

INVITED REVIEW

Extreme undersaturation in the intercellular airspace of leaves: a failure of Gaastra or Ohm?

Fulton E. Rockwell^{1,*}, N. Michele Holbrook¹, Piyush Jain², Annika E. Huber², Sabyasachi Sen² and Abraham D. Stroock²

¹Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA and ²School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14853, USA

* For correspondence. E-mail fulton.rockwell@gmail.com

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• **Background** Recent reports of extreme levels of undersaturation in internal leaf air spaces have called into question one of the foundational assumptions of leaf gas exchange analysis, that leaf air spaces are effectively saturated with water vapour at leaf surface temperature. Historically, inferring the biophysical states controlling assimilation and transpiration from the fluxes directly measured by gas exchange systems has presented a number of challenges, including: (1) a mismatch in scales between the area of flux measurement, the biochemical cellular scale and the meso-scale introduced by the localization of the fluxes to stomatal pores; (2) the inaccessibility of the internal states of CO₂ and water vapour required to define conductances; and (3) uncertainties about the pathways these internal fluxes travel. In response, plant physiologists have adopted a set of simplifying assumptions that define phenomenological concepts such as stomatal and mesophyll conductances.

• **Scope** Investigators have long been concerned that a failure of basic assumptions could be distorting our understanding of these phenomenological conductances, and the biophysical states inside leaves. Here we review these assumptions and historical efforts to test them. We then explore whether artefacts in analysis arising from the averaging of fluxes over macroscopic leaf areas could provide alternative explanations for some part, if not all, of reported extreme states of undersaturation.

• **Conclusions** Spatial heterogeneities can, in some cases, create the appearance of undersaturation in the internal air spaces of leaves. Further refinement of experimental approaches will be required to separate undersaturation from the effects of spatial variations in fluxes or conductances. Novel combinations of current and emerging technologies hold promise for meeting this challenge.

Key words: Assimilation, transpiration, undersaturation, stable isotopes, mesophyll conductance, plant hydraulics, plant water relationships, stomatal conductance, stomatal patchiness.

INTRODUCTION

*When you have eliminated all which is impossible,
then whatever remains, however improbable, must be the
truth*

Arthur Canon Doyle

In a recent series of papers, Cernusak *et al.* have combined gas exchange and online measurements of the $\delta^{18}\text{O}$ of H₂O and CO₂ to demonstrate that challenging leaves with high vapour pressure deficit (VPD) can lead to dramatic undersaturation in the leaf intercellular airspace (IAS), as low as 80 % relative humidity (RH) (Cernusak *et al.*, 2018, 2019; Holloway-Phillips *et al.*, 2019). Such low humidities undermine the standard assumption of saturation employed by gas exchange systems to estimate stomatal conductance. They also imply enormously negative liquid phase water potentials, from –7 MPa at 95 % to –31 MPa at 80 % RH (Wheeler and Stroock, 2009), raising questions of how the leaf symplast could maintain photosynthetic metabolism in coexistence with such an extreme state of

undersaturation (Buckley and Sack, 2019). If correct, the phenomenon of extreme undersaturation calls into question decades of gas exchange analysis across a variety of species under high VPD conditions, affecting interpretations of stomatal responses to water stress, and our understanding of the mechanisms of water stress management within leaves. On a more positive note, the ability of leaves to tolerate such undersaturated states would point toward the existence of a robust form of non-stomatal control of transpiration that, if understood, could provide new phenotypic and genetic targets for increasing plant water use efficiency. To help plant scientists come to terms with the issues raised by Cernusak *et al.*'s elegant application of stable isotopes, here we review the fundamental assumptions of leaf gas exchange, with particular attention to how the novel constraint on a leaf's saturation state introduced by this new method fits into the canonical analytical framework. This allows us to investigate whether assumptions in gas exchange analysis seemingly unrelated to the saturation state of leaf airspace could, in failing, lead to apparent undersaturation.

Gaastra, Gradmann and Ohm: the canonical description of leaf gas exchange and intercellular CO_2

The concentration of CO_2 within the internal airspaces of leaves, c_i , describes a fundamental quantity in the analysis of terrestrial photosynthesis at a critical juncture: the interface between a gas phase continuous with the atmosphere, and a liquid phase continuous with the sites of carboxylation within leaf chloroplasts. For most plants, this liquid phase must also be continuous with the water held within the soil. The necessity of maintaining contact with soil water derives from the familiar fact that the diffusive uptake of CO_2 is accompanied by a diffusive loss of water to the atmosphere. What may be less familiar is that the range of internal hydration tolerable by plant life is only a few per cent of the range of water availability spanning the soil, plant and air continuum (SPAC) (Honert, 1948). Furthermore, the standard practice of adopting different descriptions of the driving forces for liquid and vapour fluxes obscures how strongly this narrow hydration tolerance constrains the distribution of resistances along the SPAC. To overcome this problem, Honert (1948), drawing on Gradmann (1928), expressed the state of water in terms of the equivalent water potentials of an equilibrated liquid phase to show that stomata must constitute the dominant resistance to water flux from soil to atmosphere (Fig. 1). Repeating this exercise for a typical woody mesic plant, with a wilting point of -2 MPa and in an atmosphere at 50 % RH, brings home the point that the maximum drop in water potential between the soil and a transpiring leaf is negligible compared to a leaf-to-air difference in potential on the order of -100 MPa. This physical picture motivates an important assumption in leaf gas exchange, that the driving force for transpiration should be largely insensitive to the state of water in a leaf.

Gaastra (1959) was the first to exploit the relative insensitivity of vapour pressure to leaf water potential to estimate c_i . Assuming that the vapour pressure (or, equivalently, concentration, or mole fraction) of water vapour in a leaf is saturated at leaf surface temperature permits an estimate of the driving force for transpiration as the difference between that saturated value and a measured value in the external air (Gaastra, 1959; Moss and Rawlins, 1963). To complete the description of gas transport, we require a way to describe the effective conductance constituted by discrete stomata arrayed across a leaf in parallel, as well as the sinks and sources for CO_2 and water, and the internal conductances to both. The standard solution has been to collapse the 3D spatial distribution of sources and sinks to zero-dimensional ‘nodes’, at which concentrations, temperatures and potentials are specified along a 1D serially arranged set of resistances (Fig. 2; Table 1). Such nodes are then connected by ‘conductors’ (inverse of resistors) that describe the drop in driving force required to move the relevant flux from one node to another: in other words, the solution is to adopt an Ohm’s law analogy (Gradmann, 1928; Honert, 1948; Slatyer and Lake, 1966). Neglecting the contributions of leaf boundary layer conductance, as well as small corrections for convection (i.e. ‘ternary’ effects) for the sake of clarity, the stomatal conductance of the leaf to water vapour is then defined as the transpiration flux divided by the driving force between a node inside the leaf representing the evaporation sites, e_i , and one in the well-mixed air in the cuvette, e_a (Fig. 2, eq. 1). Adjusting for the difference in the gaseous diffusivities of CO_2 and H_2O provides the conductance to CO_2 between those same nodes (Fig. 2, eq. 2), and, finally, subtracting the known

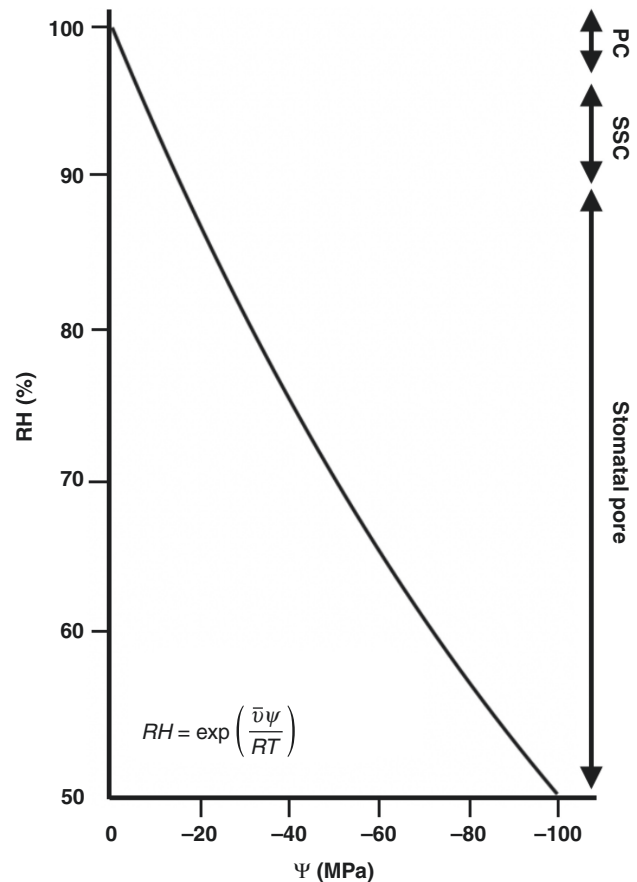


FIG. 1. The relationship between relative humidity (RH) and the water potential of an equilibrated liquid phase (Ψ), with ranges of humidity and equivalent water potential typically measured by the pressure chamber (PC) on whole leaves of mesic species, the airspace within sub-stomatal cavities (SSC), and in stomatal pores, assuming 50 % RH at the leaf surface.

(transport induced) drop in CO_2 from the known external CO_2 concentration, c_a , yields the desired estimate of c_i (Fig. 2, eq 3). Knowledge of the concentration of CO_2 at the chloroplast, inferred from fluorescence or isotopic methods and the Farquhar *et al.* (1980) model (e.g. Genty and Meyer, 1994), completes the Ohmic description of the CO_2 flux (Fig. 2, eq 5). This analysis of gas exchange continues to underlie all standard uses of the technique within the plant sciences.

The convenience of this approach provides an important motivation for plant physiologists to ignore the fact that the water potential of the liquid phase *does* affect the vapour pressure inside leaves (e.g. the ‘Kelvin effect’, Pickard, 1981). Yet, why not simply correct the driving force for the effect of water potential at the evaporation sites and dispense with Gaastra’s assumption of saturation (Vesala *et al.*, 2017)? Simply put, the expected magnitude of the correction is small relative to other uncertainties: it takes a 0.833 MPa change in water potential to equal the effect of a 0.1 °C change in temperature on vapour pressure. Leaf water potential effects on the driving force for transpiration, in addition to being generally small relative to the total leaf-to-air difference, are also expected to be within the typical uncertainty of leaf temperature measurements of ± 0.2 °C (Rockwell *et al.*, 2014).

