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# Effect of water availability on leaf water isotopic enrichment in beech seedlings shows limitations of current fractionation models

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## ABSTRACT

**Current models of leaf water enrichment predict that the differences between isotopic enrichment of water at the site of evaporation ($\Delta_e$) and mean lamina leaf water enrichment ($\Delta_L$) depend on transpiration rates (*E*), modulated by the scaled effective length (*L*) of water isotope movement in the leaf. However, variations in leaf parameters in response to changing environmental conditions might cause changes in the water path and thus *L*. We measured the diel course of $\Delta_L$ for $^{18}$O and $^{2}$H in beech seedlings under well-watered and water-limited conditions. We applied evaporative enrichment models of increasing complexity to predict $\Delta_e$ and $\Delta_L$, and estimated *L* from model fits. Water-limited plants showed moderate drought stress, with lower stomatal conductance, *E* and stem water potential than the control. Despite having double *E*, the divergence between $\Delta_e$ and $\Delta_L$ was lower in well-watered than in water-limited plants, and thus, *L* should have changed to counteract differences in *E*. Indeed, *L* was about threefold higher in water-limited plants, regardless of the models used. We conclude that *L* changes with plant water status far beyond the variations explained by water content and other measured variables, thus limiting the use of current evaporative models under changing environmental conditions.**

*Key-words*: advection–diffusion; deuterium; $\delta^2$H; $\delta^{18}$O; drought; non-steady state; oxygen; *Péclet*; transpiration; water isotopes.

## INTRODUCTION

Understanding the processes determining the isotopic composition of leaf water ($\delta^2$H and $\delta^{18}$O, for hydrogen and oxygen, respectively) is of great relevance for several scientific fields. On the one hand, it is the main source for $\delta^2$H and $\delta^{18}$O variability in organic matter (Sternberg, DeNiro & Savidge 1986; Yakir 1992a; Barbour 2007), and the latter has been used in a wide range of applications: palaeoenvironmental studies in tree rings (Gray & Thompson 1976; Libby *et al.* 1976; Epstein, Thompson & Yapp 1977, further refs. in McCarroll & Loader 2004) as a tool to explore genetic variability in stomatal conductance, transpiration and crop yields (Barbour & Farquhar 2000; Barbour *et al.* 2000a; Sheshshayee *et al.* 2005; Ferrio *et al.* 2007) and to assess physiological responses of plants to different environmental factors (Saurer, Siegwolf & Scheidegger 2001; Jäggi *et al.* 2003; Keitel *et al.* 2006; Brandes *et al.* 2007; Helliker & Richter 2008). Additionally, it can be used to support the interpretation of carbon isotope composition by allowing the separation of stomatal from photosynthetic effects (Scheidegger *et al.* 2000; Keitel *et al.* 2006; Voltas *et al.* 2008). On the other hand, leaf water also contributes to the isotopic composition of atmospheric $CO_2$ and $O_2$, which are relevant for ecological studies based on atmospheric fluxes at the ecosystem level (Flanagan *et al.* 1996; Yakir & Wang 1996; Seibt, Wingate & Berry 2007) and at the global scale (Farquhar *et al.* 1993; Luz *et al.* 1999; Bender, Sowers & Labeyrie 1994; Cuntz *et al.* 2003).

Briefly, the isotopic composition of mean lamina leaf water reflects variations in (1) source water isotope signature (i.e. xylem water) and (2) the evaporative enrichment during transpiration, which is mainly determined by the ratio of internal to atmospheric water partial pressures and the isotopic composition of atmospheric water (Yakir 1992a; Farquhar & Lloyd 1993). The isotopic enrichment at the site of evaporation can be described using the Craig & Gordon (1965) model for evaporation in water surfaces, adapted for plants by Dongmann *et al.* (1974). However, this model overestimates mean lamina leaf water enrichment during the day, as the diffusion of enriched water from the sites of evaporation to the rest of the leaf is counteracted by the input of unenriched water through the transpiration flow, which is known as the *Péclet* effect (Farquhar & Lloyd 1993). Although most recent evaporative enrichment models (e.g. Farquhar & Gan 2003; Farquhar & Cernusak

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2005; Cuntz *et al.* 2007; Ogée *et al.* 2007) provide a reasonable mechanistic understanding of the main processes determining leaf water isotopic composition, even under non-stable environmental conditions, they still require some kind of empirical calibration. In particular, one main parameter of the models, the 'scaled effective path length' ($L$, in m), is not directly measurable, and thus has to be determined empirically as a fitting parameter (Farquhar & Lloyd 1993). This parameter is defined as the product of the real distance of the water pathway and a scaling factor that accounts for leaf tortuosity, which can range from $10^2$ to $10^3$ (Farquhar & Lloyd 1993; Barbour *et al.* 2000b). Up to now, $L$ has been generally assumed to be a species-specific constant, potentially associated to the anatomical properties of the leaf. Wang, Yakir & Avishai (1998), for example, estimated $L$ by comparing modelled and measured values for leaf water enrichment in 89 species, including different life forms and habitats, and found a range from 4 to 166 mm. Barbour *et al.* (2004) showed $L$ values ranging from 19 to 34 mm in three different tree species. More recently, Kahmen *et al.* (2008) obtained $L$ values ranging from 3.2 to 220 mm in a comparison of 17 Eucalyptus species. However, short-term variations in leaf parameters in response to environmental conditions might cause changes in $L$, as has been already suggested elsewhere (Barbour & Farquhar 2003; Keitel *et al.* 2006). This has been confirmed by a recent work showing a significant relationship between $L$ and atmospheric vapour pressure deficit (VPD) for cotton leaves (Ripullone *et al.* 2008). Moreover, although some attempts have been made to relate $L$ with measurable leaf parameters (Barbour & Farquhar 2003; Kahmen *et al.* 2008), the mechanistic reasons underlying observed $L$ differences are still unclear. Thus, there is a need to characterize the variability of this parameter and, in particular, to further assess whether or not it can change with environmental conditions.

In this context, the aim of this work was to determine the effect of short-term changes in water availability on $L$, as a key parameter for water isotope models. For this purpose, we compared the diel course of water isotope enrichment in water-limited and well-watered beech seedlings grown under controlled conditions. With these data, we compared measured values with those predicted by different models, in order to determine to what extent $L$ changes could affect the interpretation of leaf water isotope composition.

## MATERIALS AND METHODS

### Plant material, growing conditions and experimental set-up

Sixty seedlings (3 years old) of beech (*Fagus sylvatica* L.), growing in small nursery containers, were placed in a climate chamber (HPS1500, Heraeus-Vötsch, Balingen, Germany), configured with a 16-hour photoperiod, 70% relative humidity and an air temperature of 20 and 18 °C during day and night, respectively. Illumination was provided by 14 daylight fluorescent lamps (Osram Fluora L58W/77, Osram GmbH, Munich, Germany; Phillips TLD 58W/950, Phillips GmbH, Hamburg, Germany) plus three incandescent light bulbs (Osram Krypton 60 W). Wind speed and daytime photosynthetic photon flux density (PPFD) at the canopy level were constant over the experiment at about 0.8 m $s^{-1}$ (0.6–1.0 m $s^{-1}$) and 150 $\mu$mol $m^{-2}$ $s^{-1}$ (100–200 $\mu$mol $m^{-2}$ $s^{-1}$), respectively. Since young beech seedlings are adapted to grow under very closed canopies, even at this low light intensity, photosynthesis is close to light saturation (Kreuzwieser *et al.* 1997).

