

## Viewpoints

# Two sides to every leaf: water and CO<sub>2</sub> transport in hypostomatous and amphistomatous leaves

### Summary

Leaves with stomata on both upper and lower surfaces, termed amphistomatous, are relatively rare compared with hypostomatous leaves with stomata only on the lower surface. Amphistomaty occurs predominantly in fast-growing herbaceous annuals and in slow-growing perennial shrubs and trees. In this paper, we present the current understanding and hypotheses on the costs and benefits of amphistomaty related to water and CO<sub>2</sub> transport in contrasting leaf morphologies. First, there is no evidence that amphistomatous species achieve higher stomatal densities on a projected leaf area basis than hypostomatous species, but two-sided gas exchange is less limited by boundary layer effects. Second, amphistomaty may provide a specific advantage in thick leaves by shortening the pathway for CO<sub>2</sub> transport between the atmosphere and the chloroplasts. In thin leaves of fast-growing herbaceous annuals, in which both the adaxial and abaxial pathways are already short, amphistomaty enhances leaf–atmosphere gas-exchange capacity. Third, amphistomaty may help to optimise the leaf-interior water status for CO<sub>2</sub> transport by reducing temperature gradients and so preventing the condensation of water that could limit CO<sub>2</sub> diffusion. Fourth, a potential cost of amphistomaty is the need for additional investments in leaf water transport tissue to balance the water loss through the adaxial surface.

### Introduction

Observations and biophysical models show that leaves with stomata on both leaf surfaces (termed amphistomatous) have an apparent gas-exchange advantage that allows for greater productivity compared with leaves with stomata on only one surface (termed hypostomatous) (Parkhurst, 1978; Mott & Oleary, 1984). Only a minority of species, however, especially in dicotyledonous families, utilise both leaf surfaces for their gas exchange. This relative rarity implies that the functional advantage of amphistomaty may come at a cost (Muir, 2015). One such cost pertains to the greater susceptibility for entry of foliar pathogens through stomata in the upper epidermis (McKown *et al.*, 2014). Recent phylogenetic insight further suggests that the occurrence of amphistomaty relates to an interaction between light environment and growth form

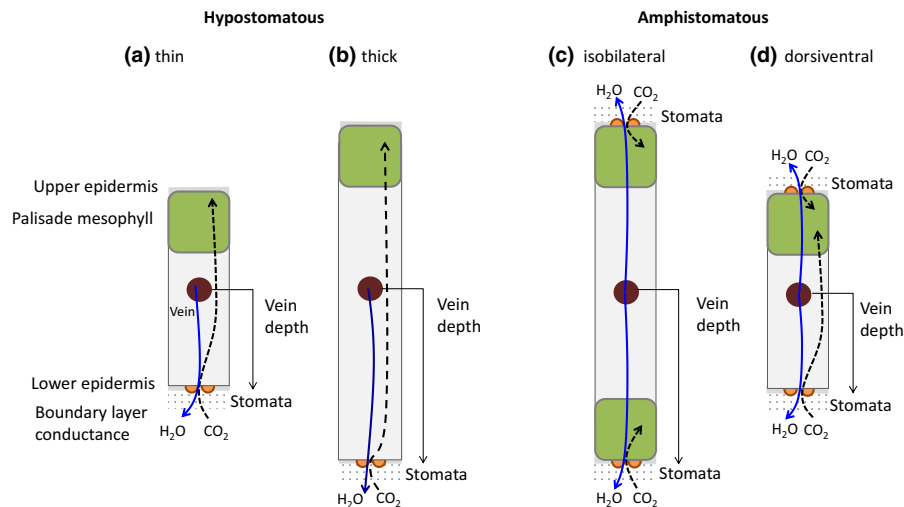
(Muir, 2018). For many families, amphistomaty appears to be the derived leaf morphology (Mott *et al.*, 1982), but a comprehensive phylogenetic analysis on the evolution of amphistomaty, including representatives from arid environments, has yet to be undertaken. Perhaps more importantly, a clear understanding of the functional costs and benefits of amphistomaty is needed to interpret evolutionary patterns.

Amphistomaty is often observed in fast-growing crops (Metcalf & Chalk, 1950), in which high light and access to a continuous supply of water can provide an adaptive advantage from high leaf conductance (Mott *et al.*, 1982). Paradoxically, amphistomatous species can also be slow growing and are common in arid environments (Parkhurst, 1978), for example many arid-zone eucalypts (Wood, 1934; de Boer *et al.*, 2016a). When considering the functional benefits and costs of amphistomaty, it is important to distinguish leaves with a dorsiventral anatomy from those with an isobilateral anatomy (Fig. 1). The dorsiventral morphology is typically found in fast-growing dicotyledonous herbaceous crops, whereas the isobilateral morphology is more commonly found in amphistomatous species adapted to arid conditions. Leaves adapted to these arid conditions are also often positioned in a near-vertical orientation, enabling light capture at low sun angles when the risks of desiccation and overheating are reduced.

In this paper, we present the current understanding of the functional costs and benefits of these distinct leaf morphologies as they pertain to water transport and carbon uptake. We specifically focus on the ecophysiological attributes of the different leaf morphologies of dicotyledonous species, and highlight recent advances in understanding leaf-interior water transport in relation to leaf hydraulic architecture and the transport pathway for CO<sub>2</sub>. Based on this view, we propose a generic explanation for the functional benefit of amphistomaty in both thin and thick leaves in relation to their specific growth environments.