The above framework for analysis of leaf gas exchange relies on three related ideas: Gaastra’s assumption of saturation, van

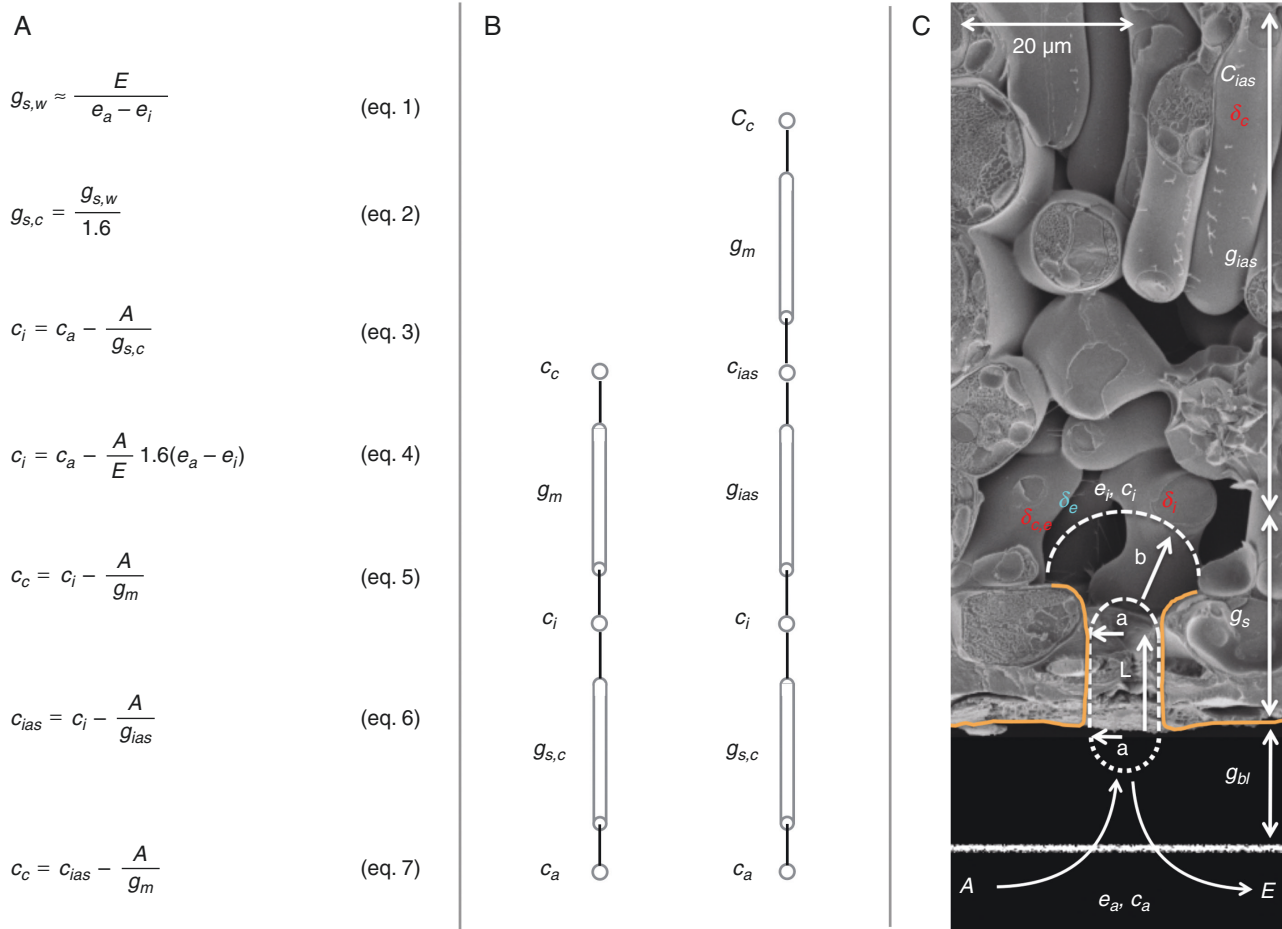


FIG. 2. Ohm's law analogies for leaf gas exchange. The fluxes of water and carbon are interpreted as the result of changes in a state variable (e.g. concentration, partial pressure or mole fractions) from node to node along a series of conductors that describe spatially averaged transport properties. (A) The equations describing the basic equations in terms of molar flux units ($\text{mol m}^{-2} \text{s}^{-1}$; A , E) and dimensionless state variables (mole mole $^{-1}$; e , c); for the sake of clarity boundary layer and ternary effects are neglected. Equations 6 and 7 present an alternative to eq. 5 that adds an additional explicit node and conductor for the mesophyll intercellular airspace (c_{ias} , g_{ias}). (B) Schematic for the nodes and conductors in A. (C) An hypothesis for a mapping of Ohmic quantities to explicit regions within the lower half of a leaf; subscripts identify the boundary layer (bl), stomata (s), intercellular air space (ias) and mesophyll surface to chloroplast liquid phase path (m). For CO_2 , the terminal nodes are taken to be the ambient air (c_a), and the chloroplasts (c_c), while for H_2O they are simply ambient air (e_a) and the interface of air space and wetted mesophyll cell surfaces (e_i). Also shown are the underlying geometries of stomatal conductance (Pickard, 1982), including the depth (L) and radius (a) of an ideal cylindrical pore, and the radius of the sub-stomatal cavity; but note that real stomatal pores are hour-glass shaped rather than cylindrical (Kaiser, 2009) (b). Delta notation (δ) refers to the $\delta^{18}\text{O}$ of H_2O (blue) and CO_2 (red) at the inner node defined for stomatal conductance (δ_s , δ_e) defined by the assumption of saturated water vapour, and for dissolved CO_2 in equilibrium with water at the sites of evaporation ($\delta_{e,c}$) and at the chloroplast surface (δ_c), as defined by Cernusak *et al.* (2017).

den Honert's related hypothesis that the stomata must represent the dominant resistance to water transport within a plant, and finally an Ohm's law analogy that dissects the SPAC into a 1D network of conductors and nodes (Slatyer and Taylor, 1966; Fig. 2). The additional information obtained by Cernusak *et al.* (2018) from isotopes allows the introduction of a new constraint: that the oxygen isotopic signature of the CO_2 at the chloroplast surface should be equal to that of CO_2 in equilibrium with water at the evaporation sites within a leaf. This new assumption allows the analyst to relax the assumption of saturation, and so check its validity (for a detailed explanation, see Cernusak *et al.*, 2018). Yet, the isotopic approach does not represent the first test of the saturation assumption: from the beginning, researchers have sought to put a solid empirical floor under Gaastra's (1959) and van den Honert's (1948) ideas. To better appreciate the experimental basis of leaf gas

exchange quantities, and so better understand the strengths and weaknesses of the standard assumptions and the isotopic innovations challenging them, we next revisit several important experiments in the history of gas exchange analysis.

HISTORICAL EFFORTS TO TEST THE CANONICAL FRAMEWORK

The question of 'wall resistance': early efforts to detect a resistance between the mesophyll symplast and the intercellular airspace

One way a failure of Gaastra could be explained is as the result of a failure of van den Honert's idea of a dominant stomatal resistance: if a large resistance exists between the symplast and

TABLE 1. Symbols for Fig. 2

Quantity	Symbol	Units
Conductance	g	$\text{mol m}^{-2} \text{s}^{-1}$
CO ₂ mol fraction	c	mol mol^{-1}
H ₂ O mol fraction	e	mol mol^{-1}
CO ₂ flux	A	$\text{mol m}^{-2} \text{s}^{-1}$
H ₂ O flux	E	$\text{mol m}^{-2} \text{s}^{-1}$
Sub-stomatal cavity (SSC) radius	b	m
Stomatal pore radius	a	m
Stomatal pore depth	L	m
Subscripts	Symbol	
Interior to stomata (in sub-stomatal cavity)	i	
Ambient	a	
Stomatal	s	
With respect to CO ₂	c	
With respect to H ₂ O	w	
Intercellular airspace	ias	
Mesophyll	m	
Boundary layer	bl	
Isotopic notation	Symbol	
δ ¹⁸ O of CO ₂ within the chloroplasts	δ_c	
δ ¹⁸ O of CO ₂ interior to the stomata	δ_i	
δ ¹⁸ O of CO ₂ in equilibrium with evaporation sites	$\delta_{c,e}$	
δ ¹⁸ O of H ₂ O at the evaporation sites	δ_e	

intercellular airspace, such that the stomata are not in fact a truly dominant resistance, then the airspace could be strongly undersaturated, violating Gaastra’s assumption. Jarvis and Slatyer (1970) compared the transport resistance of a metabolically inert gas (nitrous oxide) across an amphistomatous leaf (*Gossypium hirsutum*) to the resistance of each side of the leaf to water vapour summed in series. Assuming Gaastra holds, the difference in resistance experienced by these two gases (after accounting for their different diffusivities) would then equal the difference between any neglected ‘wall resistance’ (i.e. between a cell’s symplast and apoplast) seen by water and the extra resistance seen by the inert species due to its longer path through the mesophyll if the sites of evaporation are not in the exact centre of the leaf. These workers did find a significant wall resistance, one that increased with declines in leaf water content. However, Farquhar and Raschke (1978), in a repetition of the experiment using helium as the inert gas and three species (*G. hirsutum*, *Xanthium strumarium*, *Zea mays*), found that the total resistance experienced by helium was consistently larger than that experienced by water over all species and experimental conditions. This result meant that the extra path length travelled by the inert gas relative to water vapour, due to the evaporation sites being closer to the stomata than the centre of the leaf, created a larger resistance than any reduction in saturation that might occur at the sites of evaporation due to non-zero apoplastic water potentials.

Sub-stomatal cavities and the localization of e_i and c_i

Interestingly, for cotton (*Gossypium*) Farquhar and Raschke (1978) found a consistent difference between the total resistance across the leaf experienced by water and the inert gas of about 2 s cm^{-1} . If we assume that the wall resistance was zero, this difference is very close to the anatomy-based estimate of 1.65 s cm^{-1} for the total resistance of the mesophyll airspace, the distance between the substomatal cavities of the upper and lower surface, reported by Jarvis and Slatyer (1970) for the same species. Such near agreement would be expected if most of evaporation occurs in the substomatal cavity spaces, rather than the mesophyll. The expectation that the mesophyll surfaces bordering the sub-stomatal cavities are the principal sites of evaporation, and so is the location associated with e_i and c_i , has strong theoretical support as well (Tyree and Yianoulis, 1980; Pickard, 1982).

Pickard (1982) modelled the isothermal diffusion of gas in a sub-stomatal cavity (SSC) to understand the impact on gas exchange of cavities of different sizes. Pickard’s 1D isothermal analysis provides a mathematical description that describes the changes in the overall conductance of the SSC that occur when stomatal aperture changes (Fig. 3). As stomatal aperture increases from a $1 \text{ }\mu\text{m}$ radius to $5 \text{ }\mu\text{m}$, the gradient in humidity becomes less steep as the pore conductance increases (e.g. ‘external dryness invades the leaf’), but the gradient always remains extremely shallow at the ‘top’ of the SSC, bounded by the mesophyll cell surfaces far from the stomatal pore (Fig. 3A: note that the gradient for different pore sizes has an x -intercept equal to the pore radius; this is because transport in the SSC is modelled as beginning on the surface of a hemisphere described by the pore radius, as shown in Fig. 2C). Pickard’s analyses further show that once the height of an SSC reaches twice the stomatal pore radius, the total conductance to water vapour approaches an asymptotic value, and little more is gained in terms of controlling water loss by moving wetted mesophyll surfaces farther from the pore. It should be noted that Pickard’s analysis assumes that the cuticle in the stomatal pore extends along the inner leaf surface for a distance of two pore radii. While the extent to which inner epidermal cell surfaces may or may not be covered by an internal cuticle remains poorly known (Pesacreta and Hasenstein, 1999), here the distance of two pore radii would not require an extension of internal cuticle much beyond the guard cells themselves. Pickard’s results are also broadly consistent with more widely used models relating stomatal anatomy to conductance (Parlange and Waggoner, 1970; Brown and Escombe, 1900). While these models differ mathematically from Pickard’s analysis, they all capture the ‘resistance’ created by the concentration of the water vapour flux as it enters the mouth of the stomatal pore, but through different idealizations of the problem (e.g. R_o in eq. III of Parlange and Waggoner, 1970).

