Differential leaf expansion can enable hydraulic acclimation to sun and shade

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ABSTRACT

Although leaf size is one of the most responsive plant traits to environmental change, the functional benefits of large versus small leaves remain unclear. We hypothesized that modification of leaf size within species resulting from differences in irradiance can allow leaves to acclimate to different photosynthetic or evaporative conditions while maintaining an efficient balance between hydraulic supply (vein density) and evaporative demand. To test this, we compared the function and anatomy of leaf hydraulic systems in the leaves of a woody angiosperm (Toona ciliata M. Roem.) grown under high and low irradiance in controlled conditions. Our results confirm that in this species, differential leaf expansion regulates the density of veins and stomata such that leaf hydraulic conductance and stomatal conductance remain proportional. A broader sample of field-grown tree species suggested that differences in leaf venation and stomatal traits induced by sun and shade were not regulated by leaf size in all cases. Our results, however, suggest that leaf size plasticity can provide an efficient way for plants to acclimate hydraulic and stomatal conductances to the contrasting evaporative conditions of sun and shade.

Key-words: high irradiance; leaf hydraulic conductance; leaf size; low irradiance; stomatal conductance; stomatal density; vein density.

INTRODUCTION

Leaf size varies with mean annual temperature, rainfall, irradiation level and other local environmental factors (Nicotra et al. 2011). This is likely to reflect a combination of the effects of plastic (developmental) responses to the environment and genetic differences among species and individuals within species. These trends have applications in several biological disciplines but have been exploited in particular for reconstruction of past climates (Carpenter, Hill & Jordan 1994; Greenwood 1994; Wilf et al. 1998). Despite this, the adaptive benefits of modifying leaf size remain controversial, and to complicate matters further, there are different definitions of leaf size in the literature with potentially different physiological implications. For example, some authors use the diameter of the largest circle that can be drawn inside the leaf surface as a measure of effective leaf size (Parkhurst & Loucks 1972; Givnish 1987), while others define leaf size as total lamina area (Cornelissen et al. 2003). While these are the most common measurements of leaf size, absolute leaf width and length have also been used.

One explanation for the observed variation in leaf size is the influence of leaf width on leaf thermal properties. Boundary layer thickness increases with effective leaf size, thereby enhancing resistance to water vapour and carbon dioxide (CO2) diffusion and reducing convective heat loss in larger or broader leaves (Parkhurst & Loucks 1972). This implies that smaller or narrower leaves can maintain lower temperatures more effectively in hot and dry environments (Parkhurst & Loucks 1972). Givnish (1978) extended this hypothesis further by suggesting that smaller leaves are better suited to high irradiance conditions as they have lower transpirational costs than larger leaves. Due to inherent constraints on CO2 assimilation, the higher temperatures reached by larger leaves under high irradiance are thought to result in disproportionate increases in transpiration relative to photosynthesis. A second view is that variation in leaf size (defined in this case as lamina area) is an outcome of the optimization of resource allocation according to environmental conditions. The portion of biomass available for photosynthetic tissue is comparatively less in larger leaves as they require greater investment into support and conductive tissues (Niinemets et al. 2007). Hence, this premise also concludes that smaller leaves are favoured under stressful conditions as the construction of larger leaves becomes expensive when carbon and water is scarce. Both of these explanations are broadly consistent with general trends towards narrower leaves and leaves with lower lamina areas in dry climates (Wilf et al. 1998). However, the explanation based on transpirational costs is undermined by strong trends towards large, broad leaves in warmer climates, at least for evergreen woody plants (Carpenter et al. 1994; Jordan & Hill 1994).