### Stomatal distribution, leaf hydraulics and gas-exchange capacity

Regardless of whether a leaf is hypostomatous or amphistomatous, the core function – photosynthesis – requires uptake of CO<sub>2</sub> through the stomata and involves an inherent risk of desiccation owing to transpiration. The stomata play a dynamic role in this gas exchange by opening and closing to ensure adequate CO<sub>2</sub> supply while avoiding transpiration rates in excess of leaf water supply rates (Cowan, 1977). Maximum carbon uptake rates are, therefore, constrained by the water transport capacity of the hydraulic network, including the total leaf hydraulic conductance ( $K_{\text{leaf}}$ ) (Brodribb & Holbrook, 2003; Brodribb *et al.*, 2007; Sack & Scoffoni, 2013). Although much uncertainty exists regarding the partitioning of the leaf-interior resistance to water transport between the xylem pathway and the outside-xylem pathway



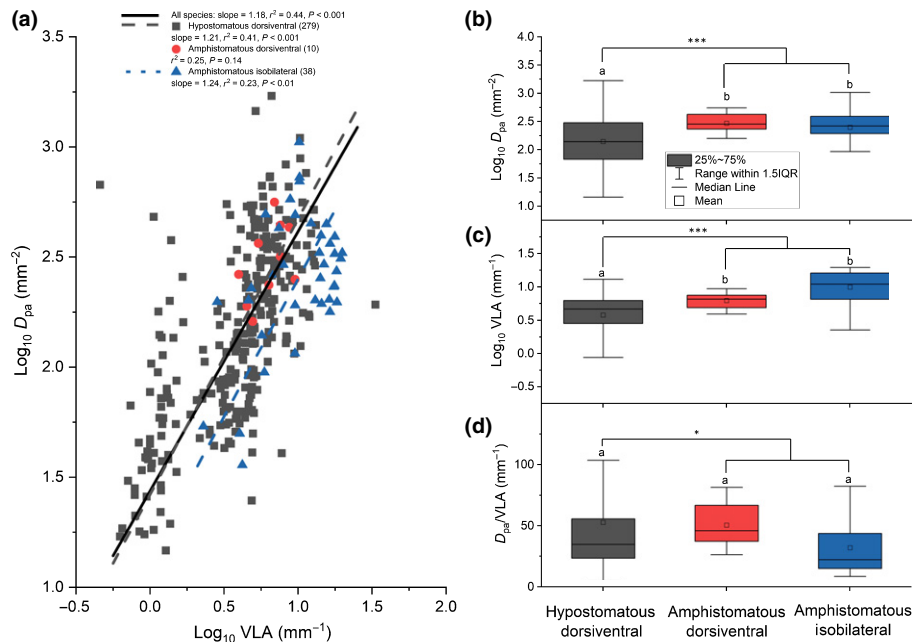
**Fig. 1** Schematised leaf cross-sections indicating lengths of pathways for water and  $\text{CO}_2$  across hypostomatous and amphistomatous leaves. (a) Thin hypostomatous and dorsiventral leaf. (b) Thick hypostomatous and dorsiventral leaf with a long diffusion pathway for  $\text{CO}_2$ . (c) Thick amphistomatous and isobilateral leaf, enabling a short diffusion pathway for  $\text{CO}_2$ . (d) In highly productive thin leaves of mesic environments, for example herbaceous crops, amphistomaty (and dorsiventrality) could also be favoured as a reduction in the pathway for  $\text{CO}_2$  enhances photosynthesis. In this scheme the difference between dorsiventral and isobilateral leaves is based on the position of the tissue performing most of the  $\text{CO}_2$  uptake (typically palisade mesophyll). The scheme focusses on  $\text{C}_3$  species. The distribution of chlorophyll-bearing cells, and hence the  $\text{CO}_2$  pathway, will differ in leaves of  $\text{C}_4$  species. Note the potential for vein depth, the distance from the vein to the lower epidermis, to vary (a, b, d). Although this scheme centres on path length, both porosity and tortuosity of the water and  $\text{CO}_2$  flow paths will influence transport.

(Rockwell *et al.*, 2014; Buckley, 2015; Buckley *et al.*, 2015, 2017; Rockwell & Holbrook, 2017), current estimates suggest that the outside-xylem pathway constitutes *c.* 30–60% of the  $K_{\text{leaf}}$  (Buckley *et al.*, 2015; Scoffoni *et al.*, 2017). Changes to the outside-xylem component of the hydraulic pathway have the largest effect on  $K_{\text{leaf}}$  during desiccation (Scoffoni & Sack, 2017). Consequently, the position of veins relative to the stomata is a key determinant of  $K_{\text{leaf}}$  and subsequent dynamics of mesophyll and guard cell water status.

