

Effects of environmental parameters, leaf physiological properties and leaf water relations on leaf water $\delta^{18}\text{O}$ enrichment in different *Eucalyptus* species

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ABSTRACT

Stable oxygen isotope ratios ($\delta^{18}\text{O}$) have become a valuable tool in the plant and ecosystem sciences. The interpretation of $\delta^{18}\text{O}$ values in plant material is, however, still complicated owing to the complex interactions among factors that influence leaf water enrichment. This study investigated the interplay among environmental parameters, leaf physiological properties and leaf water relations as drivers of the isotopic enrichment of leaf water across 17 *Eucalyptus* species growing in a common garden. We observed large differences in maximum daily leaf water $\delta^{18}\text{O}$ across the 17 species. By fitting different leaf water models to these empirical data, we determined that differences in leaf water $\delta^{18}\text{O}$ across species are largely explained by variation in the Péclet effect across species. Our analyses also revealed that species-specific differences in transpiration do not explain the observed differences in $\delta^{18}\text{O}$ while the unconstrained fitting parameter 'effective path length' (L) was highly correlated with $\delta^{18}\text{O}$. None of the leaf morphological or leaf water related parameters we quantified in this study correlated with the L values we determined even though L was typically interpreted as a leaf morphological/anatomical property. A sensitivity analysis supported the importance of L for explaining the variability in leaf water $\delta^{18}\text{O}$ across different species. Our investigation highlighted the importance of future studies to quantify the leaf properties that influence L . Obtaining such information will significantly improve our understanding of what ultimately determines the $\delta^{18}\text{O}$ values of leaf water across different plant species.

Key-words: biogeochemistry; Currency Creek Arboretum; ecophysiology; leaf hydraulics; plant water relations; stable isotopes; stomatal conductance; transpiration.

INTRODUCTION

The analysis of oxygen isotope ratios ($\delta^{18}\text{O}$) in plant material has emerged as a powerful tool in the plant, ecosystem

and earth sciences (Yakir & Sternberg 2000; Dawson *et al.* 2002; Barbour, Cernusak & Farquhar 2005; Barbour 2007; Dawson & Siegwolf 2007). Early investigations using $\delta^{18}\text{O}$ data from tree-ring cellulose have demonstrated their utility as a 'paleo-thermometer' allowing for the partial reconstruction of past climates (Gray & Thompson 1976). More recently, $\delta^{18}\text{O}$ analysis of leaf water has shown that the enrichment of the heavier oxygen isotope (^{18}O) in the leaf water and the subsequent photoassimilates formed from it hold valuable information about various aspects of plant ecophysiological performance and ecosystem biogeochemistry. Isotopic leaf water enrichment can, for example, be related to a plant's water relations in terms of the environmental evaporative demand as well as the supply or loss of water from leaves via measures of stomatal conductance (g_s) and transpiration (E) (Flanagan & Ehleringer 1991; Roden & Ehleringer 1999; Cernusak *et al.* 2003; Keitel *et al.* 2003). In turn, leaf water ^{18}O information can prove pivotal for ecophysiological interpretations and for plant breeding programmes (Barbour *et al.* 2000a; Farquhar, Cernusak & Barnes 2007). The oxygen isotope ratio of leaf organic material has also been shown to aid in the interpretation of carbon isotope ratio data ($\delta^{13}\text{C}$) by allowing the separation of stomatal from photosynthetic effects on $\delta^{13}\text{C}$ (Scheidegger *et al.* 2000; Keitel *et al.* 2006; Grams *et al.* 2007; Sullivan & Welker 2007). On the ecosystem level, the $\delta^{18}\text{O}$ of leaf water and of the water transpired from leaves as well as the $\delta^{18}\text{O}$ of CO_2 respired from leaves has been used to partition water fluxes leaving ecosystems or has allowed the separation of autotrophic and heterotrophic carbon fluxes, respectively (Bariac *et al.* 1989; Yakir & Wang 1996; Yakir & Sternberg 2000; Riley *et al.* 2003; Williams *et al.* 2004; Tu & Dawson 2005). Finally, Helliker & Griffiths (2007) have recently shown that the $\delta^{18}\text{O}$ values of water extracted from tropical epiphytes reflect the isotopic ratios of atmospheric water vapour, allowing the reconstruction of this essential component of the hydrological cycle with high spatial resolution. Given the broad range of applications for $\delta^{18}\text{O}$ in various fields of plant and ecosystem science, understanding the processes

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that govern variability in $\delta^{18}\text{O}$ in plant leaf water and organic material has recently been the focus of extensive research efforts.

Recent improvements of leaf-level models are permitting a reasonable mechanistic understanding of the environmental and physiological processes that govern the enrichment of leaf water and plant organic material (Barbour *et al.* 2007; Farquhar *et al.* 2007). Despite these improvements, the number of studies that have utilized $\delta^{18}\text{O}$ as an integrated recorder of plant water relations in plant or ecosystem studies remains limited. In part, this is due to the challenges which still remain in adequately quantifying the interactions among the factors that influence isotopic leaf water enrichment and that in turn can complicate simple interpretations of $\delta^{18}\text{O}$ signals. Moreover, most studies that have addressed the mechanistic processes that drive isotopic leaf water enrichment have focused on a very small number of species. Thus, our understanding remains limited regarding the interactions between environmental drivers (e.g. relative humidity, air temperature), leaf physiological properties (e.g. transpiration, stomatal conductance) and leaf water relations (e.g. flow of water in the leaf, i.e. effective path length) that cause variability of leaf water $\delta^{18}\text{O}$ across species. The few studies that have addressed various aspects of isotopic leaf water enrichment across different species have suggested strong and species-specific effects on leaf water $\delta^{18}\text{O}$ (Cooper & Deniro 1989; Wang & Yakir 1995; Wang, Yakir & Avishai 1998; Terwilliger *et al.* 2002). In particular, the 'effective path length' (L), which is an unconstrained fitting parameter in leaf water models and is typically interpreted as the tortuosity of the flow path for water in the transpirational stream within the leaf, has been suggested to differ substantially across different species. In addition to environmental drivers and leaf physiological properties, differences in L should therefore be of critical importance for isotopic leaf water enrichment across different species.

In order to improve our understanding and interpretation of leaf water $\delta^{18}\text{O}$ signals across different species, the study presented here addresses the interplay of environmental parameters, leaf physiological properties and L as drivers of isotopic leaf water enrichment across a large number of different *Eucalyptus* species. Specifically, our study had five objectives: (1) to quantify the variability in leaf water $\delta^{18}\text{O}$ enrichment among different *Eucalyptus* species that grow in the same ecosystem but which also possess different leaf morphologies and leaf physiological properties; (2) to test the applicability of both steady-state and non-steady-state leaf water models for different species with different patterns of leaf water enrichment; (3) to identify the role of environmental parameters, leaf physiological properties and L that may serve to explain observed differences in isotopic leaf water enrichment across different species; (4) to identify what factors of a leaf define 'effective path length' by using our across-species comparisons and (5) to perform sensitivity analyses of existing steady-state leaf water models for leaves with different effective path lengths to help improve our mechanistic understanding and

interpretation of what leads to species-specific differences in isotopic leaf water enrichment.

METHODS

We conducted our research at the Currency Creek Arboretum near Adelaide in South Australia (<http://www.dn.com.au/>). The privately owned arboretum was established in 1992 and holds over 6000 *Eucalyptus* trees belonging to over 900 species. Trees in the arboretum were typically grown from seedlings and were planted in at least 4 replicates per species. We selected 17 *Eucalyptus* species for our study that represented the wide-ranging diversity of form and function in this genus (Table 1). All trees that we selected for our study were mature, that is, they had flowered and produced fruits for several seasons prior to our experiment. All samples for this study were collected in March 2006.

Water isotopes

To characterize the diurnal isotopic enrichment of leaf water for different species, we collected leaves from six species seven times a day between 0600 and 1900 h on 10 March. For the remaining 11 species, we collected leaves once on either 8 or 9 March between 1400 and 1600 h when isotopic leaf water enrichment was typically highest (for a complete species list see Table 1). For all species, leaves were collected from three replicate trees ($n=3$). We removed the mid-vein from the leaves, sealed the leaves in extraction vials and kept the leaves frozen until water extraction in the laboratory.

