CO_2 , fractionation related to these enzymes (b), primarily from Rubisco, further depletes the heavy isotope by approximately 28‰. Fractionation effects associated with respiration have been considered to be less important. Isotopic discrimination during photosynthesis ($\delta^{13}\text{C}$) can be expressed as a function of the ratio of internal to atmospheric CO_2 partial pressure (c_i/c_a) with Eqn 1 (Farquhar *et al.* 1982; Evans

et al. 1986; von Caemmerer & Evans 1991; Gillon & Griffiths 1997)

$$\Delta^{13}\text{C} = a + (b - a)(c_i/c_a) - (b - a)A/(g_w c_a) - (eR_d/k + f\Gamma^*)/c_a \quad (1)$$

a simplified version of the complete equation, where a_i represents the fractionation during diffusion and dissolution of CO_2 into the liquid phase (1.8‰), A represents the assimilation rate, g_w is the leaf internal CO_2 conductance, e is the fractionation during day respiration, R_d is the day respiration rate, k is the carboxylation efficiency, f is the fractionation during photorespiration and Γ^* is the CO_2 compensation point in the absence of day respiration. The terms representing the effect of CO_2 transfer to the chloroplast and fractionation during respiration, $(b - a)A/(g_w c_a)$ and $(eR_d/k + f\Gamma^*)/c_a$, respectively, are often considered to be negligible. An abridged version of Eqn 1, used to calculate isotopic fractionation during photosynthesis while neglecting the effect of CO_2 transfer to the chloroplast and fractionation during respiration ($\Delta^{13}\text{C}_i$, Eqn 2), has proved useful in linking variation in $\Delta^{13}\text{C}$ with stomatal regulation and c_i/c_a (Bowling *et al.* 2002; Fessenden & Ehleringer 2003; McDowell *et al.* 2004). The $\delta^{13}\text{C}$ value of assimilated carbon ($\delta^{13}\text{C}_i$) can be calculated by subtracting $\Delta^{13}\text{C}_i$ from the $\delta^{13}\text{C}$ value of CO_2 in ambient air ($\delta^{13}\text{C}_{\text{air}}$, Eqn 3).

$$\Delta^{13}\text{C}_i = a + (b - a)(c_i/c_a) \quad (2)$$

$$\delta^{13}\text{C}_i = \delta^{13}\text{C}_{\text{air}} - \Delta^{13}\text{C}_i \quad (3)$$

During foliage respiration, plants convert previously assimilated material back into CO_2 to provide energy for cell functions. Foliage respiration occurs both in darkness by dark respiration and while exposed to light through two processes, photorespiration and day respiration (Sharkey 1988; Gillon & Griffiths 1997). Although fractionation during photorespiration is thought to be significant (Sharkey 1988; Gillon & Griffiths 1997), fractionation during dark respiration has been considered to be negligible (Lin & Ehleringer 1997).

Recent investigations have, however, indicated that fractionation-like processes appear to occur during dark respiration. Duranceau *et al.* (1999) found that the $\delta^{13}\text{C}$ of dark-respired CO_2 ($\delta^{13}\text{C}_r$) was ^{13}C -enriched by approximately 6‰ relative to bulk sucrose. Since then, other investigations have shown similar results. $\delta^{13}\text{C}_r$ has been found to be ^{13}C -enriched relative to a wide variety of metabolites,

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across multiple species, with some variability across species and environmental conditions (Duranceau, Ghashghaie & Brugnoli 2001; Ghashghaie *et al.* 2001; Xu *et al.* 2004). Tcherkez *et al.* (2003) showed $\delta^{13}\text{C}_r$ was not constant in darkness over a 5 day period and suggested that variation could be caused by changes in the respiratory substrate coupled with associated changes in the relative contribution from the different metabolic pathways, nonstatistical distribution of ^{13}C in glucose molecules (Rossman, Butzenlechner & Schmidt 1991) or by kinetic effects of respiratory enzymes (reviewed in Ghashghaie *et al.* 2003).

The effect of the nonstatistical distribution of ^{13}C in glucose molecules (Rossman *et al.* 1991; Tcherkez *et al.* 2003) and the associated ^{13}C -enriched $\delta^{13}\text{C}_r$ (Duranceau *et al.* 1999, 2001; Ghashghaie *et al.* 2001, 2003; Tcherkez *et al.* 2003; Xu *et al.* 2004) on assimilated carbon should be investigated. Also, recent findings have shown that the isotopic composition of ecosystem respiration varies on short time scales (Knobl *et al.* 2004) and that ecosystem and leaf respiration draws from at least two distinct carbon sources with different characteristics and residence times (Schnyder *et al.* 2003; Nogues *et al.* 2004), leading to the conclusion that temporal variability of leaf respiration or variation in the substrate used for respiration could be responsible for differences in reported fractionation values in the literature. Temporal variability in $\delta^{13}\text{C}_r$ is not well understood and more work is needed to answer questions regarding the implications of ^{13}C -enriched respiration.

Assimilated carbon $\delta^{13}\text{C}$ varies with environmental conditions over the course of a day (i.e. changes in c_i/c_a in response to changes in photosynthetically active radiation or vapour pressure deficit, Eqn 1) leading one to expect variation in the $\delta^{13}\text{C}$ of day-respired CO_2 , assuming that recently assimilated C is utilized for autotrophic respiration (Bowling *et al.* 2002; Mortazavi & Chanton 2002b; Mortazavi *et al.* 2005). This is regardless of any respiration-related fractionation effects (Ghashghaie *et al.* 2001, 2003) or shifts in metabolic pathways (Tcherkez *et al.* 2003). Furthermore, $\delta^{13}\text{C}_r$ in darkness has been shown to vary over the course of multiple days under constant environmental conditions, presumably because of changes in carbohydrate concentrations and shifts in the relative contributions of the two major decarboxylation processes (i.e. pyruvate dehydrogenase activity and the Krebs cycle) that occur in the dark (Tcherkez *et al.* 2003). We tested the hypothesis that $\delta^{13}\text{C}_r$ would vary over the course of a day and continue to vary overnight under field conditions, where significant changes in environmental conditions occur, by measuring $\delta^{13}\text{C}_r$ for slash pine trees (*Pinus elliotii*) under field conditions to determine the extent of diurnal variation in $\delta^{13}\text{C}_r$. Ghashghaie *et al.* (2003) suggested that discrimination during night-time respiration and the resulting ^{13}C -enriched $\delta^{13}\text{C}_r$ would cause ^{13}C depletion of the remaining plant material. We use our measured night-time respiration rates and $\delta^{13}\text{C}_r$ values, and daytime photosynthetic rates, to estimate the impact of night-time $\delta^{13}\text{C}_r$ on the $\delta^{13}\text{C}$ of the remaining plant material.