Amphistomaty may provide a gas-exchange advantage as the maximum stomatal density expressed relative to the area of the stomata-bearing epidermis ( $D_s$ ) is principally constrained by space (Franks *et al.*, 2009; de Boer *et al.*, 2016b). Accordingly, the stomatal density expressed relative to the projected leaf area ( $D_{\text{pa}}$ ) of amphistomatous leaves can be double that of hypostomatous leaves. Moreover, distribution of stomata over both leaf surfaces reduces the role of the leaf boundary layer resistance in constraining leaf gas exchange, which is of particular importance for large leaves and in environments with low air flow. To explore if amphistomatous leaves utilize the additional space for stomata and make the required investments in leaf water transport tissues to meet the associated increase in transpiration, we compiled data from the literature, supplemented with our own measurements, on  $D_{\text{pa}}$  and the vein length per unit of projected leaf area (VLA) from 279 hypostomatous species and 48 amphistomatous species (Supporting Information Table S1). We focus on stomatal density, as it correlates well with the anatomical maximum stomatal conductance, despite a negative relationship between stomatal size and stomatal density (Franks & Beerling, 2009). We emphasise that both  $D_{\text{pa}}$  and VLA are expressed per projected leaf area (for hypostomatous as well as amphistomatous leaves), in line with previous studies of stomatal density (Woodward, 1987;

Hetherington & Woodward, 2003), and consistent with the broader literature on leaf functional traits, gas exchange and leaf energy balance (Perez-Harguindeguy *et al.*, 2013; Jones, 2014). Our compilation of  $D_{\text{pa}}$  and VLA shows a generic positive correlation across species (Fig. 2a), as expected due to the functional link between potential water loss and hydraulic capacity. The scaling is not significantly different between amphistomatous isobilateral species and hypostomatous species. The regression model for the amphistomatous dorsiventral species is not significant due to small sample size. Fig. 2(a) does not support the idea that amphistomatous species use two leaf sides to achieve higher total  $D_{\text{pa}}$  than hypostomatous species, but our data set does suggest that low  $D_{\text{pa}}$  and VLA are rare in amphistomatous species. We also tested the possible phylogenetic influence on the relationship between  $D_{\text{pa}}$  and VLA by calculating phylogenetic independent contrasts (PICs) (Fig. S1). This indicates a strong phylogenetic signal for VLA, but not for  $D_{\text{pa}}$  and suggests that, within lineages, VLA is less plastic than  $D_{\text{pa}}$ . Further phylogenetic analyses of the functional coordination between stomatal and vein traits will offer more evolutionary insight into the possible environments to which specific lineages are specialised.

Exploring the morphological differences between the distinct leaf morphologies in more detail we found a higher  $D_{\text{pa}}$  in species that bear amphistomatous leaves compared with those with hypostomatous leaves (Fig. 2b). Furthermore, we observed higher VLA in amphistomatous leaves compared with hypostomatous leaves (Fig. 2c), whereas we found no clear difference in VLA or  $D_{\text{pa}}$  between amphistomatous dorsiventral leaves and amphistomatous isobilateral leaves (Fig. 2b,c). We also compared the ratio of  $D_{\text{pa}}$  to VLA as an indication of the functional consequences of the observed differences in leaf morphology (Fig. 2d). This ratio,



**Fig. 2** (a) Cross-species correlation between the total stomatal density ( $D_{pa}$ , number of stomata per unit of projected leaf area) and the vein length per unit of projected leaf area (VLA). Box plots of  $D_{pa}$  (b), VLA (c), and the ratio of  $D_{pa}$  and VLA ( $D_{pa}/\text{VLA}$ ) (d), grouped by stomatal distribution and morphological orientation (refer to Fig. 1 for a detailed description). For amphistomatous leaves the  $D_{pa}$  was taken as the sum of densities on the upper and lower surfaces. In (a), the numbers in parentheses indicate the sample size of each group. The fitted lines in (a) are a standardised major axis (SMA) regression model, derived using the R package *SMATR-3* (Warton *et al.*, 2012). The intercepts but not slopes of the hypostomatous and the amphistomatous isobilateral species were significantly different. The relationship for the amphistomatous dorsiventral group, comprising only 10 species, was not significant. Tests for differences (b–d) between hypostomatous and amphistomatous (dorsiventral and isobilateral combined) species were made via two-sample *t*-tests in which significance is assigned as \*\*\*,  $P < 0.001$ . Also, tests for differences (b, c) between the three leaf types were conducted using analysis of variance (ANOVA) and the Tukey *post hoc* test with homogenous subgroups designated. Homogeneity of variance was examined with Levene’s tests. Filled boxes (c, d) represent the 25<sup>th</sup> to 75<sup>th</sup> percentiles, the whiskers represent the interquartile range, the horizontal lines represent the median, and the open boxes represent the mean. Data were acquired from 24 previously published reports on  $D_{pa}$  and VLA and our own measurements of herbaceous crops (Supporting Information Table S1). When there were multiple measurements from the same species, the arithmetic mean was calculated to represent the species average value.

effectively a measure of the number of stomata serviced per unit of vein length regardless of the stomatal distribution, was similar in hypostomatous and amphistomatous leaves, although marginally lower in amphistomatous leaves when dorsiventral and isobilateral data were pooled (Fig. 2d). Overall, this is strong empirical evidence that a greater number of stomata require a larger investment in venation but that morphological scaling is relatively similar for amphistomatous and hypostomatous leaves. In subsequent sections we discuss the potential functional benefits of these distinct leaf morphologies in the context of recent literature on leaf-interior water and CO<sub>2</sub> transport.