We also collected xylem water from three replicate trees of every species in the early afternoon of the same day that leaf water samples were collected. Xylem sap was collected from lower branches (15–20 mm diameter) of three individual trees using the mild vacuum extraction technique developed by Jeschke & Pate (1995). In brief, the cut end of a branch was freed from its bark and inserted tightly into a 50 mL centrifugation tube using a polymer sealant (Terostat Henkel, Düsseldorf, Germany). Xylem sap was collected by applying a mild vacuum (pressure 25–60 kPa) using a vacuum pump and successive cutting of 5–10 cm segments from the proximal end of the branch to facilitate sap displacement from conducting xylem vessels. Extracted xylem fluid (0.5–2.0 mL) was collected in 2 mL crimp cap vials (Infochroma, Zug, Switzerland) and was kept frozen until analyses for stable isotopes. We assumed that the water source of the trees was constant over the sampling period because there was no moisture input (precipitation, fog) during the time of the experiment.

To characterize the isotopic composition of atmospheric moisture, we collected water vapour samples in four replicates four times a day using a cryo-trap. Air was pumped at a flow rate of 35 L h⁻¹ through glass U-tubes (12 mm inner diameter, 200 mm length) that were submerged in a liquid

Table 1. Natural habitat, xylem water $\delta^{18}\text{O}$, maximum diurnal isotopic leaf water enrichment $\delta^{18}\text{O}_{\text{M-max}}$ maximum diurnal isotopic leaf water enrichment above source water $\Delta^{18}\text{O}_{\text{M-max}}$, leaf water concentration (*LWC*) as well as effective path length (*L*) of the 17 investigated *Eucalyptus* species

	Natural habitat	Xylem water $\delta^{18}\text{O}$ (‰)	Leaf water $\delta^{18}\text{O}_{\text{M-max}}$ (‰)	Leaf water $\Delta^{18}\text{O}_{\text{M-max}}$ (‰)	<i>LWC</i> (g m ⁻²)	<i>L</i> (mm)
<i>Eucalyptus minirichi</i>	Arid	-4.51	14.75	19.35	344.35	7.0
<i>Eucalyptus incrassata</i>	Arid	-4.41	18.94	23.46	316.12	3.2
<i>Eucalyptus maculata</i>	Mesic	-4.92	16.59	21.61	210.86	6.4
<i>Eucalyptus pseudoglobulus</i>	Mesic	-4.99	8.43	13.49	256.13	119.2
<i>Eucalyptus ovata</i>	Wet	-4.49	12.00	16.56	195.17	50.9
<i>Eucalyptus tricarpa</i>	Semi-arid	-4.08	17.78	21.95	248.84	3.6
<i>Eucalyptus leucoxydon</i>	Semi-arid	-4.20	14.11	18.31	199.88	65.0
<i>Eucalyptus oleosa</i>	Arid	-5.07	13.83	18.90	246.29	60.0
<i>Eucalyptus platypus</i>	Arid	-4.35	15.60	19.95	304.58	35.0
<i>Eucalyptus tetraptera</i>	Arid	-4.28	13.08	17.35	539.93	60.0
<i>Eucalyptus saligna</i>	Mesic	-4.44	15.19	19.63	162.41	140.0
<i>Eucalyptus melliodora</i>	Semi-arid	-4.15	13.91	18.06	166.73	40.0
<i>Angophora costata</i>	Tropical	-4.59	12.51	17.09	159.25	220.0
<i>Eucalyptus astringens</i>	Semi-arid	-4.25	13.00	17.27	224.19	15.0
<i>Eucalyptus microcarpa</i>	Semi-arid	-4.53	11.52	16.05	249.29	7.0
<i>Eucalyptus pyriformis</i>	Mesic	-3.61	14.29	17.90	170.90	70.0
<i>Eucalyptus camaldulensis</i>	Phreatophytic	-4.59	14.74	19.34	241.53	5.0

nitrogen/ethanol slush. After vapour collection, the U-tubes were sealed, the frozen water thawed and transferred into 2 mL crimp cap vials.

Bulk leaf water was obtained from leaves using cryogenic vacuum distillation (Ehleringer, Roden & Dawson 2000) conducted at the Paul Scherrer Institut in Villigen, Switzerland. Leaf samples in the extraction vials were heated to 80 °C. The evaporated water was collected in glass U-tubes that were submerged in liquid nitrogen. The extraction line was subject to a vacuum of 0.03 hPa. Extractions were performed for at least 2 h per sample. After extraction, the U-tubes with the frozen leaf water were removed from the extraction line, sealed, the water thawed and transferred into 2 mL crimp cap vials.

Bulk leaf water, xylem water and water vapour samples were analysed by equilibration of 50 μL sample H_2O with CO_2 for 48 h. The isotope composition of the CO_2 gas was then determined using a gas chromatograph – isotope ratio mass spectrometer running in continuous flow mode (Finnigan MAT Delta Plus XL Finnigan, Bremen, Germany) housed at the Center for Stable Isotope Biogeochemistry at University of California, Berkeley. Calibration standards were included every sixth position to account for instrument drift during a run. Correction for instrument drift was determined by plotting the value of the reported calibration standard isotope ratio against its position in the run. Drift of the calibration standard was described by fitting a quadratic curve to the reported values. The difference between the curve fit and the ‘actual’ calibration standard value was added to the reported sample value. Instrument drift was also estimated using a ‘peak to peak’ correction. For this correction, we assumed linear drift between each pair of calibration standards and used the slope of the linear model to make corrections (Brooks, personal communication). Based on these calibration and correction procedures, the long-term external precision for these water analyses is $\pm 0.14\text{‰}$.

Leaf gas exchange and climate data

We determined the diurnal variability of leaf gas exchange and leaf temperature for all 17 *Eucalyptus* species (3 replicate trees per species) using a Li-Cor 1600 porometer (Li-Cor, Lincoln, NE, USA). Because *Eucalyptus* typically have amphistomatous leaves, we measured gas exchange on both sides of each leaf and added the values for g_s and E and determined the mean for leaf temperature (T_l). Measurements were conducted on three consecutive days and overlapped with the collection of leaf and xylem water for a particular species. We also characterized relative humidity (%) in the air of the tree canopies by taking open chamber measurements with the Li-Cor 1600 while bypassing the desiccant. In addition, we installed a climate logger (HOBO H8 Pro RH/Temp Onset, Pocasset, MA, USA) in the centre of the plantation and recorded relative humidity (%) and air temperature (°C) in 15 min intervals during the entire sampling campaign.

Leaf morphology, leaf water concentration and relative water content

At pre-dawn, we collected 3 small twigs per tree (3 trees per species) from the upper half of the canopy and stored them in plastic bags. In the field laboratory, 10–20 leaves were detached from the twigs and leaf area was determined with a portable leaf area meter (Li-Cor LI-3000C). Leaves were counted for each sample to calculate the average leaf area (LA) of an individual leaf. The maximum length and maximum width of each individual leaf were determined in millimeters with a ruler. The leaves were then dried in a drying oven at 80 °C for 48 h and dry weight (DW) determined thereafter. Leaf water concentration (LWC) and relative water content (RWC) were determined for all 17 species on 3 replicate leaves per tree. After detaching the leaves with a sharp razor blade and determining their fresh weight (FW), the leaves were then re-saturated for 3 h via the petiole. Preliminary tests with six different eucalypt species indicate that 3 h re-saturation was sufficient. After re-saturation, turgid weight (TW) was determined and the leaves dried for 48 h at 80 °C in a drying oven before DW was measured. LWC was calculated on an area basis as $LWC = (FW - DW)/LA$. RWC was calculated for each sample as $RWC = (FW - DW)/(TW - DW)$. We also determined the diurnal variability of LWC for the six diurnally sampled *Eucalyptus* species to estimate diurnal leaf water enrichment using the non-steady state model (NSS model). At four times during the day, 15–30 leaves were collected and sealed in a plastic bag. The bag including the leaves was weighed in the field laboratory and the mean area per leaf determined. Bag and leaves were then dried for 48 h at 80 °C, and both bag weight and leaf DW were determined thereafter.