MATERIALS AND METHODS

Study sites and conditions

The August and November (2002) 24 h trials of $\delta^{13}\text{C}_r$ measurements were conducted at the same height on the same slash pine tree at Tallahassee, Florida. Temperatures varied from 23 to 38 °C for the August trial and from 5 to 19 °C for the November trial. Sunrise and sunset were at 0703 and 2022 h (EDT) for the August trial and at 0657 and 1745 h (EST) for the November trial.

The April (2003) trial, which included $\delta^{13}\text{C}_r$, photosynthetic rate and respiration rate measurements, was conducted on a slash pine tree at the same height in the canopy and within 10 m of the tree used for the August and November trials. Temperatures varied from 19 to 35 °C. Sunrise and sunset were at 0706 and 2007 h (EDT) for the April trial.

The shading experiment in November (2003) included $\delta^{13}\text{C}_r$ measurements and was conducted on a slash pine tree within 2 km of the trees used for the August, November and April trials. Temperatures varied from 19 to 33 °C. Sunrise and sunset were at 0700 and 1742 h (EST).

Pine needle sampling

Leaf-chamber measurements (see below) utilized pine needles still attached to the limb, while $\delta^{13}\text{C}_r$ measurements used detached needles. For each $\delta^{13}\text{C}_r$ measurement new pine needles were detached from the tree. All of the pine needles came from approximately the same elevation. In the November (2003) shading experiment, two subgroups were determined by the shading regime, and the pine needles for each subgroup measurement came from the same limb.

Each $\delta^{13}\text{C}_r$ measurement was made with the sequenced-air-sampling (SAS) system Fig. 1, which is designed specifically for the collection of foliage respired CO_2 (see below),

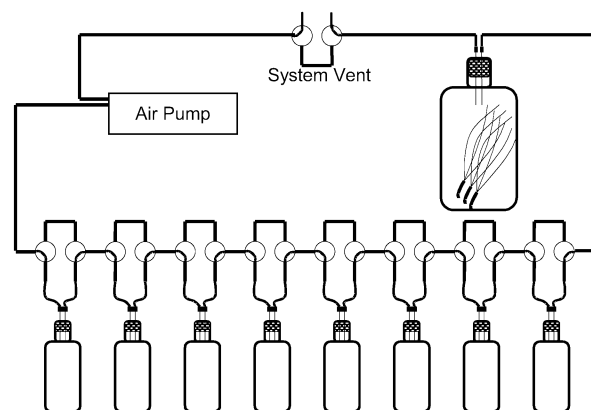


Figure 1. Schematic view of the sequenced air sampler (SAS) used for collecting CO_2 samples of foliage respiration. The 0.5 L system consists of a 120 mL serum vial containing the respiring material and eight 30 mL gas-collection vials. Circulation is provided by an air pump with a flow rate of approximately 2 L min^{-1} . The three-way valves allow the flow to be circulated through or to bypass each vial. The system neglects temperature or humidity control and is intended for measurements on short time scales.

within 15 min of needle detachment. The short time interval from detachment to measurement was necessary to minimise variations caused by dehydration or other physiological changes associated with detachment. After the $\delta^{13}\text{C}_r$ measurement was made, the needles were refrigerated and taken back to the laboratory where they were dried at 60 °C and ground to a fine powder. An isotope-ratio mass spectrometer (IRMS) coupled to a Carbon–Hydrogen–Nitrogen (CHN) analyser was used to determine $\delta^{13}\text{C}$ values of bulk-organic matter.

Leaf-chamber measurements

Photosynthetic rates, respiration rates and the c_i/c_a ratio were measured on needles still attached to the tree using a portable photosynthesis system and a 0.25 L leaf chamber (LI-6200, Li-Cor Inc., Lincoln, NE, USA). Surface areas of needles were approximated using a two-dimensional model and were determined by doubling the length multiplied by the width of the needles. Ambient CO₂ concentrations were measured with the LI-6200, and net photosynthetic rates (P) and c_i were calculated by using the LI-6200 software. Respiration rates (R) were also calculated using the LI-6200, by darkening the leaf chamber during data collection. Each rate and c_i/c_a measurement reported is the average value of eight consecutive measurements and the errors reported are one standard deviation. No isotopic measurements were made with the Li-Cor leaf-chamber system.

$\Delta^{13}\text{C}_r$ measurements

Determination of $\delta^{13}\text{C}_r$ at each time point was made through the analysis of eight consecutive air samples collected over 11 min using detached needles. Approximately 20 needles were contained in a closed system with a background CO₂ concentration of c_a and a $\delta^{13}\text{C}$ value of δ_a . The $\delta^{13}\text{C}$ of CO₂ respired into the system was assumed to be constant (δ_i). Isotopic mass balance equations are as follows:

$$c_t = c_r + c_a \quad (4)$$

$$\delta_i c_t = \delta_r c_r + \delta_a c_a \quad (5)$$

where δ_i and c_t are the $\delta^{13}\text{C}$ and CO₂ concentrations in the closed system at any relative mixture of respired and background CO₂. If Eqn 5 is solved for δ_i and Eqn 4 is solved for c_r and substituted into Eqn 5, the result,

$$\delta_i = (c_a(\delta_a - \delta_r))/c_t + \delta_r \quad (6)$$

represents the line formed when the inverse of concentration is plotted against the corresponding δ_i values, and the y-intercept will be δ_r , in this case $\delta^{13}\text{C}_r$. This technique is commonly referred to as a Keeling plot (Keeling 1958; Miller & Tans 2003; Pataki *et al.* 2003), and similar methods have been used previously to determine $\delta^{13}\text{C}_r$ (Fessenden & Ehleringer 2003; McDowell *et al.* 2004; Xu *et al.* 2004).