More recently, global trends in leaf size have been linked to plant hydraulics, or specifically, to the capacity of leaves to conduct water. Unlike the inferred relationship between effective leaf size and water limitation described previously, this relates more closely to lamina area (which will now be referred to as leaf size). Scoffoni et al. (2011) propose that having small leaves reduces the hydraulic vulnerability of species from dry environments. They suggest that the high
major vein density generally characteristic of small leaves creates redundancy in the water-transport system that may reduce the detrimental effects of major vein cavitation. However, another explanation for small, high-vein density leaves in species from dry climates is that these environments expose plants to high evaporative demands, thereby requiring greater hydraulic supply to the stomata (Sack & Froe 2006; Brodribb & Jordan 2011). Purely geometric processes mean that greater leaf expansion will result in lower vein and stomatal densities unless expansion is accompanied by increased initiation of veins and stomata. As leaf size generally responds quite plasticly to environmental conditions, and vein and stomata differentiation takes place well before the finalization of leaf size (Schoch, Zinsou & Sibi 1980; Zwieniecki, Boyce & Holbrook 2004), we hypothesize that adjusting leaf expansion can facilitate hydraulic acclimation by coordinating vein density (total length of leaf vascular tissue per unit leaf area) and stomatal density (total number of stomata per unit leaf area). Recognizing that in some species there is a weak association between leaf size and vein density (Manze 1968; Brodribb & Jordan 2011), we expect that in species with high leaf plasticity, vein and stomatal densities should be ‘diluted’ as leaves expand (Schuster 1908; Gupta 1961; Sack et al. 2003), allowing efficient acclimation to the environment. Thus, passive effects of leaf expansion have the potential to result in coordinated changes in vein and stomatal densities. This kind of coordination has been suggested as an economic means of maximizing the photosynthetic yield per unit investment in veins and stomata (Brodribb, Feild & Jordan 2007; Brodribb & Jordan 2011).

The hypothesis that vein and stomatal densities are related to leaf size is supported by empirical evidence. For example, in many tree species, leaves expanded in the sun are typically smaller and thicker than leaves expanded in the shade, and have greater stomatal density and corresponding higher rates of transpiration and photosynthesis (Ashton & Berlyn 1994; Poole et al. 1996). Although leaf veins have received much less attention than stomata, there is some evidence that leaves expanded in the sun also have greater vein density than those expanded in the shade (Uhl & Mosbrugger 1999; Brodribb & Jordan 2011). However, there is no direct evidence that vein and stomatal development is coordinated through the passive effects of leaf expansion.

Thus, we tested the hypothesis that leaf size plasticity within species induced by different levels of irradiance modulates vein and stomatal densities in a coordinated way to facilitate economic acclimation to the environment. To do this, we examined the influence of leaf size on physiology and anatomical characteristics of veins and stomata of individuals of *Toona ciliata* M. Roem grown under high and low irradiance. We also examined the relationship between leaf size and vein and stomatal anatomy within individual trees of three woody angiosperms sampled in the field [*Anodopetalum biglandulosum* (A. Cunn ex Hook. f.), *Bursaria spinosa* Cav. and *Cenarrhenes nitida* Labill.].

**MATERIALS AND METHODS**

Plant material grown under controlled conditions

Measurements were performed in 2010 on fully expanded, current-year leaves of a tropical woody angiosperm species, *T. ciliata* (Meliaceae) (Table 1), grown for 3 months in controlled conditions. This species was ideal for the purposes of this study as it is a long-lived pioneer species capable of growing under a large range of light conditions (Herwitz, Slye & Turton 1998). Four *T. ciliata* plants were grown in a glasshouse under a photoperiod of 18 h, a maximum photosynthetic photon flux density (PPFD) of 1500 µmol m⁻² s⁻¹ and a temperature range of 15 to 25 °C. Another four *T. ciliata* were grown in a growth chamber under a photoperiod of 18 h, a maximum PPFD of 50 µmol m⁻² s⁻¹ and a mean temperature of 22 °C. All plants were irrigated daily. Rates of photosynthesis, leaf hydraulic conductance and stomatal conductance were measured on two leaves from each of the eight plants. Anatomical and morphological traits, vein density, stomatal density, epidermal cell size, stomatal size and leaf size were measured on the same two leaves per plant.

**Photosynthetic rate, leaf hydraulic conductance and stomatal conductance of *T. ciliata***

Maximum instantaneous photosynthetic rate and stomatal conductance of all *T. ciliata* plants was measured using a portable infrared gas analyser (Li-6400, Li-Cor Biosciences, Lincoln, NE, USA) under controlled conditions. This species was ideal for the purposes of this study as it is a long-lived pioneer species capable of growing under a large range of light conditions (Herwitz, Slye & Turton 1998). Four *T. ciliata* plants were grown in a glasshouse under a photoperiod of 18 h, a maximum photosynthetic photon flux density (PPFD) of 1500 µmol m⁻² s⁻¹ and a temperature range of 15 to 25 °C. Another four *T. ciliata* were grown in a growth chamber under a photoperiod of 18 h, a maximum PPFD of 50 µmol m⁻² s⁻¹ and a mean temperature of 22 °C. All plants were irrigated daily. Rates of photosynthesis, leaf hydraulic conductance and stomatal conductance were measured on two leaves from each of the eight plants. Anatomical and morphological traits, vein density, stomatal density, epidermal cell size, stomatal size and leaf size were measured on the same two leaves per plant.