Leaf water models

In a stable environment, the steady-state enrichment of leaf water at the sites of evaporation above the source water ($\Delta^{18}O_e$) can be described by

$$\Delta^{18}O_e = \varepsilon^+ + \varepsilon_k + (\Delta^{18}O_v - \varepsilon_k) \frac{e_a}{e_i}, \quad (1)$$

where ε^+ is the equilibrium fractionation between liquid water and vapour at the air–water interfaces; ε_k is the kinetic fractionation that occurs during water vapour diffusion from the leaf intercellular air space to the atmosphere; $\Delta^{18}O_v$ is the isotopic enrichment of vapour in the atmosphere compared to source water, and e_a/e_i is the ratio of ambient to intercellular vapour pressures (Craig & Gordon 1965; Farquhar *et al.* 1989; Flanagan, Comstock & Ehleringer 1991). The equilibrium fractionation factor, ε^+ , is expressed as

$$\varepsilon^+ = 2.644 - 3.206 \left(\frac{10^3}{T_1} \right) + 1.534 \left(\frac{10^6}{T_1^2} \right), \quad (2)$$

where T_1 is leaf temperature in Kelvin (Bottinga & Craig 1969). ε_k is determined by the interplay of stomatal (g_s) and

boundary layer (g_b) conductances to water vapour (Farquhar *et al.* 1989; Cappa *et al.* 2003; Cernusak *et al.* 2003) and is expressed as

$$\varepsilon_k = \frac{32g_s^{-1} + 21g_b^{-1}}{g_s^{-1} + g_b^{-1}}. \quad (3)$$

Equation 1 was originally developed to estimate the isotopic enrichment of well-mixed surface waters of large water bodies such as lakes (Craig & Gordon 1965). Although the equation has also been used to model diurnal trends in leaf water, this application has been shown to overestimate the evaporative enrichment of mean lamina mesophyll water (Flanagan *et al.* 1991; Wang & Yakir 1995; Roden & Ehleringer 1999; Barbour & Farquhar 2000; Cernusak, Pate & Farquhar 2002). Farquhar & Lloyd (1993) and, subsequently, Barbour and co-workers (Barbour *et al.* 2000b, 2004) suggested that the discrepancy between the predicted leaf water enrichment at the sites of evaporation based on Eqn 1 and the observed values of mean leaf water is due to isotopic gradients in the leaf. These gradients may form as a result of the transpirational stream of unenriched water to the site of evaporation opposing the diffusion of enriched water away from the sites of evaporation. The ratio of transpirational flow over diffusion is described by the Péclet number (\wp), which relates the mean lamina mesophyll leaf water isotopic enrichment over source water ($\Delta^{18}O_L$) to $\Delta^{18}O_e$ as

$$\Delta^{18}O_L = \frac{\Delta^{18}O_e(1 - e^{-\wp})}{\wp}, \quad (4)$$

where the Péclet number is defined as

$$\wp = \frac{EL}{CD}. \quad (5)$$

In Eqn 5, E is the transpiration rate ($\text{mol m}^{-2} \text{s}^{-1}$); C is the molar concentration of water (mol m^{-3}); D is the diffusivity of H_2O in water ($\text{m}^2 \text{s}^{-1}$), and L is defined as scaled effective path length for the transpirational flow of water from the xylem through the mesophyll to the site of evaporation (m), reflecting effects of leaf water relations on isotopic leaf water enrichment. The exact nature of L remains, however, unclear so that this parameter has to be determined iteratively by fitting the model to measured values of bulk leaf water $\Delta^{18}O$. Taking the Péclet effect into account, $\Delta^{18}O_L$ of mean lamina mesophyll leaf water can be modelled for selected species under controlled environmental conditions with reasonable accuracy.

Equations 1 and 4 describe the isotopic enrichment of leaf water at steady state, that is, under constant environmental conditions. Such conditions rarely occur in nature where leaf water enrichment is subject to diurnally changing evaporative conditions. Dongmann *et al.* (1974) and, more recently, Farquhar & Cernusak (2005) therefore accounted for non-steady-state isotopic

enrichment of mean lamina mesophyll water. In their model, non-steady-state leaf water enrichment ($\Delta^{18}\text{O}_{\text{LN}}$) is expressed as

$$\Delta^{18}\text{O}_{\text{LN}} = \Delta^{18}\text{O}_{\text{L}} - \left(\frac{1 - e^{-\phi}}{\phi} \right) \left[\frac{d(W\Delta^{18}\text{O}_{\text{LN}})}{dt} \right]_{gw_i}, \quad (6)$$

where W is the water concentration of the leaf (mol m^{-2}), and w_i is the mole fraction of water vapour in the leaf intercellular air spaces (mol mol^{-1}). In essence, the deviation of leaf water enrichment from steady state was accounted for in this model by emphasizing the one-way flux of water from the leaf to the atmosphere (gw_i) (Farquhar & Cernusak 2005). This NSS model has now been tested in several studies and has shown good agreement with measured bulk leaf water values of ^{18}O (Cernusak, Farquhar & Pate 2005; Barnard *et al.* 2007; Gessler *et al.* 2007).

To separate parameters that drive differences in isotopic leaf water enrichment across the investigated *Eucalyptus* species, we used existing leaf water models (steady-state leaf water enrichment at the site of evaporation and for bulk leaf water using Eqns 1 and 4). To model non-steady-state leaf water using Eqn 6, Farquhar & Cernusak (2005) suggested using the Solver function of Microsoft Excel. To avoid this iterative approach, we transformed Eqn 6 by assuming that with sufficient accuracy over short time intervals, $\frac{d(W\Delta^{18}\text{O}_{\text{LN}})}{dt} \approx \frac{W\Delta^{18}\text{O}_{\text{LN}} - (W\Delta^{18}\text{O}_{\text{LN}})_{t-1}}{\Delta_t}$. Substituting this expression into Eqn 6 and rearranging to solve for $\Delta^{18}\text{O}_{\text{LN}}$ gives

$$\Delta^{18}\text{O}_{\text{LN}} = \frac{\Delta^{18}\text{O}_{\text{L}} + W_{t-1}\Delta^{18}\text{O}_{\text{L},t-1} \left(\frac{1 - e^{-\phi}}{\phi} \right) \left(\frac{\alpha^+ \alpha_k}{gw_i \Delta_t} \right)}{1 + W \left(\frac{1 - e^{-\phi}}{\phi} \right) \left(\frac{\alpha^+ \alpha_k}{gw_i \Delta_t} \right)} \quad (7)$$

and used this equation in our calculations, where α^+ and α_k is $1 + \varepsilon^+$ and $1 + \varepsilon_k$, respectively. Equations 6 and 7 were based on the same input parameters and produced exactly the same numbers for $\Delta^{18}\text{O}_{\text{LN}}$.

Data analyses

In a first step of our analyses, we tested the capacity of the leaf water models to predict leaf water enrichment for six *Eucalyptus* species that were sampled for leaf water enrichment diurnally. For the models, we assumed a constant boundary layer conductance of $1 \text{ m}^2 \text{ s}^{-1} \text{ mol}$, a value in the range of what has been previously published in the context of modelling leaf water enrichment of *Eucalyptus* species (Cernusak *et al.* 2005). Effective path length was determined iteratively until the best fit (minimum root mean squared error) of the NSS model with the measured leaf water $\Delta^{18}\text{O}$ values was derived for a particular species.

We then used the output of the different leaf water models to differentiate among environmental drivers, leaf

physiological properties and L as drivers of isotopic leaf water enrichment across the investigated *Eucalyptus* species. In particular, we tested if maximum daily bulk leaf water enrichment ($\Delta^{18}\text{O}_{\text{M-max}}$) as well as the deviation of $\Delta^{18}\text{O}_{\text{M-max}}$ from $\Delta^{18}\text{O}_{\text{e-max}}$ [i.e. the fraction of unenriched water in the leaf (f)] was correlated with E or L . The fraction of unenriched water in leaves, f , was determined for each plant as

$$f = 1 - \frac{\delta^{18}\text{O}_{\text{M-max}} - \delta^{18}\text{O}_{\text{s}}}{\delta^{18}\text{O}_{\text{e-max}} - \delta^{18}\text{O}_{\text{s}}}, \quad (8)$$

where $\delta^{18}\text{O}_{\text{M-max}}$ is the empirically determined maximum diurnal isotopic enrichment of bulk leaf water; $\delta^{18}\text{O}_{\text{e-max}}$ is the maximum diurnal isotopic enrichment at the site of evaporation calculated with Eqn 1, and $\delta^{18}\text{O}_{\text{s}}$ is the source water or xylem water (Leaney *et al.* 1985; Gan *et al.* 2002).

To help improve our understanding of what 'effective path length' actually is, we related L of the 17 species to leaf morphological traits and to parameters reflecting leaf water relations such as $1/g_s$, $1/E$, RWC and LWC . All analyses testing the relationships between $\Delta^{18}\text{O}_{\text{M-max}}$, f and L as well as L and leaf physiological properties or leaf water relations were tested twice: (1) including only those six species that were sampled diurnally and (2) for the combined total sample of all 17 species investigated in this study, independently if these species were sampled diurnally or only once in the afternoon. For the six diurnally sampled species, the estimation of L was constrained by seven data points for $\Delta^{18}\text{O}_{\text{M}}$. L therefore had a higher precision for these species than for the remaining 11 species that were sampled only once in the afternoon where the estimate of L was based only on a single value of $\Delta^{18}\text{O}_{\text{M}}$.

