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## Research paper

# Amphistomatic leaf surfaces independently regulate gas exchange in response to variations in evaporative demand

Freya Richardson<sup>1</sup>, Timothy J. Brodribb<sup>1,2</sup> and Gregory J. Jordan<sup>1</sup>

<sup>1</sup>School of Biological Sciences, University of Tasmania, Hobart, Tasmania 7001, Australia; <sup>2</sup>Corresponding author (Timothy.Brodribb@utas.edu.au)

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The occurrence of amphistomatic leaves (stomata on both surfaces) versus hypostomatic leaves (stomata limited to the lower or abaxial surface) has strong associations with environment. Amphistomy provides the advantage of higher conductance of CO<sub>2</sub> for photosynthesis, however, unless the stomata on both leaf surfaces can be independently controlled in response to environmental cues, amphistomy may lead to inefficient gas exchange. While previous studies have found evidence that stomata can operate independently across and between surfaces of dorsiventral leaves, we investigate whether an independent stomatal response can be induced for isobilateral leaves by largely natural conditions. Here, we exposed surfaces of isobilateral, amphistomatic *Eucalyptus globulus* Labill. leaves to natural diurnal variation in differential evaporative demand, using leaf orientation to drive differences in irradiance and heat load on leaf surfaces. We identified preferential closure of stomata on the surface exposed to higher irradiation (and therefore evaporative demand) during the afternoon under natural conditions and similarly induced differential stomatal closure under experimental conditions in the laboratory. The differential response confirms that sufficient hydraulic isolation exists for independent stomatal response to occur between surfaces of amphistomatic, isobilateral leaves, and importantly, we show that natural conditions can induce surface-specific stomatal closure.

**Keywords:** amphistomatous, hypostomatous, stomatal ratio, vapour pressure deficit.

## Introduction

Given the fundamental role that stomata play in regulating the opposing demands of carbon uptake and limiting water loss from within the leaf (Cowan and Farquhar 1977), changes in stomatal traits can show adaptive relationships to the environmental conditions in which plants grow. Stomatal traits that have been identified as adaptive or beneficial under specific conditions include changes in stomatal density ( $S_D$ ) with both light environment (Salisbury 1928, James and Bell 2000a) and CO<sub>2</sub> concentration (Woodward and Bazzaz 1988, Hetherington and Woodward 2003), and stomatal size and shape (Jordan et al. 2015), which may alter dynamic response time (Drake et al. 2013).

The location of stomata on the leaf surface is also likely to impact significantly on leaf function and there is evidence to suggest that distribution of stomata between the two surfaces of a

laminar leaf offers an adaptive advantage under certain environmental conditions (Jordan et al. 2014, Muir 2015). Most plant species have leaves that are either hypostomatic (i.e., with stomata restricted to the lower (abaxial) surface) or amphistomatic (with stomata on both abaxial and adaxial surfaces) (Muir 2015). This variation in stomatal distribution between leaf surfaces can be expressed as stomatal ratio ( $S_R$ ), which is the ratio of the frequency of adaxial to abaxial stomata.

Understanding how  $S_R$  influences the physiological processes of leaf gas exchange and water use is critical in the interpretation of  $S_R$  as a functional trait. Stomatal ratio, independent of  $S_D$ , has potential application as a mechanistic proxy for interpreting paleo-environments (Jordan et al. 2014) as well as in commercial crop selection with significant trends towards amphistomy observed in several domesticated crop species historically under selection for higher photosynthetic rates and water use (Milla et al. 2013,

Fanourakis et al. 2015). While there is some knowledge of the genetic control of stomatal development and patterning in species such as *Arabidopsis thaliana* (Dow et al. 2014), a high degree of plasticity in  $S_R$  in response to environmental conditions occurs in species such as *Ambrosia cordifolia* (Mott and Michaelson 1991) and *E. globulus* (James and Bell 2000a).

The  $S_R$  for amphistomatic leaves can be uneven, however, more evenly amphistomatic leaves (i.e.,  $S_R$  close to one) have been strongly associated with high light and/or dry environments (Jordan et al. 2014, Muir 2015). Amphistomy has also been associated with fast-growing species or herbaceousness (Muir 2015). These correlations suggest that the functional advantages of amphistomy can be primarily attributed to increased  $\text{CO}_2$  diffusion (Parkhurst 1994, Muir et al. 2014) and efficiencies in investment in vascular tissue due to the ability to simultaneously supply water to both leaf surfaces (Brodribb et al. 2013, Buckley et al. 2015). Hypostomatic leaves ( $S_R$  close to zero) are found throughout a range of light environments, are considered to be advantageous in light-limited conditions (Mott et al. 1982, Jordan et al. 2014) and have been correlated with a lower incidence of pathogen infection (McKown et al. 2014).

While the increased  $\text{CO}_2$  uptake associated with amphistomy should benefit both fast-growing plants and those in high light environments when sufficient water is available (Mott et al. 1982, Muir 2015), simultaneous regulation of stomata on both leaf surfaces may lead to inefficient gas exchange, a potential disadvantage of amphistomy. Optimization of the ratio of transpiration to assimilation is a general phenomenon in angiosperms (Cowan and Farquhar 1977, Farquhar and Richards 1984, Brodribb and Hill 1998) and requires stomatal adjustment to changes in evaporative demand. Assuming that differences in irradiance drive a thermal gradient between leaf surfaces (Rockwell et al. 2014), without independent control of stomata on leaf surfaces, amphistomatic leaves would be unable to dynamically optimize gas exchange throughout the day.

An amphistomatic leaf may display differences in stomatal conductance to water vapour ( $g_s$ ), if the surfaces vary in conditions that influence the degree of stomatal openness/closure. Important factors affecting  $g_s$  include irradiance, boundary layer conductance and leaf to air vapour pressure deficit ( $D$ ), which determine evaporative demand, as well as synthesis and/or transport of the phytohormone abscisic acid (ABA). Under natural conditions, differences in these environmental parameters would be particularly strong for leaves displayed vertically, with the light distribution changing markedly between leaf surfaces during the day, altering the distribution of heat and evaporative potential within the leaf (Rockwell et al. 2014). For such leaves, the degree of hydraulic connectivity between the two leaf surfaces is important, influencing whether stomata on the leaf surfaces have potential to respond independently to differences in  $D$ . If surfaces were not hydraulically isolated, we would expect changes in  $D$  to induce changes in the bulk leaf water potential,

resulting in synchronous stomatal responses on both surfaces, unless asymmetrical biosynthesis of ABA was occurring in close proximity to leaf surfaces.