The gas samples needed for each $\delta^{13}\text{C}_r$ calculation were collected within the closed system of the SAS, which can

be subsampled eight consecutive times without pressurization or depressurization of the system. Flushing the SAS with a consistent background air source eliminates variability associated with ambient background air conditions. Once the system is closed, the valves to each sample vial allow that vial to be bypassed and isolated from the system. Bypassing a sample vial permits the removal of the vial, and causes a decrease in the remaining system volume but no change in pressure. Leak tests have shown that the system can remain closed while circulating air for over an hour with no measurable alteration to the enclosed $\delta^{13}\text{C}$ -CO₂ value (data not shown).

We collected the samples necessary for the determination of $\delta^{13}\text{C}_r$ using the bottle method, in which pine needles were collected and placed in a 120 mL glass serum vial. The bottle was then capped with a butyl rubber stopper, wrapped in aluminium foil for the elimination of light, and placed on-line with the SAS. The needles were isolated in the foil-wrapped bottle for 2 min while the system was flushed with a constant background air source. After the system was closed, the eight samples were taken at regular time intervals while the CO₂ concentration rose as a result of respiration by the pine needles. Because relative humidity and air temperature were not regulated by the SAS during measurements, limiting the time interval between samples to 1.5 min minimised unwanted variations in the closed system's air mass, such as drastic changes in relative humidity, temperature or CO₂ concentration.

Within one week of collection, samples were analysed with a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, Inc., Palo Alto, CA, USA) coupled to a Finnigan Delta-S IRMS (GC-IRMS) for the determination of $\delta^{13}\text{C}$ (‰, relative to Vienna PDB, VPDB) and concentration of the contained CO₂ (p.p.m.). Injection of identical amounts of each sample along with known standards during GC-IRMS analysis permitted simultaneous determination of the CO₂ concentration and the $\delta^{13}\text{C}$ value by comparison of the CO₂ peak amplitude to concentrations on a standard curve (Mortazavi & Chanton 2002a). Each set of eight samples was plotted on a Keeling plot (Fig. 2), and a Model I linear regression was performed; the y-intercept represented the $\delta^{13}\text{C}$ of the CO₂ that was added to the closed system ($\delta^{13}\text{C}_r$). Errors reported for $\delta^{13}\text{C}_r$ values are standard errors from the y-intercept of the eight-sample regression. The average r^2 value for the Keeling plot data reported in this study is 0.982 ± 0.015 ($n = 49$), which is sufficient to minimise differences between results generated via a Model I or Model II regression (Miller & Tans 2003; Pataki *et al.* 2003).

To determine whether the detachment of needles or sampling with the SAS caused deviations in $\delta^{13}\text{C}_r$, we compared the bottle method of collecting respired CO₂ with two other methods; using a Mylar balloon and using a low-volume 220 mL leaf chamber. The balloon method used by McDowell *et al.* (2004) was the least intrusive sampling procedure, causing very little physical or environmental disturbance to the needles and permitting withdrawal of larger gas samples without pressurization or depressurization of

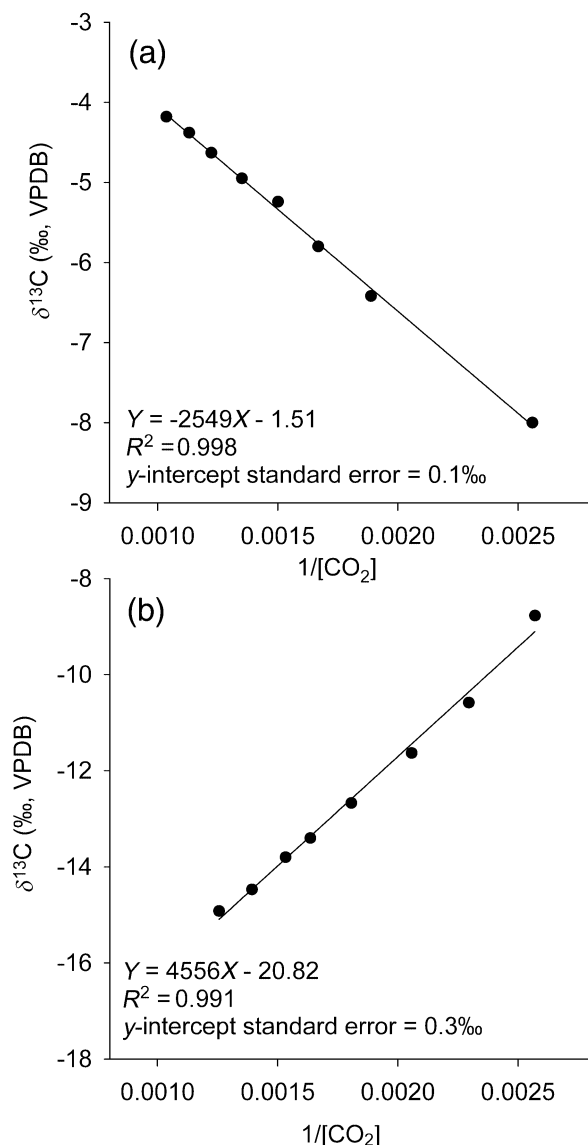


Figure 2. Keeling plot from the SAS standardization and an example of a $\delta^{13}\text{C}_r$ calculation. (a) Our sampling system, the SAS, was tested by injecting a known standard into background air ($\delta^{13}\text{C} \approx -8\text{‰}$) to simulate respiration. The Keeling plot yielded a value of $-1.5 \pm 0.1\text{‰}$ for the standard, which had a known value of -1.67‰ . (b) A typical Keeling plot $\delta^{13}\text{C}_r$ calculation, -20.8‰ in this case.

the closed system (McDowell *et al.* 2004; Xu *et al.* 2004). During the balloon procedure a portion of the limb with a group of pine needles was sealed inside a Mylar balloon with a fan inside to provide air circulation. The CO₂ concentration was monitored with an LI-6200, and eight samples were collected at 5 min intervals using a 60 cc syringe and stored in 30 mL glass serum vials capped with butyl-rubber stoppers as pressurized samples. The samples were analysed with the same procedures as those for the bottle method, and a Keeling plot was used to determine $\delta^{13}\text{C}_r$.