### Inter-vein and vein-epidermal pathways

Recent work on measuring and modelling the within-xylem and outside-xylem hydraulic conductance of leaves ( $K_x$  and  $K_{ox}$ , respectively) highlights that the length of the outside-xylem pathway is crucial in determining  $K_{leaf}$ , guard cell water status and therefore leaf gas-exchange capacity (Rockwell *et al.*, 2014; Buckley *et al.*, 2015; Scoffoni, 2015; Rockwell & Holbrook, 2017; Scoffoni *et al.*, 2017). To fully appreciate how the different leaf morphologies influence leaf-interior water transport, a three-dimensional view on the vein architecture is required. Zwieniecki & Boyce (2014), building on the theory of Noblin *et al.* (2008),

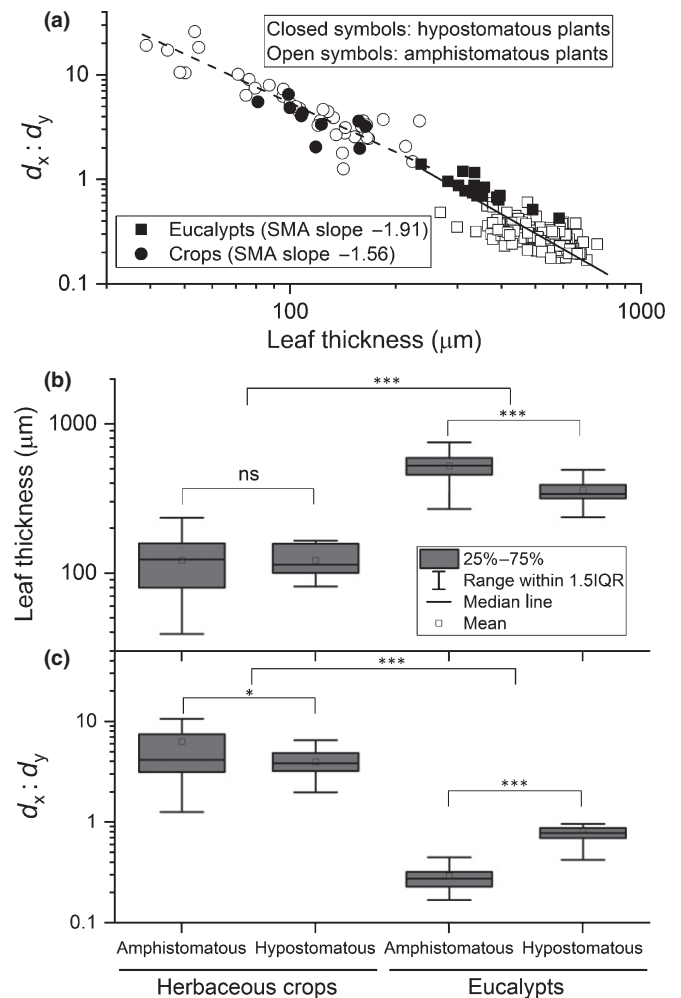
introduced the concept of a functionally optimal vein placement in leaves, in which the average distance between neighbouring veins, the inter-vein distance ( $d_x$ ), would be equal to the average distance from veins to the epidermis, the vein depth ( $d_y$ ):  $d_x : d_y \approx 1$ . Many derived angiosperms achieve a (presumed) optimal vein placement by combining a relatively high VLA with relatively thin leaves (Zwieniecki & Boyce, 2014). In other phylogenetic clades, however,  $d_x : d_y$  is significantly greater than unity (Zwieniecki & Boyce, 2014) or less than unity (de Boer *et al.*, 2016a).

One compelling observation that relates  $d_x : d_y$  to the investment in leaf structure and hydraulic function is the tendency for thick leaves to express a low  $d_x : d_y$ , shown here for a collection of eucalypts from an aridity gradient and herbaceous crops from a productive environment (Fig. 3a). Thick eucalypt leaves, which tend to be amphistomatous (Fig. 3b), exhibit a very low  $d_x : d_y$  (down to 0.2) by combining a high VLA with a long outside-xylem path length (de Boer *et al.*, 2016a) (Fig. 3c). This morphology may have evolved in thick leaves in arid environments to allow for periodically high transpiration rates, for example at times of higher soil water availability. In such circumstances, high photosynthetic rates require stomata to open, resulting in high transpiration rates supported by a high  $K_{leaf}$ . Although there are costs to developing thick amphistomatous leaves capable of high rates of water transport and gas exchange (such as a longer and potentially more

tortuous path for water and a high carbon content), there is also a benefit of reduced heat stress (Schymanski *et al.*, 2013). As a high VLA can partially compensate for a reduction in  $K_{\text{leaf}}$  caused by a large  $d_y$  (de Boer *et al.*, 2016a), this may explain the low  $d_x : d_y$  values observed here. An alternative morphology not considered in our analysis is the presence of two layers of veins, common in *Acacia* phyllodes, which may enable leaves to achieve a higher  $d_x : d_y$ .