The mean plant height was 0.79 ± 0.02 m, mean leaf area was 0.072 ± 0.003 $m^2$ and mean above-ground fresh biomass was 29.6 ± 1.3 g ($n$ = 52).

The plants were acclimated to the chamber conditions for 1 week before starting the experiment. Control, well-watered plants were watered to field capacity every day, whereas water-limited plants were not watered 60 h prior to the experiment. Measurements were taken over a 27 h cycle, with measurement and sampling rounds every 3 h. Air temperature, VPD and relative humidity within the canopy were recorded throughout the experiment with a multi-sensor probe (HYTE1308, Hygrotec Messtechnik GmbH, Titisee-Neustadt, Germany) connected to a laptop with a serial port.

### Measurements and collection of atmospheric water vapour and plant material

During each measurement round, atmospheric water vapour from the beech canopy in the climate chamber was collected by cryogenic condensation (Roden & Ehleringer 1999). Air was pumped at 1 L $min^{-1}$ for about 2 h through a trap filled with ethanol and dry ice (ca. −70 °C). The collected water was immediately transferred into sealed 2 mL crimp cap vials (Infochroma, Zug, Switzerland) and kept cool until isotope analysis.

Three plants per treatment were harvested at each measurement time. For each plant, we performed gas exchange measurements (LI-6400, LI-COR biosciences, Lincoln, NE, USA) on three leaves that were sketched to measure leaf area and then placed in glass tubes to be immediately frozen in dry ice for water extraction. For the extraction of leaf water-soluble organic matter (i.e. the short-turnover organic pool, most directly affected by leaf water isotope composition; Barnard *et al.* 2007; Gessler *et al.* 2007), three additional, adjacent leaves were harvested, sketched for their leaf area, immediately frozen and afterwards freeze-dried for 48 h. Leaf water concentration (WC; % in weight of water divided by fresh weight) and per leaf area ($m^{-2}$) were determined by comparing fresh and dry weights of the leaves harvested for organic matter. From each plant, we cut a stem section at the base of the trunk of about 5 cm length, removed the bark and transferred it into a glass tube for water extraction. Finally, stem water potential ($\Psi_s$) was determined with a Scholander pressure bomb (Scholander *et al.* 1965).

Xylem and bulk leaf water were extracted by cryogenic vacuum distillation (Ehleringer & Dawson 1992): the frozen glass tubes were placed in an 80 °C water bath, connected to a vacuum system (ca. $4 \cdot 10^{-2}$ mbar) including water traps that were cooled with liquid $N_2$. The captured water was then transferred into sealed 2 mL vials after thawing and kept cool until analysis.

Leaf water-soluble organic matter was extracted as described in Barnard *et al.* (2007). Briefly, freeze-dried leaves were milled and 1 mL of distilled water was added to 45–55 mg of the ground sample. After 1 h of agitation at 4 °C, samples were heated for 10 min at 95 °C, cooled down to room temperature and centrifuged (10 min, 12 000 *g*, 4 °C). An aliquot of 75 $\mu$L of the supernatant was then transferred to silver capsules and dried overnight at 60 °C, resulting in about 500 $\mu$g solid extract. To minimize oxygen exchange with atmospheric water, the capsules were cooled down and closed inside an argon-flushed bag, and stored in a desiccator cabinet until isotope analysis.

## Mass spectrometry measurements

An aliquot of 0.6 $\mu$L of the water sample was injected in a High Temperature Combustion Elemental Analyzer (TC/EA, Thermo Finnigan, Bremen, Germany). At 1450 °C, the water was pyrolysed on glassy carbon to $H_2$ and CO, and then these components were carried in a helium stream to the mass spectrometer (Delta plus XP, Thermo Finnigan, Bremen). The hydrogen isotope ratio was determined from the $^2H/^1H$ ratio of the $H_2$ molecule and the oxygen isotope ratio from the $^{12}C^{18}O/^{12}C^{16}O$ ratio of the CO molecule. The values are expressed as deviations in per mil (‰) from the international standard VSMOW ($\delta^2H$, $\delta^{18}O$). Each sample was injected several times, resulting in an overall precision of <1.0‰ for $\delta^2H$ and <0.2‰ for $\delta^{18}O$.

The isotopic enrichment of bulk leaf water above source water ($\Delta_{BL}$, in ‰) was calculated as $\Delta_{BL} = (\delta_{BL} - \delta_S)/(1 + \delta_S)$, where $\delta_{BL}$ and $\delta_S$ stand for the isotopic composition of bulk leaf and source water, respectively. In the present study, stem base xylem water was considered to be representative of source water. To account for xylem water included in the main vein, we took a subsample of leaves, covering the whole range of sizes, and determined the weight ratio of main vein water to bulk leaf water ($\phi_x = 0.15 \pm 0.005$, $n = 15$) as described in Cernusak, Wong & Farquhar (2003). Although there is evidence for progressive $^{18}O$ enrichment in vein water (Helliker & Ehleringer 2000; Gan *et al.* 2002), the proposed model to describe them (Farquhar & Gan 2003; Ogée *et al.* 2007) has been mainly tested for linear monocot leaves. Applying this model to our plants would require additional parameterization, which has not been done for beech leaves. As a consequence, we have not taken into account evaporative enrichment in the vein xylem water.

Taking into account the progressive $^{18}O$ enrichment would reduce the absolute values of lamina leaf water enrichment in the leaves of the water-stressed plants below the values shown here. This would further increase the fitted effective path length *L*. For simplicity, we assumed that vein water was not enriched and estimated mean lamina leaf water enrichment as $\Delta_L = \Delta_{BL}/(1 - \phi_x)$.

To determine $\delta^{18}O$ in water-soluble leaf organic matter ($\delta^{18}O_{OM}$), silver capsules were placed in an autosampler covered by a custom-made, argon-flushed hood and pyrolysed at 1450 °C as described for water samples. In order to consider the potential exchange of oxygen atoms between organic matter and extraction water, water-soluble organic matter samples extracts were produced by using water with two contrasting oxygen isotopic compositions ($\delta^{18}O = -350$‰ and $\delta^{18}O = +9.6$‰, respectively). The difference between replicates extracted with enriched and depleted water was 9.30 ± 0.34‰ on average ($n = 54$). From this, an average exchange rate with extraction water (2.6 ± 0.1%, $n = 54$) was calculated and used to correct the value of $\delta^{18}O_{OM}$ using a mass balance equation. Due to the longer residence time expected for organic matter compared with leaf water (Barnard *et al.* 2007), we calculated the enrichment of organic matter above source water ($\Delta^{18}O_{OM}$), as described for leaf water, but using the average $\delta_S$ of all time points for each treatment.

## Leaf water models

We modelled leaf water enrichment using four approaches of increasing complexity combining isotope, gas exchange and micrometeorological data:

**1** Steady-state isotopic enrichment over source water at the site of evaporation ($\Delta_e$) has been described by the Craig and Gordon model (Craig & Gordon 1965; Dongmann *et al.* 1974):

$$\Delta_e = \varepsilon^+ + \varepsilon_k + (\Delta_v - \varepsilon_k)\frac{e_a}{e_i} \tag{1}$$

where $\varepsilon^+$ is the equilibrium fractionation between liquid water and vapour (Majoube 1971), $\varepsilon_k$ is the kinetic fractionation as vapour diffuses from leaf intercellular spaces to the atmosphere (Farquhar *et al.* 1989; Cappa *et al.* 2003), $\Delta_v$ is the isotopic enrichment of atmospheric water vapour relative to plant source water and $e_a/e_i$ is the ratio of ambient to intercellular vapour pressures.