The low-volume 220 mL leaf chamber tests were designed to determine whether needle detachment or sub-

sampling with the SAS caused variation in $\delta^{13}\text{C}_r$. Procedures used for the low-volume leaf chamber test were identical to those for the bottle method, with the exception that needles were not detached from the limb and were contained in a low-volume leaf chamber specifically designed for pine needles instead of being detached and placed in a 120 mL bottle.

Twenty four hour time series

The time series measurements were initiated at sunrise; $\delta^{13}\text{C}_r$ measurements were taken at approximately 1 h intervals near sunrise and sunset, and 2 h intervals otherwise, for a 24 h period. All CO₂ samples required for calculation of $\delta^{13}\text{C}_r$ for the time series were collected by means of the bottle method described above, and each $\delta^{13}\text{C}_r$ value represents the Keeling plot y-intercept for the series of eight respired-CO₂ samples collected at the specified time point. Errors reported for $\delta^{13}\text{C}_r$ measurements are the standard errors from the y-intercept of the Keeling plot regression. Each $\delta^{13}\text{C}_r$ measurement was taken with a new set of needles from the same tree. The needles from each time point were saved, and their biomass $\delta^{13}\text{C}$ was determined when necessary.

The April (2004) time series included leaf-chamber measurements that were made at 1 h intervals during daylight and at 2 h intervals during the night. To determine the total amount of carbon fixed by needles during the April (2004) time series, the 24 h period from 0700 h 19 April to 0700 h 20 April 2003, was divided into consecutive 0.5 h intervals, and each interval was assigned a value for P (daylight hours, 0700–2000 h) or R (night-time, 2000–0700 h). During intervals where measured values of a term needed were not available, values were extrapolated from the measured values taken before and after the time interval by linear interpolation. In the case of P , sunrise and sunset end-members were each assumed to have a rate of zero. The integrated values of P and R (P_{total} and R_{night}) were determined by the sum of P or R multiplied by the interval time (0.5 h) for all 0.5 h time intervals within the time period of interest (daylight hours for P_{total} , night-time hours for R_{night}).

The impact of night-time needle respiration on assimilated carbon for the April (2003) trial was determined by a needle budget mass balance approach (Eqn 7).

$$(1-f)\delta^{13}\text{C}_{24\text{h net}} = \delta^{13}\text{C}_{P\text{ total}} - (f)\delta^{13}\text{C}_{R\text{ night}} \quad (7)$$

$\delta^{13}\text{C}_{24\text{h net}}$ is defined as the $\delta^{13}\text{C}$ value of remaining assimilated carbon after the effects of needle night-time respiration have been accounted for. Assimilated carbon $\delta^{13}\text{C}$ ($\delta^{13}\text{C}_{P\text{ total}}$) for the daylight hours was determined by substituting the rate-weighted average of c_i/c_a into Eqn 2 and the resulting $\Delta^{13}\text{C}_i$ and $\delta^{13}\text{C}_{\text{air}}$ into Eqn 3. Total night-time needle-respired carbon $\delta^{13}\text{C}$ ($\delta^{13}\text{C}_{R\text{ night}}$) is the rate-weighted average of $\delta^{13}\text{C}_r$ for all night-time data points. Fraction of assimilation used in needle night-time respiration (f) was determined by dividing R_{night} by P_{total} .

Shading experiment

The shading experiment was initiated at sunrise on 12

November 2003. Measurements were taken on two adjacent limbs at the same elevation on the same tree. Both limbs received full sun until noon, when one of the limbs was subjected to partial shading. The partial-shade limb was covered with landscaping shade cloth, which reduced photosynthetically active radiation (PAR) to 40% of the full sun value with minimal effect on temperature (2–3 °C cooler than full sun). $\delta^{13}\text{C}_r$ measurements were made on 2 h intervals and the results from the two groups were compared.

PAR and vapour pressure deficit (VPD)

Air temperature (T) was measured at the August and November trials, and relative humidity (RH) and PAR were also measured at the November trial. RH and T were measured with an LI-6200, and PAR was measured and integrated over 30 min intervals with an LI-1000 equipped with an LI-190SA quantum sensor (Li-Cor Inc.). VPD was calculated from the T and RH measurements.

RESULTS

Standardization of CO₂ collection

We tested the bottle method $\delta^{13}\text{C}_r$ measurement technique by simulating foliage-respired CO₂ with seven 1 mL injections of Ultra High Purity working CO₂ standard, calibrated against NIST NBS-19 calcite (Coplen 1996), into the system at 1.5 min intervals. Analysis of the samples was consistent with the procedures for field samples and yielded a $\delta^{13}\text{C}_r$ of $-1.5 \pm 0.1\text{‰}$ compared to -1.67‰ , the known value of the standard (Fig. 2a). This result indicates that the SAS sampling procedure does not cause fractionation and yields $\delta^{13}\text{C}$ values similar to the $\delta^{13}\text{C}$ value of introduced CO₂.