**Table 1.** Family, vegetation type, leaf habit (*E*, evergreen; *D*, deciduous), plant habit (*T*, tree; *S*, shrub), preferred light habitat (*L*, low irradiance; *H*, high irradiance) and relative drought tolerance (*L*, low; *H*, high) of the experimental species

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Vegetation type</th>
<th>Leaf habit</th>
<th>Plant habit</th>
<th>Light habitat</th>
<th>Relative drought tolerance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anodopetalum biglandulosum</em></td>
<td>Cunoniaceae</td>
<td>Temperate rainforest</td>
<td><em>E</em></td>
<td><em>T</em></td>
<td><em>L</em></td>
<td><em>L</em></td>
<td>Curtis &amp; Morris (1993)</td>
</tr>
<tr>
<td><em>Bursaria spinosa</em></td>
<td>Pittosporaceae</td>
<td>Open eucalypt woodland</td>
<td><em>E</em></td>
<td><em>S/T</em></td>
<td><em>H</em></td>
<td><em>H</em></td>
<td>Curtis &amp; Morris (1993)</td>
</tr>
<tr>
<td><em>Cenarrhenes nitida</em></td>
<td>Proteaceae</td>
<td>Temperate rainforest</td>
<td><em>E</em></td>
<td><em>S/T</em></td>
<td><em>L</em></td>
<td><em>L</em></td>
<td>Curtis (1993)</td>
</tr>
<tr>
<td><em>Toona ciliata</em></td>
<td>Meliaceae</td>
<td>Tropical and subtropical rainforest</td>
<td><em>D</em></td>
<td><em>T</em></td>
<td><em>H/L</em></td>
<td><em>L</em></td>
<td>Francis (1951)</td>
</tr>
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Leaf hydraulic conductance was also measured between 1000 and 1300 h using the evaporative flux method (Sack et al. 2002; Brodribb & Holbrook 2006). Leaves were excised under water without removing plants from the high and low irradiance treatments and leaf hydraulic conductance measured immediately after. Excised leaves were attached to a flow meter (Brodribb & Holbrook 2006) (for construction details, see: http://prometheuswiki.publish.csiro.au/tiki-index.php?page=Constructing+and+operating+a+hydraulics+flow+meter) and placed in conditions favourable to transpiration (i.e. under a light source providing a PPFD greater than 200 µmol m\(^{-2}\) s\(^{-1}\) and heated evenly by a stream of warm air maintaining leaf temperature <30 °C). Once in place, the leaves were allowed to reach a transpirational steady state (less than 10% variation over 180 s) and the resulting transpirational flux recorded. Leaf water potential was subsequently measured with a pressure chamber and leaf hydraulic conductance was calculated using the following equation:

\[ K_{\text{leaf}} = F / \Psi_{\text{leaf}} \]

where \( K_{\text{leaf}} \) is the leaf hydraulic conductance, \( F \) is the transpirational flux and \( \Psi_{\text{leaf}} \) is the leaf water potential at steady state. Leaf hydraulic conductance values were standardized for leaf size and for the viscosity of water at 20 °C, using an empirical function based on data from Korson, Drost-Hansen & Millero (1969).

**Anatomy and leaf size of T. ciliata**

The same \( T. \) ciliata leaves used for measurements of photosynthetic rate, leaf hydraulic conductance and stomatal conductance were scanned with a flatbed scanner at 300 pixels per inch, and leaf size (lamina area in mm\(^2\)) was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). Paradermal sections were then prepared from two approximately 100 mm\(^2\) pieces of lamina taken at random from the same leaves. The adaxial epidermis and palisade mesophyll were removed from each sample with a sharp razor and the sections were placed in commercial household bleach (50 g L\(^{-1}\) sodium hypochlorite and 13 g L\(^{-1}\) sodium hydroxide) until clear. Sections were rinsed and then stained with 1% toluidine blue. Five fields of view at 10× magnification (field of view area 0.56348 mm\(^2\)) were photographed from each section using a Nikon Digital Sight DS-L1 camera (Melville, NY, USA) mounted on a Leica DM 1000 microscope (Nussloch, Germany). Vein density was measured as the total length of leaf vascular tissue per mm\(^2\) of leaf area using ImageJ.