Patchy stomatal closure in heterobaric leaves (Mott et al. 1993) provides evidence for hydraulic isolation across leaf surfaces. Hydraulic isolation between surfaces has also been demonstrated under laboratory conditions in some species with dorsiventral leaves: in *Vicia faba* L. (Mott and Parkhurst 1991) using Helox and air to create differences in evaporative demand between leaf surfaces, and in *V. faba* and *Xanthium strumarium* L. in response to a humidity gradient (Mott 2007). Such hydraulic isolation has also been modelled by Buckley et al. (2015) for *Helianthus annuus* L. (also dorsiventral) with unequal transpiration rates between the leaf surfaces.

The existence of hydraulic isolation between the surfaces of the isobilateral leaves that characterize many amphistomatic trees and shrubs is unknown, as are the implications of hydraulic independence between surfaces under field conditions. A previous study measuring the stomatal conductance of individual leaf surfaces of isobilateral *Eucalyptus globulus* Labill. ssp. *globulus* leaves (Pereira et al. 1987) reported slightly higher  $g_s$  for abaxial leaf surfaces, with largely synchronous changes in  $g_s$  for both surfaces throughout the day if leaves were allowed to remain in their natural, random leaf orientation. The  $g_s$  of both surfaces was measured in response to total incident photosynthetically active radiation ( $Q$ ) to identify potential differences in stomatal behaviour between adaxial and abaxial stomata. Another study focusing on the behaviour of stomata on individual leaf surfaces under field conditions (Lange et al. 1987) found a similar response in diurnal  $g_s$  between the surfaces of the phyllodes of two species of *Acacia*, with small differences in  $g_s$  attributed to inherent differences or possibly differential long-term acclimation to light between the surfaces. While both these studies measured  $g_s$  of individual leaf surfaces under natural conditions, neither identifies whether the two surfaces of amphistomatic leaves regulate their stomata independently in response to changing conditions, allowing for amphistomatic leaves to optimize  $\text{CO}_2$  uptake when water availability is limited.

Here, we test the response of stomata on isobilateral leaf surfaces to differential evaporative demand between surfaces under natural and experimental conditions. We investigate whether the stomata on different surfaces of isobilateral, amphistomatic leaves of *E. globulus* respond synchronously when exposed to natural and artificial changes in  $D$ , or whether surfaces respond individually to conditions under natural irradiance, temperature and humidity.

## Materials and methods

Fully developed adult foliage from 10, 4-year-old *E. globulus* plants originating from three provenances in Victoria and Tasmania, Australia, was used for all components of this study.

Plants were grown, at latitude 42°54'17.9"S and longitude 147°19'27.1"E, in 25 cm diameter pots outside the glasshouse facility at the University of Tasmania and watered daily. This species was selected as the adult leaves (i.e., those of adult plants) are large, amphistomatic and isobilateral (James and Bell 2000a), and mostly hang vertically such that both leaf surfaces tend to receive an equivalent total amount of light through a daily cycle (James and Bell 2000b).

### Stomatal characteristics

To confirm that the  $S_D$  and  $S_R$  of the specific plants used for this study were within the range previously reported for the species, stomatal counts were undertaken on a total of 17 healthy, fully expanded leaves harvested from the 10 potted plants. Peels of both abaxial and adaxial surfaces were made using clear nail polish, and were mounted on glass slides. Five fields of view (FOV) ( $0.563 \text{ mm}^2$ ) per surface of each leaf were photographed using a Nikon DS Fi2 camera (Melville, NY, USA) mounted on a Leica DM 1000 microscope (Nussloch, Germany). The  $S_D$  per  $\text{mm}^2$  leaf area was counted from digital images using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The total stomatal density ( $S_{D\text{total}}$ ) reported for each leaf is the sum of the mean abaxial stomata  $\text{mm}^{-2}$  ( $S_{D\text{ad}}$ ) and adaxial stomata  $\text{mm}^{-2}$  ( $S_{D\text{ad}}$ ). Stomatal ratio was calculated as:

$$S_R = S_{D\text{ad}} / S_{D\text{ad}}$$

Length of the stomatal complex was measured as a proxy for stomatal size, in order to determine potential differences in stomatal size between the abaxial and adaxial surfaces. A mature leaf was randomly selected from each of four of the potted plants for stomatal length measurements and leaf thickness sections. Paradermal sections of  $\sim 5 \text{ mm}^2$  were prepared for both leaf surfaces following the protocol described in (Carins Murphy et al. 2012), two to three FOV ( $0.141 \text{ mm}^2$ ) were photographed with the length of 20 stomata from each surface of each leaf measured from digital images using ImageJ Software. Leaf thickness measurements were made using ImageJ from digital images of slide-mounted leaf cross sections (FOV  $0.563 \text{ mm}^2$ ).

### Stomatal conductance to water vapour responses

Two experiments were undertaken to assess whether the two leaf surfaces showed differential stomatal responses. The first was to observe changes in  $g_s$  in near natural field conditions as irradiation of the surfaces changes through the day. The second was to assess  $g_s$  (as a direct measure of stomatal opening), by manipulating evaporative demand using asymmetric temperature and irradiance in the laboratory. These methods were used in order to drive a gradient in evaporative demand between the leaf surfaces to compare stomatal response on one leaf surface relative to the response on the opposite surface.

**Leaf orientation and light direction** To determine whether conditions similar to those experienced in natural systems can induce an independent stomatal response for leaf surfaces, potted plants were placed in an open sunny area on 6 days between April 2015 and January 2016. On each day, two to four adult leaves per plant were held in place using fishing-line strung on a steel frame. The leaves were aligned so that the lamina of each leaf was vertical and in the north–south plane. As a result, one leaf surface (east facing) received direct sunlight during the morning and the opposite surface (west facing) received direct sunlight during the afternoon, and neither surface received direct sunlight during the middle of the day. The leaf surfaces were therefore exposed to differential irradiance, heat loads and vapour pressure deficits (due to differential heating of the exposed leaf surface), and these alternated during the day. The orientations of the leaf surfaces are hereafter referred to as east (E) and west (W), respectively. Adaxial and abaxial surfaces were randomly assigned to the E or W orientation on each day on which data was collected. As a result any systematic differences between surfaces cannot be attributed to anatomical variation or differences in circadian rhythm between surfaces.

The  $g_s$  was measured only on clear days with minimal cloud cover using an infra-red gas analyser (IRGA) (LI-6400; LI-COR Biosciences, Lincoln, NE, USA). A  $6 \text{ cm}^2$  portion of each leaf measured was enclosed in the IRGA chamber, completely filling the chamber space. Instantaneous gas exchange was logged as soon as conditions within the cuvette had stabilized ( $\sim 30 \text{ s}$ ), and prior to any leaf response to chamber conditions. The  $g_s$  was measured for each surface of each leaf, and for the total leaf (that is both surfaces measured together) at 10:00–11:00 h, 12:00–13:00 h and 14:00–15:00 h. Chamber conditions for the IRGA were ambient  $\text{CO}_2$  and relative humidity,  $Q$  of 1200–1500  $\mu\text{m quanta m}^{-2} \text{ s}^{-1}$ , and cuvette block temperature of 24–25 °C. The  $g_s$  of individual leaf surfaces was measured with the target surface facing upward in the IRGA chamber, and a minimum of four layers of clear polyethylene cling film (GLAD®ClingWrap) completely covering the area of cuvette beneath the leaf, blocking lower leaf surface. The porosity of this plastic film was lower than the sensitivity of the IRGA for  $g_s$ .