Comparisons with other methods further validated the technique. In two replicate trials on adjacent limbs, the Mylar-balloon, leaf-chamber and bottle methods were all used to determine $\delta^{13}\text{C}_r$. The samples were taken in the early morning, just before sunrise. The results from four out of the six measurements were consistent, and leaks are suspected to have caused the variations observed in the limb-1 leaf-chamber and balloon measurements (Table 1). The balloon and leaf-chamber methods were problematic in the field because of difficulties in sealing the system. The longer time between successive samples collected from the

Table 1. $\delta^{13}\text{C}$ values for respired CO₂ (‰, VPD). Three methods for collecting respired CO₂ were applied to needles from the same tree limb. Values shown are the eight-sample Keeling plot y-intercepts, representing the $\delta^{13}\text{C}$ of respired CO₂. The error shown is the standard error from the y-intercept of the Keeling plot regression

	Leaf chamber	Balloon	Bottle
Limb 1	-26.5 ± 0.2	-26.5 ± 0.1	-27.7 ± 0.2
Limb 2	-27.5 ± 0.2	-27.5 ± 0.2	-27.6 ± 0.1

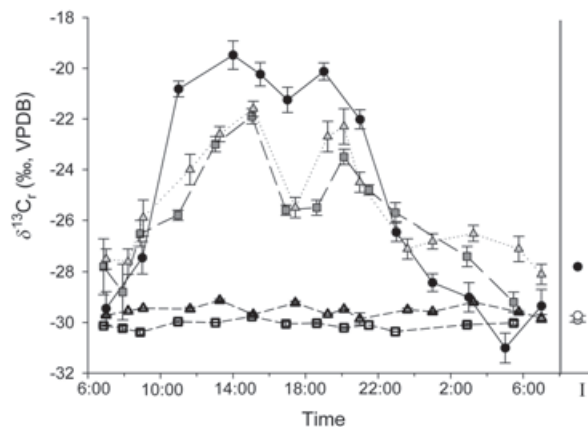


Figure 3. Twenty four hour time series of respired CO₂ from needles placed in darkness ($\delta^{13}\text{C}_r$) and biomass $\delta^{13}\text{C}$. Closed symbols represent $\delta^{13}\text{C}_r$ and open symbols represent bulk-biomass $\delta^{13}\text{C}$: August (2002) trial by triangles, November (2002) trial by squares, April (2003) trial by circles. Each $\delta^{13}\text{C}_r$ value shown represents the Keeling intercept from the regression of a set of eight samples, and errors shown for $\delta^{13}\text{C}_r$ values are standard errors from the Keeling plot regression. Biomass $\delta^{13}\text{C}$ represents pine needles collected for the respiration experiments. Biomass $\delta^{13}\text{C}$ errors are half of the range between two replicates. Sunrise was at approximately 0700 h for all trials, and sunset was at 2022 h in August, 1745 h in November, and 2007 h in April. Values of $\delta^{13}\text{C}_{P\text{ total}}$ (open circle), $\delta^{13}\text{C}_{R\text{ total}}$ (closed circle) and $\delta^{13}\text{C}_{24\text{ h net}}$ (grey circle) for the April (2003) are shown (I). The error bars associated with $\delta^{13}\text{C}_{P\text{ total}}$ represent the maximum range of variability for $0.05 < f < 0.20$ assuming $\delta^{13}\text{C}_{P\text{ total}}$ of -29‰ (see Table 3).

balloon method (5 min) aggravated the effect of any leaks. The remaining four measurements were all within $\pm 0.2\text{‰}$. The comparison indicates that detaching the pine needles did not cause an unrealistic physiological response, altering the respired-CO₂ $\delta^{13}\text{C}$ value within the short duration of the measurement (< 15 min).

Twenty four hour time series

Respired CO₂ $\delta^{13}\text{C}$ values from needles placed in darkness during $\delta^{13}\text{C}_r$ measurements varied from -30‰ to -20‰ in the August, November and April trials (Fig. 3). The $\delta^{13}\text{C}_r$ values for the August and November trials had similar magnitudes of variation and bimodal shapes, with midday maxima at 1500 and secondary maxima at around 1700 h, even though the temperatures were on average 20 °C higher and sunset was 2.5 h later for the August trial. After the second maximum, $\delta^{13}\text{C}_r$ values for the August trial returned to the August pre-dawn level (-27‰) rapidly (by 2300 h) compared to those of the November trial, where the night-time air temperature was on average 18 °C cooler and $\delta^{13}\text{C}_r$ values did not return to the November pre-dawn level (-28‰) until 0300 h. The April trial exhibited a similar pattern in diurnal $\delta^{13}\text{C}$ enrichment but the magnitude of variation exceeds that of the previous trials. The night-time return of $\delta^{13}\text{C}_r$ to the pre-dawn level (-29‰) was rapid, similar to the return to baseline in the August trial. Night

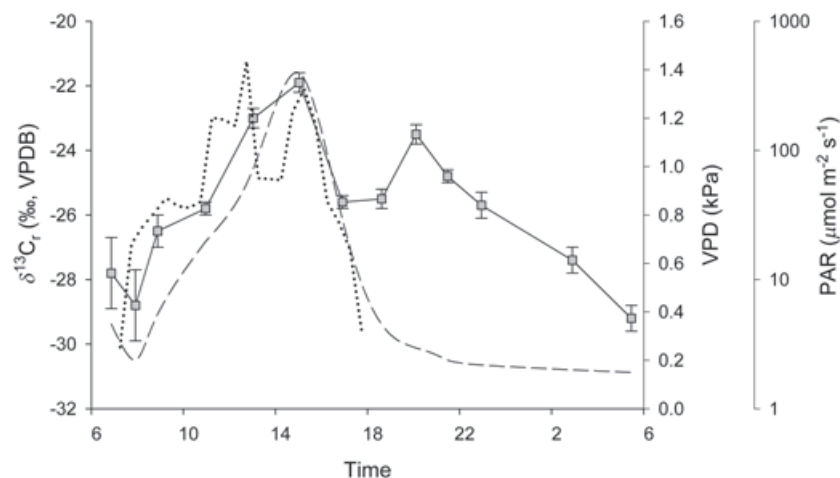


Figure 4. Respired CO_2 $\delta^{13}\text{C}$ (shaded squares), vapour pressure deficit (VPD, dashed line), and photosynthetically active radiation (PAR, dotted line) plotted against time for the November (2002) 24 h series. PAR and VPD lines are smoothed data from measurements taken at 30-min intervals during daylight hours and 2-h intervals otherwise.

temperatures were similar in April and August, and cooler in November.

The pine needles used for $\delta^{13}\text{C}_r$ measurements in the August and November trials were analysed for bulk biomass $\delta^{13}\text{C}$ (Fig. 3). No significant variation was seen over the diurnal cycle, and the average values for each trial were within 0.5‰.