**2** The steady-state isotopic enrichment of mean lamina mesophyll water ($\Delta_{LsP}$) can be described by the above steady-state Craig and Gordon model corrected for the gradient from xylem source water to enriched water at the evaporating sites, the so-called *Péclet* effect (Farquhar & Lloyd 1993):

$$\Delta_{LsP} = \Delta_e \frac{1 - e^{-\wp}}{\wp} \quad \text{with} \quad \wp = \frac{E \cdot L}{C \cdot D} \tag{2}$$

where $\wp$ is the *Péclet* number, *E* is the leaf transpiration rate (mol m$^{-2}$ s$^{-1}$), *L* is the scaled effective path length (m) for water movement from the veins to the site of evaporation, *C* is the molar concentration of water (55.56 $10^3$ mol m$^{-3}$) and *D* the tracer-diffusivity (m$^2$ s$^{-1}$) of heavy water isotopologues (either $H_2{}^{18}O$ or $^2H^1HO$) in 'normal' water.

**3** Non-steady-state effects in lamina mesophyll water enrichment ($\Delta_{LnP}$) can be approximately added to the steady-state *Péclet* description as follows (Farquhar & Cernusak 2005):

$$\Delta_{LnP} = \Delta_{LsP} - \frac{\alpha^{+}\alpha_{k}}{g_{t}w_{i}} \frac{1-e^{-\wp}}{\wp} \frac{d(V_{m}\Delta_{LnP})}{dt} \tag{3}$$

where $\alpha = 1 + \varepsilon$, ($\alpha^{+}$ and $\alpha_{k}$ are corresponding to $\varepsilon^{+}$ and $\varepsilon_{k}$, respectively), $V_m$ is lamina leaf water molar concentration (mol m$^{-2}$), $t$ is time (s), $g_t$ is the total conductance for water vapour of stomata and boundary layer (mol m$^{-2}$ s$^{-1}$) and $w_i$ is the mole fraction of water vapour in the leaf intercellular air spaces (mol mol$^{-1}$). Comparing the steady-state and non-steady-state, *Péclet* descriptions with the observations allow to estimate whether leaf water has reached isotopic steady-state.

**4** The non-steady-state *Péclet* description of Eqn 3 is a simplification of the more rigorous advection–diffusion description of leaf water enrichment ($\Delta_{LnAD}$, Cuntz *et al.* 2007; Ogée *et al.* 2007):

$$\frac{\partial \Delta_{LnAD}}{dt} = -\frac{v_{r}}{\Theta_{m}} \frac{\partial \Delta_{LnAD}}{dr} + \frac{D_{r}}{\Theta_{m}} \frac{\partial^{2} \Delta_{LnAD}}{dr^{2}} \tag{4}$$

where $r$ denotes the distance from the xylem to the evaporating site (m), $v_r$ is the advection speed of water in the mesophyll (m s$^{-1}$), $\Theta_m$ is the volumetric water content of the mesophyll and $D_r = \Theta_m \kappa_m D$ is the effective diffusivity of the water isotopologues (m$^2$ s$^{-1}$), with $\kappa_m$ (<1) as the tortuosity factor of the water path through the mesophyll. The volumetric water content in the leaf mesophyll $\Theta_m$ is simply related to the water volume $V_m$ (per unit leaf area) and the mesophyll thickness $r_m$ through $\Theta_m = V_m/(Cr_m)$ (Cuntz *et al.* 2007).

The advection–diffusion equation in porous media is complemented by flux boundary conditions at the xylem–mesophyll boundary and at the evaporating sites.

The scaled effective length is calculated in the advection–diffusion description as $L = d_L/\Theta_m\kappa_m$ with leaf thickness as $d_L$ (m). The effective length $L$ therefore varies with varying mesophyll water content $V_m = C\Theta_m d_L$ (mol m$^{-2}$) because either leaf thickness $d_L$ or volumetric leaf water content $\Theta_m$ is changing. The tortuosity factor $\kappa_m$, however, is taken constant. Comparing the advection–diffusion equation with the non-steady state *Péclet* description allows to estimate how much of the $L$ changes are simply due to changing leaf water content.

## Model parameters

For all models, equilibrium fractionation $\varepsilon^{+}$ was calculated after Majoube (1971), and kinetic fractionation $\varepsilon_k$ was calculated after Farquhar *et al.* (1989) with the diffusional fractionation factors of Cappa *et al.* (2003). The diffusional fractionation factors of Merlivat (1978) were tested and basically gave the same results with different effective lengths $L$, though. However, daytime values from the Craig and Gordon model were very close and sometimes below measured mesophyll water enrichments for $^{18}O$ in well-watered plants when using the Merlivat (1978) values. Tracer-diffusivity $D$ as depending on temperature was estimated using a Vogel–Tamman–Fulcher relationship (Cuntz *et al.* 2007):

$$D = a_{D}a_{1}\exp\left(-\frac{a_{2}}{T-a_{3}}\right) \tag{5}$$

with $a_1 = 100 \cdot 10^{-9}$, $a_2 = 577$ and $a_3 = 145$ for both $H_2{}^{18}O$ and $^{2}H^{1}HO$, and $a_D = 1/1.026$ for $H_2{}^{18}O$ and $a_D = 1/1.013$ for $^{2}H^{1}HO$.

Leaf temperature was determined as described in Barbour *et al.* (2000a), which considers both isothermal net radiation and the cooling effect of transpiration. Barbour *et al.* estimated incident radiation from PPFD measurements in the field, assuming a ratio of short wave radiation to PPFD of 0.5 MJ mol$^{-1}$ and applying an average absorption coefficient of 0.5 for the leaf (Jones 1992). However, radiation in the infrared is much higher than in the visible range with artificial lighting and thus long-wave radiation should be also considered. To estimate total incident radiation from PPFD measurements, we calculated the ratio between PPFD and both short-wave and long-wave radiation (0.61 and 2.55 MJ mol$^{-1}$, respectively), based on the measurements performed by Hamasaki & Okada (2000) in a growth chamber with comparable light conditions (PPFD = 180 $\mu$mol m$^{-2}$ s$^{-1}$; 4:1 proportion of cool-white fluorescents and incandescent lamps). Effective radiation reaching leaf surface was finally corrected by average leaf inclination angle in the upper canopy (15.9° ± 1.3°, $n = 50$) using Lambert's Law (Jones 1992). Due to the planophile nature of beech, this correction only caused minor changes to radiation estimates (3.4–6.7%). Leaf transpiration rate ($E$, mol m$^{-2}$ s$^{-1}$) was determined from measured stomatal conductance ($g_s$, mol m$^{-2}$ s$^{-1}$, plus boundary layer conductance) and from the leaf-to-air VPD [$w_a$–$w_i$, mol($H_2O$) mol$^{-1}$(air)] in the climate chamber. The effective length $L$ was determined by least square minimization of the non-steady state models and both observations, $H_2{}^{18}O$ and $^{2}H^{1}HO$, during light. This gives a direct estimate of $L$ in the case of the non-steady state *Péclet* model and an estimate of the tortuosity factor $\kappa_m$ in the case of the advection–diffusion model. In the latter, the effective length $L$ is then calculated as $L = d_L/\Theta_m\kappa_m$. Leaf thickness $d_L$ is thereby calculated once from maximum measured leaf water volume and all changes in $V_m$ are taken as changes in the volumetric leaf water content $\Theta_m$ as explained in Cuntz *et al.* (2007). Initial values for the non-steady-state models were taken as the first measured values.