In a second step of our analyses, we tested the sensitivity of the steady-state bulk leaf water model (Eqn 4) to variability in environmental drivers, leaf physiological properties and L . For this purpose, we calculated an average $\Delta^{18}\text{O}$ value using input parameters that were in the range of the values determined empirically in this study (Table 2). To test the sensitivity of the model to direct changes in specific input parameters, we individually varied each parameter by

Table 2. Values for input parameters of the sensitivity analyses

	-40%	-20%	0	+20%	+40%
Water vapour $\delta^{18}\text{O}$ (‰)	-20.3	-17.4	-14.5	-11.6	-8.7
Relative humidity (%)	27.0	36.0	45.0	54.0	63.0
Air temperature (°C)	13.2	17.6	22.0	26.4	30.8
Leaf temperature (°C)	12.0	16.0	20.0	24.0	28.0
g_s ($\text{mol m}^{-2} \text{ s}^{-1}$)	0.18	0.24	0.30	0.36	0.42
g_b ($\text{mol m}^{-2} \text{ s}^{-1}$)	0.6	0.8	1.0	1.2	1.4
E ($\text{mmol m}^{-2} \text{ s}^{-1}$)	1.2	1.6	2.0	2.4	2.8

The '0' numbers are roughly average values that were observed for the climate and *Eucalyptus* species in the Currency Creek Arboretum during the study presented in this paper.

g_s , stomatal conductance; g_b , boundary layer conductance; E , transpiration.

-40, -20, +20 and +40% while keeping all other parameters constant (Table 2). We repeated this analysis four times for leaves that differed in their initial values for L . The initial values we selected for L were 0.1, 10.0, 50.0 and 200.0 mm. These values were in the range of what had been published in previous experiments (Wang *et al.* 1998). To separate the effects of L from environmental and leaf physiological input parameters on variability in leaf water $\Delta^{18}\text{O}$, we performed a second sensitivity analysis. In this second analysis, we used the same initial values as described previously (Table 2). However, all input parameters except for E and e_a/e_i were kept constant. We gradually changed E from 1 to 10 $\text{mmol m}^{-2} \text{s}^{-1}$ at intervals of 1 $\text{mmol m}^{-2} \text{s}^{-1}$ for different values of e_a/e_i that varied between 0.1 and 1.0 at intervals of 0.1. We performed this second sensitivity analysis for four different values of L (0.1, 10.0, 50.0 and 200.0 mm).

RESULTS

Air temperatures during the three sampling days peaked in the early afternoon and were between 30 and 35 °C. Relative humidity dropped during the course of the day from ~100% to values between 25 and 40% (Fig. 1). We did not determine soil moisture, but no precipitation was observed during sampling or 6 weeks prior to the sampling campaign, so we assumed soil moisture was constant during the campaign. Diurnal E , g_s and T_l varied substantially among the 17 investigated species (Fig. 1). While some species had maximum daily transpiration rates below 1 $\text{mmol m}^{-2} \text{s}^{-1}$, others had maximum rates of more than 10 $\text{mmol m}^{-2} \text{s}^{-1}$ (Fig. 1).

Xylem water $\delta^{18}\text{O}$ values for the 17 *Eucalyptus* species varied between -3.6 and -5.1‰ (Fig. 2, Table 1) demonstrating that there was little variation in source water used among the species. Despite little variation in source water, all of our study species showed significant diurnal enrichment patterns in leaf water $\delta^{18}\text{O}$ with maximum enrichment in the afternoon (Fig. 2), and the six focus species differed substantially in their maximum diurnal leaf water $\delta^{18}\text{O}$ values (Fig. 2). Species that were only sampled once in the afternoon also differed in $\delta^{18}\text{O}$ with values that ranged between 11.5 and 15.6‰ (Table 1).

Leaf water models predicted diurnal enrichment in leaf water ^{18}O with different levels of precision (Fig. 3). The Craig-Gordon (C-G) model ($\Delta^{18}\text{O}_e$, Eqn 1) always overestimated diurnal leaf water enrichment. Further, the offset of the modelled values from the measured values differed substantially among species. The bulk leaf water model introduced by Farquhar and Lloyd in 1993 ($\Delta^{18}\text{O}_L$, Eqn 4) predicted diurnal leaf water enrichment reasonably well and better than the C-G model but typically overestimated enrichment in the morning and underestimated enrichment in the afternoon (Fig. 3). Our best results were achieved with the NSS model ($\Delta^{18}\text{O}_{LN}$, Eqns 6 and 7). The L values that were iteratively determined for a best fit of the models varied for the diurnally sampled species between 3.2 and 119.2 mm (Fig. 3) and between 5.0 and 220.0 mm for the

species that were sampled only once in the afternoon (Table 1).

Modelled leaf water enrichment at the site of evaporation ($\Delta^{18}\text{O}_e$, Eqn 1) was similar for all species (Fig. 4a). Variability in $\Delta^{18}\text{O}$ across different species increased, however, substantially when a Péclet effect was included in the model ($\Delta^{18}\text{O}_L$, Eqn 4, Fig. 4b). This indicates the importance of the Péclet effect in explaining differences in isotopic leaf water enrichment across different species.

No significant correlation was observed between maximum daily transpiration rate and $\Delta^{18}\text{O}_{M-\text{max}}$ or f (Fig. 5a,c). In contrast, a high percentage of the variability observed in $\Delta^{18}\text{O}_{M-\text{max}}$ and f was explained by L (Fig. 5b,d). This relationship was significant for both analyses, although more variability was explained when only the six diurnally sampled species were included in the analyses rather than all 17 species.

Leaf morphological variables did not correlate with L across the 17 investigated species (Table 3). Additionally, neither RWC nor LWC explained any of the observed variability in L across different species (Fig. 6). Interestingly, however, the inverse of maximum rates of stomatal conductance ($1/g_s$) and transpiration ($1/E$) were highly correlated with L (Fig. 6).

For easier interpretation of the sensitivity analyses, we grouped the manipulated input parameters into four functional units: (1) atmospheric isotope effects (water vapour $\Delta^{18}\text{O}$), (2) environmental drivers (relative humidity, air temperature, leaf temperature), (3) conductances (stomatal and boundary layer conductance) and (4) the Péclet parameters (transpiration, effective path length). We observed substantial direct effects on leaf water $\Delta^{18}\text{O}$ by all input parameters except for the conductance measures. Interestingly, the magnitude of effects of a particular functional unit varied substantially among the different L values ranging from 0.1 to 200.0 mm (Fig. 7). At small values for L , 'atmospheric isotope effects' and 'environmental drivers' were the most critical parameter units influencing leaf water $\Delta^{18}\text{O}$, while the 'Péclet parameters' became more influential at large values for L (Figs 7 & 8). In addition, increasing L led to substantially lower overall $\Delta^{18}\text{O}$ values, independent of E or e_a/e_i (compare the lower three-dimensional plots with $L = 50$ and 200 mm, respectively, with the upper two in Fig. 8).

DISCUSSION

We found strong species-specific effects on the diurnal variation in g_s , E and $\delta^{18}\text{O}$ among the 17 species of *Eucalyptus* we investigated (Table 1, Figs 1 & 2). Similar xylem water isotopic ratios among the 17 species indicated that all of these trees were using either identical or very similar water sources and that variation in source water could not explain much of the species-specific differences in leaf water $\delta^{18}\text{O}$ (Fig. 2). In addition, the diurnal fluctuations in environmental drivers on the three sampling days were relatively consistent, allowing us to attribute the observed differences in g_s , E and $\Delta^{18}\text{O}$ among species to leaf