The November trial VPD ranged from 0.2 to 1.4 kPa over the diurnal cycle, and its midday maximum corresponds well with the first peak of $\delta^{13}\text{C}_r$ (Fig. 4). After sunset, VPD quickly declined to its early morning minimum of 0.2 kPa. PAR had a bimodal shape over the diurnal cycle, caused by shading from other nearby trees. The onset and cessation of sunlight marked the initial enrichment trend and the decline from the first peak in $\delta^{13}\text{C}_r$ (Fig. 4).

Photosynthetic and respiration rate data from the April (2003) trial are shown in Table 2. The integrated value for night-time needle respiration ($R_{\text{night}} = -0.038 \pm 0.002 \text{ mol C m}^{-2}$, Table 2) is approximately one-tenth ($f = 0.101$) of the integrated value for daytime needle assimilation ($P_{\text{total}} = 0.375 \pm 0.019 \text{ mol C m}^{-2}$, Table 2). Other studies have shown similar ratios of carbon uptake and respiration in pine needles (Cropper & Gholz 1991; Will 2000). The assimilation rate-weighted average of c_i/c_a (0.73,

Table 2) corresponds to a $\delta^{13}\text{C}_{P_{\text{total}}}$ of -29.7% ($\delta^{13}\text{C}_{\text{air}} = -8.2\%$, Eqns 2 and 3). This estimate of assimilation is ^{13}C -depleted relative to the rate-weighted average of night-time needle $\delta^{13}\text{C}_r$ ($\delta^{13}\text{C}_{R_{\text{total}}}$, -27.8% , Table 2).

The $\delta^{13}\text{C}$ of remaining needle-assimilated carbon ($\delta^{13}\text{C}_{24\text{h net}}$) estimated with Eqn 7 (-29.9%) is only ^{13}C -depleted by 0.22‰ compared to the value of daytime-assimilated carbon ($\delta^{13}\text{C}_{P_{\text{total}}}$) of -29.7% . This estimate assumes that 10.1% of assimilated carbon is used for night-time needle respiration and uses the estimate for $\delta^{13}\text{C}_{P_{\text{total}}}$ that is dependent on c_i/c_a measurements. Sensitivity analysis of this calculation is shown in Table 3. Over the 24 h period when $\delta^{13}\text{C}_r$ was determined, the measurement obtained just prior to dawn ($-29.3 \pm 0.6\%$) is most representative of the ^{13}C of net assimilated carbon ($\delta^{13}\text{C}_{24\text{h net}}$) (Fig. 3).

Shading experiment

During the November 2003 shading experiment, $\delta^{13}\text{C}_r$ varied from -34% to -15% and both subgroups exhibited similar ^{13}C -enrichment until the initiation of shading at noon (Fig. 5). The limb subjected to a partial shading regime exhibited a decline in $\delta^{13}\text{C}_r$ following shading, while the full-sun control limb exhibited a slower decline in $\delta^{13}\text{C}_r$,

Table 2. Rate, c_i/c_a , and $\delta^{13}\text{C}_r$ data from the April (2003) trial. P , R and c_i/c_a were measured with a portable photosynthesis system equipped with a 0.25 L leaf chamber (LI-6200) on needles still attached to the limb. $\delta^{13}\text{C}_r$ values were measured on detached needles using the sequenced air sampler (SAS). The integrated total of respiration ($R_{\text{night}} = -0.038 \pm 0.002 \text{ mol C m}^{-2}$) is approximately one-tenth of assimilation from the daylight hours ($P_{\text{total}} = 0.375 \pm 0.019 \text{ mol C m}^{-2}$). The rate-weighted average of c_i/c_a is 0.73. The rate-weighted average of $\delta^{13}\text{C}_r$ is -27.8% . Each rate and c_i/c_a measurement reported is the average value of eight consecutive measurements and the errors reported are one standard deviation. Errors reported for $\delta^{13}\text{C}_r$ values are standard errors from the Keeling plot regressions

Time	Photosynthesis (P ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	c_i/c_a	Time	Respiration (R ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	$\delta^{13}\text{C}_r$ (‰)
1006	5.5 ± 0.2	0.76 ± 0.01	2123	-0.8 ± 0.3	-22.0 ± 0.4
1202	11.9 ± 0.8	0.63 ± 0.04	2304	-0.9 ± 0.05	-26.4 ± 1.0
1415	15.3 ± 3.4	0.76 ± 0.09	0104	-1.1 ± 0.1	-28.4 ± 0.3
1512	8.5 ± 0.7	0.73 ± 0.03	0301	-1.2 ± 0.2	-29.0 ± 0.6
1603	11.0 ± 0.8	0.73 ± 0.01	0504	-0.9 ± 0.04	-31.0 ± 0.6
1710	11.4 ± 0.7	0.72 ± 0.04	0700	-0.7 ± 0.1	-29.3 ± 0.6
1903	5.3 ± 2.4	0.87 ± 0.03			

Table 3. Change in $\delta^{13}\text{C}_{\text{P total}}$ caused by night-time respiration ($\delta^{13}\text{C}_{24\text{ h net}} - \delta^{13}\text{C}_{\text{P total}}$), where f is the fraction of assimilation used for respiration at night. $\delta^{13}\text{C}_{\text{P total}}$ is the integrated value of assimilation during daylight (dawn to dusk). Appropriate values for the April (2003) trial based on leaf-chamber measurements are $f = 0.101$ and $\delta^{13}\text{C}_{\text{P total}}$ of -29.73‰ ($c_i/c_a = 0.73$, Table 2, Eqns 2 and 3)

$\delta^{13}\text{C}_{\text{P total}}$	Change in $\delta^{13}\text{C}_{\text{P total}}$ from night-time respiration (‰)			
	-27	-29	-30	-32
f				
0.05	0.04	-0.06	-0.12	-0.22
0.1	0.09	-0.13	-0.24	-0.47
0.15	0.14	-0.21	-0.39	-0.74
0.2	0.20	-0.30	-0.55	-1.05

similar to the pattern for the previous 24 h $\delta^{13}\text{C}_r$ data (Fig. 3). However, while initial ^{13}C -enrichment and night-time ^{13}C -depletion were similar to previous 24 h trials, sampling resolution was not sufficient to show whether the bimodal pattern observed in the daytime data from the previous 24 h trials was present.