## Statistics and sensitivity analysis

The effect of treatment over the diel cycle on plant physiological variables and isotopic enrichment was assessed through repeated measures analyses of variance (ANOVA) including treatment (water-limited, control) as fixed factor (SAS 1988). Unless otherwise stated, significance was considered with $P < 0.05$, and means are reported together with the standard errors of the mean. The effect of uncertainties associated with key input variables ($g_s$, wind speed, PPFD) on $L$ was assessed by comparing the $L$ estimates calculated with the minimum and maximum extremes of variability (95% confidence interval for measured $g_s$ during daytime, and measured range for PPFD and wind speed). These variables were selected because they show a relatively high degree of uncertainty and have strong effects on the models (see model parameters section and references therein). PPFD is very important for modelled leaf temperature, which in turn determines $e_i$, and is used to calculate $\varepsilon^+$. $g_s$ is changing leaf temperature, $\varepsilon^+$, $\varepsilon_k$ and the *Péclet* number due to changed $E$. Finally, wind speed affects boundary layer resistance and therefore $\varepsilon_k$, but also leaf temperature and therefore $\varepsilon^+$.

## RESULTS

### Physiological response to the treatments

We found a significant physiological response of beech to the water limitation treatment, overall indicating moderate stress (Fig. 1). According to the repeated measures ANOVA, we found significant differences between control and water-limited plants for $\Psi_s$ (Fig. 1a), leaf temperature (Fig. 1c), $g_s$ (Fig. 1d) and $E$ (Fig. 1f), but not for assimilation rate ($P = 0.598$, Fig. 1b) and leaf WC ($P = 0.894$, Fig. 1e).

The analysis of time effects showed strong diel patterns for $\Psi_s$, leaf temperature, assimilation rates, as well as for leaf WC. However, changes over time were weak or hardly significant for transpiration rate ($P = 0.018$) and $g_s$ ($P = 0.071$) in water-limited plants, probably due to the relatively low levels of these variables during daytime, together with a rather high variability between replicates. Daytime average for $\Psi_s$ dropped from $-1.26 \pm 0.04$ MPa in the control to $-1.55 \pm 0.05$ MPa in water-limited plants, whereas during night, $\Psi_s$ recovered to similar values in both treatments, showing lower and not significant differences ($-0.81 \pm 0.11$ and $-0.98 \pm 0.11$ MPa for control and water-limited, respectively). $g_s$ and $E$ showed a strong reduction in response to water limitation (daytime $E = 2.08 \pm 0.22$ $\mu$mol m$^{-2}$ s$^{-1}$ and $E = 1.14 \pm 0.14$ $\mu$mol m$^{-2}$ s$^{-1}$ for well-watered and water-limited plants, respectively). Due to the differences in $g_s$ and $E$, daytime leaf temperature increased from $26.0 \pm 0.1$ °C in the control to $27.2 \pm 0.2$ °C under water-limited conditions, whereas no differences were found during nighttime (about 20.4 °C in both treatments).

### Diel patterns in oxygen and hydrogen isotope composition and evaporative enrichment

Table 1 shows measured values for $\delta^2$H and $\delta^{18}$O in atmospheric water vapour ($\delta^2H_V$, $\delta^{18}O_V$) and in stem ($\delta^2H_S$, $\delta^{18}O_S$) and bulk leaf water ($\delta^2H_{BL}$, $\delta^{18}O_{BL}$). As expected, bulk leaf water was significantly enriched when compared with xylem water, whereas atmospheric water vapour inside the chamber showed the most depleted values. Due to evaporative enrichment in the pots, stem water (i.e. water taken up by the trees) was slightly more enriched in water-limited than in well-watered plants, with a maximum during the afternoon, and a minimum at night. As shown in Fig. 2, lamina leaf water isotopic enrichment was higher during the light periods than in the dark, showing a significant

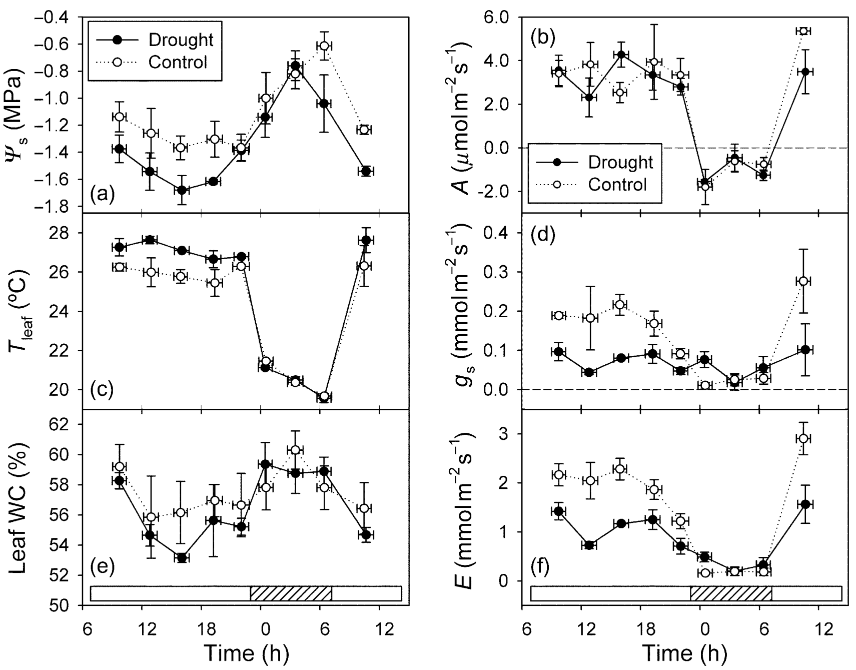


**Figure 1.** Diel course of (a) stem water potential ($\Psi_s$), (b) assimilation rate ($A$), (c) modelled leaf temperature ($T_{leaf}$), (d) stomatal conductance ($g_s$), (e) leaf water concentration (WC) and (f) transpiration rate ($E$). Vertical error bars indicate the standard error for three plants. Horizontal error bars indicate the time range for each measurement round. White/shadowed bars at the bottom denote dark/light periods.