Two sections 6 mm in diameter were hole punched from random from each leaf and placed in either a 10% aqueous solution of Cr\(_2\)O\(_3\) or commercial household bleach until the cuticle and mesophyll could be separated. Cuticles were then rinsed in water, before being stained with 1% crystal violet and mounted on microscope slides in phenol glycerine jelly. Outlines of the epidermal and stomatal cells were apparent on the resulting cuticles because pegs of cuticle extend down the anticlinal cell walls. Five fields of view at 20× magnification through a 2.5× tube (field of view area 0.02488 mm\(^2\)) were photographed from all cuticles using the same camera and microscope setup as described previously. Stomatal density was then measured as the total number of stomata per mm\(^2\) of leaf area using ImageJ. Stomatal size was determined by tracing the perimeter (including the subsidiary cells in \( B. \) spinosa and \( C. \) nitida) of five stomata per field of view and measuring the area contained within using ImageJ. Epidermal cell size was subsequently calculated by subtracting the total field of view area taken up by stomata from the total field of view area and dividing by the number of epidermal cells.

**Comparing \( T. \) ciliata plants acclimated to high and low irradiance**

The plasticity of anatomical traits, leaf size, leaf hydraulic conductance, stomatal conductance and photosynthetic rate was assessed by comparing the relative changes between plants acclimated to high and low irradiance using unpaired \( t \)-tests. The level of coordination between vein and stomatal densities was then quantified as the deviation from a proportional relationship. To test whether changes in vein and stomatal densities were passively determined by leaf size (i.e. by dilution), changes to vein density were plotted against 1/leaf area and stomatal density was plotted against 1/leaf area. The level of coordination between anatomical traits and leaf size was then quantified as the deviation from a proportional relationship. The relationship between leaf size and epidermal cell size was also tested for proportionality, as were the relationships between vein density and leaf hydraulic conductance, stomatal density and stomatal conductance, and between liquid and vapour conductances.

**Plant material sampled in the field**

To test the generality of relationships between leaf size and vein/stomatal anatomy, we collected leaves from three woody angiosperms (Table 1) growing in south-eastern Tasmania, along Rifle Range Creek within the University Reserve in Sandy Bay and within Mt. Field National Park (details listed in Table 2). The three species were selected because of the diversity of their preferred light habitats. Leaf size and anatomical traits were measured on two fully exposed and two fully shaded leaves from four plants per field-sampled species (or in the case of small-leaved species, three fully exposed and three fully shaded leaves from four plants per species). Sun and shade leaves were sampled from the same plant and minimum PPFD varied between...
plants. The mean maximum PPFD experienced in summer by shaded *B. spinosa* leaves on a cloudless day at midday was approximately 0.97% of that received in the canopy, while shaded *A. biglandulosum* and *C. nitida* leaves received approximately 0.47%. Both values of maximum PPFD in the shade were the average of 25 measurements and all measurements were made with a Quantum Meter (Apogee Instruments Inc., North Logan, UT, USA).

### Anatomy and leaf size of field-sampled species

Leaf size was measured as above, using ImageJ. Two approximately 100 mm² paradermal sections were then taken at random from each leaf, except in small-leaved species where one section was taken from three sun leaves and three shade leaves. Paradermal sections were prepared and photographed, and vein density was measured as previously described.

Cuticles were prepared from two 6 mm diameter hole punches of each leaf (or one-hole punch for small-leaved species) as described previously. Five fields of view at 20× magnification (field of view area 0.14115 mm²) were photographed for all cuticles using the same camera and microscope setup. Stomatal density and epidermal cell size were measured from the resulting images as previously described.

### Comparing sun- and shade-acclimated leaves within individual plants

The plasticity of vein density, stomatal density and leaf size in response to sun and shade was assessed by comparing relative changes within individuals, using paired *t*-tests. The degree of coordination between vein and stomatal densities was determined by the method described previously. The same approach was used to assess the relationship between anatomical traits and leaf size.