DISCUSSION

The $\delta^{13}\text{C}$ of foliage-respired CO₂ has been shown to be ^{13}C -enriched relative to the substrates used for respiration and to bulk biomass (Duranceau *et al.* 1999, 2001; Ghashghaie *et al.* 2001, 2003; Tcherkez *et al.* 2003; Xu *et al.* 2004). Tcherkez *et al.* (2003) demonstrated that the ^{13}C -enrichment of $\delta^{13}\text{C}_r$ decreased during continuous darkness over several days, presumably as a result of the decrease in the carbohydrate pool size and changes in the relative contributions of the major decarboxylation processes of dark respiration. Over a 24 h period in the dark, glucose concentrations can decline by 50–100% at temperatures ranging from 20 to 30 °C (Tcherkez *et al.* 2003). Ghashghaie *et al.* (2003) suggested that discrimination during night-time respiration and the resulting ^{13}C -enriched $\delta^{13}\text{C}_r$ would cause ^{13}C -depletion of the remaining plant material. We examined the hypothesis that significant variability of $\delta^{13}\text{C}_r$ would be observed over a diurnal cycle and used our $\delta^{13}\text{C}_r$, P and R data to estimate the effect ^{13}C -enriched night-time respiration could have on remaining assimilated carbon ($\delta^{13}\text{C}_{24\text{ h net}}$).

Diurnal variation in $\delta^{13}\text{C}_r$

Similar diurnal patterns in $\delta^{13}\text{C}$ for needles placed in darkness are evident in the August (2002), November (2002) and April (2003) 24 h series (Fig. 3). Despite temperature differences of approximately 20 °C between the August and November (2002) trials, the $\delta^{13}\text{C}_r$ values are remarkably similar, with the exception that ^{13}C -enriched CO₂ was released for a longer period under the cooler conditions of the November (2002) time series (Fig. 3). The rate of

change of night-time $\delta^{13}\text{C}_r$ values is apparently related to respiration rates, demonstrated by the comparison of the August (2002) and November (2002) trials, where the night-time decline in $\delta^{13}\text{C}_r$ values occurred at a slower pace for the November (2002) trial when air temperature was on average 18 °C cooler and associated respiration rates also would have been lower (Villar, Held & Merino 1995; Tcherkez *et al.* 2003). The rapid night-time decline in $\delta^{13}\text{C}_r$ is similar to the work of Troughton, Card & Hendy (1974) who showed respiration that was ^{13}C -depleted relative to biomass for *Pinus radiata*, possibly indicating that the respiratory metabolism of conifers produces CO₂ that is ^{13}C -depleted relative to night-time respiration of other C₃ plants. However, unlike the results of Troughton *et al.* (1974), our results show night-time respiration approaching biomass $\delta^{13}\text{C}$ values and include only one data point where respiration is ^{13}C -depleted relative to biomass (Fig. 3).

The midday $\delta^{13}\text{C}_r$ enrichment relative to plant organic matter ^{13}C during the 24 h series (Fig. 3) is similar in magnitude to the maximum enrichment of $\sim 9\text{‰}$ reported by Ghashghaie *et al.* (2001) for *Nicotina sylvestris* plants. Ghashghaie *et al.* (2001) found that under well-watered conditions, $\delta^{13}\text{C}_r$ was enriched relative to even the most ^{13}C -enriched substrates used for respiration. This enrichment could result from the non-statistical ^{13}C distribution with the glucose molecules (Rossmann *et al.* 1991). Rossmann *et al.* (1991) demonstrated that C₃ and C₄ of glucose molecules extracted from sugar beets were enriched in ^{13}C relative to the other carbon positions. During respiration, if a high percentage of catabolized carbon were used for lipid biosynthesis, for example, the ^{13}C of respired CO₂ would be highly enriched (Ghashghaie *et al.* 2003). Alternatively, if all of the catabolized carbon is used for respiration, all light and heavy carbon atoms will be carboxylated. Ghashghaie *et al.* (2003) suggested that the degree of ^{13}C -enrichment relative to the substrate used during respiration would depend on environmental conditions and the relative activities of different metabolic pathways.

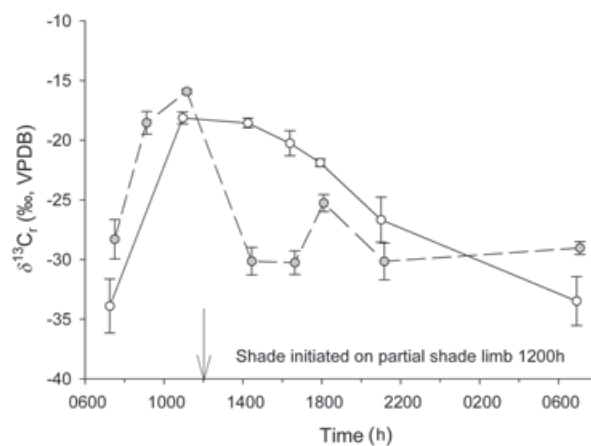


Figure 5. Results from the shading experiment, November (2003). Needles from two limbs of the same tree were subjected to full sun until noon, then one limb was left in full sun (open circles) and one was subjected to partial shading (shaded circles).

The shading experiment results are consistent with the 24 h series data in that $\delta^{13}\text{C}_r$ values become less ^{13}C -enriched with time when assimilation rates are depressed (Fig. 5). The shaded subgroup's decline in $\delta^{13}\text{C}_r$ after the midday maximum could result from respiration of ^{13}C -depleted material produced in the low light (low assimilation rate, high c_i/c_a) conditions (Knohl *et al.* 2004; Scartazza *et al.* 2004), or it could be that respiration had consumed the available assimilate and had switched to a carbon pool with a residence time of a few days, as modelled by Schnyder *et al.* (2003); see also Nogues *et al.* (2004) with a more depleted ^{13}C values.