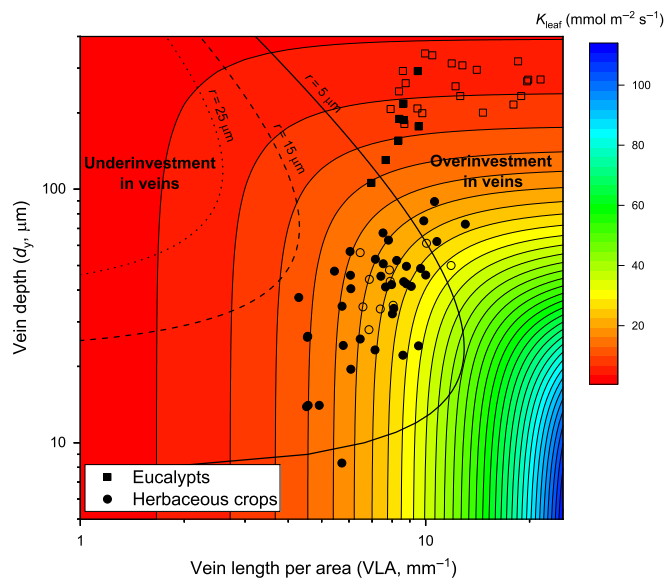
Although the thick isobilateral leaf morphology of eucalypts contrasts with the thin dorsiventral leaf morphology of herbaceous crops, (Fig. 1c vs Fig. 1d) both leaf morphologies reveal a similar relationship between  $d_x : d_y$  and leaf thickness. These patterns raise important questions on pathways and efficiencies of water and  $\text{CO}_2$  transport in leaves. In isobilateral leaves, stomatal density, mesophyll tissue anatomy and distance to veins are the same for adaxial and abaxial sides, effectively creating two functionally independent leaf surfaces (Richardson *et al.*, 2017) that are usually positioned in a near-vertical orientation (see section ‘Benefit of isobilateral amphistomatous leaves’ below). By contrast, thin herbaceous amphistomatous leaves not only have abaxial stomata with the ‘typical’ substomatal cavities and spongy parenchyma, but also adaxial stomata positioned near palisade mesophyll at similar or larger distances to veins than stomata on the abaxial surface. These leaves also usually present the upper surface to sunlight. While the (upper) adaxial stomata could potentially increase  $\text{CO}_2$  supply to the most photosynthetically active tissue (Parkhurst & Mott, 1990), they could also make this tissue prone to dehydration. Compared with the (lower) abaxial surface, hydrating the adaxial surface is likely to be more problematic, due to the greater leaf-interior water transport distance (veins are seated closer to the abaxial surface) and larger leaf-to-air vapour pressure differences. Detailed measurements of leaf hydraulics and gas exchange will be required for enhanced insight in the advantages and disadvantages of stomata on the adaxial (upper) leaf surface.

With evaporative sites in close proximity to the chlorophyll-bearing cells, amphistomatous leaves may need to closely coordinate their gas-exchange capacity and leaf hydraulics to ensure that the photosynthesising cells stay well-hydrated despite their vicinity to nearby stomatal pores. Using the semi-empirical model developed by Brodrribb *et al.* (2007) and Brodrribb & Feild (2010), covering a broad range of hypostomatous species, we can estimate the  $K_{\text{leaf}}$  of specific combinations of  $d_y$  and VLA (Fig. 4). Generally, combinations of shorter  $d_y$  and higher VLA allow the highest  $K_{\text{leaf}}$ . Overlaid on Fig. 4 are the observed values of  $d_y$  and VLA from a subset of the eucalypts and herbaceous crops presented in Fig. 3. For the range of  $d_y$  presented in Fig. 4 we modelled the optimum VLA beyond which additional investments in xylem tissue did not lead to significant further increases in  $K_{\text{leaf}}$  using the method of Rockwell & Holbrook (2017) (see Notes S1 for details). Comparing the observed data to the theoretical boundaries, representing a morphology in which  $d_y$  and VLA are optimised, suggested that the anatomy of crop leaves is approximately optimal, whereas the eucalypt leaves (with short pathways between veins, but long pathways from veins to stomata) appeared to overinvest in venation. We note that the modelling shown in Fig. 4 is based on theory for hypostomatous leaves, so the position of the amphistomatous leaves must be interpreted with caution. For a given  $d_y$



**Fig. 3** (a) Correlation between the mean ratio of the inter-vein distance ( $d_x$ ) to the mean distance from the veins to the lower (abaxial) epidermis ( $d_y$ ) ( $d_x : d_y$ ) and the leaf thickness in a collection of eucalypts and herbaceous crops. (b) Analysis of the difference in leaf thickness and (c)  $d_x : d_y$  for the species shown in (a). Values for the eucalypts are from de Boer *et al.* (2016a), representing a species-by-site dataset across an aridity gradient. For the herbaceous crops, 11 species were grown (representing 13 distinct varieties,  $n = 3$  or 4 plants per variety) in a well watered glasshouse environment (Supporting Information Table S1). Each point for the herbaceous crops therefore represents a plant. Thickness and  $d_y$  were determined from leaf cross-sections using a compound light microscope ( $n = 4$  measurements per cross-section).  $d_x$  was determined from traced images of the vein network according to de Boer *et al.* (2016a). In (a) the closed symbols represent hypostomatous leaves and the open symbols represent amphistomatous leaves. As per Fig. 2, the fitted lines in (a) are SMA regression models. The slopes, but not intercepts, of the models were significantly different ( $P < 0.05$ ). Significant differences between amphistomatous and hypostomatous leaves, and eucalypts and herbaceous crops (b, c) were determined using two-sample *t*-tests. In each case, significance is assigned: ns for  $P > 0.05$ ; \*,  $0.01 < P < 0.05$ ; \*\*,  $0.001 < P < 0.01$ ; \*\*\*,  $P < 0.001$ . Closed boxes (b, c) represent the 25<sup>th</sup> to 75<sup>th</sup> percentiles, the whiskers represent the interquartile range, the horizontal lines represent the median and the open boxes represent the mean.

