Plant, Cell & Environment

Plant, Cell and Environment (2017) **40**, 914–920 doi: 10.1111/pce.12845

Review

Leaf water stable isotopes and water transport outside the xylem

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ABSTRACT

How water moves through leaves, and where the phase change from liquid to vapour occurs within leaves, remain largely mysterious. Some time ago, we suggested that the stable isotope composition of leaf water may contain information on transport pathways beyond the xylem, through differences in the development of gradients in enrichment within the various pathways. Subsequent testing of this suggestion provided ambiguous results and even questioned the existence of gradients in enrichment within the mesophyll. In this review, we bring together recent theoretical developments in understanding leaf water transport pathways and stable isotope theory to map a path for future work into understanding pathways of water transport and leaf water stable isotope composition. We emphasize the need for a spatially, anatomically and isotopically explicit model of leaf water transport.

Key-words: hydraulic conductance; Péclet effect; stable isotopes; transpiration; water relations.

OUTSIDE-XYLEM TRANSPORT PATHWAYS

Plants are an important component of the global water cycles, controlling 50 to 90% of ecosystem evapotranspiration (Jasechko *et al.* 2013; Coenders-Gerrits *et al.* 2014; Schlesinger & Jasechko 2014). Stomata form the hydraulic valve regulating exchange of vapour between the leaf and the atmosphere and have been studied in detail for many years resulting in the development of a wide range of empirical and mechanistic models describing their function. In contrast, water movement through leaves remains poorly understood, due to the complexity of pathways and paucity of available measurement techniques (Sack & Holbrook 2006; Scoffoni *et al.* 2015). Water moves through the xylem from the roots to the leaves, the velocity of which movement may be predicted by the distribution of xylem diameters, gradients in pressure and water temperature (Roderick & Berry 2001), but once water exits the xylem in the leaf, the pathways are much less clear. Leaving the xylem, water moves through the xylem parenchyma and bundle sheath cells, and then through or around mesophyll cells via a number of potential pathways before evaporating from cell walls to finally exit as vapour through stomatal pores. Three parallel pathways are thought to exist for liquid water movement within the mesophyll (Steudle *et al.* 1993), namely (1) apoplastic flow through the cell walls; (2) symplastic movement through plasmodesmata; and (3) transcellular movement across membranes via aquaporins. To this, we need to add vapour transport, as recent studies suggest that vapour phase transport is a significant fraction of total water transport within leaves (Rockwell *et al.* 2014; Buckley *et al.* 2015), particularly in the presence of vertical temperature gradients as may be created by light absorption.

Leaf anatomy has considerable influence over outside-xylem leaf hydraulic conductance (K_{ox}). At the highest scale, vein length per unit area (VLA) is positively related to K_{ox}, due to reductions in the horizontal distance between vein and stomata at high vein density (e.g. Brodribb *et al.* 2007). High VLA may also increase K_{ox} due to lower vertical distance if VLA is related to leaf thickness (observed in some but not all studies; Noblin *et al.* 2008; Zwieniecki & Boyce 2014). The arrangement of cells within the mesophyll may also influence K_{ox} (Sack *et al.* 2015), as may the presence of bundle sheath extensions (Scoffoni *et al.* 2008; Buckley *et al.* 2011, 2015) and the thickness of cell walls. A thorough review of the influence of leaf anatomy on K_{ox} is given by Sack *et al.* (2015), who emphasize the coordinated nature of leaf anatomy between species and during leaf development. That is, diversity in leaf anatomy has been constrained both functionally and evolutionarily; a leaf capable of high rates of gas exchange must also have the hydraulic architecture to provide water to the mesophyll (Brodribb *et al.* 2013).

Apoplastic water transport, through the cell walls, has traditionally been considered as the lowest resistance pathway beyond the xylem, suggesting that hydraulic connectedness of the mesophyll should relate to K_{ox}. However, Altus *et al.* (1985) and Canny (1986, 1988) found that an apoplastic-limited dye was collected in the mestome sheath cells and was not found within the mesophyll. This was interpreted as evidence of a symplastic pathway beyond the mestome sheath cells, with water moving across the parenchyma sheath plasma membrane (Canny 1988, 1990), although this may reflect suberization of the bundle sheath alone and not the mesophyll. Biophysical modelling of water transport through the

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mesophyll suggests that observed hydraulic conductances cannot be reproduced without substantial apoplastic transport, using measured values for membrane permeability (Buckley *et al.* 2015).