Alternatively, the decline in $\delta^{13}\text{C}_r$ could result from changes in the metabolic pathway of dark respiration (Tcherkez *et al.* 2003) over the diurnal cycle. The decline in $\delta^{13}\text{C}_r$ values following sunset (Fig. 3) or shade (Fig. 5) to more ^{13}C -depleted values prior to the following sunrise is consistent with, but more rapid than, the decline in the $\delta^{13}\text{C}$ of foliage-respired CO_2 in continuous darkness over a five day period (Tcherkez *et al.* 2003). Tcherkez *et al.* (2003) observed that $\delta^{13}\text{C}_r$ values were ^{13}C -depleted by as much as 9‰ compared to initial values following the decline in carbohydrate concentrations during prolonged exposure to darkness. They concluded that the decline in ^{13}C could be accounted for by a shift in metabolism away from pyruvate dehydrogenation and towards fatty acid β -oxidation coupled to the Krebs cycle. Following depletion of the carbohydrate pool during prolonged exposure to darkness, the ^{13}C of respired CO_2 may become depleted even relative to the ^{13}C of bulk organic matter (Tcherkez *et al.* 2003). However, during our diurnal experiments (Figs 3 & 5), the ^{13}C of respired CO_2 declined only to approach values similar to that of needle organic matter ^{13}C .

The ^{13}C of needle organic matter represents the long-term integrated ^{13}C values of net carbon fixed over the needle's life. Biomass carbon $\delta^{13}\text{C}$ does not appear to represent respired carbon $\delta^{13}\text{C}$ on short time scales (Fig. 3). Any changes in reservoirs that are influenced on short time scales are buffered by structural material and reservoirs of carbon with longer residence times. This conclusion supports the results of Brendel (2001), who showed that the response of bulk-foliage $\delta^{13}\text{C}$ values to changing $\Delta^{13}\text{C}_i$ was buffered by structural material, and the results of Knohl *et al.* (2004) and Scartazza *et al.* (2004) who showed that bulk organic carbon was unresponsive to short-term variations in ecosystem respiration $\delta^{13}\text{C}$.

Effect of ^{13}C -enriched respiration

Our results suggest that $\delta^{13}\text{C}_r$ is enriched relative to assimilated carbon $\delta^{13}\text{C}$ estimated from Eqns 2 and 3 and measured c_i/c_a values (Table 2). Schnyder *et al.* (2003) observed that in mesocosm-scale experiments, integrated night-time respiration was 0.53‰ enriched relative to recent assimilate. The effect of ^{13}C -enriched respiration on total assimilated carbon estimated with Eqn 7 suggests that $\delta^{13}\text{C}_{24\text{h net}}$ would only be ^{13}C -depleted by 0.22‰ compared to $\delta^{13}\text{C}_{\text{P total}}$. The impact of night-time respiration on assimilated carbon

appears to be minimal. The limitations of our estimate are that it assumes a value for assimilated carbon at dusk derived from c_i/c_a measurements ($\delta^{13}\text{C}_{\text{P total}} = -29.7\text{‰}$, Table 2) and that 10.1% of assimilated carbon is used for night-time respiration (Table 2). We further assume that night-time respiration is fuelled by recent assimilate. Sensitivity analysis of this calculation (Table 3) shows that if f is lower than the projected 10% (i.e. that some respiration is fuelled by a longer-lived component) very little change in assimilated carbon $\delta^{13}\text{C}$ will occur. Alternatively, if f was double our estimated value and assimilated carbon was significantly more ^{13}C -depleted than our c_i/c_a measurements indicated, respiration could alter assimilated carbon $\delta^{13}\text{C}$ by more than 1‰ ($f = 0.2$, $\delta^{13}\text{C}_{\text{P total}} = -32\text{‰}$, Table 3).

Although night-time $\delta^{13}\text{C}_r$ may be ^{13}C enriched relative to assimilated carbon ($\delta^{13}\text{C}_{\text{P total}} = -29.73\text{‰}$) by as much as 7.7‰ (Table 2), it is unclear how fractionation during day respiration affects $\Delta^{13}\text{C}$. Day respiration rates may be inhibited during photosynthesis (Villar *et al.* 1995), and our measurements only determine $\delta^{13}\text{C}_r$ for dark respiration in the absence of light. Therefore further efforts are required to determine the effect of daytime respiration and associated fractionation effects on $\Delta^{13}\text{C}$.

CONCLUSIONS

Our primary objective was to examine the magnitude of variability for $\delta^{13}\text{C}$ values of needle-respired CO_2 in darkness over a diurnal cycle. Our results demonstrate that $\delta^{13}\text{C}_r$ exhibits diurnal variability and is ^{13}C -enriched compared to biomass during the day and early part of the night but approaches values similar to $\delta^{13}\text{C}_{\text{P total}}$ before sunrise. The ^{13}C -enrichment apparent in $\delta^{13}\text{C}_r$ shows a strong time dependency, with maximum enrichment immediately following assimilation and decreasing with time in darkness. Although night-time respiration has been shown to be ^{13}C -enriched relative to assimilated carbon and bulk biomass, the relatively small quantity of ^{13}C -enriched night-time respiration appears to have little effect on the ^{13}C value of the remaining assimilated carbon. The dependency of $\delta^{13}\text{C}_r$ on time and environmental conditions, such as temperature, may be responsible for some of the interspecies variation reported in the literature (reviewed in Ghashghaie *et al.* 2003). If species are to be compared, standard procedures need to be developed to address the dynamic temporal variability that exists in $\delta^{13}\text{C}_r$.

The enrichment in needle-respired CO_2 and its gradual decay with time in darkness could potentially impact measurements of the ^{13}C of ecosystem-respired CO_2 . Night-time samples of mid-canopy-respired CO_2 samples consist of inputs from both autotrophic and heterotrophic respiration. While the signature of the heterotrophic component is expected to remain invariant on short time scales (Trumbore 2000), the isotopic composition of autotrophic respiration contributed by the foliage apparently changes on a time scale of hours (Figs 3 & 5). If time series of night-time mid-canopy air samples are used to construct Keeling plots during periods when a large fraction of total ecosys-

tem respired CO₂ is contributed by foliage respiration, the intercepts of mid-canopy time series Keeling plots could potentially be biased towards more enriched values.

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