New Phytologist

Research

The influence of species and growing conditions on the 18-O enrichment of leaf water and its impact on 'effective path length'

Ansgar Kahmen[1,2], Kevin Simonin[1], Kevin Tu[1], Gregory R. Goldsmith[1] and Todd E. Dawson[1]

[1]Center for Stable Isotope Biogeochemistry, Department of Integrative Biology, University of California, Berkeley, CA, USA; [2]Institute of Plant Sciences, ETH Zurich, Switzerland

Author for correspondence:
Ansgar Kahmen
Tel: 01 510 6431749
Email: akahmen@berkeley.edu

Received: *17 April 2009*
Accepted: *23 June 2009*

New Phytologist (2009)
doi: 10.1111/j.1469-8137.2009.03008.x

Key words: gas exchange, oxygen, relative humidity, stable isotopes, transpiration, water.

Summary

• The stable oxygen isotope ratio ($\delta^{18}O$) of plant material has been shown to contain essential information on water and carbon fluxes at the plant and ecosystem scales. However, the effective path length (L_m), a parameter introduced to leaf-water models still requires a comprehensive biological characterization to allow interpretation of $\delta^{18}O$ values in plant material with confidence.

• Here, we tested the variability of L_m across and within three species that developed leaves in environments with different relative humidity. We also tested whether the L_m of fully developed leaves is affected by short-term fluctuations in relative humidity.

• We determined that significant differences in L_m exist among *Phaseolus vulgaris*, *Rizinus communis* and *Helianthus annuus*. Within a given species, however, L_m values did not differ significantly among individuals.

• These findings indicate that L_m is species specific and a relatively constant parameter and that L_m will not obscure the interpretation of $\delta^{18}O$ values in plant material of a given species. We urge caution, however, because values for L_m are derived from fitting leaf-water models to measured values of $\delta^{18}O$, so care must be taken in assigning a 'cause' to values of L_m as they likely capture a combination of different biological leaf properties

Introduction

The stable oxygen and hydrogen isotope ratios ($\delta^{18}O$ and δD, respectively) of plant materials have been shown to contain essential information for understanding plant and ecosystem water and carbon fluxes (Dawson *et al.*, 2002; Barbour, 2007). Applications of $\delta^{18}O$ and δD data range from the reconstruction of paleoclimates and neoclimates using tree rings (Schiegl, 1974 Epstein & Yapp, 1977) or plant-derived organic compounds in lake sediments (Sachse *et al.*, 2004, 2006), with the heavy isotope (i.e. ^{18}O) to the analyses of carbon and water fluxes at the global (Farquhar & Lloyd, 1993) and ecosystem scales (Yakir & Wang, 1996; Moreira *et al.*, 1997; Williams *et al.*, 2004). In addition, $\delta^{18}O$ and δD data collected at the leaf level can provide critical information on plant ecophysiological responses to environmental variability (Barbour *et al.*, 2000a; Cernusak *et al.*, 2007, 2008; Ripullone *et al.*, 2008) and anthropogenic factors such as air pollution (Grams *et al.*, 2007; Jaggi & Fuhrer, 2007; Bassin *et al.*, 2009).

A primary cause of $\delta^{18}O$ and δD variability in plant materials (e.g. water and organic matter) is the evaporative enrichment of leaf water with the heavy isotope (i.e. ^{18}O). With the exception of halophytic (Lin & Sternberg, 1994) and a few extreme-ophylic plants (Ellsworth & Williams, 2007), no fractionation has been observed during the uptake of soil water by plant roots and its subsequent transport through the xylem to the leaves (White *et al.*, 1985; Dawson & Ehleringer, 1991). Once water arrives at the leaf, enrichment through evaporative water loss in ^{18}O can be substantial (Dongmann *et al.*, 1974). Recent improvements in mechanistic leaf water models have significantly advanced our understanding of the physiological and environmental factors that lead to leaf water enrichment in ^{18}O (Flanagan *et al.*, 1991; Farquhar & Lloyd, 1993; Farquhar & Cernusak, 2005; Cuntz *et al.*, 2007; Kahmen *et al.*, 2008).

However, even with these theoretical advances, the complexity of interactions among the different factors known to influence the magnitude of ^{18}O enrichment of leaf water has hampered efforts to interpret data obtained from the $\delta^{18}O$ of organic matter.

Of particular interest in this regard is the 'effective path length' (L_m), because it can substantially complicate the interpretation of oxygen isotope values in plant material (Kahmen *et al.*, 2008). The L_m roughly describes the flowpath of water in the leaf lamina from the veinlets to the sites of evaporation. Despite the importance of L_m, the factors contributing to variation in L_m still require a comprehensive description. Recent efforts to understand biological controls over leaf water have revealed the significance of L_m as a driver for variation in leaf water $\delta^{18}O$ across species (Wang *et al.*, 1998; Barbour & Farquhar, 2003; Kahmen *et al.*, 2008). As a result, plant $\delta^{18}O$ signals compared across species are not only driven by environmental and ecophysiological parameters, but also by L_m, which makes the interpretation of $\delta^{18}O$ signals across different species complicated. Furthermore, it has recently been proposed that L_m could not only vary across, but also within, any given species (Keitel *et al.*, 2006; Barnard *et al.*, 2007; Ferrio *et al.*, in press). If true, L_m could therefore also obscure the environmental or ecophysiological interpretation of $\delta^{18}O$ signals within individuals of a given species; this in turn would make using $\delta^{18}O$ data in organic matter (e.g. in tree ring or breeding studies) particularly problematic.

Because a comprehensive investigation that addresses the presence and extent of variation in L_m within individuals of a given species is still missing, we specifically designed the investigation presented here to explore whether L_m varied within individuals of a given species in response to different environmental treatments. For three different plant species, we tested if (1) L_m varies as a result of different environmental conditions during leaf development or (2) as a result of short-term environmental fluctuations.

Materials and Methods

To evaluate the effects of different environmental conditions during leaf development on L_m, we grew three different plant species that varied in a number of leaf characteristics: common bean (*Phaseolus vulgaris* L.), castor bean (*Ricinus communis* L.) and sunflower (*Helianthus annuus* L.). Plants were grown from seed to mature, flowering plants in two adjacent glasshouses with contrasting environmental conditions that we refer to here as 'long-term treatments'. Plants were either grown in 'wet' conditions defined by high relative humidity (RH; ranging from 70% to 100%) and high soil water availability, or 'dry' conditions defined by low RH (40–65%) and low soil water availability (Fig. 1). Soil water availability in the long-term wet treatment was kept at a constant level using drip irrigation, whereas plants in the long-term dry treatment were watered only every other day. In addition to the long-term treatments, we tested the effects of short-term environmental fluctuations on L_m of fully developed mature leaves by growing plants under the wet conditions described above and transferring these plants to an additional dry glasshouse 7 d before conducting our sampling. Our treatment results are therefore expressed as 'dry' (long-term dry growth conditions), 'wet' (long-term wet growth conditions) and 'transfer' (short-term transfer from wet to dry).