There is evidence of active regulation of K_{ox} in response to short-term changes in environmental conditions through aquaporin expression and/or activity (Siefritz *et al.* 2002; Cochard *et al.* 2007). Some aquaporins are gated in response to osmotic gradients (Steudle *et al.* 1982; Shatil-Cohen *et al.* 2011) or phosphorylation (Daniels *et al.* 1994), and there is also evidence of rapid changes in the abundance of aquaporin in membranes altering membrane permeability (Robinson *et al.* 1996). Aquaporins in bundle sheath cells have been shown to provide a regulation point between xylem and mesophyll, which is responsive to drought and applied abscisic acid, with strong reductions in leaf hydraulic conductance in plants with lowered bundle sheath aquaporin abundance (Shatil-Cohen *et al.* 2011; Sade *et al.* 2014). Water may also move from cell to cell via plasmodesmata, although the relative sizes of water molecules (0.26 nm) and plasmodesmata transport channels (4 nm diameter, and length of at least 400 nm) suggest that such water transport is likely to be negligible (Fricke 2000).

The complexity of outside-xylem water transport and the strongly interrelated nature of leaf anatomical traits mean that causal relationships between leaf anatomical properties, K_{ox} and pathways of water movement are difficult to establish (Sack *et al.* 2015). Modelling approaches have explored relationships between K_{ox} and anatomy and shed light on the pathways of water movement (Rockwell *et al.* 2014; Buckley 2015). In a two-dimensional model of water flow under varying environmental conditions for leaves of varying anatomy, Buckley *et al.* (2015) were able to predict that apoplastic bulk flow will dominate outside-xylem water transport under most conditions for many leaves, with trans-membrane flow being of lesser importance, given available estimates for the prevailing values of membrane permeability. This was due to the higher intrinsic conductance of bulk apoplastic flow compared with other liquid flow or to vapour diffusion. Further, their integrated water potential and transport model predicted that vapour phase transport may account for 16% of water transport when no temperature gradient is assumed within the leaf and that this increases to 65% if the temperature gradient is assumed to be 0.2 °C across the leaf.

Further complicating the picture is uncertainty regarding the location of the sites of phase change from liquid to vapour (Barbour & Farquhar 2004). Modelling studies have variously concluded that evaporation primarily occurs close to stomata (Tanton & Crowdy 1972; Cowan 1977; Yianoulis & Tyree 1984), deep within the mesophyll close to the vascular system (Boyer 1985) or both (Rockwell *et al.* 2014). Pieruschka *et al.* (2010) also raised the possibility that water may actually condense onto inner epidermal cell walls when the evaporation from cells is out of balance with the leaf transpiration rate, a phenomenon observed by Sheriff (1979), although Mott & Peak (2011) later suggested that this interpretation may relate to artefacts in leaf temperature measurement. Additionally, a high proportion of vapour phase transport as predicted by Rockwell *et al.* (2014) and Buckley *et al.* (2015) suggests that some evaporation may occur deep within the mesophyll of the leaf rather than only from the sub-stomatal cavity. Buckley *et al.* (2015) suggest that evaporation may occur at three locations (namely adjacent to the stomata, from the upper spongy mesophyll at the boundary with the palisade mesophyll and at the bundle sheath), with the relative importance varying considerably between species, particularly between hypo-stomatous and amphi-stomatous species, and with evaporative environment. Simulations by using models developed by Rockwell *et al.* (2014) and Buckley *et al.* (2015) further predict that the portion of evaporation that occurs from the mesophyll may increase as light absorption increases.