More recently, models that account for the energy consumed by evaporation have challenged the isothermal view that all evaporation occurs at the mesophyll–SSC interface, pointing toward some proportion of evaporation occurring deeper in the leaf. These fully coupled heat and molecular transport models predict that significant proportions of evaporation could be originating at the vascular plane (i.e. the spongy to palisade mesophyll transition; Rockwell et al., 2014; Buckley et al.,

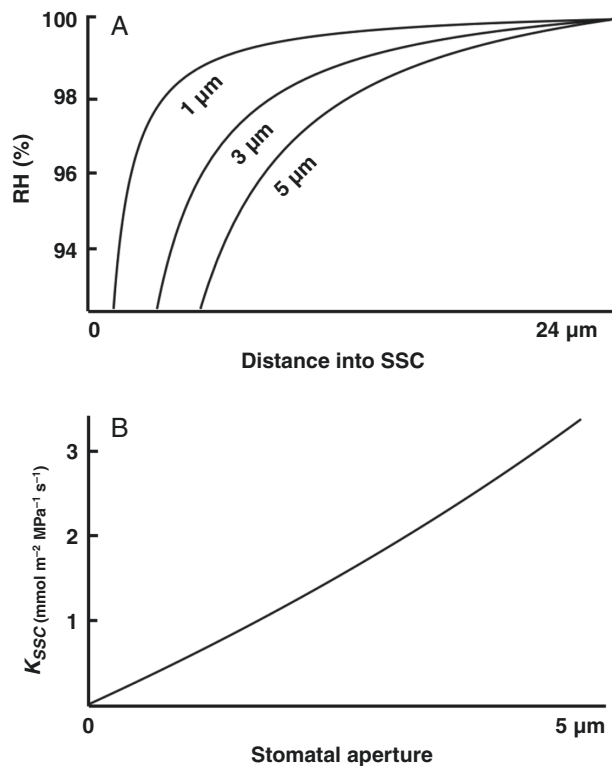


FIG. 3. Pickard's (1982) solution for the effective Ohmic conductance of gaseous diffusion in a sub-stomatal cavity (SSC). (A) The gradient in relative humidity (RH) from the stomatal pore to the wetted surfaces at the top of the SSC (where the mesophyll begins), for stomatal apertures (assuming a circular pore idealization) of 1, 3 and 5 μm radii. Note that x -intercepts are non-zero due to the fact that the SSC begins at the top of the hemisphere defined by the radius of the pore: that hemisphere is part of the 'end correction' included in the pore conductance by convention (Pickard, 1980; Nobel, 2005). (B) Total Ohmic conductance of the SSC path (K_{SSC}) to the diffusion of water vapour as a function of stomatal aperture, for an SSC bounded by spongy mesophyll cells located 24 μm from the stomatal pore. Note that the driving force has been linearized to the equivalent MPa of a functional equilibrated liquid phase, in order to make it comparable to liquid-phase conductances through the mesophyll.

2017; Fig. 4). For example, for *Quercus rubra* (northern red oak), an ecologically dominant woody canopy tree in North America, about 25 % of the water transpired is predicted to evaporate at the palisade–spongy transition at the vascular plane (Rockwell et al., 2014), with temperature and water potential minima occurring at the transition to 100 % vapour transport at the top of the SSC (Fig. 4B). Such models have also been used to predict precisely where in a leaf the water vapour is at the concentration assumed by Gaastra (Buckley et al., 2017), but the important point is simply this: accounting for heat and mass transport does not appear to undermine the assumption in gas exchange calculations that vertical mesophyll gradients (i.e. through the leaf thickness), in both water potential and temperature, are negligible for the purposes of calculating 'leaf to air' VPD (Fig. 4B). Irrespective of the exact locations of the phase change of transpired water, large gradients in humidity inside the leaf are predicted to be confined to the SSC and stomatal pore (Fig. 4C, D).

The first reason for this confinement is that the thermal conductivity of the mesophyll cells is much more efficient at moving thermal energy than is latent heat transport through

the airspace, so temperature variations and their effects on the local vapour concentration are small (Rockwell et al., 2014). A second reason is that symplastic water potentials, and their influence on the local vapour concentration, are in turn bounded by the whole leaf turgor loss 'point', at which point stomata are generally closed (Brodribb and Holbrook, 2003; Hochberg et al., 2017; Knipfer et al., 2020). Yet importantly, these results for the humidity gradient, as well as other results from the models of Rockwell et al. (2014) and Buckley et al. (2017), depend on an assumption of local equilibrium between the symplast, cell wall and air space, akin to the idea of zero wall resistance above. The assumption of local equilibrium is not arbitrary, but rests on a foundation that is both physical and biological. Neglect of humidity gradients outside of those in the through-thickness direction is supported by the fact that air spaces are small in diameter relative to the leaf thickness, while the assumption of local equilibrium between a mesophyll cell's symplast and its apoplastic wall space is justified by a composite model of water transport through the symplast, apoplast and cell-to-cell (cross-membrane) paths that is consistent with cell pressure probe measurements (Rockwell et al., 2014).

As long as local equilibrium holds, choosing the top of the SSC as a discrete localization of e_i , though approximate, offers enormous advantages in simplicity over attempting to model the 'true' localization of e_i at those places within a leaf where, through whatever combination of temperature and water potential, e_i happens to take the exact value predicted by Gaastra (Buckley et al., 2017). Another advantage is that assuming saturation of water vapour at the top of the SSC causes the variable conductance of the SSC (Fig. 3B) to be lumped into the calculation of g_s , with which it varies. With this view of e_i , as long as the gradients in CO_2 through the mesophyll airspace are 'small,' such that $c_i \sim c_{ias}$ (Fig. 2C), spatial differences in the sites of evaporation and carboxylation should not introduce errors into gas exchange analysis. The challenge lies in testing whether c_i provides a good estimate of the concentration of CO_2 on the mesophyll cell surfaces responsible for the bulk of assimilation.

Describing CO_2 gradients in leaf airspaces: direct measurements of c_i by dual gas exchange experiments

Sharkey et al. (1982) developed a novel approach for measuring the drop in CO_2 across an amphistomatous leaf: making an equilibrium measurement of c_i using a 'closed' gas exchange system on one side of an amphistomatous leaf, while estimating c_i at the other leaf surface mated to a standard 'open' gas exchange system and assuming Gaastra. At steady state, the concentration of CO_2 in the closed system in contact with the lower leaf surface should equal c_i just inside the leaf, and there is no net exchange of CO_2 across the lower surface. The closed and open measurements of c_i at the lower and upper surfaces should differ only to the extent that there is a measurable draw-down of CO_2 within the leaf, Gaastra is in error or both. This study, and similar work (Mott and O'Leary, 1984; Parkhurst et al., 1988), found generally small drawdowns on the order of 10 ppm across the leaf mesophyll [for Parkhurst et al. (1988), this magnitude corresponds to amphistomatous leaves taking CO_2 across both surfaces, see their fig. 1]. Two related

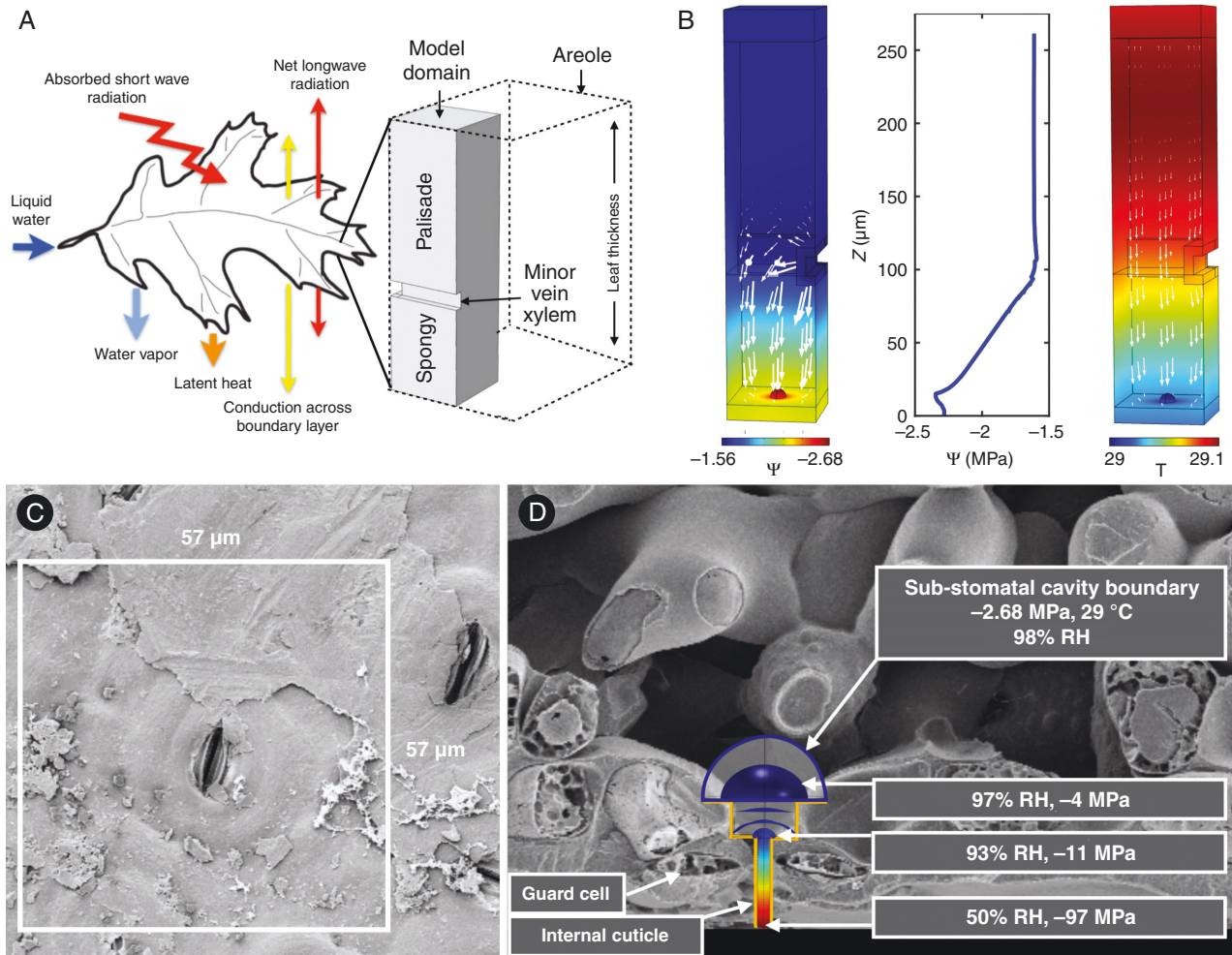


FIG. 4. (A) Coupled heat and molecular transport model for an oak leaf. The model couples a macroscopic leaf energy balance to a microscopic transport model for vapour and liquid phase transport in a 3D domain extending through the leaf thickness. Through symmetry considerations, the model domain corresponds to one-quarter of an areole, irrigated by minor vein xylem on two sides just below the mid-plane. (B) Colour maps of water potential and temperature on the domain surfaces, with white vectors showing the gradients in the mesophyll, and the epidermis rendered as solid. The dome in the centre of the lower epidermis marks the top of the sub-stomatal cavity, at which all transport switches to the vapour phase only: this region corresponds to the coolest and driest cell surfaces in the domain. The line plot presents the average water potential of each plane through the thickness (Z); note the minimum potential occurs at the plane containing the top of the SSC, relaxing slightly in the epidermal planes. (C) On the leaf surface, the model domain corresponds to an area 57 μm by 57 μm , an area associated with approximately one stoma. (D) Isosurfaces for relative humidity from the top of the sub-stomatal cavity to the stomatal pore exit, expressed relative to pure water at 29 $^{\circ}\text{C}$. Water potentials are those that would occur for an equilibrated liquid phase.

conclusions can be drawn: one, that any errors associated with Gastra in these leaves were small, and two, that the conductance of the intercellular air space was large enough to suppress large differences in CO_2 between the unknown sites of evaporation for the flux of water out of the upper leaf surface, and the other end of the mesophyll inside the lower leaf surface. This result supports neglecting the difference between c_i and c_{ias} , and absorbing g_{ias} into the liquid phase conductance g_m (i.e. using eq. 5 rather than eq. 6 in Fig. 2). But whether this approximation is justified for a particular leaf is not simply predictable from knowledge of the anatomy of the internal airspace (and so its physical conductance to an inert gas), as it should also depend on the magnitude and distribution of assimilation.