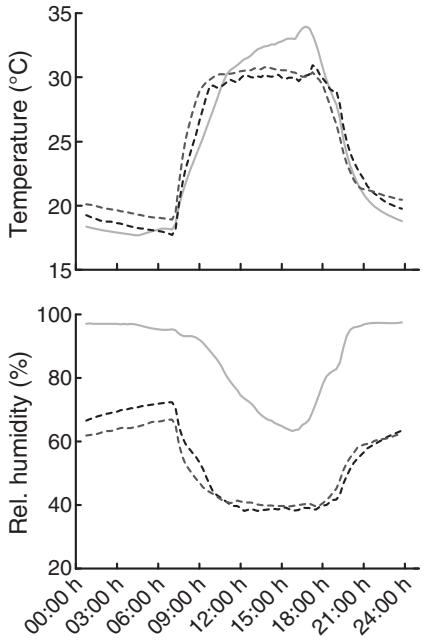

Fig. 1 Average diurnal variability of air temperature and relative humidity in the three glasshouse chambers (8-wk average shown for dry (dashed–dotted line) and wet (grey line) glasshouse; 1-wk averages shown for transfer (black/grey dashed line) glasshouse).

We grew and sampled five replicate individuals for every species in each of the three treatments. Environmental conditions were constantly monitored with permanent climate sensors installed in each of the glasshouses. In addition, we installed four additional climate sensors (RH/TempLog Datalogger; Oakton Instruments, Vernon Hills, IL, USA) in each glasshouse during our intensive sample collection period to obtain a more precise estimate of the variation in relative humidity and air temperature that plants experienced for each treatment.

All plant samples were collected on 20 September 2007, 8 wk after seeds were planted. The diurnal changes in stomatal conductance (g_s), transpiration (E) and leaf temperature were measured seven times each day for all three species in the three treatments using a LI-COR 6400 (LI-COR Biosciences, Lincoln NE, USA). Measurements started at sunrise and ended after sunset. For the

gas-exchange measurements, we adjusted light and atmospheric conditions within the cuvette of the LI-COR 6400 to reflect the environmental conditions of the respective glasshouse. Nevertheless, environmental conditions within the cuvette always deviated slightly from ambient conditions in the glasshouse. To avoid cuvette artifacts on our estimates of E and leaf temperature, we did not use cuvette-derived values for E and leaf temperature in our analyses. Instead, we adjusted values of E and leaf temperature to ambient RH and air temperature using the Penman–Monteith equation (Monteith, 1965). In our calculations, we first solved the Penman–Monteith equation for leaf net radiation (Rn) using E, g_s RH and air temperature from within chamber measurements. In a second step, we again used the Penman–Monteith equation to recalculate E with Rn and ambient values for RH and air temperature that we obtained from the four climate loggers in a glasshouse. In this second step, we used g_s values obtained from chamber measurements in our calculations, assuming that the slight differences between chamber and ambient atmosphere RH and air temperature had little instantaneous effects on g_s. The adjusted values for E represent the rate of transpiration that would be observed inside the leaf chamber at ambient RH and air temperature. In a final step, leaf temperature at ambient RH and air temperature was calculated with the Penman–Monteith corrected values for E by solving the leaf energy balance (Jones, 1992).

To test for the effect of growth conditions on plant and leaf morphology, we determined total plant height, leaf size (length, width and area), leaf weight and specific leaf area (SLA) for every species in the wet and the dry treatment. Leaf area was measured using a LI-COR 3100 leaf area meter. We determined leaf water concentration (mol H_2O m^{-2}) for leaves from all species in the three treatments by measuring FW, DW and leaf area twice a day (once in the morning shortly after sunrise and once in the evening just before sunset). To evaluate the effect of growth conditions on plant water status, we measured pre-dawn and midday water potentials using a pressure chamber (PMS Instruments, Albany OR, USA) on the day of the experiment. We also determined maximum leaf hydraulic conductance (K_l; mol s^{-1} m^{-2} MPa^{-1}) for plants in the wet and in the dry treatment. Leaves were sampled for determination of maximum K_l within 2 d of the experiment. Maximum K_l was determined using the evaporative flux method described by Sack *et al.* (2002). Briefly, leaves were collected in the glasshouse by cutting the base of the petiole under water and these were then allowed to rehydrate by placing the cut ends in a 10 mM aqueous KCL solution overnight in the dark. The next morning, petioles were attached to an evaporative flux apparatus, which consisted of low resistance PVC tubing, filled with KCl solution running to a plastic cup on a balance (± 0.01 mg; Metler-Toledo AG245, Columbus, OH, USA). Mineral oil was used to eliminate evaporation from the KCl solution in the cup on the balance. A light source was suspended above the leaf, producing > 1200 μmol m^{-2} s^{-1} of photosynthetically active radiation at the leaf surface. Fans were placed around the leaf to minimize the boundary layer resistance. Leaves were allowed to transpire until a steady-state flow through the lamina was achieved over a 10-min interval. They were then removed from the tubing and placed in a pressure chamber until the balancing pressure was recorded (Soil-moisture Equipment Corp., Santa Barbara, CA, USA). Maximum K_l was calculated as the stable flow rate/balancing pressure for leaves with a balancing pressure ≥ −0.65 MPa.

Since L_m cannot be directly measured and can only be determined by fitting leaf water models to measured values of leaf water $\delta^{18}O$, we determined leaf water $\delta^{18}O$ of the three plant species in the three treatments on diurnal timescales. To do so, we collected one leaf from each replicate species seven times each day, starting at sunrise and ending after sunset. After the leaves were clipped from the plant, the primary veins were removed and the remaining leaf lamina sealed in 5 ml PVC vials and immediately frozen. Bulk leaf lamina water was extracted from the leaves using cryogenic vacuum distillation at the Center for Stable Isotope Biogeochemistry, UC Berkeley, USA, following the method described by West *et al.* (2006).

To determine the isotopic composition of the plant's source water, we collected the water used for irrigation three times during the sampling day. To avoid isotopic enrichment of irrigation water by evaporation from the soil, all pots were covered with 3 cm quartz sand; this sand 'cap' decouples the soil from the atmosphere and prevents evaporation.

All measurements of leaf and plant morphology, as well as leaf water status and leaf water isotopic composition, were performed on five replicate individual plants per species and treatment. Repeated destructive sampling of leaves from the plants had no noticeable effects on the plants' physiological performance.