LEAF WATER ISOTOPE THEORY

Stable isotopes of water, both deuterium and ^{18}O, have been widely used to trace water through biophysical systems including aquifers (e.g. Darling *et al.* 2003), continental water recycling (e.g. Aemisegger *et al.* 2014) and identification of sources of plant water uptake (e.g. Dawson & Ehleringer 1991). We have previously suggested that leaf water isotopes may provide information on pathways of water movement in leaves (Barbour & Farquhar 2004), with a modelling approach finding that gradients in leaf water isotope enrichment may vary with differing pathways of water movement between the vein and the evaporating surface. In order to explore these predictions in greater detail here, we start by describing key elements of current mechanistic understanding of leaf water isotope enrichment. We have chosen to focus on $H_2^{18}O$ rather than DHO, but the principles apply to DHO as well.

Water containing the heavier oxygen isotope has a lower diffusivity as a gas than does water containing the common isotope (Merlivat 1978; Luz *et al.* 2009), described by a fractionation effect associated with stomatal and boundary resistances to water vapour (r_s and r_b, respectively; Farquhar *et al.* 1989):

$$\varepsilon_k\left(^0/_{00}\right) = \frac{28r_s + 19r_b}{r_s + r_b}, \tag{1}$$

and $\alpha_k = 1 + \varepsilon_k$. The other important fractionation effect on leaf water isotopes is the proportional depression of vapour due to the heavy isotope, which is dependent on leaf temperature (T_l, in °C; Bottinga & Craig 1969; Majoube 1971):

$$\varepsilon^+\left(^0/_{00}\right) = 2.644 - 3.206\left(\frac{10^3}{T_l}\right) + 1.534\left(\frac{10^6}{T_l^2}\right). \tag{2}$$

and $\alpha^+ = 1 + \varepsilon^+$. Beyond fractionations, leaf water isotope composition is also affected by the isotope composition of the source water taken up by the plant, the isotope composition of the water vapour in the air surrounding the leaf and the ratio of the vapour pressures outside and inside the leaf. Variation in stable oxygen isotope composition can be expressed in three ways, using the mole ratio of the rare to the common isotope (R), relative to the Vienna Standard Mean Oceanic Water standard ($R = 0.0020052$) in ‰ (δ^{18}O), or as an enrichment above the source water isotope composition (Δ, again in ‰).

The influences on the isotope composition of water at the evaporating surfaces (R_e or Δ_e) were first described by Craig & Gordon (1965), and later adapted for transpiration from leaves as (Dongmann *et al.* 1974; Farris & Strain 1978; Farquhar *et al.* 1989; Flanagan *et al.* 1991; Farquhar & Lloyd 1993; Harwood *et al.* 1998; Farquhar & Cernusak 2005)

$$R_e = \alpha^+\left[\alpha_k R_s\left(1 - \frac{w_a}{w_i}\right) + R_v\frac{w_a}{w_i}\right], \tag{3a}$$

where R_s and R_v are the isotope ratios in source water and water vapour, respectively, and w_a and w_i are the mole fractions (mol mol^{-1}) of water vapour in the ambient air and inside the leaf, respectively. Equation 3a can be expressed equivalently as enrichment above source water (Farquhar *et al.* 2007):

$$\Delta_e = \alpha^+\left[\alpha_k\left(1 - \frac{w_a}{w_i}\right) + \frac{w_a}{w_i}(1 + \Delta_V)\right] - 1. \tag{3b}$$

Equation 3a can also be rearranged to make R_s the subject and, in the steady state, gives the isotope composition of transpired water vapour (R_E):

$$R_E = \frac{R_e - \alpha^+ R_V\frac{w_a}{w_i}}{\alpha^+\alpha_k\left(1 - \frac{w_a}{w_i}\right)}. \tag{4}$$

The Craig–Gordon model predicts broad trends in Δ_e well, but often overestimates measurements in bulk leaf water (i.e. water from the whole leaf, with or without the mid vein and associated tissue; see Cernusak *et al.* 2016 for a discussion of sampling leaf water and that paper and Holloway-Phillips *et al.* 2016 for a summary of studies comparing the Craig–Gordon model to measurements). This observation led Farquhar & Lloyd (1993) to propose a Péclet effect within leaves, in which convection of unenriched water via the transpiration stream is opposed by backward diffusion of enrichment from the sites of evaporation. Hence, gradients in isotope composition would exist between the sites of phase change in the leaf, at R_e (or Δ_e), and water entering the leaf at R_s ($\Delta = 0$). The Péclet effect is characterized by a Péclet number (P), the ratio of convection to diffusion, vl/D, where v is the velocity of water movement (m s^{-1}), l is the distance between the vein and the evaporating sites over which the Péclet effect occurs (m) and D is the diffusivity of $H_2^{18}O$ in water (m^2 s^{-1}). D is dependent on temperature (°C) and is given by (Cuntz *et al.* 2007)