and VLA, we expected a higher  $K_{\text{ox}}$  (but not  $K_x$ ) in amphistomatous leaves, due to the additional adaxial flow pathway. We therefore predicted that amphistomatous leaves increase VLA relative to  $d_y$  as that will increase  $K_x$  more than  $K_{\text{ox}}$ , especially in thick leaves, and



**Fig. 4** Vein depth, the mean distance from the veins to the lower (abaxial) epidermis ( $d_y$ ) and vein length per unit leaf projected area (VLA) for a collection of hypostomatous (closed symbols) and amphistomatous (open symbols) eucalypts and herbaceous crops (see Fig. 3 for the methodology). The total leaf hydraulic conductance ( $K_{\text{leaf}}$ ) is estimated for each combination of  $d_y$  and VLA using the semi-empirical approach of Brodrribb *et al.* (2007) and Brodrribb & Feild (2010). The solid, dashed and dotted lines are isolines for the optimal ratio between the inter-vein distance ( $d_x$ ) and  $d_y$  beyond which additional increases in VLA do not significantly increase  $K_{\text{leaf}}$ . These are presented for vein radii ( $r$ ) of 5, 15 and 25  $\mu\text{m}$ , respectively. The theoretical optimal  $d_x : d_y$  ratios are based on the approach of Rockwell & Holbrook (2017), who extended the concept of 'optimal' vein spacing based on the poly(dimethylsiloxane) gel experiments of Noblin *et al.* (2008). The mean radii of the eucalypt and herbaceous crop samples were 18.3 and 7.4  $\mu\text{m}$  respectively ( $n = 20$  veins per leaf).

will also increase  $K_{\text{leaf}}$  to scale with the higher potential transpiration rates of amphistomatous leaves. Interestingly, there is no indication in Fig. 4 that amphistomatous crop species differ from hypostomatous crop species. By contrast, in the thicker eucalypt leaves, VLA is considerably higher in amphistomatous leaves. In these thick leaves from harsh environments, high VLA may be the only feasible way to increase  $K_{\text{leaf}}$  to match high transpiration rates.

The results presented in Fig. 4 also highlight that other evolutionary pressures may outweigh the effects of leaf thickness and VLA on  $K_{\text{leaf}}$ . For example, herbaceous crops with a relatively small  $d_y$  could significantly increase  $K_{\text{leaf}}$  with a marginally higher VLA. However, as suggested by Zwieniecki & Boyce (2014), greater investment in veins would reduce the space available for photosynthetic (mesophyll) tissue, such that the additional hydraulic capacity would not be used effectively due to a lack of photosynthetic capacity, especially in thin leaves. Denser packing of mesophyll cells could potentially compensate for lost space, but this might influence  $K_{\text{ox}}$  and mesophyll conductance. Potentially, the associated loss of productivity due to fewer chlorophyll-bearing cells outweighs the additional gas-exchange capacity in thin, short-lived leaves. Similarly, a marginal reduction in  $d_y$  would significantly increase  $K_{\text{leaf}}$  in eucalypt leaves. However, veins are typically seated mid-depth in eucalypt leaves. Hence, any reduction in  $d_y$  could only be achieved by producing thinner leaves, which

could be considered disadvantageous in arid environments where thick leaves offer greater thermal and hydraulic capacitance and are associated with a longer life span.

## Prospective future research

Although the available quantitative evidence is incomplete, the distinct internal morphologies and stomatal distributions of amphistomatous and hypostomatous leaves would have functional costs and benefits in relation to leaf-interior water and  $\text{CO}_2$  transport and leaf temperature. An improved quantitative understanding of these potential costs and benefits could provide an insight into the ecophysiological relevance of amphistomaty and guide specific breeding goals for crops. Our view on specific topics to explain the potential costs and benefits of amphistomaty in this regard are discussed in the following sections by formulating three testable hypotheses. These hypotheses could stimulate future research into this important topic.

## Cost of amphistomaty

Leaves with stomata on both sides can potentially transpire more, as they have twice the transpiring surface area and a reduced boundary layer effect (see section 'Benefit of dorsiventral amphistomatous leaves'). Are there situations in which this entails a greater risk of dehydration? Stomata in upward-facing leaf surfaces (and the mesophyll tissue they service) typically experience higher radiation and greater evaporative demand, caused by higher temperatures on the sunlit surface than those of leaf surfaces facing downwards. This contrast would be most common in amphistomatous dorsiventral species that typically present their upper leaf surfaces to the sun. However, even amphistomatous isobilateral leaves with predominantly vertical leaf angles could experience one-sided illumination at low solar angles, for example in the morning and later afternoon hours. Although stomatal aperture can be adjusted to reduce the effect of the associated large leaf-to-air vapour pressure deficits, such responses lag behind rapid microclimatic fluctuations (Drake *et al.*, 2013; Schymanski *et al.*, 2013), which may expose fast-transpiring leaves in dry air to potentially harmful desiccation, especially on the sunlit surface. Unless stomatal regulation of adaxial and abaxial leaf sides is fully independent, which may not be the case (Mott & Peak, 2018), stomatal aperture may not be optimised to the conditions of each leaf surface. Estimating potential risks of exposure to desiccation, therefore, also requires knowledge of stomatal regulation and leaf internal water transport.