The  $Q$  intercepting the leaf surface was measured using a Li-Cor LI190SZ quantum sensor (Li-Cor Biosciences) connected to the IRGA chamber that was positioned directly adjacent to the leaf surface with the same orientation for instantaneous light measurements prior to measuring  $g_s$ .

Maximum daily temperatures, and air temperatures and % relative humidity for 9:00 and 15:00 h for days on which measurements were taken were obtained from the Bureau of Meteorology (Australian Government Bureau of Meteorology 2016) for the Ellerslie Road weather station (station 094,029),  $\sim 1.3 \text{ km}$  from the site.  $D$  at 9:00 and 15:00 h were calculated from the measurements for each day on which  $g_s$  was measured.

**Manipulation of evaporative demand** In the laboratory, leaf surfaces were exposed to differential evaporative demand in order to establish whether leaf surfaces were sufficiently hydraulically isolated from one another for stomata to respond independently. Leaves attached to potted plants were enclosed in a small-volume, purpose-built Perspex chamber, which made it possible to expose the entire leaf to relatively rapid changes in humidity. The Perspex chamber had two inlets at the base of one end that were connected to an industrial compressed air cylinder and two outlets at the base of the other that were connected to dew point hygrometer (Dewprime I, EdgeTech Instruments, Hudson, MA, USA). A Schott KL1500 fibre optic light source above the chamber lit the upper leaf surface to a  $Q$  of  $\sim 1200 \mu\text{m quanta m}^{-2} \text{s}^{-1}$ , and two lamps with 60 w blue globes were used to control the upper leaf temperature. Upper leaf temperature was monitored via a fine wire thermocouple in contact with the leaf surface in order to calculate an approximate (due to potential for radiation to effect the temperature of the thermocouple)  $D$  within the chamber. The upper leaf temperature was maintained between 26 and 27 °C throughout both 'mild' and 'high'  $D$  treatments. Approximate  $D$  was calculated from the saturation vapour pressure at the upper leaf temperature and the dew point temperature of the air exiting the chamber.

The  $g_s$  was measured for each leaf surface with an IRGA as described above. Each leaf had a minimum of 30 min to adjust to chamber conditions and for a stomatal response to  $D$  to commence, prior to measurements being taken. Thirty minutes was considered an adequate time based on previously reported dynamic stomatal response of angiosperms to  $D$  change (McAdam and Brodribb 2016). Leaves were removed from the chamber for measurement of  $g_s$  on each surface. This measurement took  $\sim 1\text{--}2$  min to complete. The  $g_s$  was measured initially after a mild  $D$  treatment of between 1.5 and 1.6 kPa, leaves were then returned to the whole leaf chamber ensuring that the original orientation was maintained. The leaf was then exposed to a high  $D$  treatment of 2.9 kPa for 30 min before re-measuring  $g_s$ . Finally, leaves were returned to the mild  $D$  of between 1.5 and 1.8 kPa for 30 min to determine whether stomata were re-opening following the exposure to increased  $D$ . The  $g_s$  was measured with chamber conditions of ambient  $\text{CO}_2$ ,  $Q$  of  $1500 \mu\text{m quanta m}^{-2} \text{s}^{-1}$  and cuvette block temperature of 25 °C.

To measure the difference in leaf surface temperature between the upper and lower leaf surfaces, a total of five additional leaves (from two plants) were enclosed within the perspex chamber with fine wire thermocouples placed in contact with both the upper and lower leaf surfaces. The upper leaf surface was illuminated (as described above) with upper leaf surface temperature maintained between 26 and 27 °C. Both upper and lower leaf temperatures were recorded following stabilization of the dew point, and leaf temperatures. Based on calculations using the upper leaf temperature,  $D$  ranged from 2.9 to 3.2 kPa.

**Temperature, vapour pressure deficit and stomatal conductance** A likely explanation for independent stomatal regulation of leaf surfaces during the day would be if there were thermal gradients in the leaf that caused the two leaf surfaces to experience different evaporative demand (leaf to air vapour pressure gradients). To determine whether the difference in  $g_s$  observed between the leaf surfaces could be attributed to an irradiance-driven temperature gradient through the leaf, the relationship between  $g_s$  and  $D$  was determined in the laboratory using the IRGA, with a single leaf surface blocked, as described in the 'Leaf orientation and light direction' methods, under a range of  $D$  conditions. In this case we used leaves from 4 of the same 10 well-watered, potted plants used in other experiments. We measured  $g_s$  of an individual leaf surface ( $n = 17$ ) using 'low to high'  $D$  transitions in the IRGA ( $D$ s ranging from 0.86 to 3.4 kPa). Leaves were exposed to  $D$  conditions for a minimum of 30 min prior to recording  $g_s$ . Chamber conditions were ambient  $\text{CO}_2$ ,  $1500 \mu\text{m quanta m}^{-2} \text{s}^{-1}$  and cuvette block temperature of 26 °C.

The relationship between  $g_s$  and  $D$  for single leaf surfaces was used to calculate the approximate magnitude of temperature difference required to account for differences in  $g_s$  observed between leaf surfaces if differential heating of leaf surfaces was causing independent closure.

### Statistical analysis

Paired  $t$ -tests were made to determine significant differences in  $S_D$  and stomatal length between the abaxial and adaxial surfaces, and changes in ratio of  $g_s$  undertaken by different leaf surfaces (upper vs lower) following  $D$  change. Unpaired  $t$ -tests were used to compare the ratio of  $g_s$  undertaken by the adaxial and abaxial surfaces facing E, relative to the  $g_s$  of the total leaf throughout the day. Linear regression models were fitted to pairwise scatter plots to analyse the relationship between the sum of  $g_s$  measured for each individual surface, and the  $g_s$  measured for the entire leaf. ANOVAs were used to compare relative changes in  $g_s$  of the leaf surfaces. These ANOVAs were based on the square root of the ratio of  $g_s$  between morning and midday ( $\sqrt{g_{s \text{ am/mid}}}$ ) and between midday and afternoon ( $\sqrt{g_{s \text{ mid/pm}}}$ ), and had leaf orientation (E vs W), day of observation, and the interaction between orientation and day as factors. The transformation was necessary to satisfy the assumptions of the ANOVA. The relationship between  $g_s$  and  $D$  for a single leaf surface was fitted with a generalized additive model, and used to infer the temperature difference between leaf surfaces required for  $D$  to explain the difference in  $g_s$  measured between surfaces. Analyses were undertaken in R (R Development Core Team 2014).