**Table 1.** Mean values within each round for air temperature ($T_{air}$) and relative humidity ($RH$), and measured isotope compositions of atmospheric water vapour ($\delta^2H_V$, $\delta^{18}O_V$), stem water ($\delta^2H_S$, $\delta^{18}O_S$) and bulk leaf water ($\delta^2H_{BL}$, $\delta^{18}O_{BL}$)

| Time (hh:mm) | $T_{air}$ (°C) | $RH$ (%) | $\delta^2H_V$[a] (‰) | $\delta^{18}O_V$[a] (‰) | $\delta^2H_S$[b] (‰) | $\delta^{18}O_S$[b] (‰) | $\delta^2H_{BL}$[c] (‰) | $\delta^{18}O_{BL}$[c] (‰) |
|---|---|---|---|---|---|---|---|---|
| Control | | | | | | | | |
| 09:05–10:19 | 23.0 (0.2) | 67.8 (4.1) | −120.1 | −18.0 | −51.7 | −5.9 | −20.2 ± 1.0 | 5.0 ± 0.4 |
| 12:23–13:44 | 22.5 (0.4) | 69.9 (1.9) | −111.7 | −16.3 | −48.8 | −5.4 | −16.7 ± 0.7 | 6.3 ± 0.3 |
| 15:08–16:25 | 22.8 (0.7) | 68.6 (5.0) | −106.3 | −15.6 | −48.0 | −5.2 | −15.7 ± 1.0 | 7.0 ± 0.5 |
| 18:46–20:14 | 21.9 (0.6) | 70.1 (4.6) | −103.8 | −14.7 | −49.5 | −5.5 | −13.6 ± 2.0 | 7.2 ± 0.7 |
| 21:31–22:57 | 22.0 (1.0) | 71.4 (8.4) | −106.9 | −15.6 | −50.0 | −5.5 | −13.2 ± 2.6 | 7.1 ± 0.8 |
| 00:09–00:57[d] | 21.5 (1.1) | 69.3 (6.0) | −108.2 | −15.5 | −51.9 | −5.6 | −16.3 ± 1.1 | 6.4 ± 0.1 |
| 03:02–03:56[d] | 20.5 (0.5) | 73.3 (3.8) | −115.9 | −16.3 | −50.8 | −5.6 | −19.0 ± 1.1 | 5.3 ± 0.3 |
| 06:06–06:50[d] | 19.8 (0.4) | 73.0 (2.3) | −125.6 | −17.8 | −50.3 | −5.6 | −25.0 ± 0.6 | 3.6 ± 0.5 |
| 09:37–10:58 | 23.2 (0.2) | 66.3 (0.9) | −124.5 | −18.2 | −51.2 | −5.7 | −26.0 ± 0.9 | 5.4 ± 0.4 |
| Mean | 21.9 (3.8) | 70.1 (10.2) | −113.7 ± 8.2 | −16.4 ± 1.3 | −50.3 ± 1.3 | −5.5 ± 0.2 | −18.4 ± 4.6 | 5.9 ± 1.2 |
| Water-limited | | | | | | | | |
| 09:09–10:09 | 23.1 (0.3) | 67.5 (3.4) | −120.1 | −18.0 | −43.3 | −4.3 | −14.4 ± 3.7 | 6.9 ± 1.5 |
| 12:00–13:20 | 22.7 (0.4) | 69.2 (2.9) | −111.7 | −16.3 | −44.4 | −4.4 | −15.5 ± 2.8 | 6.6 ± 0.7 |
| 15:30–16:51 | 22.9 (0.5) | 69.3 (0.9) | −106.3 | −15.6 | −44.5 | −4.3 | −12.1 ± 1.4 | 7.8 ± 0.4 |
| 18:18–19:48 | 22.2 (1.4) | 71.3 (3.6) | −103.8 | −14.7 | −42.2 | −3.8 | −12.0 ± 0.9 | 7.7 ± 0.8 |
| 21:41–22:28 | 21.9 (0.2) | 72.3 (2.5) | −106.9 | −15.6 | −41.0 | −3.5 | −10.9 ± 2.0 | 8.0 ± 0.8 |
| 00:04–00:53[d] | 21.5 (1.1) | 69.3 (6.0) | −108.2 | −15.5 | −42.3 | −4.0 | −10.3 ± 1.9 | 8.1 ± 0.5 |
| 03:07–03:56[d] | 20.6 (0.3) | 72.4 (4.6) | −115.9 | −16.3 | −44.1 | −4.5 | −16.0 ± 1.4 | 4.8 ± 0.6 |
| 06:02–06:44[d] | 19.8 (0.4) | 73.0 (2.3) | −125.6 | −17.8 | −43.9 | −4.5 | −20.3 ± 0.1 | 4.4 ± 0.3 |
| 10:00–11:29 | 23.3 (0.3) | 66.2 (1.9) | −124.5 | −18.2 | −43.2 | −4.3 | −19.6 ± 1.8 | 7.7 ± 0.3 |
| Mean | 22.0 (3.8) | 70.1 (9.3) | −113.7 ± 8.2 | −16.4 ± 1.3 | −43.2 ± 1.2 | −4.2 ± 0.3 | −14.6 ± 3.6 | 6.9 ± 1.4 |

[a]Water vapour trapped along the whole duration of each round in the climate chamber in which trees from both treatments were growing. Therefore, the same values are given for the control and the water-limitation treatment.
[b]Pool of three stems.
[c]Mean of three replicates.
[d]Night-time measurements.
When applicable, either range (between brackets) or standard deviation (±) within and across time points is indicated.

diel pattern and no significant interaction between time and treatment. On the other hand, we found slightly higher isotopic enrichment in well-watered than in water-limited plants (daytime $\Delta^{18}O_L = 13.8 \pm 0.3$‰, $\Delta^2H_L = 40.4 \pm 2.2$‰ for the control and $\Delta^{18}O_L = 13.5 \pm 0.2$‰, $\Delta^2H_L = 35.6 \pm 1.6$‰ under water limitation), although differences were significant only for $^2$H ($P = 0.022$) but not for $^{18}$O ($P = 0.270$). The same pattern was observed for the isotopic enrichment of water-soluble leaf organic matter ($\Delta^{18}O_{OM}$) averaged over the whole experiment, which was slightly (but not significantly) higher in the control ($\Delta^{18}O_{OM} = 38.5 \pm 0.8$‰) than in water-limited plants ($\Delta^{18}O_{OM} = 37.4 \pm 0.5$‰).

The diel cycle of modelled evaporative enrichment followed the same patterns observed in measured values: relatively high enrichment during the day and lower

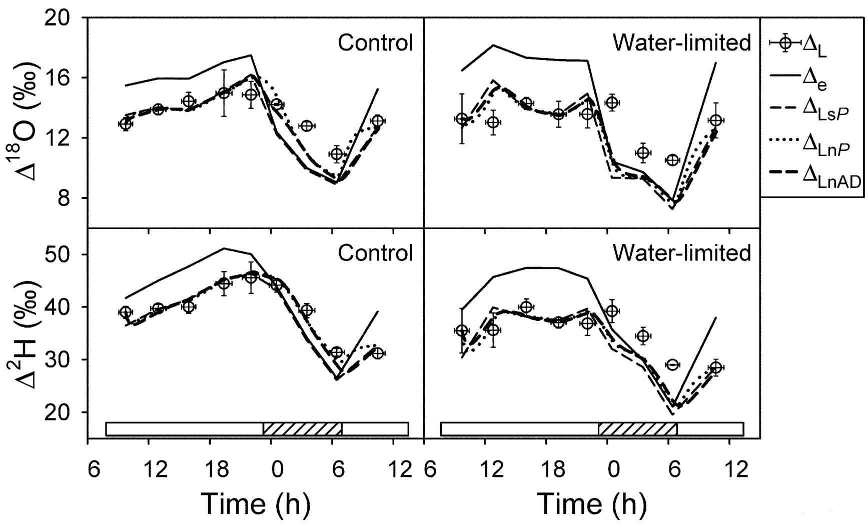


**Figure 2.** Comparison between the diel course of isotopic enrichment of hydrogen ($\Delta^2$H) and oxygen ($\Delta^{18}$O) as observed in the lamina leaf water ($\Delta_L$) and the values estimated from models for isotopic enrichment at the site of evaporation (Craig and Gordon model, $\Delta_e$) and for mean lamina leaf water: $\Delta_{LsP}$ and $\Delta_{LnP}$, *Péclet*-based steady-state and non-steady-state models, respectively; $\Delta_{LnAD}$, advection–diffusion non-steady-state model. Vertical error bars indicate the standard error of the mean of three plants. Horizontal error bars indicate the time range for each measurement round. White/shadowed bars at the bottom denote dark/light periods.