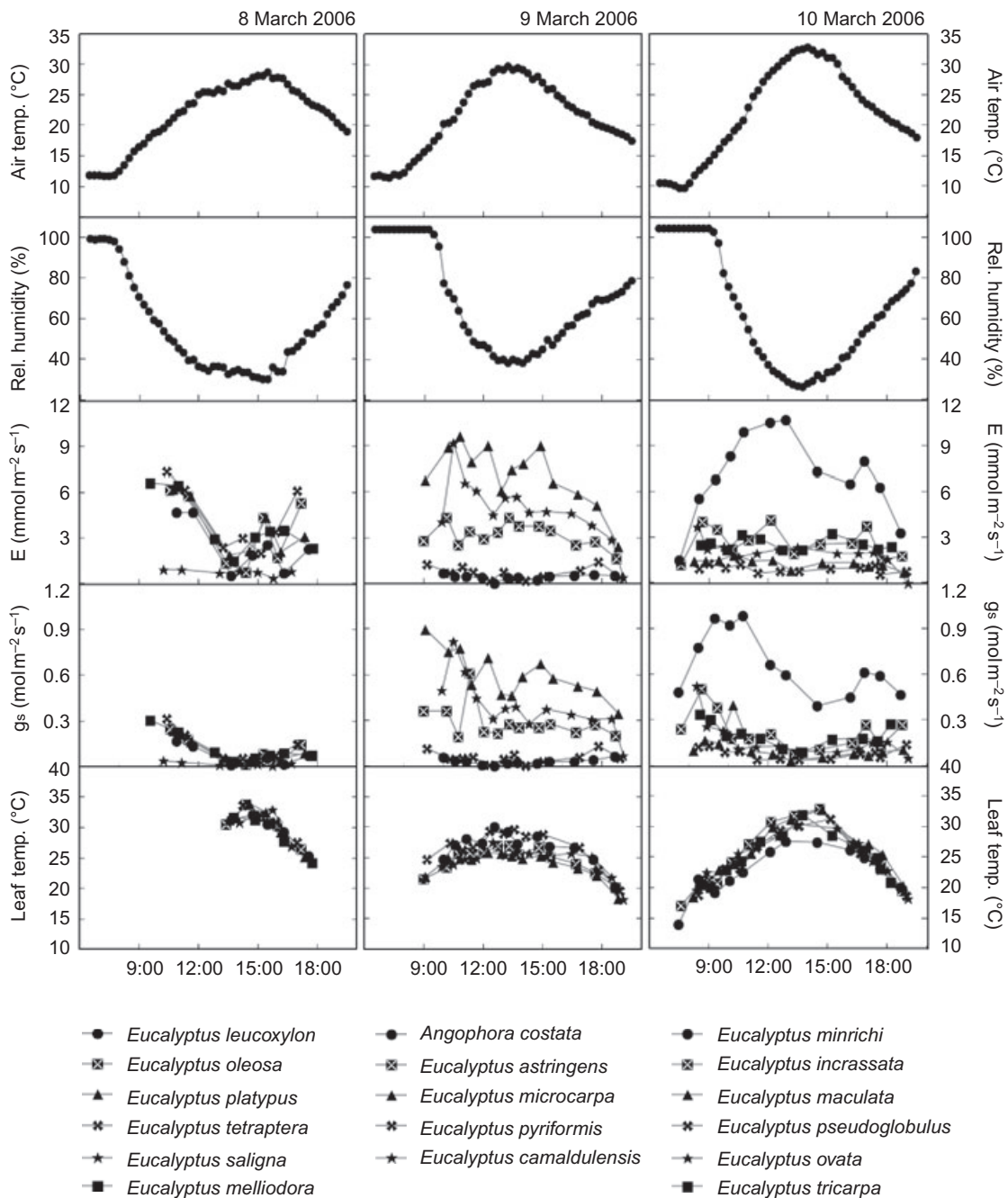


Figure 1. Diurnal variability of air temperature, relative humidity, leaf gas exchange and leaf temperature of 17 *Eucalyptus* species in the Currency Creek Arboretum during the tree day sampling period. For leaf gas exchange and leaf temperature, each point on the graph represents the average value of measurements from three individual trees.

physiological properties or to differences in leaf water relations rather than different environmental drivers (Fig. 1).

The range of values found for $\Delta^{18}\text{O}_L$ values is about the same order of magnitude as reported in other studies that have compared $\Delta^{18}\text{O}_L$ across different species under ambient environmental conditions (Cooper & Deniro 1989; Wang & Yakir 1995; Wang *et al.* 1998; Helliker & Ehleringer 2000; Terwilliger *et al.* 2002). All three leaf water models did a reasonable job of predicting the

observed diurnal patterns of $\Delta^{18}\text{O}_L$ in the six species we focused on for our diurnal measurement campaign (Fig. 3). However, if we included a Péclet effect, this significantly improved the accuracy of the models for predicting the magnitude of diurnal $\Delta^{18}\text{O}_L$ in all six species. Interestingly, the predictive power of the models was only slightly improved when the NSS model was used when compared with the steady-state model. This finding is in line with previous studies that have shown only small deviations

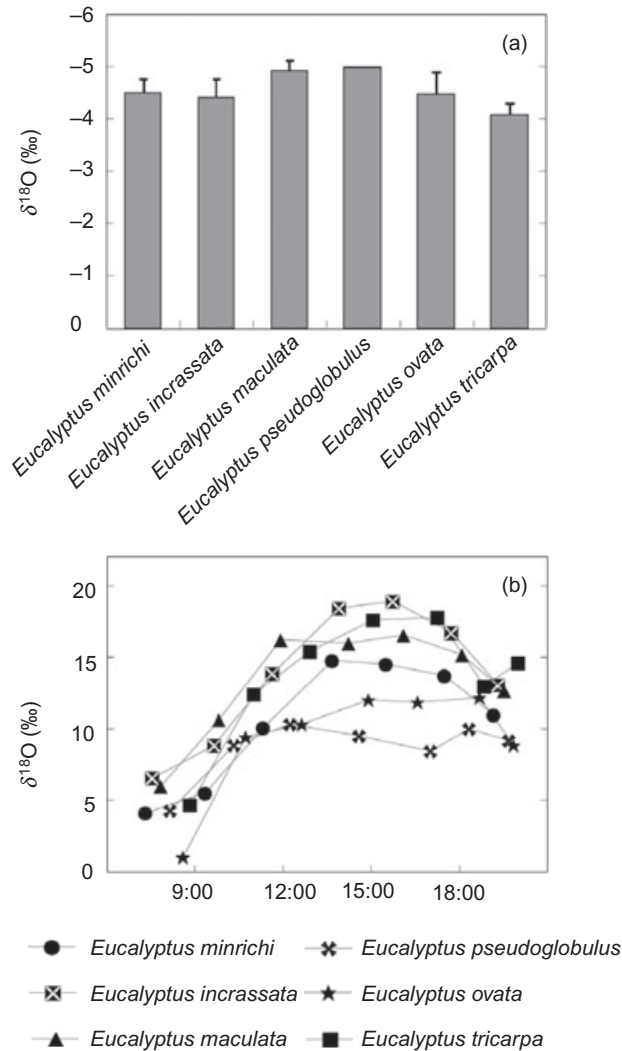


Figure 2. Xylem water $\delta^{18}\text{O}$ values and diurnal leaf water $\delta^{18}\text{O}$ values of six *Eucalyptus* species sampled on 10 March 2006. Bars represent mean values of $\delta^{18}\text{O}$. Error bars represent 1 SD from the mean ($n = 3$), except for *Eucalyptus pseudoglobulus*, where only one replicate was collected. Within-species variability of leaf water $\delta^{18}\text{O}$ obtained from measurements on 3 replicate trees per species is not shown in the graph. Variability in leaf water $\delta^{18}\text{O}$ is, however, in the same range as the variability of leaf water $\Delta^{18}\text{O}$ given in Fig. 3.

between modelled steady-state and non-steady-state daytime values for the bluegum *Eucalyptus globulus* (Cernusak *et al.* 2005) as well as for other broad leaf species (Cernusak *et al.* 2002; Farquhar & Cernusak 2005; Gessler *et al.* 2007) and a range of coniferous tree species (Lai *et al.* 2006; Seibt *et al.* 2006; Barnard *et al.* 2007).

Leaf water enrichment at the site of evaporation as predicted by the modified C-G model ($\Delta^{18}\text{O}_e$, Eqn 1) was almost identical across different species (Fig. 4a). In contrast, when a Péclet effect was included in the model, strong species-specific differences in leaf water enrichment appeared. Species-specific differences in $\Delta^{18}\text{O}$ generated by the model including the Péclet effect resembled the

magnitude of differences in $\Delta^{18}\text{O}$ we empirically determined across species (Fig. 4b). This shows that the strong species-specific differences we observed in this study can mainly be attributed to parameters included in the Péclet effect and that environmental drivers or the direct influences of g_s or g_b are less important.