## Results

### Stomatal density and stomatal ratio

The adult *E. globulus* leaves measured were amphistomatic, with stomata present on both adaxial and abaxial surfaces

(Figure 1a). Mean leaf thickness was 385  $\mu\text{m}$ . The mean abaxial stomatal density  $S_{D_{ab}}$  (157  $\text{mm}^{-2}$ ) was significantly greater than mean adaxial stomatal density  $S_{D_{ad}}$  (105  $\text{mm}^{-2}$ ) ( $P \leq 0.0001$ ), with a mean  $S_{D_{total}}$  of 261  $\text{mm}^{-2}$ . There was no significant difference in stomatal complex length between the abaxial and adaxial surfaces ( $P = 0.5$ ), which was used as a proxy to identify potential differences in stomatal size. The mean length of the stomatal complex was 49.3  $\mu\text{m}$ . Despite a >2-fold range in  $S_{D_{total}}$  within the sampled leaves,  $S_R$  remained between 0.57 and 0.76, with a mean value of 0.67 (Figure 1b).

To validate the efficacy of the method used for measuring the  $g_s$  of individual surfaces, that is blocking the 'non-target' leaf surface with cling-film within the IRGA cuvette,  $g_s$  measured for the whole leaf with neither surface blocked ( $g_{s_{total}}$ ) was compared with total  $g_s$  calculated by summing  $g_s$  for the two sides measured individually ( $g_{s_{ad}} + g_{s_{ab}}$ ). The calculated  $g_{s_{ad}} + g_{s_{ab}}$  closely corresponded with  $g_{s_{total}}$  ( $r^2 = 0.95$ ,  $P \leq 0.0001$ , slope = 0.90, which was not significantly different from 1; Figure 2). This validation was done for all  $g_s$  measurements under natural conditions and in the laboratory (Figure 2), as well as separately for gas exchange measurements following manipulation of evaporative demand in the laboratory, where  $g_{s_{ad}} + g_{s_{ab}}$  also closely corresponded with  $g_{s_{total}}$  ( $r^2 = 0.99$ ,  $P \leq 0.0001$ , slope = 1.02, which also was not significantly different from 1).

### Stomatal conductance to water vapour responses

**Leaf orientation and light direction under natural conditions** In leaves exposed to natural diurnal variation in light angle,  $D$  and temperature,  $D$  was consistently higher during the afternoon than the morning (Figure 4) over the 6 days of the experiment. The mean  $g_s$  for leaves on each day was highest during the morning and decreased throughout the rest of the day (Figure 3). This decrease in  $g_s$  represents overall closure of stomata throughout the day from the morning to the afternoon.

The  $g_{s_{E/W}}$  is the ratio of  $g_s$  between the east surface (receiving light in the morning) and west surface (receiving light in the afternoon) and expresses the contribution to the  $g_{s_{total}}$  undertaken by the individual leaf surfaces, and hence whether the decrease in total  $g_s$  throughout the day was ascribed to stomata closing equally on both sides or preferential closure of stomata on one surface (Figure 4). The  $g_{s_{E/W}}$  varied between the individual leaves measured on all days.

On all mornings, stomata on both surfaces were open and therefore contributed to the  $g_{s_{total}}$ , with mean  $g_{s_{E/W}}$  ranging from 0.69 (Figure 4f) to 1.45 (Figure 4b). The mean  $g_{s_E}$  was similar to mean  $g_{s_W}$  during the morning despite the east surface receiving direct sunlight.

There was no evidence for differential responses of the leaf surfaces from morning to midday, with no consistent trend in mean  $g_{s_{E/W}}$  and both minor increases (Figure 4c, e and f) and decreases in  $g_{s_{E/W}}$  (Figure 3a, b and d). The  $\sqrt{g_{s_{am/mid}}}$  (data were transformed to satisfy the assumptions of the ANOVA) varied significantly for individual leaves between days ( $P = 0.003$ ), with no significant difference between surfaces orientated east and west ( $P = 0.092$ ). Across all measurements, interaction between day and surface orientation was not significant for the  $\sqrt{g_{s_{am/mid}}}$  ( $P = 0.18$ ), nor  $\sqrt{g_{s_{mid/pm}}}$  ( $P = 0.22$ ).

However, there was clear evidence for differential stomatal closure in the afternoon, in all cases (6/6 days) the ratio mean  $g_{s_{E/W}}$  increased from midday to afternoon, implying preferential closure of stomata on the west (irradiated) surface during the afternoon (Figure 4). The  $\sqrt{\text{ratio of } g_s}$  from midday to afternoon measured for individual leaves varied significantly between both days ( $P \leq 0.0001$ ), and notably, orientation ( $P \leq 0.0001$ ), with no interaction effect (indicating that the response was relatively consistent across days). There was a significant relationship between the afternoon  $D$  across the days (Figure 4) and the

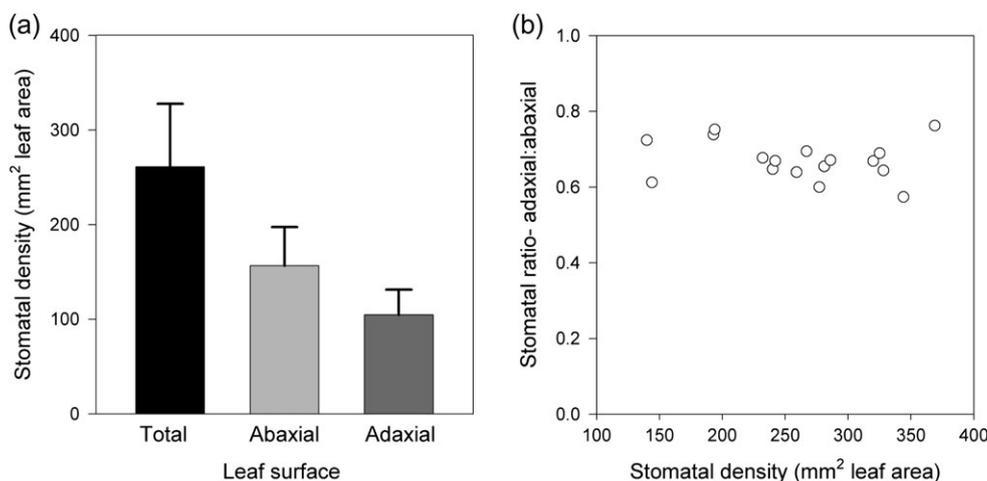


Figure 1. Stomatal density of *E. globulus* leaves and allocation of stomata between leaf surfaces ( $n = 17$ ). Mean stomatal densities ( $\pm$  standard deviation) (a) and relationship between total stomatal density and stomatal ratio (b).