### RESULTS

#### Response of anatomy and leaf size to high and low irradiance in *T. ciliata*

*T. ciliata* leaves expanded under low irradiance were 116% larger in area than leaves expanded under high irradiance (*P* < 0.001) (Fig. 1). As expected, low irradiance also induced a significant decrease in vein density, 33% (*P* < 0.01), and stomatal density, 51% (*P* < 0.01) (Fig. 1). However, there was a significant deviation from the expected proportional relationship as vein density in the plants acclimated to low irradiance was 41% greater than would be expected if vein and stomatal densities were directly proportional (*P* < 0.05) (Fig. 2).

#### Relationship between anatomy and leaf size in *T. ciliata*

As predicted, vein density was proportional to 1/leaf area and stomatal density to 1/leaf area in *T. ciliata* (Fig. 3). In addition, epidermal cell size was almost proportional to leaf size, with epidermal cells from leaves acclimated to low irradiance being 19% smaller than expected if epidermal cell size and leaf size were directly proportional (*P* < 0.05) (Fig. 4). Stomatal size did not vary significantly between *T. ciliata* plants acclimated to high and low irradiance (Fig. 4).

#### Response of leaf physiology to high and low irradiance in *T. ciliata*, and coordination with anatomy

Low irradiance induced a significant decrease in leaf hydraulic conductance (56%; *P* < 0.001) and stomatal conductance (66%; *P* < 0.001) in *T. ciliata*. There was also a significant decrease (74%) in photosynthetic rate from a mean of 9 μmol CO₂ m⁻² s⁻¹ under high irradiance to 2 μmol CO₂ m⁻² s⁻¹ under low irradiance (*P* < 0.001). Anatomical traits (vein and stomatal densities) were proportional to the corresponding liquid and vapour conductances (leaf hydraulic conductance and stomatal conductance) (Fig. 5). Stomatal density and stomatal conductance were directly proportional, but leaf hydraulic conductance was 34% lower than expected for the observed vein density (*P* < 0.01). The acclimation of liquid and vapour conductances was also coordinated (Fig. 5).

#### Response of anatomy and leaf size to sun and shade in field-sampled species

Shade induced significant decreases in vein density and stomatal density in all field-sampled species. Vein density decreased by 13% in *A. biglandulosum* (*P* < 0.01), 11% in *B. spinosa* (*P* < 0.05) and 22% in *C. nitida* (*P* < 0.01), while
stomatal density decreased by 28, 23 and 18%, respectively ($P < 0.05$ in all cases). Changes to vein and stomatal densities were coordinated within plants across all three species (Fig. 6). The scale of this coordination, however, varied with species, such that there was a 21% deviation from proportionality in $A. biglandulosum$ ($P < 0.05$), an 18% deviation in $B. spinosa$ ($P > 0.05$) and only a 5% deviation in $C. nitida$ ($P > 0.05$).

Shade leaves of $C. nitida$ were 103% larger than sun leaves ($P < 0.05$), but in $B. spinosa$ and $A. biglandulosum$ there were no detectable changes to leaf size. Irradiance-induced changes to vein and stomatal densities were only related to leaf size in $C. nitida$ (Fig. 7), with vein density being proportional to $1/\sqrt{\text{leaf area}}$ and stomatal density being proportional to $1/\text{leaf area}$ as found in $T. ciliata$. However, in both cases there was a significant deviation from proportionality ($P < 0.05$ and $P < 0.001$). Epidermal cell size was proportional to leaf size in $C. nitida$, although epidermal cells from shade leaves were 21% smaller than expected if epidermal cell size and leaf size were directly proportional ($P > 0.05$) (Fig. 8). Epidermal cell size was not proportional to leaf size in $A. biglandulosum$ or $B. spinosa$.