We collected atmospheric water vapor five times during each sampling day in the dry and transfer treatments, and six times in the wet treatment. Vapor was trapped using polyethylene tubing that was looped three times, with the bottom two-thirds of the loops submerged in an ethanol–dry ice slurry (*c.* −80°C), and attached to a small diaphragm pump that pulled air through the traps. The airflow through the cryogenic traps was monitored by flow meters and set at 0.5 l min^{-1}. While we were able to collect two replicate samples at each time-point from the wet treatment, our sampling system allowed for the collection of only one sample per point in time from the dry and the transfer treatment.

Bulk leaf water, source water and water vapor samples were analysed for $\delta^{18}O$ by equilibration of a 50 μl sample of H_2O with 2% CO_2 for 48 h. The isotope composition

of the CO_2 gas was then determined using an isotope ratio mass spectrometer running in continuous flow mode (Finnigan MAT Delta Plus XL; Thermo Instruments Inc., Bremen, Germany) housed at the Center for Stable Isotope Biogeochemistry at UC Berkeley. Calibration standards were included every sixth position to account for instrument drift during a run. The long-term external precision for all of our water analyses was ± 0.14‰.

Leaf water models

We calculated effective path length using isotopic leaf water models. The development and precision of the models has been discussed in depth in the literature (Barbour *et al.*, 2004; Cernusak *et al.*, 2005; Cuntz *et al.*, 2007; Ogee *et al.*, 2007; Kahmen *et al.*, 2008). Briefly, the models are based on equations that mechanistically describe the steady-state enrichment of leaf water in ^{18}O at the sites of evaporation above the source water ($\Delta^{18}O_e$) as:

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

ε^+ is the equilibrium fractionation between liquid water and vapor at the air-water interfaces (Bottinga & Craig, 1969); ε_k is the kinetic fractionation that occurs during water vapor diffusion from the leaf intercellular air space to the atmosphere (Farquhar *et al.*, 1989; Cappa *et al.*, 2003; Cernusak *et al.*, 2003a); $\Delta^{18}O_v$ is the isotopic value of water vapor in the atmosphere compared with source water; e_a/e_i is the ratio of ambient to intercellular vapor pressures (Craig & Gordon, 1965; Dongmann *et al.*, 1974; Farquhar *et al.*, 1989; Flanagan *et al.*, 1991).

Equation 1 was originally developed to estimate the ^{18}O enrichment of well-mixed surface waters of large water bodies such as lakes (Craig & Gordon, 1965) and has been shown to overestimate the evaporative enrichment of mean lamina mesophyll water (Flanagan *et al.*, 1991; Wang & Yakir, 1995; Roden & Ehleringer, 1999b; Barbour & Farquhar, 2000; Cernusak *et al.*, 2002). As such, Farquhar & Lloyd (1993) and Barbour *et al.* (2000b) have suggested that the discrepancy between the predicted leaf water enrichment based on Eqn 1 (i.e. the enrichment at the sites of evaporation) and the observed values of mean leaf water is caused by isotopic gradients within the leaf. These gradients may form as a result of the mixing of the transpirational stream of unenriched (source) water with enriched water moving backwards by diffusion in the opposing direction from the sites of water evaporation and therefore ^{18}O enrichment. The ratio of transpirational flow over back-diffusion is described by the Péclet number (□; after Farquhar & Lloyd, 1993), which relates the mean lamina mesophyll leaf water isotopic enrichment over source water ($\Delta^{18}O_L$) to $\Delta^{18}O_e$ as

$$\Delta^{18}O_L = \frac{\Delta^{18}O_e(1 - e^{-\wp})}{\wp} \qquad \text{Eqn 2}$$

where the Péclet number is defined as,

$$\wp = \frac{EL_m}{CD} \qquad \text{Eqn 3}$$

In Eqn 3, E is transpiration rate (mol m^{-2} s^{-1}), C is the molar concentration of water (mol m^{-3}), D is the diffusivity of H_2O in water (m^2 s^{-1}), and L_m is the effective path length for the transpirational flow of water from the xylem veinlets through the mesophyll (m) to the site of evaporation. Since the exact nature of L_m remains unclear, this parameter must be determined by iteratively by fitting the model to measured values of bulk leaf water $\Delta^{18}O$.

Equations 1 and 2 describe the enrichment of leaf water in ^{18}O at steady state (i.e. under constant environmental conditions). Such conditions rarely occur in nature, where leaf water enrichment in ^{18}O is subject to diurnally changing evaporative conditions. Dongmann *et al.* (1974), and more recently Farquhar & Cernusak (2005), therefore accounted for nonsteady-state enrichment of mean lamina mesophyll water in ^{18}O. In their model, nonsteady-state leaf water enrichment ($\Delta^{18}O_{LN}$) is expressed as

$$\Delta^{18}O_{LN} = \Delta^{18}O_L - \left(\frac{1 - e^{-\wp}}{\wp}\right)\left(\frac{\frac{d(W\Delta^{18}O_{LN})}{dt}}{gw_i}\right) \qquad \text{Eqn 4}$$

W is the water concentration of the leaf (mol m^{-2}); w_i is the mole fraction of water vapor in the leaf intercellular air spaces (mol mol^{-1}). In essence, the deviation of leaf water enrichment in ^{18}O from the steady state is 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 nonsteady-state model has now been tested in several studies and has shown good agreement with measured bulk leaf water values of ^{18}O (Cernusak *et al.*, 2005; Barnard *et al.*, 2007; Gessler *et al.*, 2007; Kahmen *et al.*, 2008).

To estimate values of L_m for the different species in the different treatments, we used the nonsteady-state leaf water model (Eqn 4). We fitted the model to measured diurnal values of bulk leaf water $\Delta^{18}O$ of a given species and treatment by adjusting L_m until the sum of the differences between modeled $\Delta^{18}O_{LN}$ and measured $\Delta^{18}O_M$ values reached a minimum. For our estimates of L_m, we used the solver function in Microsoft Excel as suggested by Cernusak *et al.* (2005). As we fitted the nonsteady-state model to an entire set of diurnally measured bulk leaf water $\Delta^{18}O$ values, we obtained a single value for L_m for each replicate plant (n = 5) in each of the three treatments.