By locating the chlorophyll-bearing cells and stomata at opposite sides of the water supply system (adaxial and abaxial to the vein), the hypostomatous leaf morphology provides relative isolation of the most photosynthetically active tissue (the palisade mesophyll) from the evaporative demand of the outside environment. The contribution of this tissue to the total leaf transpiration rate is consistently small in hypostomatous leaves modelled by Buckley *et al.* (2017). The hypostomatous leaf morphology therefore constitutes a spatially compartmentalised hydraulic architecture that presumably helps buffer rapidly fluctuating water potentials while stomata are open for gas exchange. By contrast, the close proximity of

stomata to adaxial mesophyll in amphistomatous leaves that have otherwise similar morphology (especially thickness) requires a higher  $K_{\text{leaf}}$  to ensure that the mesophyll does not desiccate in response to short-term changes in transpiration. Although the addition of an adaxial pathway will roughly double  $K_{\text{ox}}$ , it will not reduce the vulnerability of the amphistomatous leaf to short-term changes in evaporative demand and it may require a proportional increase in  $K_x$ . We therefore hypothesise that amphistomatous leaves, compared with hypostomatous leaves of the same thickness, need a higher VLA to increase both  $K_x$  and  $K_{\text{ox}}$ , enabling higher transpiration rates without excessive tissue dehydration, especially on sunlit sides of the leaf during periods of high evaporative demand. Our observations (Fig. 4) show that thick amphistomatous leaves have a particularly high VLA. It would be of interest to investigate the relationships between VLA,  $K_x : K_{\text{ox}}$  and leaf thickness in a wide range of species and environments, including specific consideration of the amphistomatous leaf morphologies.

### Benefit of isobilateral amphistomatous leaves

The anatomical structure of leaves has a strong influence on the diffusion of  $\text{CO}_2$  from the stomata to the chloroplasts (Tomas *et al.*, 2013). However, it appears that in thick leaves with veins seated mid-depth, which are most common in arid environments, the amphistomatous and isobilateral leaf morphology constitutes a distinct advantage for  $\text{CO}_2$  transport, shortening the diffusion path length (Fig. 1c). As this morphology does not reduce the distance between veins and stomata, but does reduce the distance between stomata and mesophyll, the overall effect may be greater water-use efficiency due to increased mesophyll conductance for  $\text{CO}_2$  (Evans *et al.*, 2009; de Boer *et al.*, 2016a). Although diffusion of  $\text{CO}_2$  in the liquid phase (mesophyll cell wall to thylakoids) is much slower than in the gas phase, and ecological evidence for a clear relationship between mesophyll conductance for  $\text{CO}_2$  and leaf thickness remains elusive (Muir *et al.*, 2014), first principles suggest that for similar leaf morphologies an increase in the leaf-interior diffusion path length for  $\text{CO}_2$  should increase the resistance of the intercellular airspaces (Parkhurst, 1994).

A shorter path length for  $\text{CO}_2$  diffusion inside amphistomatous isobilateral leaves relies upon the stomata on the light-exposed surface to remain open so that sufficient gas exchange can occur (Evans & Vogelmann, 2006). The near-vertical orientation of amphistomatous and isobilateral leaves is usually regarded as an important adaptation in this respect as it reduces radiation load around midday and maximises light capture and therefore photosynthesis during early morning and late afternoon when the risks of desiccation and overheating are reduced (King, 1997). Stomata on adaxial and abaxial sides can respond independently to differences in evaporative demand (demonstrated for *Phaseolus vulgaris* and *Eucalyptus pauciflora* by Wong *et al.* (1985) and for *Eucalyptus globulus* by Richardson *et al.* (2017)). Such independent control may help to prevent excess water loss from the mesophyll positioned in close proximity of open stomata on the sunlit leaf side. Substantial differences in surface temperatures between sunlit (nontranspiring) and shaded transpiring sides of leaves have been found through detailed modelling of the leaf energy balance (0.2–

0.3°C; Buckley *et al.*, 2017) and through experiments with leaf replicas (Schymanski *et al.*, 2017). The wider occurrence of independent control on adaxial vs abaxial stomatal conductance, as well as the potential benefit in terms of water-use efficiency, remain to be determined. A critical element of research into this control is the effect of leaf flutter due to wind (Zhang *et al.*, 2016), which may equalise light intensity and temperature across the leaf surfaces at short time scales.

Thick leaves (with a high leaf mass per unit area, LMA) are often observed in arid and high-light environments, probably due to their greater longevity, defence against herbivory, and thermal and hydraulic capacitance (Schymanski *et al.*, 2013). Despite these benefits, increased thickness may potentially constrain photosynthesis in a hypostomatous morphology because it lengthens the distance for leaf-interior  $\text{CO}_2$  transport. From experiments with air and helox, Parkhurst & Mott (1990) concluded that the intercellular diffusion limitation for leaves of six amphistomatous species was only 3.5% compared with 21% for leaves of five hypostomatous species. Amphistomaty may therefore mitigate the reduced diffusive  $\text{CO}_2$  transport efficiency of thick leaves. We hypothesise that amphistomaty enables leaves to have higher leaf-interior  $\text{CO}_2$  concentrations in the airspace surrounding the mesophyll cells compared with equivalent locations in hypostomatous leaves, therefore alleviating this limitation on photosynthesis in thick leaves.