The Péclet effect is driven by two variables: E and the unconstrained fitting parameter L (Eqn 5). To understand which of these two variables drive the observed variability in isotopic leaf water enrichment across the different *Eucalyptus* species, we tested the effect of E and L on $\Delta^{18}\text{O}_{\text{M-max}}$ and f . Interestingly, we found no significant correlation between E and either $\Delta^{18}\text{O}_{\text{M-max}}$ or f (Fig. 5a,c). This is remarkable given that several previous studies have reported a strong effect of E [or g_s under constant vapour pressure deficit (VPD)] on both $\Delta^{18}\text{O}_{\text{M-max}}$ and f (Walker *et al.* 1989; Flanagan *et al.* 1991; Barbour *et al.* 2000b, 2004; Cernusak *et al.* 2003; Thompson *et al.* 2007). The relationship between E and $\Delta^{18}\text{O}_{\text{M-max}}$ or f has typically been viewed as evidence for the importance of the Péclet effect, where the input of unenriched source water via the transpirational stream mixes with leaf water that in turn leads to the dilution of the isotopically enriched water at the sites of evaporation (Barbour *et al.* 2000b; Farquhar *et al.* 2007). We believe that our results differ from the previous findings because in all of these studies, the significant relationships between E and $\Delta^{18}\text{O}_{\text{M-max}}$ or f were only found for *within*-species rather than *across*-species comparisons as shown here. In fact, the only study that has addressed across-species variability in $\Delta^{18}\text{O}_{\text{M}}$ found no significant relationship between E and $\Delta^{18}\text{O}_{\text{M}}$ (Wang *et al.* 1998). Because in our study we did not test for the degree of variability in $\Delta^{18}\text{O}_{\text{M}}$ or f within individual species but instead focused on across-species comparisons, our results do not necessarily contradict previous findings. Rather, they are in agreement with the results reported by Wang *et al.* (1998) and highlight the importance of across-species investigations in helping to explain what can lead to variability in $\Delta^{18}\text{O}_{\text{M}}$ or f across plants in general.

We found a strong correlation between L and $\Delta^{18}\text{O}_{\text{M-max}}$ and f across the different *Eucalyptus* species (Fig. 5b,d). Because the empirically determined values for E do not relate to either $\Delta^{18}\text{O}_{\text{M-max}}$ or f in this study, the relationship between L and $\Delta^{18}\text{O}_{\text{M-max}}$ or f is a logical mathematical consequence as either L or E are used to adjust the Péclet number (Eqn 5) to get the best fit between the modelled and the observed isotopic leaf water data. The strong correlation between L and $\Delta^{18}\text{O}_{\text{M-max}}$ or f , however, highlights the critical need to understand what L actually is from an anatomical and/or physiological perspective, particularly if we want to enhance our mechanistic understanding of the parameters that drive variability in leaf water enrichment within and across different plant species.

L has typically been described as the 'effective path length' or, in other terms, as the distance that water flows in a leaf from the source to the site of evaporation (l) corrected by a scaling factor (k) that accounts for the tortuosity of this path in the leaf (Barbour & Farquhar 2003). This is expressed as

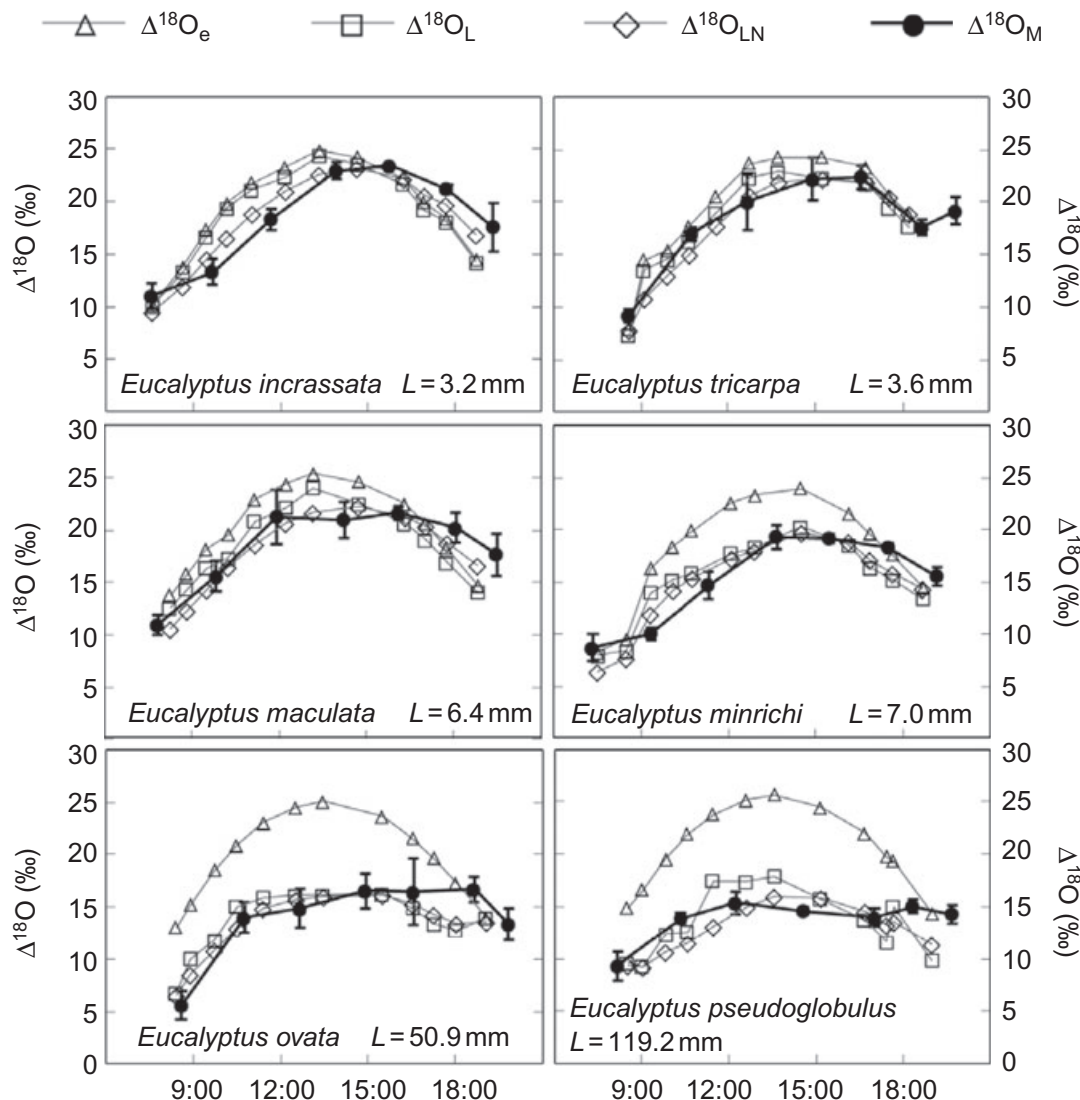


Figure 3. Measured and modelled diurnal leaf water $\Delta^{18}\text{O}$ values of 6 *Eucalyptus* species. Modelled values are shown for $\Delta^{18}\text{O}$ values at the sites of evaporation [Craig–Gordon (C-G) model, Eqn 1, $\Delta^{18}\text{O}_e$], for mean lamina leaf water at steady state (bulk leaf water model, Eqn 4, $\Delta^{18}\text{O}_L$) and for mean lamina leaf water at non-steady state (Eqns 7 & 8, $\Delta^{18}\text{O}_{LN}$). Error bars indicate one SD of the measured values ($\Delta^{18}\text{O}_M$). L , effective path length.

$$L = l \times k. \quad (9)$$

Although some attempts have been made to determine what L is empirically (Barbour & Farquhar 2003), evidence that L is in fact just the effective path length is still wanting (Barbour & Farquhar 2003; Farquhar *et al.* 2007). In addition to the actual effective path length, iteratively determined values of L might also contain other influences that derive from leaf morphological/anatomical properties, leaf water relations and/or the physiological properties of leaves. We tested to see if L was related to any of the leaf morphological properties we measured among the 17 *Eucalyptus* species but found no significant correlations (Table 3). Similarly, leaf water relations such as RWC and LWC were not related to L (Fig. 6). As mentioned, only $1/g_s$

and $1/E$ showed a highly significant relationship with L . Given that E is not correlated with f , the relationship between $1/g_s$ and $1/E$ with L is, however, a necessary mathematical consequence. The correlations between L and $1/g_s$ or $1/E$ nevertheless highlight the need for a more detailed investigation of the mechanistic reasons underlying these relationships. Without further information, our understanding of the importance of the Péclet effect and thus the interpretation of isotopic leaf water enrichment across different species may prove much too challenging.