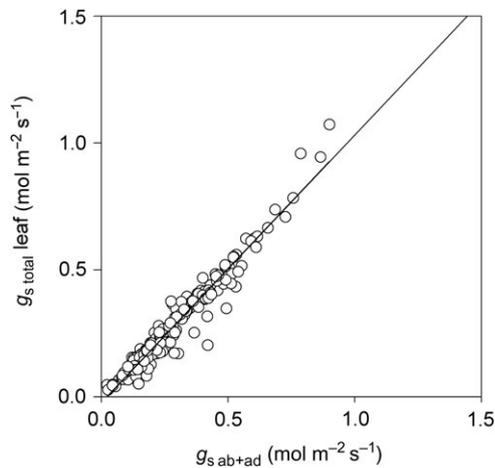


Figure 2. Stomatal conductance for total leaf ( $g_{s\text{ total}}$ ) versus sum of abaxial and adaxial surface ( $g_{s\text{ ab+ad}}$ ). Data from field leaf orientation measurements ( $n = 200$ ), over 6 days in April 2015–January 2016.

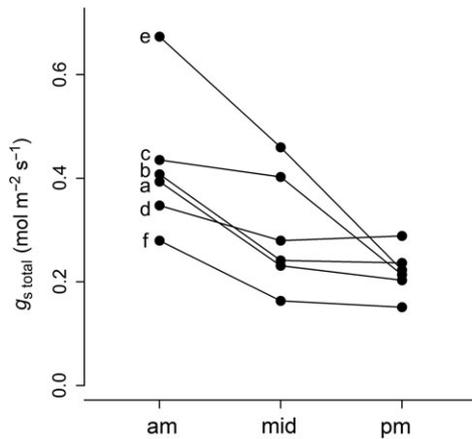


Figure 3. Mean  $g_{s\text{ total}}$  throughout the day measured between 10:00 and 11:00 h (am), 12:00 and 13:00 h (mid), and 14:00 and 15:00 h (pm). Letters a–f represent individual days between April 2015 and January 2016 and correspond with letters and dates used in Figure 4. Values are the mean of six leaves measured on days 'b', 'c', 'e' and 'f', five leaves on day 'a', and four leaves on day 'd'.

change in  $g_{s\text{ E/W}}$  from midday to afternoon, with higher afternoon  $D$  associated with greater closure of the west (exposed to direct sunlight) surface relative to the east surface ( $g_{s\text{ E/W}}$ ) throughout the afternoon ( $r^2 = 0.67$ ,  $P \leq 0.04$ ).

Based on the relationship between the  $g_s$  and  $D$  for a single *E. globulus* leaf surface (see Supplementary Data at *Tree Physiology Online*), the approximate difference in leaf surface temperature required for  $D$  to fully explain the differences in  $g_s$  between surfaces during the afternoon ranged from 1.1 °C (day a, 4 April) to 3.4 °C (day e, 13 Jan) (Table 1).

The abaxial and adaxial leaf surfaces were randomly assigned to face either east (22 leaves) or west (22 leaves). In order to establish whether the greater density of stomata on the abaxial

surface (Figure 1a), or inherent differences between surfaces influenced the  $g_{s\text{ E/W}}$  recorded, the  $g_{s\text{ E}}$  relative to the  $g_{s\text{ total}}$  for leaves with east facing abaxial surfaces was compared with values for the leaves with east facing adaxial surfaces. There were no significant differences in  $g_{s\text{ E/total}}$  between abaxial and adaxial surfaces during the morning ( $P = 0.1$ ,  $t = -1.65$ ) mid-day ( $P = 0.98$ ,  $t = 0.02$ ) or afternoon ( $P = 0.77$ ,  $t = -0.28$ ).

**Manipulation of evaporative demand** Stomata on both upper and lower surfaces of leaves enclosed in a Perspex chamber in the laboratory opened for all individual leaves under 'Mild 1'  $D$  conditions (1.5–1.6 kPa), although there was considerable variation in the ratio of  $g_{s\text{ upper/lower}}$  between leaves (Figure 5c).

Upon the increase to 'High'  $D$  (2.9 kPa), with light/heat applied to the upper surface only, there was a significant decrease in  $g_{s\text{ upper}}$  ( $P = 0.02$ ) signifying partial closure of upper stomata (Figure 5a). However, there was no associated decrease in  $g_{s\text{ lower}}$  ( $P = 0.74$ ) (Figure 5b), causing the ratio  $g_{s\text{ upper/lower}}$  to decrease from 'Mild 1' to 'High'  $D$  ( $P = 0.02$ ) (Figure 5c). The mean difference in  $g_s$  between the upper and lower leaf surfaces under 'High'  $D$  was  $0.086\text{ mol H}_2\text{O m}^{-2}\text{ s}^{-1}$ .

A subsequent decrease in  $D$  from 'High' to 'Mild 2' (1.5–1.8 kPa) resulted in some re-opening of the upper stomata ( $P = 0.03$ ), with an increase in  $g_{s\text{ upper}}$  (Figure 5a). The change in  $g_{s\text{ upper/lower}}$  from 'High' to 'Mild 2'  $D$  suggests a degree of equalization of  $g_s$  between surfaces (Figure 5c), however this change was not statistically significant ( $P = 0.1$ ). Following the closure of upper stomata induced with high  $D$ , the upper leaf surface did not reopen to the same mean  $g_s$  as prior to the closure suggesting a small degree of hysteresis in the recovery.

Upper and lower leaf temperature varied between 0.18 and 0.99 °C, with the temperature of the irradiated surface consistently higher than that of the lower, shaded surface. The greatest difference in temperature between the surfaces (0.99 °C), was recorded with a  $D$  calculated for the upper leaf surface of 2.4 and 2.2 kPa for the lower surface.

The mean difference in temperature recorded between the leaf surfaces was 0.64 °C, the maximum temperature difference recorded between leaf surfaces was 1.25 °C. Based on the relationship between the  $g_s$  and  $D$  for a single *E. globulus* leaf surface (see Supplementary data at *Tree Physiology Online*), the difference in leaf surface temperature required for  $D$  alone, to fully explain the differences in  $g_s$  between surfaces under the 'High'  $D$  treatment, ranged from 0.23 °C (L2) to >5 °C (L4) (Table 2).