**Table 2.** Scaled effective length $L$ estimated for different non-steady-state leaf water enrichment models, for control and water-limited conditions

| Sensitivity case | PPFD ($\mu$mol m$^{-2}$ s$^{-1}$) | $g_s$ (mol m$^{-2}$ s$^{-1}$) Control | $g_s$ (mol m$^{-2}$ s$^{-1}$) Limited | Wind speed (m s$^{-1}$) | Effective length $L$ (mm) *Péclet* Control | *Péclet* Limited | Adv.–Diff. Control | Adv.–Diff. Limited |
|---|---|---|---|---|---|---|---|---|
| Standard | 150 | 0.187 ± 0.088 (0.075–0.395) | 0.077 ± 0.045 (0.033–0.210) | 0.8 | 17 ± 2 (14,18) | 52 ± 5 (52,51) | 15 ± 2 (15–19) | 45 ± 5 (49–55) |
| Low PPFD | **100** | = | = | = | 6 ± 2 (0,11) | 33 ± 6 (22,40) | 6 ± 2 (6–7) | 29 ± 5 (32–36) |
| High PPFD | **200** | = | = | = | 22 ± 2 (24,22) | 61 ± 5 (69,56) | 20 ± 2 (20–26) | 53 ± 4 (58–65) |
| Low gs/$E$ | = | **0.156** | **0.061** | = | 23 ± 2 (22,23) | 70 ± 7 (69,70) | 20 ± 2 (20–26) | 60 ± 6 (65–73) |
| High gs/$E$ | = | **0.219** | **0.092** | = | 14 ± 2 (12,16) | 45 ± 5 (41,47) | 13 ± 2 (13–17) | 39 ± 4 (43–48) |
| Low wind | = | = | = | **0.6** | 19 ± 2 (17,20) | 56 ± 5 (59,54) | 17 ± 2 (17–21) | 49 ± 5 (55–62) |
| High wind | = | = | = | **1.0** | 15 ± 2 (12,17) | 48 ± 5 (47,49) | 13 ± 2 (13–17) | 42 ± 5 (45–51) |

The first line of the table (Standard) gives the values for $L$ as calculated from measured and modelled physiological/environmental parameters as shown in Fig. 1. The following lines show sensitivity analyses. Changing daytime input variables for the sensitivity cases are highlighted in bold, whereas variables labelled with an equal sign (=) keep the values shown in the first row: photosynthetic photon flux density (PPFD), stomatal conductance ($g_s$) and wind speed. Effective lengths $L$ for the advection–diffusion model (Adv.–Diff.) stem from estimated tortuosity factors $\kappa_m$ and calculated with the maximum water content $\Theta_m$ of all plants (control plus water-limited). The numbers in parentheses of the advection–diffusion model give the range of actual lengths $L$ with the measured water contents. The effective length $L$ of the *Péclet* model is constant and is normally in-between the range of the variable lengths of the advection–diffusion model. Numbers in parentheses of the *Péclet* model are estimates with firstly only $\delta^{18}$O and secondly with only $\delta^2$H.

enrichment at night (Fig. 2). As expected, this pattern was stronger for modelled enrichment at the site of evaporation ($\Delta_e$) than for the rest of the models predicting mean lamina water. We did not find significant differences between treatments for daytime $\Delta_e$ ($\Delta^{18}O_e = 16.2 \pm 0.4$‰, $\Delta^2H_e = 45.8 \pm 1.9$‰ for the control and $\Delta^{18}O_e = 17.2 \pm 0.2$‰, $\Delta^2H_e = 43.9 \pm 1.7$‰ under water limitation). We only found slight differences between the outputs of the two *Péclet* models, steady state ($\Delta_{LsP}$) and non-steady state ($\Delta_{LnP}$). Both models corrected the daytime overestimation of $\Delta_e$ but still slightly departed from measured values at certain time points, especially for water-limited plants at night and less pronounced at midday. The values predicted by the advection–diffusion model ($\Delta_{LnAD}$) were comparable to those given by the non-steady-state *Péclet* model ($\Delta_{LnP}$), being slightly less sensitive to variations in input conditions. In agreement with the measured values, modelled values for $\Delta_L$ were slightly higher in control than in water-limited plants.

### Effect of treatment on the effective path length *L*

We found strong differences in $L$ between the treatments, with $L$ values in water-limited plants being about threefold higher than in well-watered plants, regardless of the modelling approach used (Table 2). The sensitivity analysis showed that changes in $g_s$ (together with $E$) and wind speed had relatively little effect on $L$ estimations, while the effect of PPFD was considerable (Table 2). Nevertheless, in all cases, $L$ under water-limited was much higher (2.7- to 5.5-fold) than in well-watered plants. Even comparing the most extreme cases, $L$ under water-limited conditions with low PPFD estimates was still higher than $L$ in control conditions with high PPFD estimates.

## DISCUSSION

### Changes in evaporative enrichment in response to water limitation

According to the Craig and Gordon model (Eqn 1), and within the same atmospheric conditions, plants with higher $g_s$ (i.e. lower $\varepsilon_k$; see Farquhar *et al.* 1989) and lower leaf temperature (thus higher $e_a/e_i$) are expected to show smaller enrichment at the site of evaporation ($\Delta_e$). Despite the synergistic effect of $g_s$ and temperature, modelled $\Delta_e$ was however comparable in both treatments (Fig. 2). This was due to the less negative values for $\delta^2H_S$ and $\delta^{18}O_S$ in water-limited plants (see Table 1) indicating higher evaporative enrichment of source water and leading to a more negative $\Delta_v$ (see Eqn 1), thus compensating for the differences in $e_a/e_i$ between treatments. It should be noted that, despite being in a relatively closed system, water vapour in the chamber was strongly uncoupled from source (i.e. xylem) water. It apparently resulted from a mixture of different evaporation processes (e.g. evaporation from the leaves, the substrate and the humidifier). Given that temperature effects on evaporative fractionation differ for $\delta^{18}$O and $\delta^2$H (see e.g. Cappa *et al.* 2003), relative differences between treatments in $\Delta_v$ were smaller for $^2$H, and hence, their counteracting effect against $e_a/e_i$ was smaller. Nevertheless, neither for $^{18}$O nor $^2$H were significant differences in $\Delta_e$ observed.