### Benefit of dorsiventral amphistomatous leaves

The advantage for amphistomaty in thin leaves of productive environments, common in herbaceous crops, is more obscure as the resistance for  $\text{CO}_2$  transport is small regardless of the entry point (Evans *et al.*, 2009). The tendency for a dorsiventral tissue asymmetry in these leaves does, however, point to potential benefits. Unlike thick amphistomatous isobilateral leaves, thin amphistomatous dorsiventral leaves tend to present their adaxial surface to light. The risk of overheating is likely to be low in these highly productive plants, due to moderate growing season temperatures and a reliable water supply. Under these conditions amphistomatous leaves can achieve much higher conductances for  $\text{CO}_2$  than hypostomatous leaves, as they effectively halve the leaf boundary layer resistance by using both leaf surfaces for gas exchange, which can be particularly significant in calm conditions and for large leaves. This may allow for greater rates of photosynthesis, which correlates with fast leaf turnover in denser canopies, therefore reducing the need for investment in tough durable leaves.

An alternative, and more generic, potential benefit of amphistomaty for leaf-interior  $\text{CO}_2$  transport stems from the prediction of Buckley *et al.* (2017) that condensation may occur around the transition between palisade and spongy mesophyll in an amphistomatous dorsiventral leaf as a result of the temperature gradient between the sunlit and shaded leaf sides. The formation of films of liquid water on mesophyll cell surfaces would increase the resistance to diffusion of  $\text{CO}_2$  into these cells, and liquid water in the intercellular airspaces could potentially block  $\text{CO}_2$  diffusion pathways or increase tortuosity. Although predicted rates of condensation are low, cumulative deposition could be

significant. It is worth noting, however, that physical evidence of accumulation of condensed water is lacking. In simulated leaves of *Helianthus annuus*, condensation was less apparent when stomata were open on both leaf surfaces, compared with the adaxial surface only (Buckley *et al.*, 2017). In reality, the transition between tissue types is more gradual, and so temperature and water vapour pressure will not change abruptly, meaning that over-saturated air is unlikely to occur in specific locations. Regardless of whether the Buckley *et al.* (2017) model reflects the actual location of over-saturation, amphistomaty may help to reduce the incidence of condensation in the intercellular spaces and, when illuminated stomata supply CO<sub>2</sub> to nearby mesophyll, provide an additional pathway for CO<sub>2</sub> without the necessity to pass through the relatively wet leaf interior. This functional explanation for amphistomaty is especially appealing for thick leaves that are likely to have even more scope for condensation, because the temperature differences between sunlit and shaded sides are likely to be larger in thick leaves compared with thin leaves.

The interaction between stomatal distribution and leaf temperature also has the potential to influence photosynthetic efficiency under specific conditions. By spatially separating evaporative cooling from light absorption and photosynthesis, hypostomatous leaves could reduce transpiration, thereby increasing longwave and sensible heat emissions (Schymanski *et al.*, 2017) and, when leaf temperature is below optimal, slightly increase photosynthetic efficiency on the slightly warmer sun-exposed leaf side. By contrast, the amphistomatous leaf morphology may achieve slight increases in photosynthetic efficiency when leaf temperature is above optimal (as is more likely in arid environments) by spatially coupling evaporative cooling to the photosynthetically active tissue. A potentially larger favourable effect at high air temperatures is offered by the more effective evaporative cooling of amphistomatous leaves compared with hypostomatous leaves, because their relevant boundary layer conductance is increased two-fold, although this presumes ample water availability to sustain high transpiration rates. We hypothesise that leaf temperature differences between the irradiated and shaded surfaces are significantly higher in hypostomatous leaves compared with their amphistomatous counterparts, at similar leaf thickness, with potentially important implications for liquid and vapour transport.

## Conclusions

Our review of recent literature in relation to the distinct amphistomatous leaf morphologies highlights the potential benefit of this leaf type for improving the supply of CO<sub>2</sub> to chloroplasts. This improved supply may serve as an adaptive advantage in habitats occupied by fast-growing herbaceous annuals and slow-growing sclerophyllous perennials. The potential for amphistomaty to reduce temperature gradients and potentially prevent condensation in the intercellular spaces may explain the occurrence of this morphology in contrasting habitats that select for either high leaf conductance or thick leaves. Disadvantages to the amphistomatous morphology include the need to increase  $K_{\text{leaf}}$  via additional investments in the xylem to ensure that stomata of both surfaces are supplied with water.

Renewed interest in the study of leaf structure–function relationships and, in particular, those involving hydraulics and gas exchange, will improve our understanding of plant adaptations and assist with targeting traits for enhanced crop productivity and water-use efficiency in different environments. Further research on these relationships in grasses will be of significant interest as specific vein and stomatal morphologies are a prerequisite for suites of leaf traits aimed at improving productivity in these important agricultural species.

## Data availability

The raw data underlying each figure is deposited in the following perpetual data repository: <https://research-repository.uwa.edu.au/en/datasets/> (doi: 10.26182/5c4fc6735e1da).

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## Author contributions

PLD conceived the study, collected, analysed and interpreted the data and wrote the manuscript. HJdB, EJV and SJS interpreted the data and wrote the manuscript.

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

**Fig. S1** Phylogenetic independent contrast of stomatal density ( $D_{pa}$ ) and vein length per unit area (VLA).

**Notes S1** Predicted optimal relationship between VLA and vein-epidermal distance.

**Table S1** Database compiled from the published literature on vein length per unit area (VLA) and stomatal density ( $D_{pa}$ ).

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**Key words:** amphistomatous, hydraulic conductance, hypostomatous, leaf thickness, mesophyll conductance, stomata, stomatal ratio, vein.

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