To understand this dependence, consider an experiment where a steady flow of an inert gas through an amphistomatous leaf defines a total conductance across the leaf. By hypothesis,

subtracting the stomatal conductances of the upper and lower surfaces, and correcting for the difference in diffusivities, provides an estimate of the diffusive conductance of the mesophyll airspace to CO_2 , as in Farquhar and Raschke (1978); call it g_c . The drop in CO_2 within a leaf is then just $A/(n g_c)$, where n is a pre-factor determined by the specifics of the problem. For example, if the rate of photosynthesis is uniform through the leaf thickness, and CO_2 is fed from one side of the leaf only, then $n = 2$, and this result is independent of whether CO_2 is fed from the illuminated or non-illuminated side. Physically, $n = 2$ because the average CO_2 molecule only travels half the distance through the leaf before being assimilated, and so the effective conductance is double that experienced by an inert gas (Sharkey *et al.*, 1982). Yet, it seems unlikely that assimilation is truly uniform through the leaf thickness, and is instead highest near the illuminated surface and declines with depth (Vogelmann and

Evans, 2002). As a lower limit on the effect of non-uniform A , we can ask what happens if CO_2 is fed into the spongy mesophyll side, and all assimilation occurs at the top of the palisade on the illuminated side: in this case all CO_2 diffuses through the full leaf thickness, and $n = 1$. Alternatively, if for this same leaf all the CO_2 were fed from the illuminated side, n would be infinite, and there would be no draw down in CO_2 across the mesophyll. That plants have evolved to make efficient use of incident light, and so achieve something close to uniform assimilation through the leaf thickness, is perhaps attested to by a comment in Sharkey et al. (1982) that switching the light source between the CO_2 -fed and non-fed sides produced results that were ‘nearly identical’ (data were not shown), as well as by the report of Parkhurst et al. (1988) that switching the illuminated side of *Eucalyptus* leaves had only minor effects on the difference between the CO_2 -fed and non-fed sides. Nevertheless, in all the dual gas exchange studies described above, it appears to be generally the case that the leaves were illuminated from the CO_2 -fed side (Farquhar and Raschke, 1978; Sharkey et al., 1982; Mott and O’Leary 1984; Parkhurst et al., 1988), which to the extent that assimilation is biased toward the illuminated surface should lead to values of n greater than 2, and so perhaps smaller than expected differences in c_i across a leaf. Cryptic differences in n between nominally similar experiments, as might arise from spectral differences changing the distribution of assimilation with depth (Vogelmann and Evans, 2002), might help explain why Sharkey et al. found a value of intercellular resistance for *X. strumarium* a third that reported by others (Farquhar and Raschke, 1978; Mott and O’Leary, 1984).

Here, the difference we are interested in is not across the entire mesophyll, but between the Gaastra-based c_i of a transpiring surface and the average mesophyll CO_2 , c_{ias} . For this difference n , and thus the effective conductance $n g_c$, is higher, with (assuming uniform assimilation) $n = 3$ for an amphistomatous leaf and $n = 6$ for an hypostomatous leaf with equal conductances for both surfaces (Parkhurst et al., 1988). While such high values of n support the approximation that $c_i \sim c_{ias}$, how good the approximation proves in practice still depends on the ratio of A to the actual conductance of the total intercellular airspace (g_c), a parameter whose measurement has so far depended on the assumption of Gaastra. Recently, a new approach for estimating g_c from structure has been demonstrated that combines X-ray computed tomography and mathematical simulations of diffusion, to arrive at pathlengths and tortuosities for gas transport through the photosynthetic mesophyll (Earles et al., 2018). Comparing dual gas exchange with structure-based estimates of g_c could open up a new perspective on the relationship between the dominant sites of evaporation and carboxylation, and the importance of distinguishing c_{ias} from c_i .

This limited defence of $c_i \sim c_{ias}$ concludes our exploration of the basic Ohmic framework of leaf gas exchange, as given in Fig. 2 (eqs 2–5). In summary, as long as local equilibrium between symplast, apoplast and airspace holds, the variations in humidity deeper inside than the SSC–mesophyll boundary cannot deviate importantly from the Gaastra assumption of saturation at leaf surface temperature, at least for most leaves. If so, then given current knowledge of g_c , and values of n probably larger than 3, taking c_i as estimated by Gaastra as the CO_2 concentration throughout the mesophyll airspace seems a safe assumption. And yet, there is evidence that some part of this framework may fail at high VPD.

NOVEL APPROACHES CHALLENGING THE CANONICAL FRAMEWORK

Isotopic inferences of undersaturation

Leaving aside the complexities of isotopic calculations, the essential logic of the Cernusak et al. (2018) method can be understood as follows: measurement of gas fluxes and the assumption of Gaastra lead in the usual way to e_i , g_s and c_i . The addition of isotopic measurements of CO_2 in the cuvette leads to δ_i , the $\delta^{18}\text{O}$ of intercellular air space CO_2 . With knowledge of g_m , one could arrive at an estimate of the concentration of CO_2 at the sites of carboxylation, c_c , and its isotopic ratio δ_c . To find g_m , a second chain of inferences is needed, and this is supplied by analysing the water flux: with e_i again from Gaastra, an estimate of the $\delta^{18}\text{O}$ of the liquid water at the evaporation site, δ_e , can be made. One can then calculate the $\delta^{18}\text{O}$ for dissolved CO_2 in equilibrium with that water, $\delta_{c,e}$. Cernusak et al. (2018) then assume that the evaporation sites correspond to the cell surfaces proximal to the chloroplasts, and therefore $\delta_{c,e}$ is equal to δ_c . This assumed constraint, $\delta_{c,e} \sim \delta_c$, joins the two lines of inference based on the carbon and water fluxes, and fixes the value of g_m .

To fix a value for g_m , we have had to assume Gaastra: how then can this approach be used to check the validity of Gaastra? Cernusak et al. (2018) first perform the experiment at low VPD, under conditions under in which they assume Gaastra to hold, and then test whether that value of g_m continues to reconcile the equilibrium assumption, $\delta_c \sim \delta_{c,e}$, and the saturation assumption of Gaastra as VPD increases. Importantly, failure may be interpreted as a failure of $\delta_c \sim \delta_{c,e}$, a change in g_m or evidence that the leaf air space is undersaturated. For Cernusak et al. (2018), undersaturation is simply the ‘improbable result’ that remains after eliminating, if not quite impossible, arguably *less plausible* alternatives. The first alternative, that a change in g_m occurs, would require g_m to increase more steeply with temperature than expected, and to *increase* with higher VPD. A difficulty here is that the plausible behaviour of g_m is itself difficult to characterize independently from Gaastra, and no doubt this problem motivated the authors to refine their method in later studies (albeit on different species) to provide additional constraints on g_m (Holloway-Phillips et al., 2019), finding weak *declines* in g_m as VPD increased. While questions remain about the variability of g_m , these are essentially empirical rather than analytical, and so we will turn our attention to the assumption of isotopic equilibrium between evaporation sites and chloroplasts ($\delta_c \sim \delta_{c,e}$).

At first glance that assumption appears highly vulnerable. Asserting that the δ of CO_2 in equilibrium with water at the sites of evaporation is equivalent to the δ of CO_2 at the chloroplasts would seem to involve an assumption that the cell surfaces evaporating water are the same surfaces absorbing CO_2 into solution. Yet, the distribution of chlorophyll and light intensity place the dominant sites of carboxylation in the palisade mesophyll (Vogelmann and Evans, 2002), while coupled heat and molecular transport models place the *principal* sites of evaporation at the wetted surfaces closest to the stomatal pores, with the possible exception of leaves with the highest internal air volumes (Rockwell et al., 2014; Buckley et al., 2017). The typical distance between evaporation sites and chloroplast surfaces are

then not a mere tens of nanometres, but on the order of tens of micrometres, or more in thicker hypostomatous leaves. For the assumption that there are no gradients in the $\delta^{18}\text{O}$ of water between δ_c and $\delta_{c,e}$ to hold, the entire mesophyll would then have to behave like a single well-mixed pool of water. Against this idea of uniformity, isotope physiologists expect that back diffusion of ‘heavy’ isotopologues (enriched in ^{18}O) from evaporation sites occurs against the convection of unenriched (‘light’) water from the xylem. The competition between back diffusion and forward convection, a Peclet effect, is expected to lead to gradients in $\delta^{18}\text{O}$ through the mesophyll (Farquhar and Gan, 2003; Barbour and Farquhar, 2004). We should therefore expect that δ_c should be less enriched, or close to xylem water, while $\delta_{c,e}$ remains enriched at the hypothetical value given by equilibration with water at the sites of evaporation, as described by the Craig–Gordon model (Craig and Gordon, 1965; Flanagan *et al.*, 1991). To the extent this analysis is correct, forcing $\delta_c \sim \delta_{c,e}$ embeds an error in the estimate of g_m .

And yet, this error only matters to the extent that its magnitude changes at high VPD, and even then, to account for apparent undersaturation, it must change in a particular way: the evaporation site water must get ‘lighter’ (less H_2^{18}O), and the carboxylation site water deeper in the leaf ‘heavier’ (more H_2^{18}O). Thus, an increase in the Peclet effect with VPD (i.e. higher evaporation increasing a convective flux of light water that works against the diffusion of heavy water from the evaporation site) goes the wrong way as a potential alternative to undersaturation. This point was made by Cernusak *et al.* (2018) in considering flow and diffusion between a chloroplast and its host cell surface, but it holds equally well at the larger scales considered in our analysis. This does not mean that the large separation between δ_c and $\delta_{c,e}$ we have identified here is ultimately irrelevant. Separation of evaporation and carboxylation over a distance of many cells could provide an alternative to undersaturation if, with higher VPD, the palisade δ_c became heavier even as the SSC $\delta_{c,e}$ lightened, as could happen if transpiration began to *decrease* with increasing VPD in a feed-forward [e.g. abscisic acid (ABA)-driven] reduction of g_s . In the limit of low g_s and high energy loads, there is also the potential for an internal vapour flux larger than the transpiration flux that escapes through the stomata. In this case the leaf acts as a heat pipe, with evaporation deep in the leaf and condensation at the epidermal surfaces leading to recirculation of liquid back toward the evaporation sites (Pieruschka *et al.*, 2010; Chen *et al.*, 2014; Rockwell *et al.*, 2014). How exactly $\delta^{18}\text{O}$ would vary spatially in a condensing leaf has not been studied to our knowledge, but it seems possible that $\delta_{c,e}$ could actually become lighter than δ_c . Whether a condensing state could have been reached cannot be evaluated from the published data in Cernusak *et al.* (2018), but as increasing VPD is not favourable for attaining this state, except perhaps in the case of significant reductions in E due to stomatal closure, it does appear to be an improbable explanation.

For the species studied in Cernusak *et al.* (2018), Pinyon pine and juniper, what remains is the authors preferred hypothesis, the ‘merely improbable’ result that $\delta_c \sim \delta_{c,e}$ holds, Gaastra fails and the air at the mesophyll cell surfaces is strongly undersaturated. Yet, as pointed out by Buckley and Sack (2019), the reported degrees of undersaturation are difficult to reconcile with our understanding of the liquid phase in leaves. For extreme

undersaturation to coexist with non-lethal symplastic water potentials would require the development of a large resistance between the symplast and cell wall surfaces, and so a failure in local equilibrium between the symplast and apoplast. The question then arises, could plasma membrane permeabilities be down-regulated to the point where they become the controlling resistance for the liquid path through the mesophyll?

Can plant cell water relationship theory and undersaturation be reconciled?

Measurements of individual cell permeabilities with the pressure probe, and swelling assays with oocytes, suggest aquaporin activity could cause membrane conductance to vary over two to three orders of magnitude (Kramer and Boyer, 1995; Ramahaleo *et al.*, 1999), thereby supplying the dynamic range required for the hypothesized change in the resistance separating symplast and apoplast. Yet, such a resistance will have no effect if it does not occur in the principal path of transpiration through the mesophyll, which some authors have hypothesized to occur in the cell wall apoplast (Brodrick *et al.*, 2010; Buckley, 2015). Relevant data for apoplastic flow are hard to find, but against the idea of a dominant apoplastic flow is at least one measurement, from potato parenchyma, that estimates the intrinsic hydraulic conductivity of the cell wall as of the same order of magnitude as for flow in the cell-to-cell path (Fig. 5A; symbols are defined in Table 2, values for conductivities in Table 3). Given a typical mesophyll cell wall thicknesses of about 250 nm, for a 25- μm -diameter cell the area available for cell-to-cell flow is about 25 times that for flow in the walls, and in this scenario the cross-membrane path would dominate [Fig. 5D, compare (4) and (5)]. In support of the competence of a cell-to-cell path, the high end of estimates of the conductivity of the cell-to-cell path, derived from protoplast swelling assays and cell pressure probe experiments (Table 3), are more than sufficient to explain transpiration rates on the order of $10 \text{ mmol m}^{-2} \text{ s}^{-1}$ over paths as long as five cells, while keeping the gradient in potential across the mesophyll to less than 1 MPa (i.e. a constraint to maintain turgor).