$$D = 97.5\times10^{-9}e\left(-\frac{577}{T - 145}\right). \tag{5}$$

To scale the Péclet effect to that relevant for bulk leaf water sampling, both the tortuousity of the water pathways within the leaf and the water associated with the veins must be considered. The true velocity of water in the leaf is faster than if water moved as a slab perpendicularly to the leaf surface, and we use a scaling factor (k) to describe the ratio of true to slab velocities. The slab velocity of water, E/C (where E is transpiration rate in mol m^{-2} s^{-1} and C is the molar concentration of water; 5.55×10^4 mol m^{-3}), then becomes kE/C. For convenience, a Péclet effective length (L) is commonly used, where $L = kl$. Then (Farquhar & Lloyd 1993)

$$P = {}^{EL}\!/_{CD}. \tag{6}$$

Applying the Péclet model to leaf water enrichment above source water (Δ_L), and ignoring water in the veins, gives (Farquhar & Lloyd 1993)

$$\Delta_L = \Delta_e\left(\frac{1 - e^{-P}}{P}\right). \tag{7}$$

Equation 7 predicts that as transpiration rate increases, the proportional difference between Δ_L and Δ_e ($1 - \Delta_L/\Delta_e$) increases and that as L increases $1 - \Delta_L/\Delta_e$ increases.

However, most leaves have a significant portion of water in their veins and associated tissue, which is less affected by evaporative enrichment and likely has its own Péclet effect. Farquhar & Gan (2003) developed an extended model that included both lamina radial Péclet effect (as described earlier) and a Péclet effect associated with water in the large veins and surrounding tissue, and assuming the water in the minor veins contributed little to the volume of water in the leaf:

$$\Delta_L = \Delta_e\left(\varphi_x e^{-P_r} + (1 - \varphi_x)\frac{1 - e^{-P}}{P}\right), \tag{8}$$

where φ_x is the proportion of water in the large veins and P_r is the total radial Péclet number, which is equal to the sum of the radial lamina Péclet number (P) and the veinlet Péclet number. Equation 8 has not been widely used due to the requirement of values for φ_x and P_r. Using measurements of bulk leaf water isotope composition (studies variously included or excluded the major veins) as Δ_L and either calculating Δ_e from Eqn 3b using measurements of R_s, R_v and leaf gas exchange, or calculating Δ_e from measurements of R_E, a number of studies have assessed the relationship between transpiration rate and $1 - \Delta_L/\Delta_e$. Cernusak *et al.* (2016) summarized these studies by observing that $1 - \Delta_L/\Delta_e$ was 0.12, on average, and that there was no significant relationship between E and $1 - \Delta_L/\Delta_e$ across all studies. In the most detailed study to date, Song *et al.* 2015a found a slope between E and $1 - \Delta_L/\Delta_e$ of just 0.006, which was not significantly different to zero, among more than 50 cotton leaves. They suggested that a two-pool model (lamina tissue at Δ_e and unenriched water in the veins) adequately described variation in Δ_L and that very steep gradients in enrichment may occur over short spatial scales, perhaps within the vascular tissue. Further experimental studies over a wide range of species are required to reconcile the strong evidence of a Péclet effect in some studies (e.g. Barbour *et al.* 2000; Ripullone *et al.* 2008; Loucos *et al.* 2015) and limited evidence in other studies (Roden & Ehleringer 1999; Cernusak *et al.* 2003; Song *et al.* 2013; Roden *et al.* 2015; Song *et al.* 2015a). A study using the fuller Eqn 8 (Holloway-Phillips *et al.* 2016) showed the importance of the Péclet effect in the veins.

Part of the variation in observed 'strength' of the Péclet effect may be due to (1) differences in leaf anatomy between species (i.e. the particular anatomy of the cotton leaf resulted in the 'weak' Péclet effect reported by Song *et al.* 2015a); (2) changes in the pathways of water movement within the leaf as transpiration rate changes; (3) changes in the sites of evaporation as transpiration rate or other conditions change;

and (4) isotopic non–steady state of leaf water pools (Cernusak *et al.* 2016).