The importance of L in predicting isotopic leaf water enrichment across different plant species is also shown in our sensitivity analyses. For these analyses, we used the more simplistic steady-state leaf water enrichment model (Eqn 4). Because the goal of this study was to test the

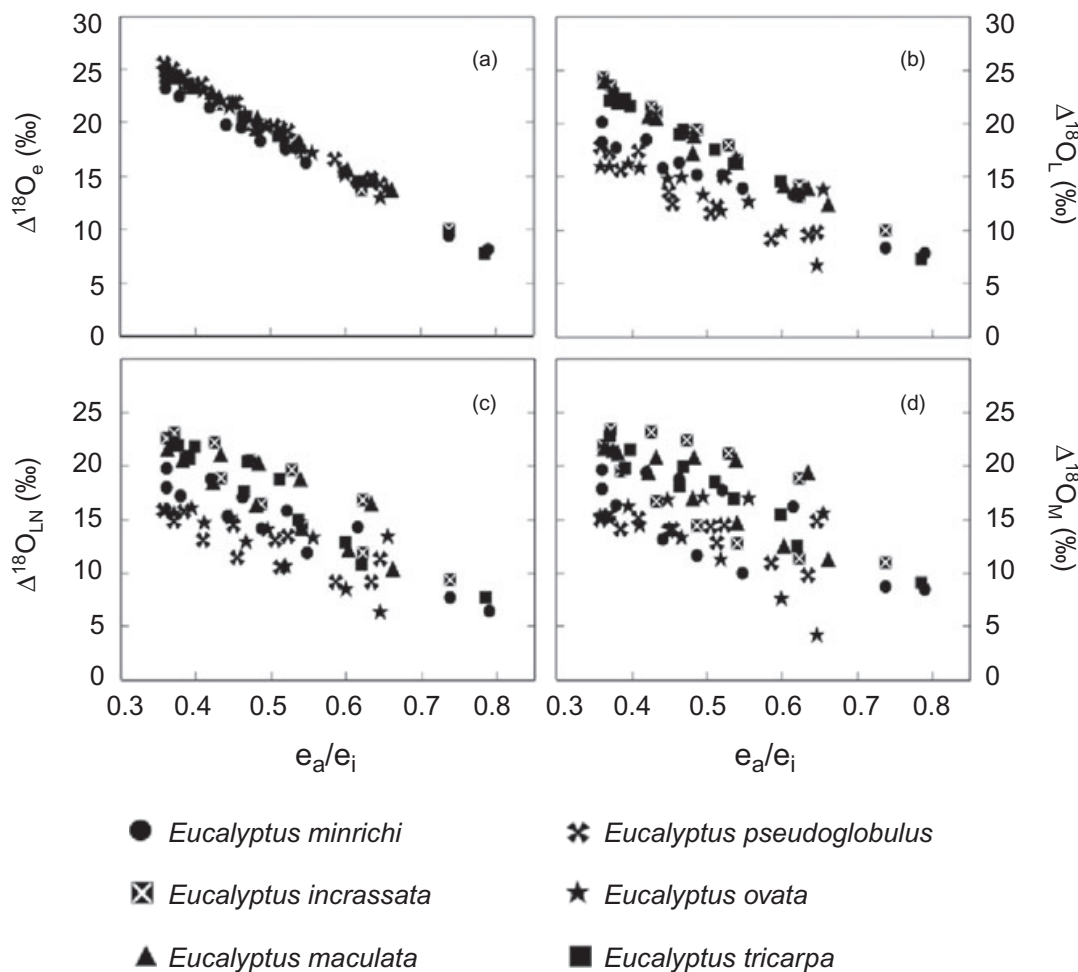


Figure 4. Relationship between ambient and intercellular vapour pressure (e_a/e_i) and leaf water isotopic enrichment calculated for the site of evaporation [Craig–Gordon (C-G) model, Eqn 1, $\Delta^{18}O_e$], for mean lamina leaf water at steady state (bulk leaf water model, Eqn 4, $\Delta^{18}O_L$), for mean lamina leaf water at non-steady state (Eqns 6 & 7, $\Delta^{18}O_{LN}$) as well as measured leaf water ($\Delta^{18}O_M$) for the six diurnally sampled *Eucalyptus* species.

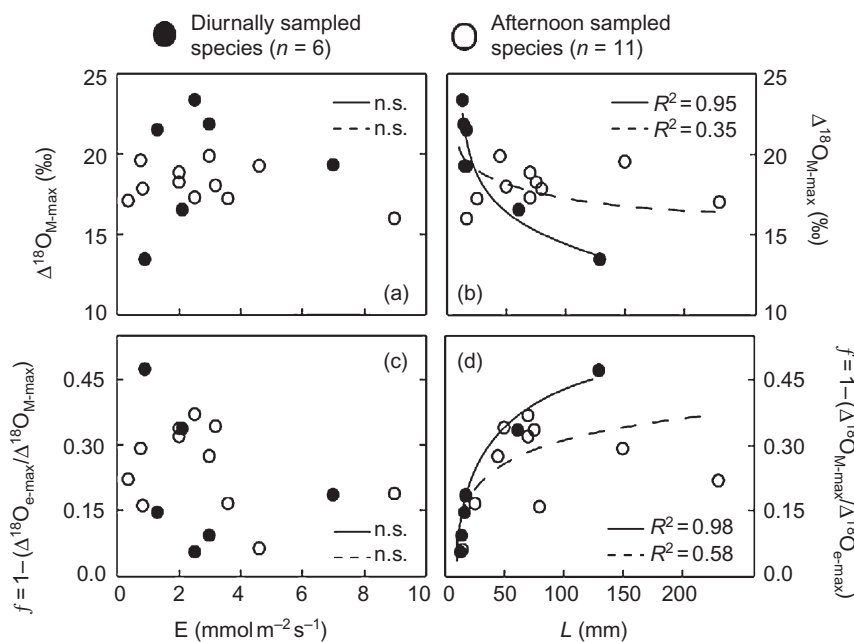
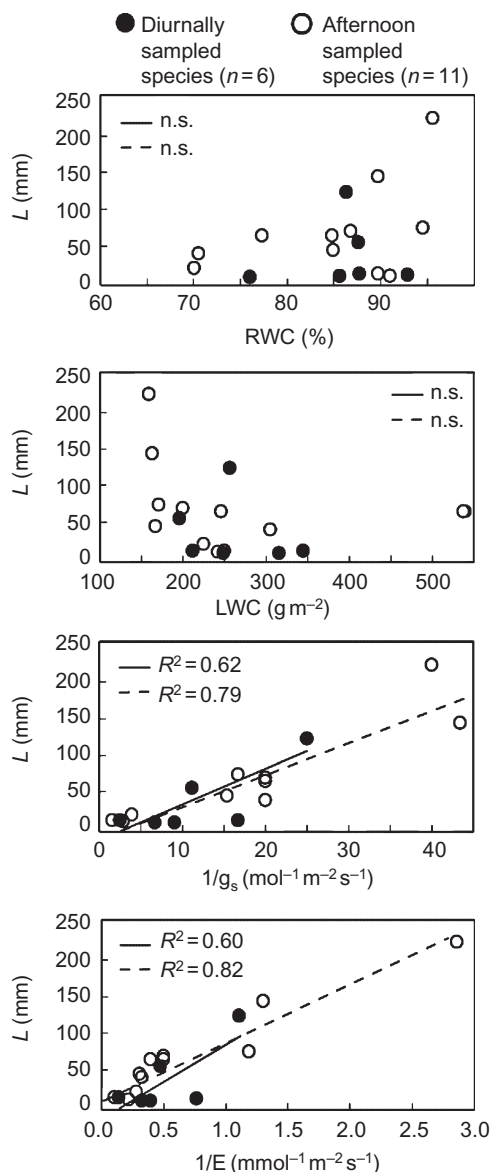
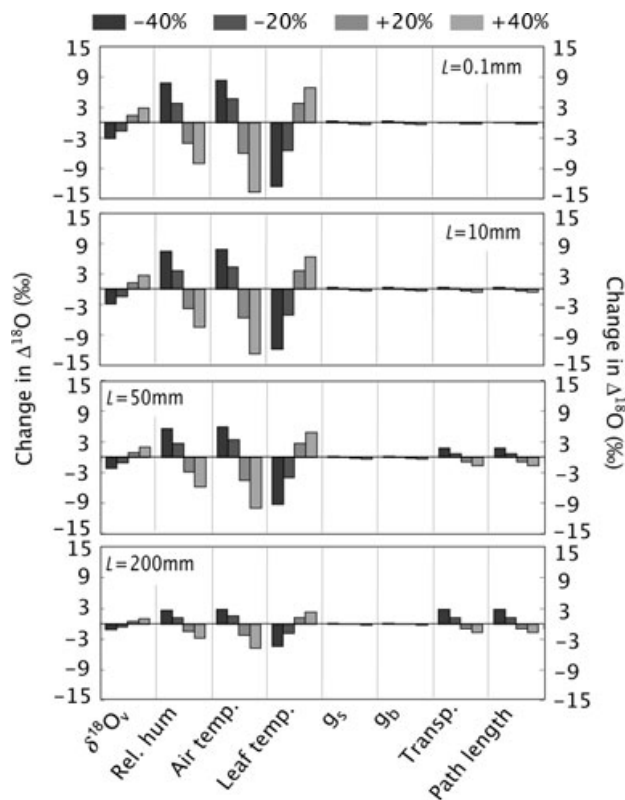