If membrane conductance falls by orders of magnitude to some minimum, the conductivity of the cell-to-cell path would collapse, but the cell wall and vapour path have enough conductivity (Table 3) that cross-membrane flow would be ‘bypassed’, and it would seem impossible to create large local differences in potential between symplast and apoplast at steady state in this model (Figure 5A). Alternatively, there is some evidence that angiosperms have an apoplastic barrier between bundle sheath cells that forces flow from the vasculature to the photosynthetic mesophyll to occur in the symplast (through plasmodesmata) or across membranes, similar to the foliar endodermis of gymnosperms (Canny, 1990). This opens up the possibility that flow through plasmodesmata could keep the symplast at one potential, with flow to the apoplast occurring across the entire mesophyll surface area (Fig. 5B). Given a membrane resistance (r_p) at the low end of the range (Table 3), and a ratio of mesophyll surface area to leaf surface area on the order of 10, a transpiration rate of $10 \text{ mmol m}^{-2} \text{ s}^{-1}$ per transpiring surface would give a $\Delta\Psi$ between symplast and apoplast of ~ 3 and 6 MPa for a hypostomatous and amphistomatous leaf respectively. Together

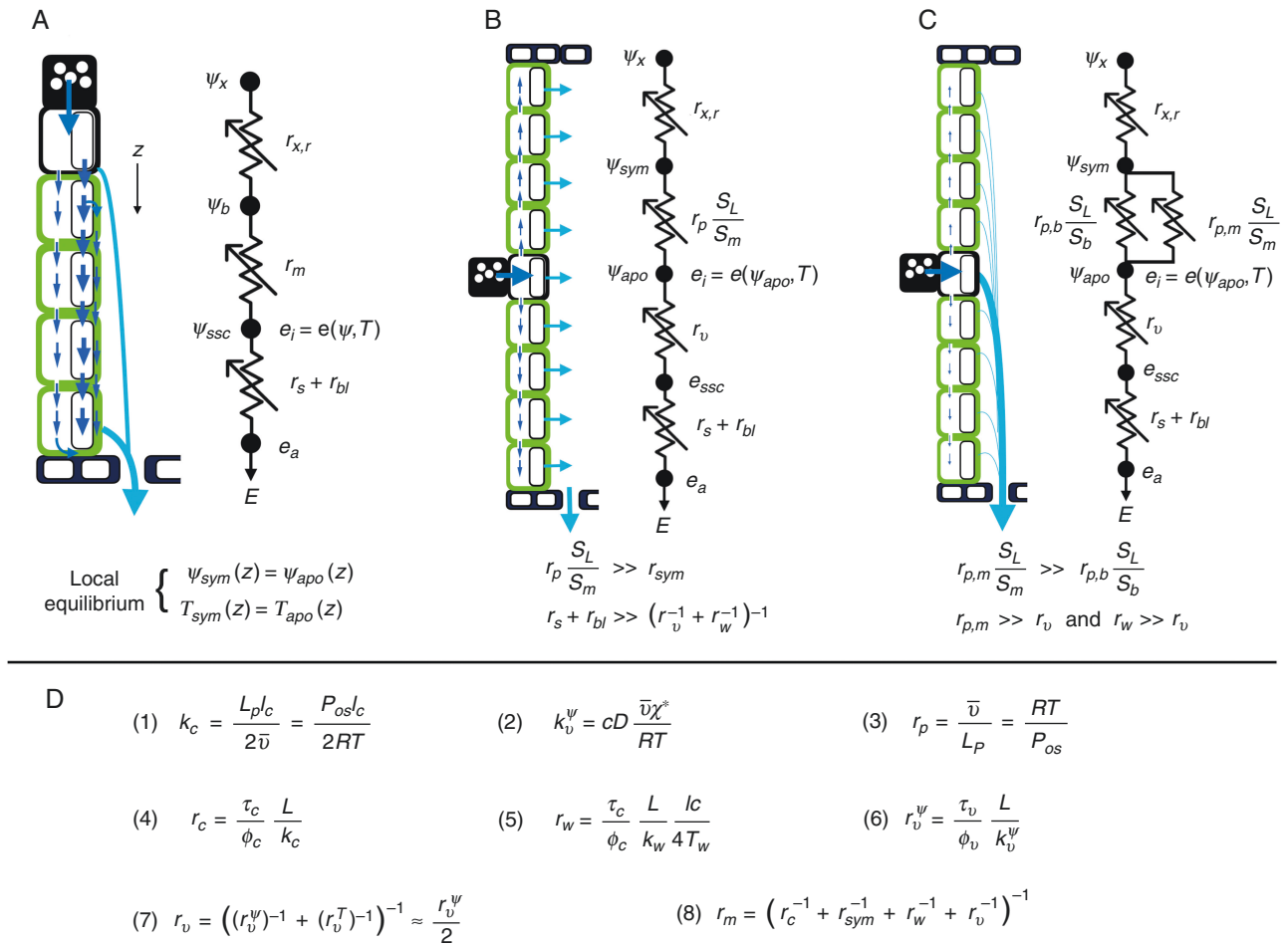


FIG. 5. Ohmic hypotheses for the local decoupling of symplast and apoplast water potential; for symbol definitions see Table 2. (A) At the high-end of membrane permeabilities (Table 3), local equilibrium between symplast and apoplast is expected, and flow occurs in four nearly unidirectional parallel symplastic and apoplastic paths through the leaf thickness: this is an Ohmic 1D reduction of the 3D fully coupled heat and mass transfer model in Fig. 3. (B) At the lowest plasmamembrane permeabilities, if the movement of water within the symplast encounters little resistance relative to that of the plasmamembrane, then a simple model placing the symplast in series with the apoplast can be justified. (C) Alternatively, if symplastic hydraulic resistance is high, keeping membrane permeability relatively high near the vascular surfaces can still produce local disequilibrium between a stagnant symplast and its apoplast near the transpiring surfaces, if flow through cell walls cannot ‘short’ the creation of large apoplastic water potential gradients by vapour phase transport. (D) Relationships between material properties (i.e. k , L_p , P_{os}) and Ohmic resistances (r) for various potential paths for water movement through leaves.

with a $\Delta\Psi$ from the soil to the leaf, this could lead to mesophyll air space humidities of 97 %, perhaps as low as 95 %, but it would require order of magnitude lower membrane permeabilities than those collected from the literature here (Table 3) to reach 80 % RH. While possible, the low ends of the ranges shown here derive from the lower bounds of histogram bins found in published studies that are themselves sparsely populated. It should also be noted that the range of permeabilities in Table 3 probably confounds changes in phosphorylation with density of aquaporins, which change on different time scales (Chaumont and Tyerman, 2014). A final difficulty with this model, as noted by Buckley and Sack (2019), is that the hydraulic conductivity of the plasmodesmata, while not well characterized, appears unlikely to support such high transpiration without symplastic turgor loss.

This last objection regarding plasmodesmata need not be fatal. Maintaining a symplastic compartment above turgor loss will not require a high conductance path through plasmodesmatal connections if most of the flow bypasses most

of the symplast (Fig. 5C). In this model, we conceive of the bundle sheath and mesophyll (i.e. spongy and palisade) membranes as resistors in parallel. If the area-corrected resistance of the bundle sheath is one or more orders of magnitude less than the mesophyll, the latter becomes a stagnant pool by-passed by a transpiration flux that evaporates from the bundle sheath (Fig. 5C). Given that mesophyll surface area may be about 10 times greater than leaf surface area, and given a bundle sheath (and proximal mesophyll) surface area similar to leaf surface area, then mesophyll membrane permeability would have to be down-regulated 100-fold compared to bundle sheath membranes to satisfy this condition. This would leave the bundle sheath membranes with only a (theoretically) 10-fold dynamic range in permeability, capable of creating a local potential drop from symplast to apoplast on the order of 1 MPa for the above transpiration rate of $10 \text{ mmol m}^{-2} \text{ s}^{-1}$ per transpiring surface. The bundle sheath apoplast would therefore remain close to saturation. However, if flow in the cell walls remains negligible, then the resistance to vapour transport could create a

large gradient in humidity from the bundle sheath surface to the top of the SSC. For example, for a half leaf thickness of 100–200 μm , the total internal resistance to isothermal vapour transport grows to 400–800 $\text{MPa m}^2 \text{s mol}^{-1}$, large enough to create a 4- to 8-MPa drop from the midplane to the top of the SSC for our chosen transpiration rate (here we are assuming that the

temperature contributions to internal vapour flow are roughly offset by volume fraction and tortuosity effects). This resistance is low enough that the resistance of the mesophyll membranes near the top of the SSC, as high as 3000 $\text{MPa m}^2 \text{s mol}^{-1}$ (Table 3), might still prevent a flux out of the local mesophyll symplast from ‘shorting’ the vapour path. Yet, while this model seems viable, it struggles to explain humidities as low as 80 %, unless plasmamembrane permeabilities (P_{os}) can be pushed to even lower values than $1 \mu\text{m s}^{-1}$.

Both of the scenarios in Fig. 5(B,C) – one with symplastic flow and one without – if analysed with a standard gas exchange model that takes e_i to be saturated at leaf temperature, would lead to errors in inferred c_p , but they would do so for different reasons. In the first case, where symplastic resistance is low and the evaporative surface becomes the entire mesophyll, the error arises simply because e_i is overestimated due to the effects of apoplastic water potential on vapour pressure becoming non-negligible. As a result, stomatal conductance is underestimated, as well as c_p , which (given an implicit assumption in this model that r_v is small, or in the terms of the standard framework in Fig. 2, g_{ias} is large) represents the entire intercellular airspace. In this model, the retreat of evaporation from the SSC to the mesophyll surfaces does not itself lead to errors. In the second model, where evaporation occurs at the vascular plane, the errors come not from a saturated value of e_i at the evaporation site being wrong, but from the change in the physical location of the evaporation site itself. When the evaporation sites move from the SSC to the bundle sheath, r_v effectively gets folded into the estimate of stomatal conductance, with the result that c_i is estimated in the middle of the leaf rather than just inside the stomata. The resulting error is the same though – c_i is again too low, as the value estimated is more properly considered c_{ias} (Fig. 2, eq. 6), and g_{ias} is small enough to matter.

The different types of error in the two models (Fig. 5B,C) also have different effects on the isotopic calculations as well. In the first case, equilibrium fractionation at the evaporation site is mis-estimated if saturated vapour pressure is assumed, while in the second case that approximation holds while the kinetic fractionation due to g_m and vapour diffusion through the airspace is mis-estimated. However, it might be difficult to know based on isotopes alone which case applied, especially if at isotopic steady-state the entire symplast is near the level of enrichment of the evaporation sites (Peclet effects are small). Alternatively, the two models should be distinguishable by local water potential measurements, made possible by a nano-scale hydrogel reporter of water potential that can be infused into leaves to coat mesophyll cell surfaces (Jain et al., 2021). In the first model, we would expect that the probe would report

TABLE 2. Symbols for Fig. 5

Quantity	Symbol	Value (25 °C)	Units
Molar volume of water, liq.	$\bar{v}$	1.807×10^{-5}	$\text{m}^3 \text{mol}^{-1}$
Diffusivity, water in air	D	2.5×10^{-5}	$\text{m}^2 \text{s}^{-1}$
Molar concentration of air	c	40.86	mol m^{-3}
Gas constant	R	8.3145	$\text{m}^3 \text{Pa mol}^{-1} \text{K}^{-1}$
Reference temperature	T	298.15	K
Reference vapour mole fraction	χ^*	0.0313	–
Transpiration	E	–	$\text{mol m}^{-2} \text{s}^{-1}$
Hydraulic conductivity	k	–	$\text{mol m}^{-1} \text{Pa}^{-1} \text{s}^{-1}$
Stomatal conductance	g_s	–	$\text{mol m}^{-2} \text{s}^{-1}$
Boundary layer conductance	g_{bl}	–	$\text{mol m}^{-2} \text{s}^{-1}$
Tortuosity	τ	1.5	–
Volume fraction	φ	–	–
Cell diameter	l_c	–	m
Subscripts and superscripts			
Wall path	w	–	–
Cell-to-cell path	c	–	–
Symplast	sym	–	–
Apoplast	apo	–	–
Plasmamembrane	p	–	–
Vapour path	v	–	–
Mesophyll	m	–	–
Stomata	s	–	–
Boundary layer	bl	–	–
Bundle sheath	b	–	–
Isothermal vapour path	ψ	–	–
Leaf	L	–	–
Surface area	S	–	–
Xylem	x	–	–
Radial (from xylem)	r	–	–