## Discussion

Differential stomatal responses between the individual surfaces of isobilateral amphistomatic leaves occurred when leaves were exposed to both artificial and natural changes in irradiance and  $D$ . In general, stomata on the surface exposed to higher evaporative demand closed relatively more than stomata on the opposite

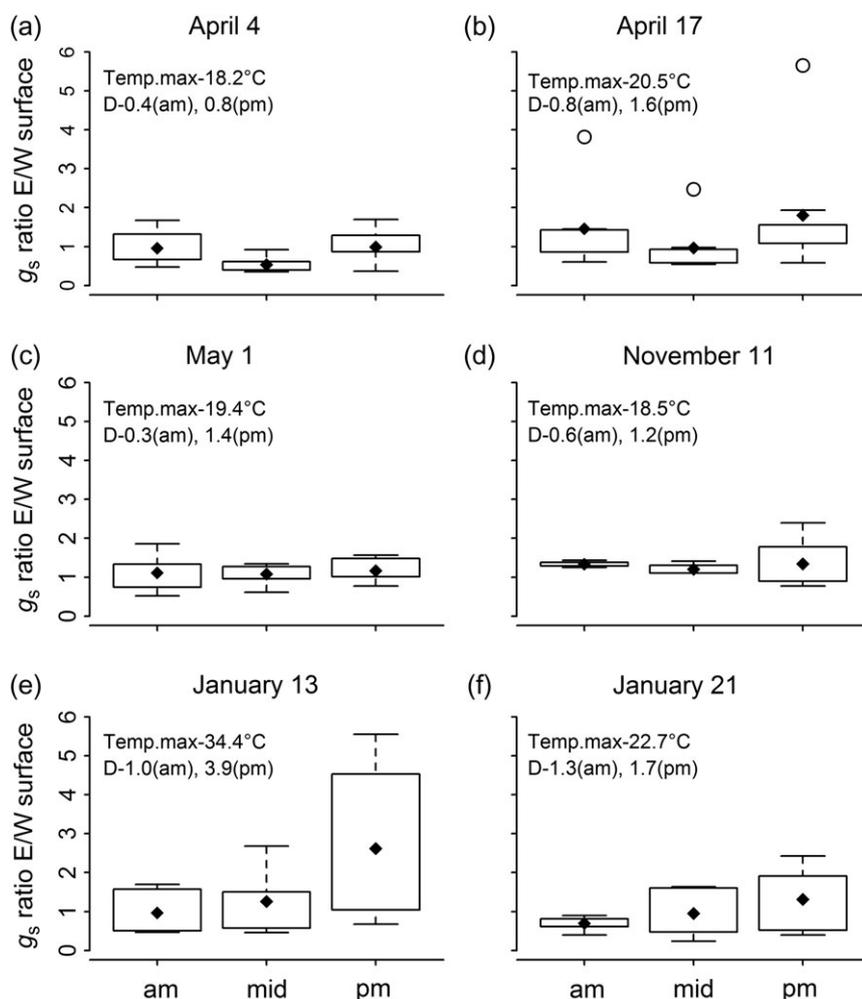


Figure 4. Ratio of  $g_s$  of east-facing leaf surface relative to west-facing surface throughout the day. Symbols represent the mean ratio  $g_s$   $E/W$ , with the value '1' representing an equal contribution from each surface to the  $g_s$  total. The box plots show the range of ratio  $g_s$   $E/W$  across the individual leaves measured on each day. Panels (a)–(f) represent individual days between April 2015 and January 2016.  $N = 6$  leaves for days (b), (c), (e) and (f), 5 on day (a), and 4 on day (c). 'Temp.max' is the maximum daily temperature, and  $D$  is shown for 9:00 h (am) and 15:00 h (pm) on each day.

Table 1. The difference in temperature ( $^{\circ}\text{C}$ ) required for  $D$  alone to explain the observed difference in  $g_s$  between leaf surfaces oriented E and W over six afternoons. Air temperature and relative humidity are from the Bureau of Meteorology data (Australian Government Bureau of Meteorology 2016). The expected difference in  $D$  between leaf surfaces was calculated from the relationship between  $g_s$  and  $D$  for a single leaf surface (see Supplementary Data at *Tree Physiology* Online).

Date (day)	Air temp. (15:00 h)	RH% (15:00 h)	$\Delta$ mean $g_s$ between leaf surfaces (p.m.)	$\Delta D$ between leaf surfaces (kPa)	$\Delta$ temp. between leaf surfaces ( $^{\circ}\text{C}$ )
4 Apr (a)	16.2	70	0.007	0.13	1.1
17 Apr (b)	20.3	31	0.022	0.39	2.5
1 May (c)	18.7	35	0.012	0.21	1.5
15 Nov (d)	17.9	40	0.020	0.37	2.7
13 Jan (e)	33.2	24	0.059	1.06	3.4
21 Jan (f)	21.7	36	0.009	0.15	2.3

surface (Figures 4 and 5). This independent regulation of stomata suggests there is a degree of hydraulic isolation between the leaf surfaces, which enables differential stomatal closure to occur should variation in conditions be large enough to induce such behaviour. Although independent regulation of stomata on

different leaf surfaces has been demonstrated for dorsiventral leaves (Mott and Parkhurst 1991, Mott 2007), our results show that a similar response can occur for isobilateral leaves and that natural irradiance, heat load and humidity can provide sufficient diurnal variation in between surfaces to induce differential

stomatal closure. While temperature differences recorded between leaf surfaces in the laboratory are sufficient to explain the difference in  $g_s$  observed in a number of cases, there also examples of  $g_s$  variation between leaf surfaces where temperature gradient alone would need to exceed the gradient observed.

### Hydraulic isolation in isobilateral leaves

The stomata on hypostomatic leaves respond to bulk leaf signals and to evaporative demand on the abaxial, or lower surface, the

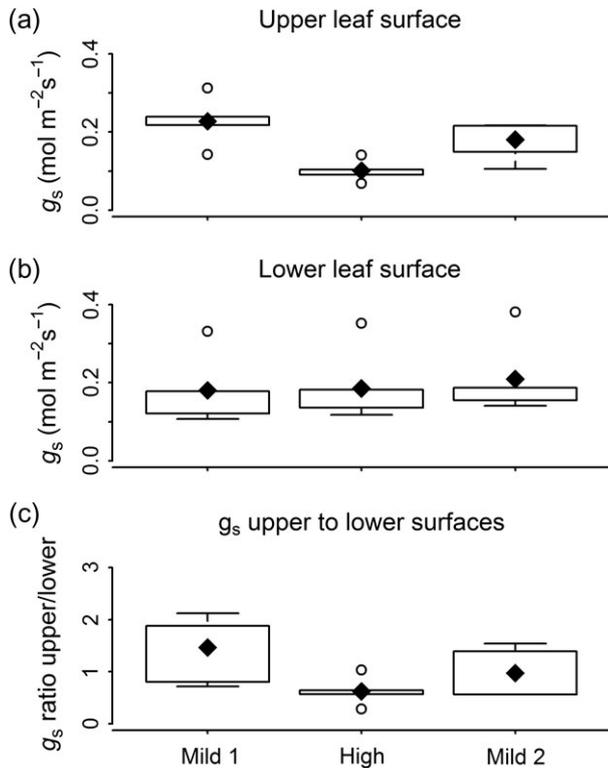


Figure 5. The box plots (a) and (b) show the range of  $g_s$  of individual leaf surfaces (upper and lower, respectively), and (c) the ratio of  $g_s$  upper surface to  $g_s$  lower surface ( $n = 5$ ), following changes in  $D$  with heat and light applied to the upper surface only. Symbols show the mean  $g_s$  for (a) and (b), and the mean ratio of  $g_s$  upper to  $g_s$  lower (c) with the value '1' representing an equal contribution from each surface to the  $g_s$  total. Treatments are 'Mild 1' ( $D$  1.5–1.6 kPa), 'High' ( $D$  2.9 kPa) and 'Mild 2' ( $D$  1.5–1.8 kPa).