In a final evaluation of what parameters ultimately explain the enrichment of leaf water in ^{18}O for a given

species across the different treatments, we tested if the deviation of $\Delta^{18}O_M$ from $\Delta^{18}O_e$ (i.e. the fraction of unenriched water in the leaf (f) for a given species was correlated with E or L_m. The fraction of unenriched water in leaves, f, was determined for each plant as:

$$f = 1 - \frac{\delta^{18}O_M - \delta^{18}O_s}{\delta^{18}O_e - \delta^{18}O_s} \quad \text{Eqn 5}$$

$\delta^{18}O_M$ is the measured isotopic composition of bulk leaf water; $\delta^{18}O_e$ is the isotopic composition at the site of evaporation calculated with Eqn 1; $\delta^{18}O_s$ is the isotopic composition of source water or xylem water (Leaney *et al.*, 1985; Gan *et al.*, 2002).

Statistical effects on plant and leaf morphological properties, leaf water potential, leaf water concentration and mean daily values of $\Delta^{18}O_M$ were tested using a one-way ANOVA with an LSD *post-hoc* test.

Results

The climate in the three different treatments differed significantly and consistently for the entire duration of plant growth and development. Figure 1 shows the mean diurnal patterns of air temperature and RH for each of the three treatments. Temperatures and RH were comparable between the dry and the transfer treatments. The wet treatment, however, was on average 30% more humid than the dry and transfer treatments. Photosynthetic radiation did not differ across treatments and peaked at 1000 μmol m^{-2} s^{-1} on sunny days during midday. The climatic patterns on the day of the experiment were comparable to the mean values shown in Fig. 1.

The different environmental growth conditions in the wet and the dry treatments had significant effects on growth and morphology of the three species (Fig. 2). Plants grown in the wet treatment were all significantly taller by more than a factor of two. Also, plants developed significantly larger leaves in wet conditions compared with the dry growing conditions. Specific leaf area (SLA; m^2 g^{-1}) was significantly higher in the wet treatment for sunflowers. Beans also showed a trend towards higher SLA in the wet treatment, but this trend was nonsignificant. The SLA for castor bean did not differ between the two treatments. Maximum K_l differed across species, but did not differ for any species as a result of wet or dry growing conditions.

Pre-dawn leaf water potential was most negative for sunflower and least negative for castor bean (Fig. 3). For any given species, pre-dawn leaf water potentials did not differ across the three treatments. At midday, however, leaf water potentials in the dry and transfer treatments were more negative for a given species when compared with the wet treatment (Fig. 3). Unfortunately, data for midday leaf water potential of bean were lost during the analysis.

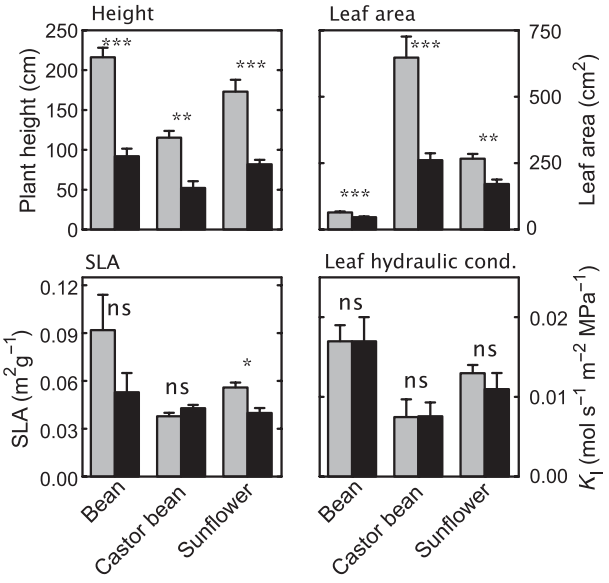

Fig. 2 Effects of dry (closed bars) and wet (tinted bars) growing conditions in the two long-term treatments on leaf morphological and leaf hydraulic properties of the three species (bean, castor bean and sunflower) (n = 5). Significance: ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$, n.s., not significant. Error bars, 1 SE. SLA, specific leaf area; K_l, maximum leaf hydraulic conductance.

Morning and evening values for leaf water concentration did not differ for any of the three plant species. We therefore used morning and evening values to calculate daily means. Castor bean showed the lowest leaf water concentration, while bean and sunflower had a higher but comparable leaf water concentration (Fig. 4). For any given species, leaf water concentration did not differ between wet and transfer treatment but was significantly higher in the dry treatment.

For all three species, diurnal stomatal conductance was substantially higher in the wet treatment compared with the dry and transfer treatments (Fig. 5). Across all species, in all treatments, sunflower grown in the wet treatment had the highest stomatal conductance. Diurnal patterns and the magnitude of stomatal conductance in the dry and in the transfer treatments were roughly comparable for bean and castor bean, but differed substantially for sunflower, where stomatal conductance was higher in the transfer treatment compared with the dry treatment. Transpiration rates for all three species showed similar diurnal pattern in the wet treatment, with maximum transpiration rates in the early afternoon (Fig. 5). Transpiration rates in the dry and in the transfer treatment showed very similar diurnal patterns and magnitudes for bean and castor bean. For both species in both the dry and the transfer treatment, transpiration rates peaked in the late morning and then gradually declined over the course of the day. By contrast, sunflower showed lower transpiration rates in the dry treatment and these were consistently low over the course of the day but were consistently

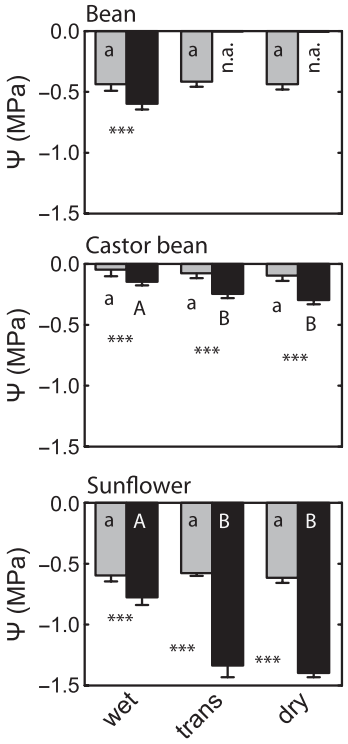

Fig. 3 Pre-dawn (tinted bars) and midday (closed bars) leaf water potential for the three plant species (bean, castor bean and sunflower) in the different treatments (n = 5). Midday values for the dry and transfer treatment of bean were not available (n.a.). Significance: ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; n.s., not significant. Different lower case letters indicate significantly different pre-dawn water potential values across treatments for a given species and different upper case letters indicate significantly different midday water potential values across treatments for a given species. Error bars, 1 SE.