TABLE 3. Estimates for the hydraulic conductivities k_c , k_w and k_v^ψ in Fig. 5

Source	Tissue type	Symbol	Range/expression	Cell size	Estimated k range $\text{mol m}^{-1} \text{MPa}^{-1} \text{s}^{-1}$	Estimated r_p range $\text{m}^2 \text{MPa s mol}^{-1}$
Ramahaleo et al. (1999)	Petunia leaf	P_{os}	$1\text{--}330, \mu\text{m s}^{-1}$	30 μm	$6 \times 10^{-9}\text{--}2 \times 10^{-6}$	2485–7.5
Martre et al. (2002)	Arabidopsis leaf	L_p	$5 \times 10^{-9}\text{--}4 \times 10^{-6} \text{ m MPa}^{-1} \text{s}^{-1}$	45 μm	$6.2 \times 10^{-9}\text{--}5 \times 10^{-6}$	3604–4.5
Kim and Steudle (2007)	Maize leaf	L_p	$2.6 \times 10^{-8}\text{--}5 \times 10^{-6} \text{ m MPa}^{-1} \text{s}^{-1}$	70 μm	$5 \times 10^{-8}\text{--}9.9 \times 10^{-6}$	693–3.5
Michael et al. (1997)	Potato parenchyma	k_w	–	n/a	2.7×10^{-6}	n/a
Rockwell et al. (2014)	Model (isothermal)	k_v^ψ	$cD\bar{v}\chi^*(RT)^{-1}$	n/a	2.3×10^{-7}	n/a

similar, highly undersaturated apoplastic water potentials near both the transpiring and non-transpiring surfaces. In the second model, however, the non-transpiring side would be expected to be at water potentials close to that of the symplast.

Could mechanisms other than down-regulation of membrane permeability explain the coexistence of turgor and 80 % RH in the adjacent airspaces? One hypothesis might be that ‘excessive’ evaporation drives an extension of internal cuticle (IC) formation (Pesacreta and Hasenstein, 1999) from inner epidermal cell wall and guard cell surfaces to mesophyll cell wall surfaces. This process would, in theory, begin in the cells closest to the SSC. Suppressing evaporation from these surfaces would force evaporation deeper into the mesophyll, potentially bringing cuticle precursors to the mesophyll cell surfaces, until all the photosynthetic surfaces were covered. One strength of this idea is that it would not require that plasmodesmata provide the path for transpiration, as the shared cell wall surfaces between adjacent cells would remain unblocked. A major weakness of this idea is that an internal cuticle, if similar in chemistry to the external cuticle, would represent a resistance not just to water, but to CO₂ as well. The models of Cernusak *et al.* (2018, 2019) do not allow for any such resistance: to satisfy the constraint $\delta_c \sim \delta_{c_e}$ the barrier to water loss would have to have no effect on the flux of CO₂ into the symplast. This begs the question, if there *was* such a substance available to plants, one blocking water loss without impeding CO₂ entry, why would plants not simply deploy it on their outer surfaces, and dispense with stomata altogether?

This paradox implicit in the IC scenario underlines an important structural feature of the isotope and transport model employed to detect undersaturation: the implicit requirement that we have a resistance to water not seen by CO₂. This requirement derives from the fact that in the Ohmic analogy employed by Cernusak *et al.*, c_i is both the source-side concentration seen by g_m , as well as linked directly to e_i through their shared dependence on stomatal conductance. If we must choose the least improbable mechanism that could make the ‘interfacial resistance’ model true, aquaporin gating that effects membrane permeability to water but not CO₂ would therefore appear more plausible than internal cuticle formation.

ALTERNATIVE EXPLANATIONS TO UNDERSATURATION

Stomatal patchiness, heterogeneity and failure of the Ohmic analogy

The above explanations, in various ways, all involve failures of the Gaastra and van der Honert assumptions. There is another family of possible explanations for apparent undersaturation that derive from a failure of the third canonical assumption, the Ohmic analogy. The potential failures of the Ohmic analogy in question here do not arise from problems with the way the circuit analogy breaks the continuum into a series of nodes, the structure of which we have analysed already, but from the way that each node represents an average describing the states and behaviours of the vast number of areoles, stomata, cells and chloroplasts that exist in parallel (for a thoughtful discussion of the potential problems, in all their dimensions, arising

from an Ohmic discretization of the SPAC, see Philip, 1966). The Ohmic description of gas exchange assumes that a single value for the fluxes and driving forces across the area of a leaf captures the local behaviour of most stomata. The justification of this assumption in gas exchange analysis rests on the uniformity of light and well-mixed air and minimal boundary layer effects typically achieved in cuvette-based measurement systems: given a uniformity of imposed conditions, the assumption that biological responses such as stomatal aperture and assimilation are uniform as well, or at least unimodal such that the most frequent value is close to the mean, follows naturally. Evidence that this assumption can fail emerged in the 1980s to late 1990s from numerous studies of midday depressions in assimilation under high VPD. Gas exchange under high midday VPD showed that assimilation decreased more than expected based on the Gaastra-inferred c_i , and the A/c_i curve generated by manipulating external CO₂ (e.g. Fig. 6A, B; Farquhar and Raschke, 1978; Raschke and Resemann, 1988), motivating a hypothesis that high VPD somehow reduced g_m . However, an alternative explanation subsequently appeared in the form of ‘patchy’ stomatal behaviour, which can lead to a bimodal distribution in apertures whose ensemble behaviour deviates from that of a unimodal distribution even when it retains the same overall mean (Downton *et al.*, 1988; Beyschlag *et al.*, 1992).

To understand why a bimodal distribution breaks Ohm, consider the extreme case in which stomata are either wide open or shut (Kraalingen, 1990). In this case both fluxes, A and E , occur only through the reduced leaf area containing the open stomata (Fig. 7). As the calculation for c_i involves the ratio of E/A , such that the area normalization common to both A and E cancels out, c_i is in fact correctly calculated for the air space interior to the *open* stomata, but obviously not for the portion of the leaf covered by *closed* stomata. However, c_c (or g_m if c_c is known from other information) depends on both A and c_i , and as A represents a single value believed to describe the entire measured area, it is in fact an underestimate of the fluxes through the open stomata involved in gas exchange. One possible result of this error in the Ohmic description is that, if the stomatal conductance distribution becomes bimodal due to complete closure of numerous stomata under high VPD, there is an apparent decline of g_m with increasing VPD that is not real (e.g. fig. 6 in Laisk, 1983).

Could this scenario in fact explain the extreme undersaturation observed by Cernusak *et al.* (2018)? No, because in this case the effect goes the wrong way: to rescue saturation in those experiments would require an apparent increase in g_m , not decrease. Moreover, concerns about ABA-induced closure inducing bimodal distributions of conductance have been in part allayed by studies showing generally unimodal conductance (Mott, 1995; Meyer and Genty, 1998), and non-stomatal limitation of assimilation under water stress appears to be real, if incompletely understood (Tang *et al.*, 2002; Lawlor and Tezara, 2009). Conductance-based errors in c_i can arise from factors other than patchiness. Meyer and Genty (1998) report that failing to account for cuticular transpiration caused larger errors in c_i than the distribution of g_s following ABA-induced closure; but again the effect of cuticular conductance goes the wrong way to explain the results for pinyon pine and juniper (Cernusak *et al.*, 2018). We will return to the question of

whether some form of heterogeneity could provide alternative explanations to undersaturation in that study. First, however, we turn to a related case in which a form of ‘patchiness’ in *A* seems both likely to have occurred and would shift the data in the right direction to eliminate apparent undersaturation.

ABA-insensitive mutants

A concrete demonstration of the potential for heterogeneity to explain apparent undersaturation is available with the data provided by Cernusak *et al.* (2019) on *Populus* wild-type and ABA-insensitive mutants (*abai*). In these experiments, using the dual isotopic method and relaxing Gaastra in order to enforce $\delta_c \sim \delta_{c,e}$ results in extreme undersaturation in the *abai* plants at moderate VPD (2.5 kPa), but not in the wild-type even at very high VPD (up to 7 kPa). That the *abai* leaves did experience extreme stress is evident from their desiccation and death, despite being still attached to the plant, within hours after removal from the cuvette. That the responses of the *abai* leaves are indeed due to a failure of stomata to shut, and not some pleiotropic effect, is attested to by A/c_i curves constructed from the VPD experiment data and calculated with or without the Gaastra assumption (Fig. 6C,D). For the *abai* leaves, *A* falls with only slight increases in c_i as VPD increases, as would be expected for stomata that remain wide open even as desiccation drives *A* lower.

These VPD experiments appear to offer a strong proof-of-concept for the method, in that they show exactly what we might expect: the development of undersaturation in a leaf whose stomata do not shut in response to increasing VPD and *E*, but not in one (the wild-type) whose stomata regulate *E* so tightly as to be independent of VPD (fig. 2B in Cernusak *et al.*, 2019). That *A* declines in the mutant despite high light and high c_i can also be rationalized as the deleterious effect of desiccation on the ability of the drying mesophyll to sustain assimilation (Tang *et al.*, 2002). Yet if so, surely the observed effect on *A* is too small: at 60 % RH in the airspace, *A* remains between 3 and 4 $\mu\text{mol m}^{-2} \text{s}^{-1}$, even as the equilibrium water potential reaches -71 MPa, far beyond a stress level which any of the strategies discussed above for decoupling symplastic from apoplastic water potential would permit.

Alternatively, the declines in *A* may have occurred in a patchy manner across the leaf (i.e. ‘Patchy Desiccation’, Fig. 6). In this conception, cavitation (and or minor vein collapse) would serve as the trigger toggling an areole from maintenance of *A* and symplastic water potential in the vicinity of turgor loss to total loss of assimilation capacity due to catastrophic drying. As even catastrophic drying only partially suppresses the driving force for evaporation (e.g. Cernusak *et al.*, 2019; Fig. 2B), *E* would remain more evenly distributed than *A*. That the ohmic estimate of *A* underestimates assimilation for the still functional areas of the leaf would mean that c_i was overestimated for those areas. In this case, imposing $\delta_c \sim \delta_{c,e}$ ‘corrects’ the true c_i to an overestimated value by revising g_s upward, and e_i downward, while conserving *E*. Instead, one can break the Ohmic assumption of uniformity, and simply fit a patchiness factor describing the true (but unknown) proportion of leaf area over which *A* occurs, iterating to find the value that satisfies both saturation and $\delta_c \sim \delta_{c,e}$. This procedure results in a

patchiness factor that is essentially one-to-one with the undersaturation in humidity fitted by Cernusak *et al.* assuming no patchiness ($y = 0.9989x + 0.0122$, $R^2 = 0.9$; Supplementary Data). Patchiness therefore offers an entirely substitutable alternative hypothesis to undersaturation, one capable of rescuing Gaastra as a useful approximation, at least for those areas of the leaf still photosynthetically competent. It is also perhaps easier to verify, and indeed we can see that patchiness must have occurred to some degree during the VPD experiments of Cernusak *et al.* (2019) from the supplemental images of an *abai* leaf after its removal from the cuvette. These images show both green and discoloured areas. Furthermore, images taken several hours later, in which most of the leaf is dark brown, show distinct patches of green areoles that remain along the midrib, some of the secondaries, and at the blade petiole junction.