Even without having differences in isotopic enrichment at the site of evaporation, average leaf water is expected to be depleted in $^{18}$O and $^2$H with respect to the site of evaporation due to the *Péclet* effect (Eqn 2), and such depletion would be related to the mass flow of water from the xylem to the stomata, which in turn depends on $E$ (Farquhar & Lloyd 1993). Effectively during daytime when stomata are open and transpiration is higher, $\Delta_L$ was clearly depleted when compared with modelled $\Delta_e$ (Fig. 2). However, despite having double the transpiration rate $E$ in control than in water-limited plants, the difference between $\Delta_e$ and $\Delta_L$ was not significantly higher in well-watered plants, but just the opposite. To further assess the magnitude of this effect, we calculated the fractional isotopic difference between $\Delta_e$ and $\Delta_L$ ($1-\Delta_L/\Delta_e$), which is positively related to the *Péclet* number, and thus to $E$ when the other variables are constant (Barbour & Farquhar 2003). Despite higher $E$, daytime fractional differences were significantly lower in the control than in water-limited plants ($0.13 \pm 0.01$ and $0.20 \pm 0.01$, respectively; average for both isotopes and all daytime points; $n = 12$). Since $C$ is a physical constant and variations in $D$ are well characterized, a big change in $L$ is necessary in order to counteract the differences in $E$ between treatments, to the extent that *Péclet* numbers were about halved despite doubled $E$: $\wp = 0.27 \pm 0.05$ and $\wp = 0.49 \pm 0.08$ in control and dry conditions, respectively. Accordingly, estimated $L$ using the *Péclet* models was about threefold greater in water-limited than in well-watered plants (Table 2). Such differences were strong enough to overrule measurement uncertainties in model input parameters, and particularly those affecting modelled leaf temperature. Indeed, the sensitivity analysis showed that even comparing the most extreme cases (which translated into differences in leaf temperature of up to 3.5 °C), $L$ differences between treatments were maintained. On the other hand, non-steady-state effects are expected to cause greater deviation under drought stress, mostly due to changes over time in WC (Farquhar & Cernusak 2005), and thus, the observed differences might be just an artefact due to deviations from steady-state conditions. However, during most of the day, the outputs from steady-state and non-steady-state *Péclet* models were almost identical and both, environmental conditions and physiological variables, were nearly constant. So, we cannot expect big deviations from steady-state conditions neither in control nor in water-limited plants. Moreover, the differences between treatments reported here are considerable and comparable to interspecies variations reported elsewhere (Wang *et al.* 1998; Barbour *et al.* 2004; Kahmen *et al.* 2008).

We did not account for the potential evaporative enrichment of leaf vein xylem water. If such effects were present and included in our calculations, the fitted effective path length $L$ would further increase, especially in the leaves of water-stressed plant, and thus strengthen our argumentation.

## Environmental and physiological effects on *L*

One of the main differences between the non-steady-state *Péclet* model and the advection–diffusion model is that the latter separates the influence on the effective path length $L$ of the water status represented by $V_m$ or $\Theta_m$, respectively, from of the influence of the different water pathways, such as symplastic, apoplastic and transcellular (Cuntz *et al.* 2007). Despite this, $L$ calculated from the advection–diffusion approach was still three times higher in water-limited plants than in control plants, thus indicating that $L$ can be rather sensitive to environmental and physiological factors, far beyond the variations explained by volumetric water change alone. Barnard *et al.* (2007), for example, studied the diel course of $\delta^{18}O$ in pine needles and obtained $L$ values three times higher in 1-year-old needles than in current needles (150 and 50 mm, respectively). Ripullone *et al.* (2008) showed an about threefold increase in $L$ over a VPD range from 5 to 30 mbar. Considering the wide range of VPD assayed, this might appear as a small change, but again, it emphasizes the potential sensitivity of $L$ to environmental conditions. Our study suggests an even higher sensitivity of $L$ since the relative differences in $g_s$ and $E$ between treatments were relatively small when compared with changes in $L$. However, a VPD/temperature treatment (Ripullone *et al.* 2008) and a water-limitation treatment (our study) would affect the mechanisms controlling leaf water evaporative enrichment in a different way. In the former case, changes in $g_s$ occur in response to big changes in $e_a/e_i$ (both input variables for the models), resulting in a poor relationship between $E$ and both VPD and $L$ (Ripullone *et al.* 2008). On the contrary, in our case, changes in $g_s$ were independent of VPD, and $e_a/e_i$ was only slightly affected due to the increase in leaf temperature associated with lower conductance. On the other hand, the sensitivity of $L$ to environmental and physiological conditions may vary among species, and this might be related to the strategies adopted to regulate plant water balance (e.g. water saving versus fast-growing, opportunistic species).

## Possible causes for *L* variations under drought

In our experiment, $L$ increased when reduced $g_s$ led to lower $E$ in water-limited plants and evidence so far in the literature suggests that this is a real physiological response, and not an artefact due to the fitting procedure. Up to now, most experiments have been performed with fast-growing crop species, i.e. with high potential $g_s$ and $E$ (e.g. *Gossypium hirsutum* L., *Ricinus communis* L, *Phaseolus vulgaris* L.), giving relatively low mean $L$ values (6.25–13.5 mm) (cf. Flanagan *et al.* 1994; Barbour & Farquhar 2000; Barbour *et al.* 2000b). In contrast, fitted $L$ values above 35 mm seem to be a common case for trees with an inherently lower transpiration rate, regardless of leaf dimensions (13 out of 16 species with $E < 4$ mmol m$^{-2}$ s$^{-1}$; Barnard *et al.* 2007; Brandes 2007; Kahmen *et al.* 2008). Again, in the work from Barbour *et al.* (2004), the $L$ values decreased from birch (*Betula occidentalis* Hock) to alder (*Alnus incana* L. Moench) and cottonwood (*Populus fremontii* Wats) corresponding to increasing $g_s$ and $E$, and all three species showed apparently higher $L$ when $g_s$ and $E$ dropped in the low humidity treatment. Furthermore, Kahmen *et al.* (2008) found $L$ to be inversely correlated with $E$ and $g_s$ across 17 Eucalyptus species ($r^2 = 0.60–0.82$). Short-term variations in $L$, compensating or even exceeding the stomatal response, might be the cause of contrasting responses of $\Delta^{18}O$ (sometimes apparently opposed to theory) to changes in $E$ and $g_s$ as induced by abscisic acid (Barbour & Farquhar 2000; Sheshshayee *et al.* 2005). Thus, although the data available are scarce to drive definitive conclusions, current evidences suggest that $L$ values tend to increase when transpiration rates are limited by total leaf conductance. However, a mechanistic understanding for such observation is still lacking.