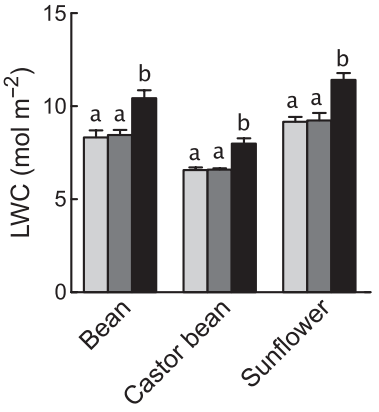

Fig. 4 Leaf water concentration (LWC) for the three different plant species (bean, castor bean and sunflower) in the three different treatments (wet, light grey bars; transfer, dark tinted bars; dry, closed bars). No significant differences for LWC were found in leaves collected just after sunrise and leaves collected just before sunset. The values shown are therefore mean daily values. Different lower case letters indicate significantly different LWC concentrations across treatments for a given species ($P \leq 0.05$). Error bars, 1 SE.

high in the transfer treatment, with a substantial peak in the late afternoon.

The different treatments also had substantial effects on bulk leaf water enrichment in ^{18}O above source water ($\Delta^{18}O_M$; Fig. 5). In the wet treatment, leaf water $\Delta^{18}O_M$ was comparable across species and significantly lower compared with the dry and the transfer treatments with minimal diurnal patterns. Statistical testing of mean daily $\Delta^{18}O_M$ values of a given species revealed no significant differences between $\Delta^{18}O_M$ values from the dry and the transfer treatment, but $\Delta^{18}O_M$ values from the wet treatment were significantly different from $\Delta^{18}O_M$ values from the dry and the transfer treatment. This pattern was consistent for all three species.

The isotopic composition of atmospheric water vapor was comparable in all three treatments (Fig. 6). The only variation was in the morning, when atmospheric vapor in the wet treatments was *c.* 2‰ less enriched compared with the dry and the transfer treatments. Overall, diurnal variability was less than 3‰ in either treatment.

Predictions by the isotopic leaf water models as described in Eqns 1–4 closely matched the diurnal patterns of isotopic leaf water enrichment for all three species in the different treatments (Fig. 7). As expected, the basic Craig and Gordon (CG) (Craig and Gordon, 1965) model overestimated leaf water enrichment in ^{18}O except for the first three samples collected for castor bean in the wet treatment. The inclusion of a Péclet effect improved model performance. The best fit between the measured and modeled $\Delta^{18}O$ values was achieved with the nonsteady-state model (Fig. 7).

The effective path lengths obtained by fitting the nonsteady-state model to the measured values of leaf water $\Delta^{18}O$ were significantly different for the three species (Fig. 8). However, no significant differences were observed for L_m within a species across different treatments (Fig. 8).

Our analyses revealed that the mean daily ratio of atmospheric to leaf internal vapor pressure (e_a/e_i) was the key driver of both the mean leaf water enrichment in ^{18}O at the site of evaporation ($\Delta^{18}O_e$) and $\Delta^{18}O_M$ (Fig. 9). In addition, transpiration also explained some of the variability in $\Delta^{18}O_M$ by driving the fraction of unenriched water in a leaf (f). However, the relationship between f and transpiration was not significant for the daily mean values. As indicated earlier, daily transpiration rates for common bean and castor bean showed complementary diurnal patterns across treatments but did not differ in their average daily rates (Fig. 6). We therefore determined the relationship between E and f separately for mean morning and mean afternoon values. Except for castor bean in the morning, all species showed a significant relationship between E and f (Fig. 9).

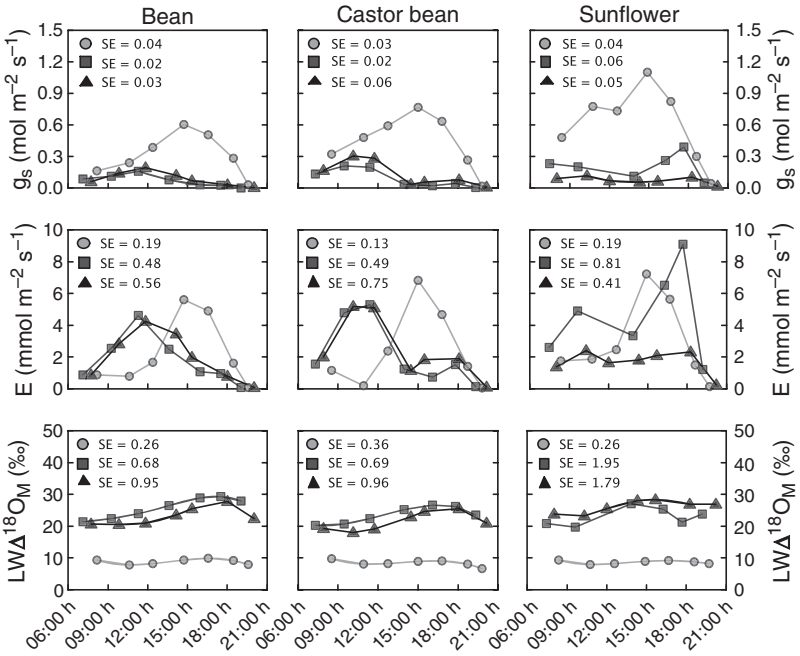

Fig. 5 Diurnal variability in stomatal conductance (g_s), transpiration (E), and enrichment of bulk lamina leaf water in ^{18}O above source water ($LW\Delta^{18}O_M$) of the three plant species (bean, castor bean and sunflower) in the different treatments (wet, circles; transfer, squares; dry, triangles). Data shown represent the means of five replicates. Numbers in figures indicate mean daily standard error.

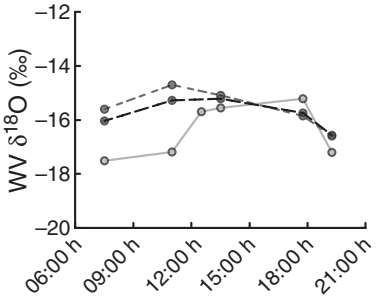

Fig. 6 Diurnal patterns of water vapor (WV) $\delta^{18}O$ values in the three glasshouse chambers at the day of the experiment. Wet, solid grey line; transfer, dashed line; dry (dashed–dotted line).

Discussion

Treatment effects and gas exchange

Greater height growth and larger leaf sizes for wet treatment plants illustrate that the different growth conditions in the wet and dry glasshouses significantly affected the morphology of the three plant species (Fig. 2). Pre-dawn water potentials did not differ across treatments for a given plant species (Fig. 3), even though plants in the wet glasshouse were subject to constant water supply via a drip irrigation system, while plants in the dry and transfer glasshouse were watered only every other day. In contrast to pre-dawn, the different growth conditions in the wet, dry and transfer glasshouses had significant effects on the midday leaf water potentials of two plant species (Fig. 3; data for bean were not available). Growing conditions also affected the leaf water concentrations of all three plant species (Fig. 4). In combination, these data suggest that differences in RH in the wet, dry and transfer glasshouse strongly not only affected the leaf morphology but also leaf water relations of the plant species investigated.