**DISCUSSION**

Leaf expansion can enable the acclimation of leaf anatomy to sun and shade

Acclimation of vein and stomatal densities to high and low irradiance was found to be coordinated in the woody angiosperm $T. ciliata$. Furthermore, the variation of these traits with leaf size was consistent with them being determined by differential leaf expansion (i.e. they are proportional to $1/\sqrt{\text{leaf area}}$ and $1/\text{leaf area}$, respectively). Thus, our results provide evidence to support the hypothesis that modification of leaf size within species between sun and shade allows leaves to balance vein and stomatal densities during acclimation to different photosynthetic or evaporative conditions. Sun leaves, for example, experience much greater evaporative demand than shade leaves due to greater heat load and increased energy for electron transport which creates higher photosynthetic yields resulting in increased stomatal conductance (Wong, Cowan & Farquhar 1979). Hence, these findings provide a new perspective to view the commonly observed changes in size between sun and shade leaves of tree species.
The perfectly coordinated acclimation of vein density and $1/\sqrt{\text{leaf area}}$ in *T. ciliata* conforms to the expected geometric relationship if vein spacing is determined passively by differences in leaf expansion. Likewise, changes to stomatal density are proportional to changes in $1/\text{leaf area}$, implying that stomata are passively ‘diluted’ by leaf expansion. The sensitivity of epidermal cell size and the apparent insensitivity of stomatal size to changes in leaf size also suggest that stomata are moved apart by leaf expansion, but not actively expanded in proportion to leaf size (as epidermal cells appear to be). The insensitivity of stomatal size to changes in leaf size and stomatal density has also been observed within temperate deciduous tree species (Sack et al. 2006). This premise is further supported by the differentiation of veins and stomata before the completion of leaf expansion in some species (Schoch et al. 1980; Zwieniecki et al. 2004), and the plasticity of leaf size in *T. ciliata*.

Modification of leaf anatomy facilitates the acclimation of leaf physiology to sun and shade

The coordinated acclimation of liquid and vapour conductances (leaf hydraulic conductance and stomatal...
conductance) with the corresponding anatomical traits (vein density and stomatal density) in *T. ciliata* leaves provides further evidence that the modification of vein and stomatal densities by leaf expansion facilitates hydraulic acclimation to high and low irradiance in this species. Higher stomatal densities in leaves acclimated to high irradiance produced higher vapour-phase conductances to water. This greater demand for water was met by a higher liquid-phase conductance to water produced by higher vein density. Vein density is closely related to leaf hydraulic conductance because greater densities of minor veins bring water delivery closer to the sites of evaporation in the leaf (Brodribb et al. 2007). Hence, as leaf hydraulic conductance and stomatal conductance are clearly associated across a range of plant species (Brodribb et al. 2005), the control of hydraulic acclimation by leaf expansion-mediated changes to vein and stomatal densities may be widespread in other plant species.

Although leaf hydraulic conductance was proportional to stomatal conductance in *T. ciliata*, in leaves acclimated to low irradiance it was lower than expected if directly proportional to vein density. A similar relationship between leaf hydraulic conductance and vein density was observed in *Nothofagus cunninghamii* (Hook.) Oerst. (Brodribb & Jordan 2011). This suggests that an extra-vascular component of total leaf hydraulic conductance is also responsive to high and low irradiance in this species. These results support the findings of an earlier study in which five sun-establishing tropical tree species were found to have a higher proportion of leaf hydraulic conductance outside the...
xylem than five shade-establishing species (Sack, Tyree & Holbrook 2005). This discrepancy may be due to the variable presence of aquaporins (Agre, Sasaki & Chrispeels 1993) or anatomical differences in the mesophyll of leaves acclimated to high and low irradiances.

**Acclimation of anatomy to sun and shade in field-sampled species**

The vein and stomatal densities of the three field-sampled species included in this study (A. biglandulosum, B. spinosa and C. nitida) also displayed coordinated acclimation to sun and shade. However, the degree of coordination between vein and stomatal densities varied between them. In contrast to C. nitida, the plasticity of vein density in A. biglandulosum and B. spinosa was lower than of stomatal density leading to disproportionately high ratios of vein density to stomatal density. Variability in the degree of coordination between anatomical traits is likely to have ecological or evolutionary significance. For example, species with disproportionate changes in vein and stomatal densities are likely to have suboptimal allocation of vein density under changing light/evaporative conditions (Brodribb & Jordan 2011). The significance of this variation may be resolved by investigating the relationship between vein and stomatal densities in a broad range of plant species and groups.

Although there were significant irradiance-induced changes to leaf size in C. nitida, they were only partially related to vein and stomatal densities. Changes to leaf size, however, were proportional to epidermal cell size, suggesting that coordinated changes to vein and stomatal densities in this species were, to some extent, the result of differential leaf expansion. Sun and shade did not induce significant changes to leaf size in A. biglandulosum or B. spinosa.