Figure 5. Relationship between effective path length (L) and maximum daily transpiration rate and maximum daily leaf water enrichment ($\Delta^{18}O_{M-max}$) and the fraction of isotopically unenriched water in a leaf (f). The solid line stands for regression results based on the six diurnally sampled species (full circles); the dotted line stands for regression results based on the six diurnally sampled species plus the 11 afternoon sampled species (open circles). Only significant regression lines are shown. $\Delta^{18}O_M$, measured leaf water; $\Delta^{18}O_e$, Craig–Gordon (C-G) model. n.s., non-significant.

Table 3. Correlations between leaf morphological parameters and effective path length (L)

	r	P
Leaf length	0.383	0.129
Leaf width	0.027	0.919
Leaf length/width	0.322	0.208
Leaf area	0.247	0.338
Specific leaf area	0.363	0.152

**Figure 6.** Relationship between effective path length (L) and $1/g_s$, $1/E$, relative water content (RWC) and leaf water concentration (LWC). The solid line stands for regression results based on the six diurnally sampled species (full circles); the dotted line stands for regression results based on the six diurnally sampled species plus the 11 afternoon sampled species (open circles). Only significant regression lines are shown. n.s., non-significant.**Figure 7.** Results for the sensitivity analyses using the steady-state leaf water enrichment model (Eqn 4) and input variables shown in Table 2. For the sensitivity analyses, each variable was independently varied by $\pm 20\%$ and $\pm 40\%$, while other variables were kept constant. $\delta^{18}\text{O}_a$, $\delta^{18}\text{O}$ of atmospheric vapour; g_s , stomatal conductance; g_b , boundary layer conductance; L , effective path length.

individual direct effects of different ecophysiological and morphological parameters on leaf water $\Delta^{18}\text{O}$ rather than diurnal dynamics, the steady-state model is more suitable for this analysis. Using this model, we first tested the direct effect of each individual input parameter on leaf water $\Delta^{18}\text{O}$. Except for conductances, all parameter units showed significant effects on the order of between -13.4 and 8.4% (for $\Delta^{18}\text{O}$) when varied by $\pm 40\%$ (Fig. 7). Most interestingly, the effects of the different parameter units on $\Delta^{18}\text{O}$ were strongly dependent on effective path length. At short path lengths, changes in 'environmental drivers' had the strongest effects on $\Delta^{18}\text{O}$, while 'Péclet parameters' were insignificant. However, with increasing path lengths, the 'Péclet parameters' became more important factors for $\Delta^{18}\text{O}$ in leaf water (Fig. 7). In a similar but more simplistic analysis, where all variables except for E , e_a/e_i and L were kept constant, the strong interplay of physiological (E) and environmental (e_a/e_i) parameters with L in their effects on $\Delta^{18}\text{O}$ becomes even more evident (Fig. 8). This again highlights that a critical assessment and understanding of L is essential for the interpretation of leaf water isotopic ratios across different species (Figs 7 & 8).

The purpose of the sensitivity analyses we performed in this study was to evaluate the direct influences of different

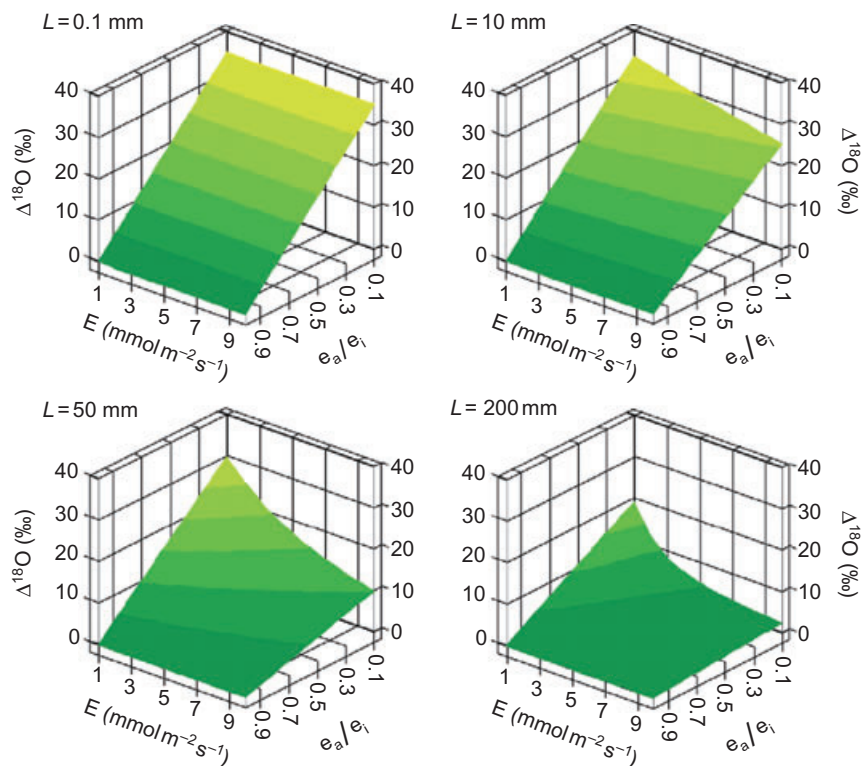


Figure 8. Results for the sensitivity analyses using the steady-state leaf water enrichment model (Eqn 4) and input variables shown in Table 2. For the sensitivity analyses, transpiration and e_a/e_i were varied from 1 to 10 $\text{mmol m}^{-2} \text{s}^{-1}$ and from 0.1 to 1.0, respectively, while all other variables were kept constant at the '0' value. L , effective path length; E , transpiration; e_a/e_i , ratio of ambient to intercellular vapour pressures.

environmental and physiological drivers on leaf water $\Delta^{18}\text{O}$ and to demonstrate the mediating effects that L has on these drivers with respect to their impact on leaf water $\Delta^{18}\text{O}$. While a sensitivity analysis can be very useful in understanding the direct mechanistic effects of individual parameters on isotopic leaf water enrichment, one must be cautious that, under natural conditions, the individual input parameters do not act independently as they do in the sensitivity analyses we performed here. For example, a change in stomatal conductance at a constant relative humidity will also result in changes in transpiration rate, leaf temperature and thus VPD under natural conditions. Changes in conductance will therefore not only effect leaf water enrichment directly by decreasing kinetic fractionation (Eqn 3) but also indirectly by influencing VPD, E and the Péclet effect (Eqn 5). The overall indirect effects of changes in g_s on isotopic leaf water enrichment can therefore be much larger than the direct effects as tested here. The strong effects of stomatal conductance of *Fagus sylvatica* trees on $\Delta^{18}\text{O}$ of phloem and bulk leaf material reported by Keitel *et al.* (2003) or Keitel *et al.* (2006) or the effects of stomatal conductance on $\Delta^{18}\text{O}$ of cellulose and grain in spring wheat shown by Barbour *et al.* (2004) or cotton leaves (Barbour & Farquhar 2000) are therefore in agreement with our study.

In summary, our study shows that leaf water enrichment across different species – even within a single genus – that grow in the same ecosystem and under similar environmental conditions can be substantial. We found effective path length, L , to be a critical driver of the observed differences in leaf water $\Delta^{18}\text{O}$ across these different species. Although we were not able to link L with any specific and

independent leaf physiological properties or leaf water relation characteristics across the different species, we believe that these underlie the differences we and other studies have observed. Future studies should therefore investigate the effects of the mesophyll anatomy and leaf hydraulic properties on isotopic leaf water enrichment within and across different species. Such information is needed to assist us in better defining L while also helping us clarify the underlying factors that 'set' the oxygen isotope ratios in plant organic material, CO_2 and H_2O .

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