It is known that water, after leaving the leaf xylem, is not only moving on apoplastic pathways but also (mediated by aquaporins) via cell vacuoles (transcellular pathway) to the sites of evaporation (Steudle & Frensch 1996; Sack, Streeter & Holbrook 2004). Due to changes in aquaporin expression and activity, mesophyll hydraulic conductance (and potentially related parameters, such as mesophyll conductance for $CO_2$ and hydraulic conductivity) can be highly dynamic and respond rapidly and reversibly to changes in temperature, irradiance and water supply (Flexas *et al.* 2002; Sack & Holbrook 2006; Cochard *et al.* 2007). Thus, we can assume that changes in mesophyll hydraulic properties (e.g. proportion between symplastic, transcellular and apoplastic water movement) would affect $L$. For example, an increase in water compartmentation (e.g. through closure of intracellular water channels) would increase the tortuosity of water pathways, leading to an effective increase in $L$, but may also cause the uncoupling between evaporation sites and part of the leaf water (Yakir 1992b; Yakir *et al.* 1993). Such uncoupling is expected to be higher in water-stressed plants, where 'empty' (i.e. gas-filled) apoplastic spaces between leaf water pools might appear. In our case, however, this effect alone is not likely to be responsible for the apparent increase in $L$ observed in water-limited plants, as we did not observe significant differences in molar leaf WC. However, even without changes in water content, the closure of water channels would reduce the proportion of water affected by the backward diffusion of evaporative enrichment, and this would cause an apparent increase in $L$. Additionally, the fitted $L$, as an 'effective length', may not be only affected by the length of the water flow paths, but also by their (potentially variable) total section. If water flow inside the leaf is restricted to a limited number of narrow channels, the mesophyll flux rate (i.e. the one that effectively determines the *Péclet* number in Eqn 2) can be much higher than measured $E$ (Yakir 1992b). Under such conditions, an increase in the effective mesophyll flux rate relative to $E$ will cause an increase in the effective path length $L$, without implying changes neither in path length nor tortuosity. Thus, additional efforts are needed to characterize $L$ empirically and to mechanistically assess its relationship with measurable physiological parameters in order to understand the ultimate source of its variability. Alternatively, a combined model considering both the *Péclet* effect together with changes in leaf compartmentalization and water pathways might help to minimize the effect of $L$ parameterization in the models. Unfortunately, and despite recent technical advances, e.g. in magnetic resonance imaging (Van As 2007), measuring short-term changes in water content and conductivity within the mesophyll is still a challenging issue.

## Implications for the use of water isotopes as physiological indicators

The observed changes in $L$ in response to moderate drought stress may compromise some of the potential applications for $\delta^2H$ and $\delta^{18}O$, such as their use as indicators of $g_s$ and $E$ (Barbour & Farquhar 2000; Barbour *et al.* 2000a; Wang & Yakir 2000; Farquhar, Cernusak & Barnes 2007) or as integrators of leaf temperature (e.g. Helliker & Richter 2008). In our case, an increase in $g_s$ and $E$ did not result in a subsequent decrease in leaf water enrichment, but the opposite was true, and the same trend was observed in leaf-soluble organic matter, which is a quite good proxy for new assimilates (Gessler *et al.* 2007). On the other hand, genetic variability in $L$ can also lead to relatively little response of leaf water enrichment to differences in $g_s$ and $E$, if they are compensated by differences in $L$ (Kahmen *et al.* 2008). Additionally, given that changes in leaf hydraulic properties, and thus in $L$, might occur within a few hours (Lo-Gullo *et al.* 2005; Cochard *et al.* 2007), the assumption that $L$ is stable over the diel cycle (Farquhar & Cernusak 2005) or responds only to changes in water content (Cuntz *et al.* 2007) is probably wrong. This may be an extra source of discrepancies between modelled and measured data, which cannot be solved by current enrichment models.

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## APPENDIX

*List of symbols*

| | |
|---|---|
| $a_1$ | Constant in temperature dependent description of diffusivity [$m^2\ s^{-1}$] |
| $a_2$ | Constant in temperature dependent description of diffusivity [K] |
| $a_3$ | Constant in temperature dependent description of diffusivity [$K^2$] |
| $a_D$ | Tracer dependent constants in diffusivity description |
| $C$ | Molar water concentration [$mol\ m^{-3}$] |
| $d_L$ | Leaf thickness [m] |
| $D$ | Diffusivity [$m^2\ s^{-1}$] |
| $D_r$ | Effective diffusivity [$m^2\ s^{-1}$] |
| $E$ | Transpiration rate [$mol\ m^{-2}\ s^{-1}$] |
| $g_s$ | Stomatal conductance [$mol\ m^{-2}\ s^{-1}$] |
| $g_t$ | Total conductance [$mol\ m^{-2}\ s^{-1}$] |
| $L$ | Scaled effective length of water path in mesophyll [m] |
| $\wp$ | *Péclet* number |
| PPFD | Photosynthetic photon flux density [$mol\ m^{-2}\ s^{-1}$] |
| $r$ | Radial coordinate [m] |
| $r_m$ | Mesophyll thickness |
| $RH$ | Relative humidity corrected to leaf temperature |
| $t$ | time dimension [s] |
| $T_{air}$ | air temperature [K] |
| $V_m$ | Mesophyll water volume [$mol\ m^{-2}$] |
| $v_r$ | Effective advection velocity in $r$-direction [$m\ s^{-1}$] |
| $e_a$ | Water vapour pressure in the atmosphere [Pa] |
| $e_i$ | Water vapour pressure in the leaf intercellular air spaces [Pa] |
| $w_a$ | Water vapour mole fraction of the atmosphere [mol ($H_2O$) $mol^{-1}$ (air)] |
| $w_i$ | Water vapour mole fraction in the leaf intercellular air spaces [mol ($H_2O$) $mol^{-1}$ (air)] |
| $WC$ | Leaf water concentration [%] |
| $\alpha^+$ | Equilibrium water-vapour fractionation factor |
| $\alpha_k$ | Kinetic fractionation factor |
| $\delta_{BL}$ | Isotope ratio of bulk leaf water [VSMOW] |
| $\delta_S$ | Isotope ratio of source/xylem water [VSMOW] |
| $\delta^2H_{BL}$ | Deuterium isotope ratio of bulk leaf water [VSMOW] |
| $\delta^2H_S$ | Deuterium isotope ratio of source/xylem water [VSMOW] |
| $\delta^2H_V$ | Deuterium isotope ratio of water vapour [VSMOW] |
| $\delta^{18}O_{BL}$ | $^{18}O$ isotope ratio of bulk leaf water [VSMOW] |
| $\delta^{18}O_S$ | $^{18}O$ isotope ratio of source/xylem water [VSMOW] |
| $\delta^{18}O_V$ | $^{18}O$ isotope ratio of water vapour [VSMOW] |
| $\delta^{18}O_{OM}$ | $^{18}O$ isotope ratio of water-soluble leaf organic matter [VSMOW] |
| $\Delta_{BL}$ | Isotope ratio of bulk leaf water relative to source water |
| $\Delta^{18}O_{OM}$ | Isotope ratio of water-soluble leaf organic matter relative to source water |
| $\Delta_L$ | Isotope ratio of mean lamina mesophyll water relative to source water |
| $\Delta_V$ | Isotope ratio of air water vapour relative to source water |
| $\Delta_e$ | Isotope ratio at evaporative site relative to source water (Craig and Gordon model) |
| $\Delta_{LsP}$ | Isotope ratio of mean lamina mesophyll water relative to source water(steady-state Péclet model) |
| $\Delta_{LnP}$ | Isotope ratio of mean lamina mesophyll water relative to source water(non-steady-state *Péclet* model) |
| $\Delta_{LnAD}$ | Isotope ratio of mean lamina mesophyll water relative to source water(advection–diffusion model) |
| $\varepsilon^+$ | Equilibrium water-vapour fractionation |
| $\varepsilon_k$ | Kinetic fractionation |
| $\Phi_x$ | Ratio of main vein water to bulk leaf water |
| $\Psi_s$ | Stem water potential [MPa] |
| $\Theta_m$ | Volumetric liquid water content |