LEAF WATER TRANSPORT PATHWAYS AND THE PÉCLET EFFECT

Consideration of gradients in leaf water isotope composition predicted by the Péclet effect with typical distances between veins and stomata of order 0.1 mm and typical fitted values for L of 8 mm suggests k (the scaling factor between slab and actual velocities) would be around 160 or that water transport is restricted to 1/160 of the volume of the leaf (Barbour & Farquhar 2004). With air space ratios in the mesophyll between 0.07 and 0.60 (Buckley *et al.* 2015), liquid water transport must be limited to narrow pathways within cells where transport velocities are high enough to produce L in the order of mm. We suggested (Barbour & Farquhar 2004) that a leaf with 35% air space in the mesophyll would have a value for k of not less than 1.5 if water moves as a slab through all cells and that this would translate to $L = 0.13$ mm, or roughly an order of magnitude less than published values for L. We went on to estimate k for liquid water movement through only the cell walls, only aquaporin in the membranes and only plasmodesmata (323 to 394, 69 to 128 and 819 to 2154, respectively, depending on assumptions regarding cell-to-cell transport and sites of phase change), with associated L values (around 30, 10 and 150 mm, respectively) that were within an order of magnitude of L estimates from experiments on wheat leaves. The interesting point is that if the relative importance of each liquid transport pathway was to change, then the value for L would change. For example, if increased transpiration rate increased the importance of the apoplastic pathway at the expense of the plasmodesmatal pathway, then the effective length would decrease. Conversely, if increased transpiration rate increased the importance of the transmembrane pathway (i.e. via aquaporin) and apoplastic and plasmodesmatal pathways became less important, then the effective length would increase.

These predictions have been explored experimentally. Song *et al.* (2013) found that L decreased with increasing transpiration rate (E) perhaps due to higher apoplastic transport at high E. Other studies have suggested the reverse – that lower L at high E may relate to increased aquaporin activity and reduced apoplastic transport at high E (Zhou *et al.* 2011; Ferrio *et al.* 2009, 2012). However, Loucos *et al.* (2015) point out that the negative relationship between E and L may simply be a mathematical imperative of the Péclet model. That is, an inverse relationship is expected between E and L if $1 - \Delta_L/\Delta_e$ is constant with E.

The recent prediction of significant water transport in the vapour phase within leaves from two independent models (Rockwell *et al.* 2014; Buckley *et al.* 2015) challenges interpretations of leaf Péclet effects, because a Péclet effect will not be present in the vapour phase (rates of diffusion in the vapour phase are much higher than diffusion in the liquid phase, allowing rapid mixing of vapour isotopes and little opportunity for development of gradients in enrichment). One interpretation is that the Péclet effect will be easier to discern in dense leaves with low air space ratios and proportionally lower transport vapour transport, so that the closeness of fit of the $1 - \Delta_L/\Delta_e$ to E relationship will be negatively related to mesophyll air space ratio. However, if multiple parallel pathways in the liquid phase (all with differing values for L) obscure the Péclet effect at the bulk leaf level, then leaves with a high proportion of vapour phase transport and few parallel liquid pathways would have a 'stronger' Péclet effect. This would result in a positive relationship between mesophyll air space ratio and the closeness of fit of the $1 - \Delta_L/\Delta_e$ to E relationship. These interpretations could be tested experimentally by measuring $1 - \Delta_L/\Delta_e$ at a range of transpiration rates for species differing in mesophyll air space ratio.