**Figure 6.** Vein and stomatal densities of sun leaves (white circles) and shade leaves (black circles) of four (a) A. biglandulosum, (b) B. spinosa and (c) C. nitida plants. Solid lines connecting circles show the within-plant trends. Stomatal density changed in proportion to vein density in all three species. The slope of the mean species response (solid line) was not significantly different (ns, P > 0.05) from the expected proportional relationship (broken line) in B. spinosa and C. nitida. However, in A. biglandulosum, the slope of the mean species response was significantly different (*, P < 0.05) from the expected proportional relationship.
Figure 7. Vein density and \(1/\sqrt{\text{leaf area}}\), and stomatal density and \(1/\text{leaf area}\) of sun leaves (white circles) and shade leaves (black circles) of four (a, b) *A. biglandulosum*, (c, d) *B. spinosa* and (e, f) *C. nitida* plants. Solid lines connecting circles show the within-plant trends. Leaf area traits changed in proportion to anatomical traits in *C. nitida* only. However, the slope of the mean species response (solid line) was significantly different from the expected proportional relationship (broken line) in both cases (*, \(P < 0.05\); ***, \(P < 0.05\)).
despite substantial shifts in vein and stomatal densities. This implies that the coordinated acclimation of vein and stomatal densities in these species was not a passive outcome of irradiance-induced leaf expansion, but the result of independent developmental processes. Recent research found that coordinated changes to vein and stomatal densities in another woody Tasmanian angiosperm, *N. cunninghamii*, were similarly unrelated to leaf expansion (Brodribb & Jordan 2011). These deviations from the coordination observed between vein and stomatal densities and leaf size mean that leaf expansion is one of several mechanisms coordinating the development of veins and stomata within plant leaves.

CONCLUSIONS

The independent processes governing the differentiation of veins and stomata have been the focus of some research in recent times; however, the developmental link between these traits remains unknown. For example, it is widely accepted that vascular development is regulated by the movement of auxin through embryonic plant tissue (ScarPELLA, BARKOULAS & TSANTIS 2010), while in contrast, the number and distribution of stomata is regulated by a series of asymmetrical cell divisions mediated by intercellular signalling (Serna 2011). A simple coordination of these disparate mechanisms can occur through leaf expansion as observed here in *T. ciliata* plants during acclimation to high and low irradiances. However, our results also support the concept of independent developmental control in two field-sampled species lacking the same level of leaf size plasticity observed in *T. ciliata*. Hence, it seems likely that there is variation between species in the mechanisms coordinating the development of veins and stomata or in the degree to which they exploit these mechanisms. *Cenarrhenes nitida*, for instance, provides an example of a species with a high

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**Figure 8.** Epidermal cell area and leaf area of sun leaves (white circles) and shade leaves (black circles) of four (a) *A. biglandulosum*, (b) *B. spinosa* and (c) *C. nitida* plants. Solid lines connecting circles show the within-plant trends. Leaf area changed in proportion to epidermal cell area in *C. nitida* only. The slope of the mean species response (solid line) was not significantly different (ns, $P > 0.05$) from the expected proportional relationship (broken line).
level of leaf size plasticity in which leaf anatomy appears to be only partially coordinated by leaf expansion. Future studies may consider utilizing plant development mutants to explore the role of signalling molecules in the synchronized development of veins and stomata.

The coordination of vein and stomatal densities in our experimental species reflects an optimization of the trade-off between transpirational costs to plants and CO₂ assimilation. Assuming the production of veins and stomata is energetically expensive, the most efficient use of resources should occur when vein density matches stomatal density, balancing water supply with transpirational demand. This was observed in T. ciliata grown in controlled conditions and within individuals of three species (A. biglandulosum, B. spinosa and C. nitida) sampled in the field, as well as within individuals and across populations of N. cunninghamii in a previous study (Brodribb & Jordan 2011). Thus, for T. ciliata at least, leaf expansion represents an economic way to acclimate hydraulically to local conditions. Furthermore, the process of leaf-size adaptation of the hydraulic system to local evaporative conditions may contribute to global trends in leaf size, in that the superior hydraulic capabilities of small leaves may explain their prevalence in conditions of high photosynthetic and evaporative demand.

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