Patchiness could also contribute to some of the features seen in the wild-type data. Although Cernusak *et al.* (2019) report they found no undersaturation for the wild-type, we do see a difference in the A/c_i curves calculated with (Fig. 6C) and without (Fig. 6D) the Gaastra assumption: with Gaastra, at around 200 ppm c_i , *A* falls without a change in c_i . This occurs under high VPD, as the stomata shut, and indeed appears similar to the pattern of apparent non-stomatal limitation of *A* under high VPD that attracted the interest of Raschke and others. In the Cernusak analysis, breaking Gaastra and enforcing $\delta_c \sim \delta_{c,e}$ lowers the c_i for these points, but not their *A*, moving them to the left and so restoring them to the trend one would expect for an unstressed A/c_i curve driven by changes in c_a (Fig. 6A, dashed line). However, forcing $\delta_c \sim \delta_{c,e}$ also results in oversaturation for these points, with humidities ranging from 100 to 140 %.

Alternatively, patchiness in stomatal aperture could be contributing to the pattern in Fig. 5(C). With an assumption of patchiness, re-normalizing *A* and *E* to the reduced leaf area associated with open stomata results in shifting points upwards in the A/c_i plot, as *A* is increased but c_i remains unchanged. Assuming Gaastra, one can again fit a patchiness coefficient that gets closer to $\delta_c \sim \delta_{c,e}$. For the points with c_i around 200 ppm that are at issue here, those coefficients correspond to an ‘active’ area of assimilation and transpiration ranging from 17 to 78 % of the actual leaf area (Supplementary Data).

Finally, for the sake of completeness, we should consider the fact that, unlike for the Pinyon pine and juniper in Cernusak *et al.* (2018), for both the *Populus* wild-type and *abai* mutant in Cernusak *et al.* (2019) the δ of CO_2 in equilibrium with water at the evaporation site is heavier than at the chloroplasts ($\delta_{c,e} > \delta_c$), meaning a Peclet effect could be contributing to apparent undersaturation. For the *Populus* data taken over both leaf types, we can then point to three possible interpretations:

1. Gaastra is correct, there is a Peclet effect due to spatial separation of evaporation and carboxylation, and non-stomatal limitation of *A* occurs with increasing VPD.
2. Gaastra is wrong, there is neither a Peclet effect ($\delta_c \sim \delta_{c,e}$ holds) nor non-stomatal limitation of *A*, but at high VPD the leaf air space can become oversaturated as stomata shut and reduce *E*.
3. Gaastra is correct, there is no Peclet effect or non-stomatal limitation of *A* aside from that due to extreme drying stress, and patchiness results in *A* being mis-estimated in relation to c_i .

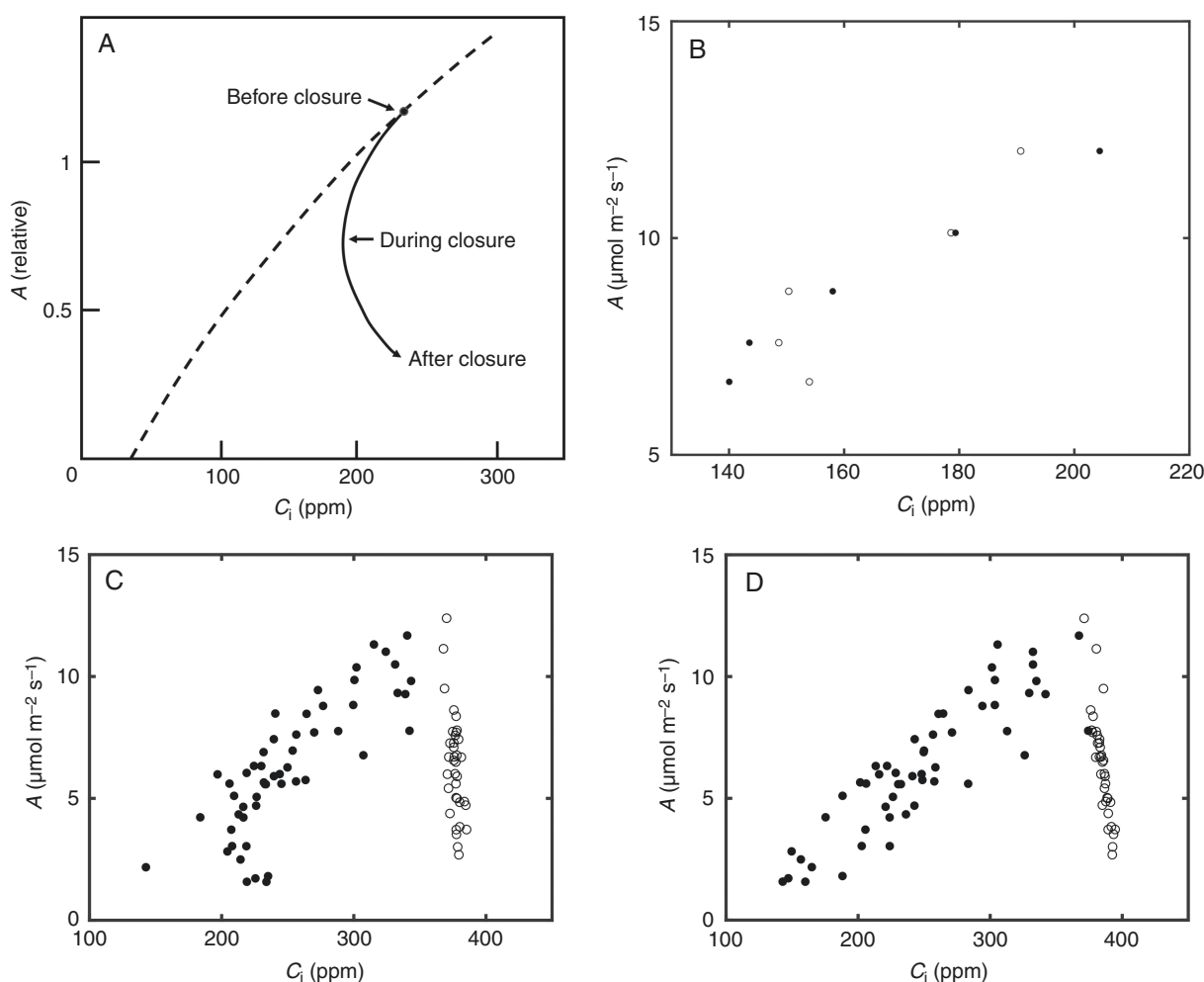


FIG. 6. Patchiness and anomalous A/c_i curves. (A) Theoretical effect of bimodal distributions of stomatal aperture on an A/c_i curve, re-plotted from Kraalingen (1990). (B) Assimilation, A , versus intercellular CO_2 , c_i , for *Gossypium hirsutum* (cotton) leaves subject to increasing VPD; open circles are as measured by a passive ('closed') gas exchange system in contact with the abaxial side, and closed circles represent the estimate for the adaxial side of the leaf assuming saturation at leaf surface temperature (Gaastra); data are re-plotted from Sharkey et al. (1982). Assimilation versus c_i for wild-type (closed circles) and *abai* (open circles) *Populus* leaves, assuming saturation of internal vapour pressure (C), or allowing for undersaturation (D); data are from Cernusak et al. (2019).

These are limiting behaviours: the truth could involve each of these possibilities to varying degrees. Clearly, Gaastra in the strong form of 100 % saturation is always wrong, stomatal aperture is never truly uniform and some degree of a Peclet effect may be expected, while at the same time $\delta_{c,e}$ cannot be too far from δ_c . Yet, there is reason to hope that the dual-isotope approach can be augmented to place some bounds on the magnitudes of these effects. The first and third scenarios recapitulate the controversy over non-stomatal limitation versus bimodal distributions of g_s , and such questions have been successfully addressed by fluorescence imaging (Meyer and Genty, 1998), as well as simple leaf infiltration assays (Beyschlag and Pfanz, 1990). With respect to the second scenario, that evaporation deep in the leaf and condensation at the lower epidermis (Pieruschka et al., 2010; Rockwell et al., 2014) could create a region of oversaturation near the stomata, it is helpful to note that such condensing states require that the internal vapour flux be larger than that which escapes the leaf as E . In the *Populus* study, we see that, for the wild-type leaf, E remained essentially

constant as VPD changed, making it hard to imagine a mismatch in internal and external vapour transport could have created over-saturation, especially without dramatic increases in energy load.

Lastly, we can ask on a theoretical basis whether we should ever expect detectable Peclet effects ($\delta_{c,e}$ more enriched than δ_c) based on the length scales and hydraulic properties typical of leaf mesophyll. In the context of leaf mesophyll, Barbour and Farquhar (2004) defined the Peclet number as $Pe = UL/D$, where U is the 'slab' velocity of transpiration through the mesophyll (i.e. the velocity that would exist if E was spread uniformly across a cross-section), D is the self-diffusivity of H_2^{18}O in pure water, and L is the 'effective length' of the path of transpiration that takes full account of the tortuosity and hinderance of that path. Alternatively, one can choose the Euclidian (straight line) distance through the leaf thickness as the length, and define an 'effective diffusivity' for the mesophyll that then contains all the information on tortuosity and hinderance. In this spirit, using a thermodynamic argument that extracts an effective

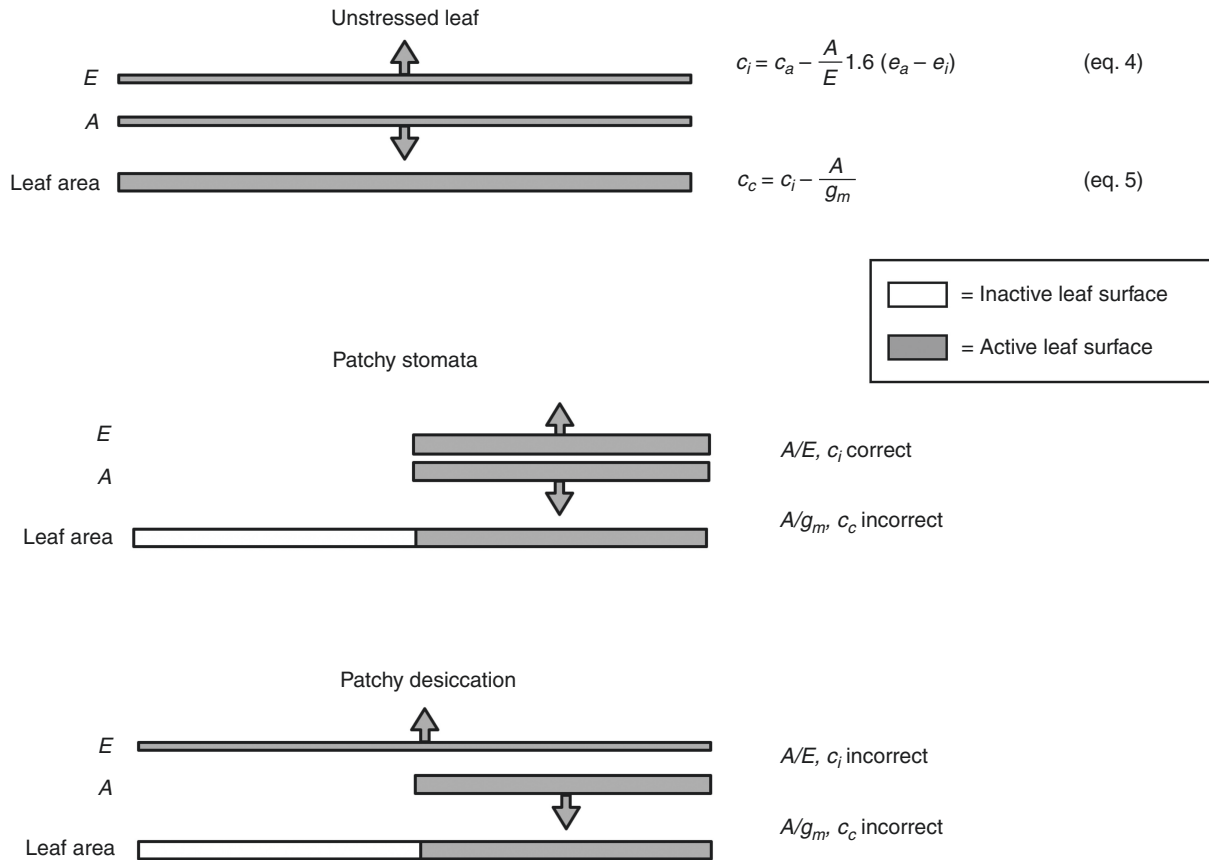


FIG. 7. Conceptual diagram showing the effects of patchiness on gas exchange calculations. In an unstressed leaf with uniform conductances and concentrations, the fluxes of water and carbon can be conceptualized as a layer whose thickness (vertical dimension) corresponds to the magnitude of the flux, and whose area (i.e. lateral width in this 2D depiction) corresponds to the leaf area. In an extreme case of ‘patchy stomata’, stomatal pores are open over half the leaf (shaded), but are closed over the other half (white), and thus the true fluxes over the active leaf area are twice that reported by a gas exchange system. In ‘patchy desiccation’, stomata insensitive to ABA fail to close, and the expectation that assimilation is more sensitive to desiccation than evaporation (see text) results in the ratio of A/E being incorrectly estimated from gas exchange data.

diffusivity from empirical measures of mesophyll hydraulic conductivity, we have estimated a Peclet number on the order of 0.1–0.01 for leaf mesophyll (Supplementary Data). If this idea is close to correct (the reliance on a ‘slab’ velocity is an obvious weakness), diffusion should dominate convection in the mesophyll, and $\delta_c \sim \delta_{c,e}$ should hold even in cases where the evaporation sites are concentrated at the SSC and assimilation principally occurs in the palisade layer. A small Peclet number for the mesophyll does have some empirical support: Farquhar and Gan (2003) report that enrichment in ^{18}O can be detected in the secondary veins of the leaf vasculature, which suggests that in isotopic steady state, given the long flow path and concentrated flux (so high velocity) in the xylem between the secondary veins and mesophyll, the mesophyll outside the xylem may be close to the $\delta^{18}\text{O}$ of the evaporation sites.