Stomatal conductance and transpiration showed a clear response to the different environmental treatments (Fig. 5). Notably, mature leaves from common bean and castor bean plants grown in a wet environment and transferred into a dry environment 1 wk before sampling (transfer treatment) showed almost identical patterns in their gas exchange to leaves that had experienced continuously dry growing conditions (dry treatment). This suggests that fully developed leaves from these two species were quickly able to adjust their gas exchange physiology to the dramatic changes in the environmental conditions that plants experience in dry environments. By contrast, sunflower leaves that fully developed in the wet environment and were then transferred to the dry environment had consistently higher stomatal conductance and transpiration when compared with leaves that had developed in the dry environment, suggesting an inability to adjust.

Diurnal patterns of $\Delta^{18}O_M$ also varied substantially across the different treatments (Fig. 5). Values for $\Delta^{18}O_M$ were significantly higher in the dry and transfer treatments for all three species compared with $\Delta^{18}O_M$ values from the wet treatment plants. Interestingly, the $\Delta^{18}O_M$ values for leaves in the dry and transfer treatment were roughly comparable. This again suggests that fully developed leaves were quickly able to acclimatize physiologically to the marked changes in the environmental conditions exper-

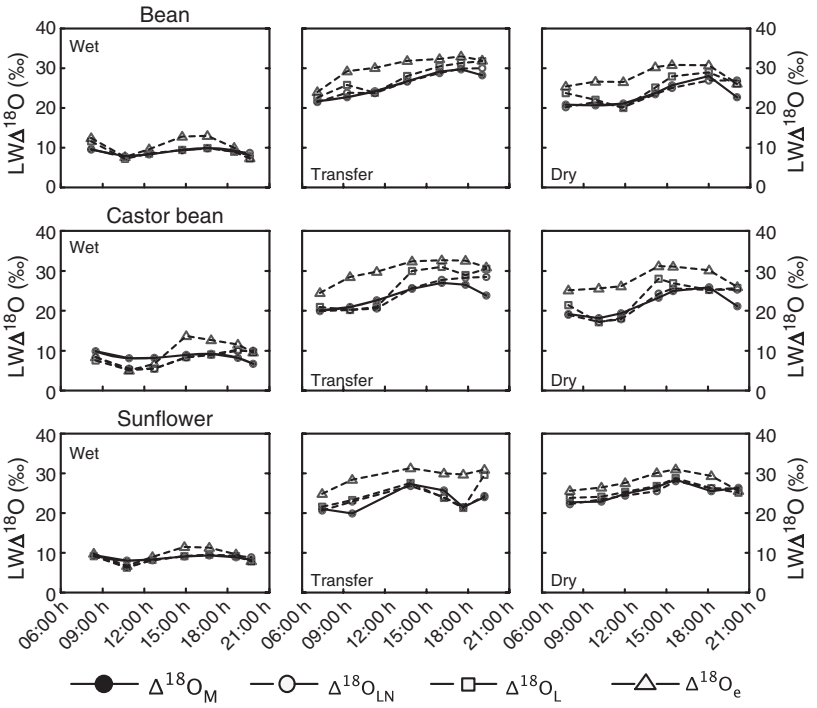

Fig. 7 Modeled and measured enrichment of bulk lamina leaf water in ^{18}O above source water of the three plant species (bean, castor bean and sunflower) in the different treatments. Data shown represent the means of five replicates. $LW\Delta^{18}O_M$ is the ratio of measured leaf water, $LW\Delta^{18}O_e$ is the enrichment in ^{18}O at the sites of evaporation as predicted by (Eqn 1), $LW\Delta^{18}O_L$ is the enrichment in ^{18}O of bulk lamina leaf water in the steady-state (Eqn 2) and $LW\Delta^{18}O_{LN}$ is the enrichment of bulk lamina leaf water in ^{18}O in the non-steady-state (Eqn 4). Each individual value shown represents the mean of five replicate samples.

ienced when transferred from the wet to the dry treatment. The overall patterns in $\Delta^{18}O_M$ are similar to several previous studies where leaves in dry environments were more enriched in ^{18}O compared with leaves in humid environments (Yakir *et al.*, 1990; Flanagan *et al.*, 1991; Roden & Ehleringer, 1999a; Barbour & Farquhar, 2000; Helliker & Ehleringer, 2002; Santrucek *et al.*, 2007; Ripullone *et al.*, 2008). The relative contribution of different environmental and ecophysiological drivers to the observed patterns of $\Delta^{18}O_M$ across different species and treatments is discussed below.

Modeling leaf water $\delta^{18}O$ and estimating L_m

We estimated values for effective path length by fitting the nonsteady-state isotopic leaf water model to diurnally measured values of leaf water $\delta^{18}O$. The precision and uncertainties of the different models that we used to determine L_m have been discussed in depth in other literature and will therefore not be discussed here (Barbour *et al.*, 2004; Cernusak *et al.*, 2005; Cuntz *et al.*, 2007; Ogee *et al.*, 2007). The L_m values obtained by fitting the leaf water models were significantly different for the three species and were within the range of previously determined effective path lengths across a broad range of different plant species (Wang *et al.*, 1998; Kahmen *et al.*, 2008). However, we found no significant differences for L_m values for a given species across different treatment types (Fig. 8). In other words, for the species investigated, L_m appeared to be a constant factor that was independent of the long- or short-term environmental variability used in setting up our treatments. This result is surprising given that several previous studies, including our own, have speculated that L_m should be related to leaf anatomical/morphological properties (Barbour & Farquhar, 2003; Cuntz *et al.*, 2007; Kahmen *et al.*, 2008). The SLA, plant height, leaf area, midday leaf water potential and leaf water concentration all showed significant responses to the three treatments in this study. However, contrary to what we had expected, values for L_m remained constant across treatments for a given species (Fig. 8).