Table 2. The difference in temperature (°C) required for  $D$  alone to explain the observed difference in  $g_s$  between upper and lower leaf surfaces following high  $D$  treatment (2.9 kPa based on upper leaf temperature) in the laboratory. The expected difference in  $D$  between leaf surfaces was calculated from the relationship between  $g_s$  and  $D$  for a single leaf surface (see Supplementary Data at *Tree Physiology* Online).

Leaf	Upper leaf temp. (°C)	Dew point temp. (°C)	$\Delta$ mean $g_s$ between leaf surfaces (high $D$ ) (mol m <sup>-2</sup> s <sup>-1</sup> )	$\Delta D$ between leaf surfaces (kPa)	$\Delta$ temp. between leaf surfaces (°C)
L1	26.2	-2.7	0.05	0.35	1.8
L2	26.0	-2.9	0.005	0.03	0.2
L3	26.2	-2.4	0.05	0.35	1.8
L4	26.6	-2.0	0.25	1.75	>5
L5	26.4	-2.2	0.08	0.55	2.8

only surface with stomata. Assuming that irradiance produces a thermal gradient through the leaf, if amphistomatic leaves were limited to synchronous regulation of  $g_s$  on leaf surfaces, the transpiration rate ( $E$ ) relative to carbon assimilation rate ( $A$ ) would be greater for the surface exposed to higher evaporative demand than the opposite surface. Independent stomatal regulation of  $g_s$  between surfaces would allow each surface to independently track a similar optimal ratio of  $E/A$ . However, relying simply on a temperature gradient through the leaf to explain the mean difference in  $g_s$  between the E- and W-facing leaf surfaces on the days measured would require a temperature difference of 1.1–3.4 °C between the surfaces (Table 1, also see Supplementary Data at *Tree Physiology* Online), not accounting for evaporative cooling associated with transpiration.

The concept of a vertical thermal gradient through a leaf has been discussed and modelled in relation to the liquid and vapour boundaries and transport within leaves, with temperature gradients as small as 0.1 °C between illuminated palisade mesophyll and the transpiring epidermis of a hypostomatic leaf likely to affect vapour transport within the leaf (Rockwell et al. 2014, Buckley et al. 2015). One study measured the surface temperatures for leaves of two species with one surface illuminated (Sheriff 1979). Of the species measured, temperature gradients of >0.9 °C were reported for *Commelina cyanea* R.Br., and <1.9 °C for *Tradescantia virginiana* L., which had a thicker mesophyll layer (Sheriff 1979). In addition to a potential temperature gradient through the leaf with asymmetrical irradiation, it is possible that additional processes are involved in the independent stomatal behaviour, for example, potential asymmetry in synthesis or transport of ABA between leaf surfaces.

The differential stomatal response between surfaces supports the idea that the stomatal response is driven by changes at or in close proximity to the epidermis. Furthermore, it confirms that there is a significant hydraulic resistance between the veins and the sites of evaporation (El-Sharkawy et al. 1985, Mott and Michaelson 1991, Buckley et al. 2015) such that an increase in evaporative demand on one surface could produce a local change in water potential without an overall change to bulk leaf water potential, which would induce a synchronous stomatal response on both surfaces.

### Differential stomatal response under natural conditions

Stomatal conductance for leaves measured outside under natural irradiance, humidity and temperature decreased from the morning, when the highest  $g_s$  was recorded, to the afternoon (Figure 3). This is consistent with diurnal trends reported for *E. globulus* (Pereira et al. 1987) and other eucalypts (Prior et al. 1997, Pfautsch et al. 2011). The change in the ratio  $g_{s\ E/W}$  was minimal on some days (1 May 2015 and 15 November 2015) (Figure 4), which appears consistent with the response reported for randomly orientated leaves of *E. globulus* (Pereira et al. 1987), and phyllodes of *Acacia* spp. (Lange et al. 1987). However, preferential closure of stomata on the west surface over the east surface was consistently measured here across all days to varying degrees during the afternoon, when  $D$  was higher (Figure 4) and heating of the west surface would have been a maximized by direct irradiance.

Differences in the afternoon  $D$  across the days may explain the variation in the differential stomatal response between surfaces. However, more localized factors influencing  $D$  are also likely to affect the stomatal response such as temperature, humidity and wind. An increase in stomatal sensitivity to  $D$  with water stress has been observed in a number of species (Osonubi and Davies 1980, Johnson and Ferrell 1983). The idea of water stress affecting the stomatal sensitivity to  $D$  could also explain the large variation for individual leaves in both  $g_s$  and  $g_s$  ratio between surfaces on any given day. While Pereira et al. (1987) found no link between water stress and  $g_s$  ratio, the random orientation of leaves measured may not have offered a gradient in irradiance large enough to induce differential closure.

The lack of significant difference in  $g_s$  between similarly oriented abaxial and adaxial leaf surfaces measured outside differs from the higher  $g_s$  reported for abaxial leaf surfaces by Pereira et al. (1987). The  $S_R$  of 0.67 (Figure 1b) for the plants used within study falls between  $S_{RS}$  previously published for the species of 0.58 (James and Bell 2001) and 0.75 (Pereira et al. 1987). Given that stomatal size was consistent between the surfaces (using length of stomatal complex as a proxy) and abaxial leaf surfaces have a higher density of stomata than the adaxial surfaces,  $g_{s\ abaxial}$  should be higher than  $g_{s\ adaxial}$  if maximum  $g_s$  was reached during the experiment. As the difference in  $g_s$  between surfaces was not significantly different, it is likely that the operating  $g_s$  was limited by factors other than light intensity, such as local water potential.

The differential closure of stomata on leaf surfaces in response to both changes in evaporative demand and leaf orientation relative to irradiance throughout the day indicates that specific environmental conditions can induce independent behaviour of leaf surfaces in *E. globulus* leaves. However, for non-manipulated *E. globulus* plants, random orientation, shading and wind would likely limit the number of leaves exposed to a

gradient in irradiance and evaporative demand sufficient to induce differential stomatal closure between individual surfaces.

To minimize costs associated with non-optimal gas exchange on different leaf surfaces, would we not only expect amphistomatic leaves to have surfaces that are hydraulically isolated from one another (to enable gas exchange at each surface to be independently optimized), but also to observe differential responses between the surfaces on a day to day level, under natural light, temperature and humidity. Here we established that the surfaces of an isobilateral amphistomatic leaf can respond independently to a gradient in evaporative demand. Importantly, natural conditions that influence evaporative demand (temperature, humidity and irradiance) can induce differential closure.

### Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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### References

- Australian Government Bureau of Meteorology. Weather and Climate data. <http://www.bom.gov.au/climate/data/index.shtml> (13 June 2017, date last accessed)
- Brodribb T, Hill R (1998) The photosynthetic drought physiology of a diverse group of southern hemisphere conifer species is correlated with minimum seasonal rainfall. *Funct Ecol* 12:465–471.
- Brodribb TJ, Jordan GJ, Carpenter RJ (2013) Unified changes in cell size permit coordinated leaf evolution. *New Phytol* 199:559–570.
- Buckley TN, John GP, Scoffoni C, Sack L (2015) How does leaf anatomy influence water transport outside the xylem? *Plant Physiol* 168: 1616–1635.
- Carins Murphy MR, Jordan GJ, Brodribb TJ (2012) Differential leaf expansion can enable hydraulic acclimation to sun and shade. *Plant Cell Environ* 35:1407–1418.
- Cowan IR, Farquhar GD (1977) Stomatal function in relation to leaf metabolism and environment. *Symp Soc Exp Biol* 31:471–505.
- Dow GJ, Berry JA, Bergmann DC (2014) The physiological importance of developmental mechanisms that enforce proper stomatal spacing in *Arabidopsis thaliana*. *New Phytol* 201:1205–1217.
- Drake PL, Froend RH, Franks PJ (2013) Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *J Exp Bot* 64:495–505.

- El-Sharkawy MA, Cock JH, Hernandez ADP (1985) Stomatal response to air humidity and its relation to stomatal density in a wide range of warm climate species. *Photosynth Res* 7:137–149.
- Fanourakis D, Giday H, Milla R et al. (2015) Pore size regulates operating stomatal conductance, while stomatal densities drive the partitioning of conductance between leaf sides. *Ann Bot* 115:555–565.
- Farquhar G, Richards R (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Funct Plant Biol* 11:539–552.
- Hetherington AM, Woodward FI (2003) The role of stomata in sensing and driving environmental change. *Nature* 424:901–908.
- James SA, Bell DT (2000a) Influence of light availability on leaf structure and growth of two *Eucalyptus globulus* ssp. *globulus* provenances. *Tree Physiol* 20:1007.
- James SA, Bell DT (2000b) Leaf orientation, light interception and stomatal conductance of *Eucalyptus globulus* ssp. *globulus* leaves. *Tree Physiol* 20:815.
- James SA, Bell DT (2001) Leaf morphological and anatomical characteristics of heteroblastic *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *Austral J Bot* 49:259–269.
- Johnson JD, Ferrell WK (1983) Stomatal response to vapour pressure deficit and the effect of plant water stress. *Plant Cell Environ* 6:451–456.
- Jordan GJ, Carpenter RJ, Brodribb TJ (2014) Using fossil leaves as evidence for open vegetation. *Palaeogeogr Palaeoclimatol Palaeoecol* 395:168–175.
- Jordan GJ, Carpenter RJ, Koutoulis A, Price A, Brodribb TJ (2015) Environmental adaptation in stomatal size independent of the effects of genome size. *New Phytol* 205:608–617.
- Lange OL, Ullmann I, Tenhunen JD, Bannister P (1987) Stomatal conductance and transpiration of the two faces of *Acacia phyllodes*. *Trees* 1:110–122.
- McAdam SAM, Brodribb TJ (2016) Linking turgor with ABA biosynthesis: implications for stomatal responses to vapour pressure deficit across land plants. *Plant Physiol* 171:2008–2016.
- McKown AD, Guy RD, Quamme L et al. (2014) Association genetics, geography and ecophysiology link stomatal patterning in *Populus trichocarpa* with carbon gain and disease resistance trade-offs. *Mol Ecol* 23:5771–5790.
- Milla R, de Diego-Vico N, Martin-Robles N (2013) Shifts in stomatal traits following the domestication of plant species. *J Exp Bot* 64:3137–3146.
- Mott KA (2007) Leaf hydraulic conductivity and stomatal responses to humidity in amphistomatous leaves. *Plant Cell Environ* 30:1444–1449.
- Mott KA, Michaelson O (1991) Amphistomy as an adaptation to high light intensity in *Ambrosia cordifolia* (Compositae). *Am J Bot* 78:76–79.
- Mott KA, Parkhurst DF (1991) Stomatal responses to humidity in air and Helox. *Plant Cell Environ* 14:509–515.
- Mott KA, Gibson AC, O'Leary JW (1982) The adaptive significance of amphistomatic leaves. *Plant Cell Environ* 5:455–460.
- Mott KA, Cardon ZG, Berry JA (1993) Asymmetric patchy stomatal closure for the two surfaces of *Xanthium strumarium* L. leaves at low humidity. *Plant Cell Environ* 16:25–34.
- Muir CD (2015) Making pore choices: repeated regime shifts in stomatal ratio. *Proc Biol Sci* 282:20151498.
- Muir CD, Hangarter RP, Moyle LC, Davis PA (2014) Morphological and anatomical determinants of mesophyll conductance in wild relatives of tomato (*Solanum* sect. *Lycopersicon*, sect. *Lycopersicoideae*; Solanaceae). *Plant Cell Environ* 37:1415–1426.
- Osonubi O, Davies W (1980) The influence of plant water stress on stomatal control of gas exchange at different levels of atmospheric humidity. *Oecologia* 46:1–6.
- Parkhurst DF (1994) Tansley review No. 65. Diffusion of CO<sub>2</sub> and other gases inside leaves. *New Phytol* 126:449–479.
- Pereira JS, Tenhunen JD, Lange OL (1987) Stomatal control of photosynthesis of *Eucalyptus globulus* Labill. trees under field conditions in Portugal. *J Exp Bot* 38:1678.
- Pfautsch S, Keitel C, Turnbull TL, Braimbridge MJ, Wright TE, Simpson RR, O'Brien JA, Adams MA (2011) Diurnal patterns of water use in *Eucalyptus vitrix* indicate pronounced desiccation–rehydration cycles despite unlimited water supply. *Tree Physiol* 31:1041–1051.
- Prior LD, Eamus D, Duff GA (1997) Seasonal and diurnal patterns of carbon assimilation, stomatal conductance and leaf water potential in *Eucalyptus tetrodonta* saplings in a wet & dry savanna in Northern Australia. *Aust J Bot* 45:241–258.
- R Development Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Rockwell FE, Holbrook NM, Stroock AD (2014) The competition between liquid and vapor transport in transpiring leaves. *Plant Physiol* 164:1741–1758.
- Salisbury EJ (1928) On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. *Philos Trans R Soc Lond Ser B* 216:1–65.
- Sheriff DW (1979) Water vapour and heat transfer in leaves. *Ann Bot* 43:157–171.
- Woodward FI, Bazzaz FA (1988) The responses of stomatal density to CO<sub>2</sub> partial pressure. *J Exp Bot* 39:1771–1781.