Another challenge to understanding the importance of the Péclet effect in leaf water transport pathways is the possibility that the location of the evaporating surfaces within the leaf shift depending on the leaf energy balance. For example, if the sites of evaporation moved from close to the stomatal pore to evenly throughout the mesophyll (and water transport is strictly apoplastic), we calculated that L would decrease a little (from 40 to 29 mm in the wheat leaf example given; Barbour & Farquhar 2004). Interestingly, the two spatially explicit models of water transport in leaves (Rockwell *et al.* 2014; Buckley *et al.* 2015) both predict changes in the location of the evaporating sites due to increased absorbed energy under high light or when changes in transpiration rate are driven by changes in external humidity. Changes in the location of evaporating surfaces have the potential to alter the Péclet effect and hence the shape of the E versus $1 - \Delta_L/\Delta_e$ relationship. Taking the prediction of retreating evaporation sites with increased transpiration, the simple interpretation is that shortening the distance between the vein and the evaporating sites would reduce L, lower $1 - \Delta_L/\Delta_e$ at high E and so flatten the E versus $1 - \Delta_L/\Delta_e$ relationship. Conversely, if reducing the number of parallel liquid transport pathways between veins and evaporation sites (in effect, removing some of the mesophyll cells from the transpiration stream by shifting evaporation closer to the veins) makes the Péclet effect more apparent, then a plot of E versus $1 - \Delta_L/\Delta_e$ may be highly variable at low E but become 'clearer' at high E. In both cases, a key question becomes what happens to the isotopic composition of water in mesophyll cells close to the stomata when evaporation from these cells becomes proportionally less important as E increases. This is difficult to answer without a spatially explicit isotope model of leaf water transport. Despite considerable uncertainty in the details of water isotopes at the scale of the vein to the evaporating sites, it is clear that a Péclet effect must apply at some scale within leaves, because there is very good experimental evidence for spatial patterns of increasing enrichment towards the tips and margins of leaves (Bariac *et al.* 1994; Wang & Yakir 1995; Helliker & Ehleringer 2000; Gan *et al.* 2002; Santrucek *et al.* 2007). Such patterns suggest that evaporatively enriched water diffuses back into the veins to be carried forward to the next leaf portion (Cernusak *et al.* 2016).

MODELLING TRANSPORT PATHWAYS AND ISOTOPE COMPOSITIONS

The complexity of the leaf interior and transport pathways makes it difficult to apply simple Péclet equations and suggests that a numerical approach may be appropriate. We suggest that a spatially explicit numerical model along the lines of those presented by Rockwell *et al.* (2014) and Buckley *et al.* (2015), which can account for both liquid and vapour phase transport and the potentially important effect of vertical temperature gradients, is required to further resolve these issues. An isotope-enabled version of such models may provide a powerful tool to explore the complexity of leaf water transport and stable isotope enrichment. Stable isotopes provide a tracer, allow model validation against measurements and may provide greater constraint of key model parameters. Key concepts of an isotope-enabled, spatially explicit model of water transport pathways within leaves include

1. predicted spatial distribution of water potential;
2. predicted temperature gradients among leaf tissues;
3. predicted spatial distribution of phase change;
4. description of parallel, exchanging liquid and vapour transport pathways, to describe the spatial distribution of evaporating cells and their neighbouring air space and the bulk flow of $H_2^{18}O$ and diffusional fractionation within each phase;
5. inclusion of equilibrium fractionation between adjacent phases;
6. the isotope composition of vapour immediately adjacent to an evaporating surface reflecting an equilibrium with water at that specific evaporating surface within the leaf;
7. the isotope composition of vapour at the stomatal pore reflecting both that in the air surrounding the leaf and that in the transpired by the leaf; and
8. inclusion of a method for integrating to values for R_e and R_L (or Δ_e and Δ_L) for comparison with measurements.

In the development and validation of such an isotope-enabled numerical model, there are a number of conceptual and experimental hurdles to be overcome. The first relates to the isotope composition of vapour within the leaf intercellular air spaces. Specifically, the isotope composition of the flux of evaporated water through the intercellular air spaces may differ considerably from the actual isotope composition of the vapour in the air spaces, just as the isotope composition of transpired vapour may differ considerably from the isotope composition of vapour surrounding a leaf enclosed in a leaf chamber (Simonin *et al.* 2013), except when the air stream entering the leaf chamber is completely dry (Song *et al.* 2015b). This creates the requirement to predict the isotopic vapour flux from the leaf and the isotopic vapour pool within the leaf, and their interdependence.