We were unable to detect a significant relationship between leaf morphological traits and L_m in the study we present here. The true morphological, anatomical or physiological nature of L_m therefore remains unclear. Barbour *et al.* (2004) tried to link effective path length values derived from ^{18}O leaf water models to the physical distance between the main vein and the site of evaporation in wheat leaves. While this early study showed that physically measured and model-derived values for L_m were within an order of magnitude, the study was also limited to a single species and thus did not allow any correlative investigation relating leaf functional traits to L_m. Kahmen *et al.* (2008) specifically tested the relationship between L_m of and a range of different leaf traits such as SLA, leaf area and leaf size across a large number of different *Eucalyptus* species, but found no significant correlation. It has also been suggested that L_m should be related to aquaporin expression and activity and consequently to K_l (Keitel *et al.*, 2006; Ferrio *et al.*, in press). In a recent study, Ferrio *et al.* (in press) have shown that L_m in leaves of slightly water-stressed beach saplings

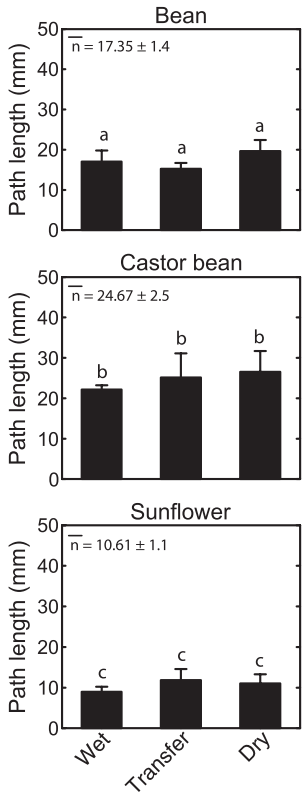

Fig. 8 Effective path-length as predicted by the nonsteady-state leaf water model (Eqn 4) for the three species (bean, castor bean and sunflower) in the three treatments (dry, transfer and dry). The total mean for effective path-length including 1 SE is given for each species in the respective plot. Letters indicate significant differences across species and treatments. Error bars represent 1 SE.

was threefold higher than L_m in leaves of well-hydrated beach saplings. The authors speculate that reduced aquaporin expression and leaf-internal cavitation resulted in a loss of conductivity for water in the leaf and thus an increase in L_m. In our study, we specifically tested effects of leaf hydraulic conductance on L_m. We detected significant differences in maximum K_l among the three species investigated in this study, but maximum K_l of the three different species was not correlated with L_m. Interestingly, we did not find treatment effects on maximum K_l for any species in this study (Fig. 2). It could therefore be argued that L_m for a given species was not affected by our treatments since maximum K_l was not affected by the treatments. We wish to urge caution here, however, because maximum K_l was determined under standardized conditions for the different species and treatments. Previous research has shown that K_l can respond quickly to changes in environmental conditions such as light and water availability (Cochard *et al.*, 2007). Therefore, the maximum K_l values that we measured may not reflect K_l values that plants experienced at the time when isotopic leaf water enrichment was determined. Our data therefore do not necessarily allow us to establish a mechanistic link between K_l and L_m across treatments for a given species.

The general properties of L_m

As indicated above, there have now been several attempts to link model-derived values for L_m to leaf morphological, anatomical or leaf hydraulic traits, yet, there is no clear indication that these types of leaf traits determine the nature of L_m. A reason for this could be hidden in the method employed for determining L_m. Since direct measurements of L_m are not possible, L_m must be estimated as a fitting parameter in isotopic leaf water models. As a result, values for L_m will not only capture effects of the 'true' effective path length, but also other biological characteristics that may influence the isotopic enrichment of leaf water that are yet to be identified and therefore are not accounted for in current leaf water enrichment models. What is more, L_m as the fitting parameter in isotopic leaf water models will not only contain the unexplained biological information, but will also capture the sum of all measurement errors in the input parameters that are used to fit the isotopic leaf water models. This adds further complications and could explain why L_m values that we publish for a given species here differ to some extent from L_m values that have been published for the same species in previous studies. For *R. communis*, for example, previous studies have published L_m values of 13.5 mm (Barbour *et al.*, 2000b), 15.0 mm and 11.1 mm (Cernusak *et al.*, 2003b). It is likely that different instrumentation, different calibration precision and different methodological routines of the researchers are all likely to introduce small measurement errors to the individual parameters used to model leaf water enrichment in ^{18}O and to calculate L_m.

We tested the sensitivity of L_m to small variations (± 2.5%) in input parameters using the leaf water models that we parameterized with mean environmental and physiological data from the wet and the dry treatment. This sensitivity analyses revealed that a small variation of only ± 2.5% in some input parameters had substantial effects on estimates of L_m (Fig. 10). This shows that values for L_m that are derived from fitting leaf water models capture a combination of different biological leaf properties but also the measurement errors associated with the model input parameters. Such multidirectional influences on L_m could explain why no individual leaf morphological or hydraulic parameter has yet been identified to explain L_m.

Despite the uncertainties involved with determining values for L_m, the data presented here strongly indicate that L_m is a constant factor for a given species. This finding is interesting, given that other studies have suggested that L_m should vary with environmental conditions as a result of changes in the leaves hydraulic properties (Keitel *et al.*, 2006; Ripullone *et al.*, 2008; Ferrio *et al.*, in press). Despite