The ubiquity of Ohm

All the above arguments taken together suggest that patchiness (scenario 3) should be accounted for prior to rejecting Gaastra. We also need to keep in mind that Ohmic failure is not so much a potential artefact of the dual isotope method as

it is of the gas exchange systems upon which the isotopic approach is based. Ohmic failure may also confound the dual gas exchange approach, as exemplified by Sharkey *et al.* (1982), which was developed expressly to check the Gaastra estimate, or Ward and Bunce (1986), who used dual open systems on amphistomatous sunflower leaves to find negative transpiration rates when the opposing side of a leaf was subject to high VPD. In the dual gas exchange experiments of Sharkey *et al.* (1982), under increasing VPD the passive measurement of c_i on one side of a cotton leaf showed c_i following a normal A/c_i curve, while the Gaastra estimate of c_i at the other surface showed a decline in A as c_i held firm or even increased (Fig. 4B). As we have seen, this pattern can be explained by patchiness, which takes the estimated c_i as correct, and revises the local A upward. Except, in this method, c_i is also measured directly, and the discrepancy in c_i may force us to conclude that Gaastra is wrong. Yet, given that stomata on opposing surfaces of an amphistomatous leaves behave independently (Mott and O’Leary, 1984), and that high VPD was only applied to the leaf surface in contact to the ‘open’ gas exchange system, if stomatal conductance was bimodal on that surface, but more uniform on the ‘closed’ surface, then the c_i measured by the closed system would have been an average across *all* the areoles, while

the c_i estimated by Gaastra on the open system side would only have described those areoles with open stomata. As the leaf was uniformly illuminated, those areoles that have closed stomata on the open-system side due to high VPD would still have had their c_i pulled down lower. Under these conditions we should expect that measured c_i on the lower side would be lower than that estimated by Gaastra for the upper side.

Yet, even if patchiness in stomatal aperture does contribute to the surprising persistence of assimilation at extreme undersaturation in *abai* *Populus* leaves, could it have contributed to the undersaturation detected in Pinyon pine and juniper? No, given that for those data $\delta_{c,e}$ is lighter than δ_c . However, forms of heterogeneity other than bimodal stomatal distributions could produce an error making data $\delta_{c,e}$ only appear lighter than δ_c . The Pinyon and juniper data were derived from gas exchange experiments on topologically complex shoots, in which heterogeneities in light, energy loading and temperature probably occurred. For example, self-shading of leaves in the conifer chamber, arising from the 3D topography of the shoots, could have resulted in most of the A occurring in the directly illuminated leaves, even as E , driven as it was by high VPD and energy transfer from the warm air at high fan speeds, and not simply by short-wave solar radiation, was more evenly distributed. As a result, c_i calculated assuming Gaastra would be wrong because the average A/E would be too small, not because e_i was too large (Fig. 2, eq. 4). Moreover, this bias would be expected to grow as VPD increased, and E transitioned from being mostly ‘pushed’ out of the illuminated leaf regions into humid air by the short-wave energy load, to being mostly ‘pulled’ out of all leaf regions by the increasing dryness of the external air. While this explanation is mere speculation, the important point is that the failure of the Ohmic analogy encompasses a whole family of potential heterogeneities within which patchiness in stomatal aperture is just one instance, and any of which could save plant physiologists from having to accept improbable levels of undersaturation in leaves. Even in the ideal topological case of flat leaves uniformly illuminated in a cuvette, we should recognize that the onset of large stresses can drive the development of heterogeneity by introducing spatially variable hydraulic failures (Brodrribb *et al.*, 2016; Zhang *et al.*, 2016) that amplify local stress.

CONCLUSION

Fifty years after Jarvis and Slatyer (1970) presented evidence of interfacial resistances in leaf mesophyll, the dual isotope approach is providing a new and powerful tool for probing the saturation state in leaf mesophyll. Continued refinements to the method, including the simultaneous estimate of g_m , promise to bring the question of undersaturation into clearer focus (Holloway-Phillips *et al.*, 2019). The prospect that, at least in some species, high VPD can set in motion a cascade of stresses that remodel the internal hydraulic resistance of leaves, and so create undersaturated conditions, introduces the potential for improvement to crop water use efficiencies through the exploitation of such non-stomatal control of E . To that end, emerging technologies such as *in planta* and minimally invasive reporters of mesophyll cell surface water potential could provide an independent source of information on leaf air space saturation state and the dynamics of mesophyll hydraulic architecture (Jain *et al.*, 2021). Nevertheless, the potential

for Ohmic failure in all its possible forms must be explicitly confronted if we are to build a robust understanding of leaf gas exchange under extreme conditions.

SUPPLEMENTARY INFORMATION

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. ‘Populus_data_Cernusak_2019.xlsx’: an Excel data sheet of gas exchange data from Cernusak *et al.* (2019), edited to include a patchiness factor that describes the fraction of the leaf surface actively participating in gas exchange. ‘Peclet and K_{ssc} calculation.pdf’: a pdf containing the details for estimating a Peclet number for leaf mesophyll from a hydraulic conductivity.

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Supporting calculations

*F.E. Rockwell et al.*¹

¹Harvard University, Dept. of Organismic and Evolutionary Biology

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Should we expect $\delta H_2^{18}O$ gradients in the leaf mesophyll?

The question has been raised whether we should expect transpiration to induce a gradient in leaf water $H_2^{18}O$ to develop through the mesophyll between the xylem minor veins and evaporation sites in leaves (Barbour and Farquhar, 2004). Here we present an argument for estimating the Peclet number that describes the balance of convection and diffusion that controls the development of gradients due to depleted bulk flow from the xylem and back-diffusion of enriched water from the evaporation sites.

A general thermodynamic description of the flux of a species i is given by (Howard A. Stone, Princeton University, pers. comm.),

$$q_i = \frac{c_i D_i}{RT} \frac{\partial \mu_i}{\partial x} \quad (1)$$

Here the driving force is the gradient in chemical potential, $\partial \mu_i / \partial x$, while the transport coefficient (the proportionality between the flux and driving force) is given by the product of the diffusivity and the local concentration of the species of interest divided by RT , and so describes the mobility of free energy due to diffusion. In plant water relations, the convention is to express the free energy per mole as an energy per volume, which has the dimensions of pressure. Substituting $\mu_i = \bar{v} \psi$, where $\bar{v}$ is the volume per mole of liquid water, and eliminating subscripts to focus only on water, yields,

$$q = \frac{c_w D_w \bar{v}}{RT} \frac{\partial \psi}{\partial x} \rightarrow \frac{D_w}{RT} \frac{\partial \psi}{\partial x}, \quad (2)$$

where we recognize that $c_w \bar{v} = 1$ by definition. D_w is taken as the self-diffusivity of water, and the above equation describes the mobility of water molecules in pure water. In a leaf, the effects of the presence of cell walls and membranes, and the tortuosity of the path, can be combined into a single term describing the hinderance experienced by a single water molecule, ζ :

$$q = \frac{\zeta D_w}{RT} \frac{\partial \psi}{\partial x}, \quad k = \frac{\zeta D_w}{RT}, \quad (3)$$

where we have defined k , the hydraulic conductivity of the mesophyll to the forced permeation of water molecules. The Peclet number $\mathcal{P}$ that describes the relative importance of convection to diffusion is given as,

$$\mathcal{P} = \frac{UL}{D}, \quad D = \zeta D_w, \quad (4)$$

where U is the velocity of the water flux, L is the distance over which the competition of diffusion and convection occurs, and we identified the relevant diffusivity as the hindered self-diffusivity of water. Identifying transpiration as the relevant flux, $E = q$, the ‘slab velocity’ of water (e.g., volume per unit leaf area per second) is $U = \bar{v}E$, leads to,

$$\mathcal{P} = \frac{\bar{v}EL}{\zeta D}. \quad (5)$$

This is the form employed by Barbour and Farquhar (2004, eq. 9), where the quantity L/ζ constitutes the effective length for transpiration and back-diffusion through the mesophyll. Here we proceed by combining eqn’s 3 and 4,

$$\mathcal{P} = \frac{\bar{v}k \frac{\Delta\psi}{\Delta x} L}{k RT}, \quad \rightarrow \quad \mathcal{P} = \frac{\bar{v}\Delta\psi}{RT}, \quad (6)$$

where taking $\Delta x = L$ identifies $\Delta\psi$ as the water potential drop from the minor veins to the sites of evaporation. In the final expression, we see that all of the information about the hinderance of the movement of water molecules and path-length is embedded, through the hydraulic conductivity k , in the water potential drop $\Delta\psi$. For $R = 8.3145 \text{ J/mol/K}$, $\bar{v} = 1.807 \times 10^{-5} \text{ m}^3/\text{mol}$, $T = 300 \text{ K}$, and a $\Delta\psi$ from 0.1 to 1 MPa (Rockwell et al., 2014; Buckley et al., 2017), results in $\mathcal{P} = 0.07 \rightarrow 0.007$, and diffusion would appear to dominate.

Conclusion

A ‘forced permeation’ description of the transpiration flux through the mesophyll, which is arguably appropriate to the extent that single-molecule traffic through aquaporins constitutes an important part of the hinderance to transport, leads directly to a conclusion that the relative strength of back diffusion would make the mesophyll, in isotopic steady state, a single (locally, at the areole scale) well-mixed pool.

Calculating K_{ssc}

Pickard (1982) gives the Ohmic conductance of a single substomatal chamber as,

$$K = \frac{2\pi cD ab}{b - a}, \quad (7)$$

where c is the concentration of air, in moles per m^2 , and D the diffusivity of water vapor, and a and b are the radii of the stomatal pore and substomatal chamber respectively. To convert to a conductance normalized to the leaf surface area, and to convert to an expression of the driving force as the water potential of an equilibrated liquid phase, we write,

$$K_{ssc} = \frac{1000}{w^2} \frac{2\pi cD ab}{(b - a)} \frac{\partial\chi}{\partial\psi}, \quad (8)$$

50 which has the required units of $\text{mmol m}^{-2} \text{MPa}^{-1} \text{s}^{-1}$. Here, w is the side length of the
51 square patch of leaf area associated with a single stomate, the factor of 1000 arises from the
52 conversion from mol to mmol, and $\partial\chi/\partial\psi$ describes the sensitivity of the mole fraction of
53 water vapor to the water potential of an equilibrated liquid phase.