Comparisons between the model and measurements are complicated by uncertainty regarding the isotopic composition of water within the vascular tissue. The xylem isotope composition of relevance to an isotope-enabled spatially explicit numerical model is that in the smallest veins, which cannot be measured with current technology. Holloway-Phillips *et al.* (2016) recently demonstrated the importance of consideration of variation in isotope composition of water within different tissues within the leaf to bulk leaf water isotope composition. Leaves from species with differing density of minor veins also differed in the effect of transpiration rate on the fractional difference between Δ_L and Δ_e ($1 - \Delta_L/\Delta_e$). The species with the highest minor vein density also displayed the highest sensitivity of $1 - \Delta_L/\Delta_e$ to increasing transpiration rate, while cotton leaf samples from which the large veins had been removed showed only a slight increase in $1 - \Delta_L/\Delta_e$ as transpiration rate increased. A composite model that included the fraction of water present in the xylem, veinlets and lamina, and Péclet effects associated with each pool (as described in Farquhar & Gan 2003) was required to accurately model the observations (Holloway-Phillips *et al.* 2016). Rigorous testing of an isotope-enabled spatially explicit leaf water model against measurements will require careful consideration of the isotope composition of water in the veins and veinlets.

A more immediate problem is the current lack of appropriate data to directly test the model. There are currently no overlapping species for which there is both published leaf anatomy and stable isotope measurements. Rockwell *et al.* (2014) and Buckley *et al.* (2015) predicted important differences in the relative importance of vapour versus liquid transport between leaves with differing mesophyll air space ratio, and differences in the sites of evaporation between hypostomatous and amphistomatous leaves with otherwise similar anatomy, both expected to influence Δ_e and Δ_L. Species contrasting in these traits seem a good place to start in testing the proposed model.

A spatially explicit isotope model will also allow prediction of changes over time in isotope composition of water pools and fluxes in response to changes in environmental conditions. Studies have shown that leaves are unlikely to be at isotopic steady state under natural conditions (Wang & Yakir 1995; Harwood *et al.* 1998; Farquhar & Cernusak 2005; Simonin *et al.* 2013; Dubbert *et al.* 2014), particularly when stomatal conductance is low (Cernusak *et al.* 2016). Further, Loucos *et al.* (2015) demonstrated the importance of non-steady-state effects on interpretation of variation in $1 - \Delta_L/\Delta_e$. Existing models at the whole leaf level accurately predict leaf water turn-over times and approach to isotopic steady state under stable environmental conditions inside a leaf cuvette (Song *et al.* 2015b). The proposed model would allow parameter sensitivity testing under non-steady-state scenarios of interest because the model includes explicit treatment of volume flows across different tissues within the leaf.

An extension of existing leaf water models along the lines described earlier would be useful for testing hypotheses about mechanisms of isotopic effects during water transport and for interpreting divergences in bulk leaf water isotopic composition from simpler models such as Craig–Gordon. Such a model is indeed vital for extending current understanding about patterns of isotopic enrichment to meaningful comparisons across species and also in relation to environmental conditions such as temperature and illumination that likely affect the spatial distribution of evaporative enrichment. However, such a model could also be used to generate testable predictions about mechanisms of leaf water transport. For example, such a model would predict different effects of environmental conditions on liquid and vapour phase water transport, and therefore on the spatial distribution and magnitude of evaporative and diffusive enrichment. This in turn would likely cause the predicted sensitivity of L to environmental conditions to depend strongly on the proportion of vapour phase transport – providing a way to test the hypothesis that vapour transport contributes substantially to K_{ox} and potentially providing an isotopic proxy for the vapour phase proportion of leaf water transport.

CONCLUSIONS

Stable isotopes have been suggested to provide useful insights into the pathways of water movement within leaves, but interpretation of experimental data is highly speculative at present. We anticipate progress through comparison of predictions from an isotope-enabled spatially explicit leaf water model with experimental data. However, there are considerable challenges to overcome in the development of such a model, challenges with which we are currently grappling.

ACKNOWLEDGMENTS

This work was supported by the Australian Research Council (DP150103863 to M.M.B. and T.N.B. and DP150100588 to G. D.F.) and the National Science Foundation (award no. 1146514 to T.N.B.). The authors declare no conflicts of interest.

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Received 5 May 2016; received in revised form 12 September 2016; accepted for publication 19 September 2016