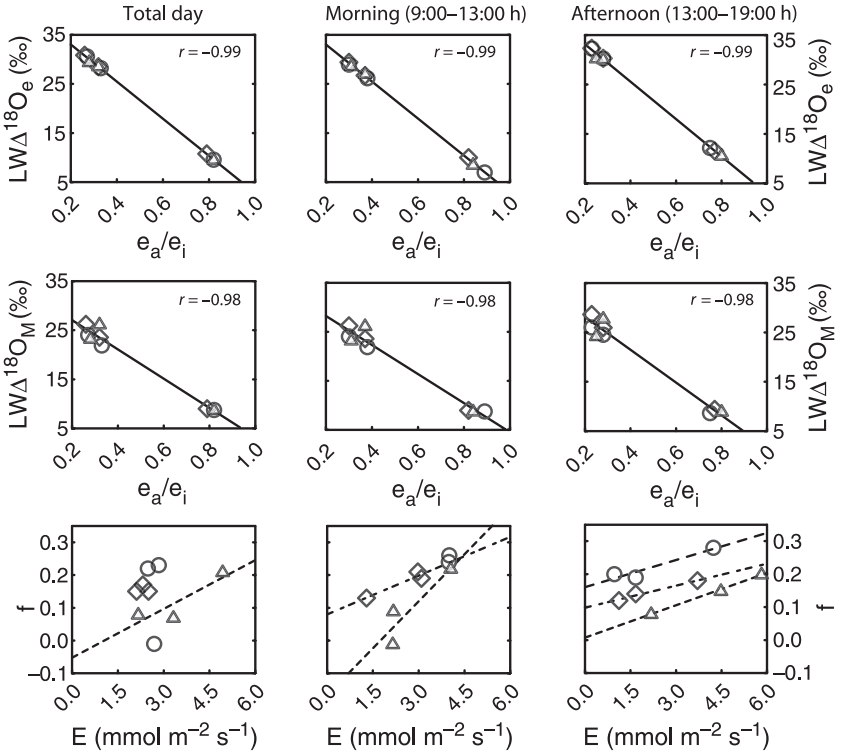

Fig. 9 The relationship between the ratio of atmospheric and leaf internal vapor pressure (e_a/e_i) and leaf water enrichment in ^{18}O at the sites of evaporation ($LW\Delta^{18}O_e$, upper panels) and measured lamina leaf water ($LW\Delta^{18}O_M$, middle panels) for the three species (bean, diamonds and dashed–dotted lines; castor bean, circles and dashed line; sunflower, triangles and dotted line) in the three treatments. The lower panels show the relationship between *f*, the fraction of unenriched source water in a leaf, to transpiration (*E*), where $f = 1 - (\Delta^{18}O_M/\Delta^{18}O_e)$. The relationships are shown for average values across the entire day (07:00–19:00 h) as well as separated in average morning values (07:00–13:00 h) and afternoon values (13:00–19:00 h). All regressions that are shown are significant. The relationship between *f* and transpiration for entire day averages was significant for only sunflower ($r = 0.88$), for morning average values it was significant for sunflower ($r = 0.90$) and bean ($r = 0.95$) and for average afternoon values it was significant for sunflower ($r = 0.99$), bean ($r = 0.95$) and castor bean ($r = 0.99$). Effective path-length (L_m): bean, 17.35; castor bean, 24.67; sunflower, 10.61).

substantial differences in long- and short-term growing conditions and the resulting effects on leaf morphology and leaf water relations, we found consistent values of L_m for individuals of three different species. This extensive and rigorous test gives us confidence that – at least for the species investigated here – L_m is a species-specific parameter and should therefore not obscure the response of a plant's $\delta^{18}O$ values to environmental or ecophysiological drivers. To test this assertion we evaluated, for a given species, the relationship between $\Delta^{18}O_M$ and major environmental and ecophysiological parameters used to influence $\Delta^{18}O$ across the different treatments. As expected, $\Delta^{18}O_e$ can be explained almost exclusively by the ratio of atmospheric to leaf internal vapor pressure (e_a/e_i) for all species across all three treatments (Fig. 9). Similarly, $\Delta^{18}O_M$ was also strongly related to e_a/e_i, but was substantially less enriched in ^{18}O than $\Delta^{18}O_e$ (Fig. 9), an observation that has largely been attributed to the Péclet effect in previous studies (Barbour *et al.*, 2000b, 2004). The influence of the Péclet effect on isotopic leaf water enrichment depends on *E* and L_m (Eqn 3). As we have asserted that L_m is constant for a given species, *E* should then largely influence *f*, the fractional difference between $\Delta^{18}O_M$ and $\Delta^{18}O_e$ for any given species (Fig. 9). We found that all species showed a significant relationship between *E* and *f* for mean morning and afternoon values with the exception of castor bean during the morning (Fig. 9). The strong relationship between *E* and *f* demonstrate that *E*, and not L_m, is the parameter that drives the variability of leaf water $\Delta^{18}O$ and of the Péclet effect for a given species (Flanagan *et al.*, 1991; Barbour *et al.*, 2000b, 2004; Ripullone *et al.*, 2008).

Conclusions

The data presented here show that L_m, or what has been suggested to be the effective path-length of water flux in the leaf lamina, differs significantly across species but not for individuals that are within a given species (even when subject to dramatically different environmental conditions). This finding is important because it facilitates the interpretation of $\delta^{18}O$ values in plant material. For example, variation in $\delta^{18}O$ values of plant material that has been generated under different climatic conditions but originates from the same species, a situation typically found in tree ring time series, will reflect the air to leaf vapor pressure ratio and transpiration but will not be obscured by variation in effective path length. Further, constant values for L_m will allow one to use the variability of $\delta^{18}O$ values in plant material that originates from plants of the same species that have grown under the same climatic conditions as an indicator for transpiration. This will be particularly useful in studies where integrative measures of transpiration are needed to compare, for example, the ecophysiological performance of plants in common garden evaluations of different varieties of agriculturally important species (Barbour *et al.*, 2000a; Morison *et al.*, 2008), or in large-scale envir-

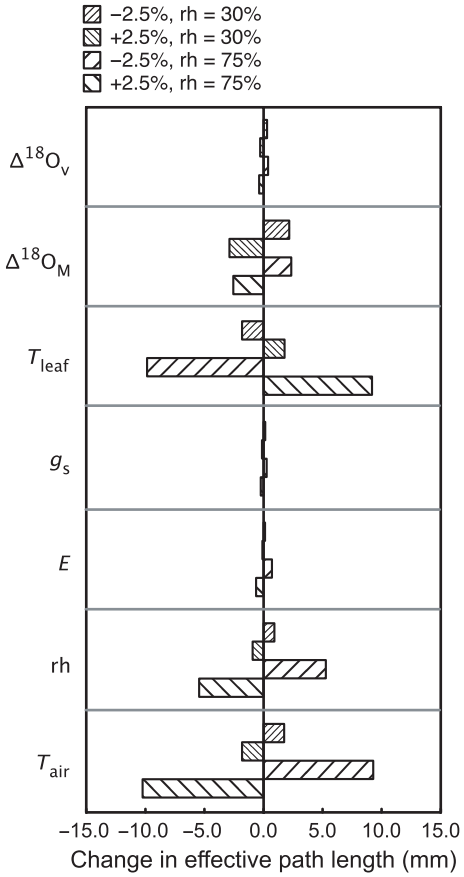

Fig. 10 The result of a sensitivity analysis testing the effects of small variations in input parameters (2.5%) on effective path length, L_m. The sensitivity analysis was performed with models that were parameterized with average environmental and physiological values for the wet treatment (densely hatched bars) and dry treatment (coarsely hatched bars). $\Delta^{18}O_v$, isotopic value of water vapor in the atmosphere compared with source water; $\delta^{18}O_M$, measured isotopic composition of bulk leaf water; T_{leaf}, leaf temperature; g_s, stomatal conductance; E, transpiration; rh, relative humidity; T_{air}, air temperature.

onmental experiments such as in ozone or CO_2 (FACE) fumigation experiments (Grams *et al.*, 2007; Jaggi & Fuhrer, 2007; Bassin *et al.*, 2009).

Acknowledgements

The authors would like to thank Kim DeLong for growing and maintaining the plant specimens used in this study and David Barajas for his assistance with sample collection in the glasshouses and cryogenic leaf water extractions in the laboratory. We would also like to thank Paul Brooks for analysing water samples at the Center for Stable Isotope Biogeochemistry at UC Berkeley. A.K. was supported by an Erwin–Schroedinger Postdoctoral fellowship of the Austrian Science Foundation (FWF) and a Marie-Curie Outgoing International Fellowship of the EU.

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