A novel optimization approach incorporating non-stomatal limitations predicts stomatal behaviour in species from six plant functional types

Teresa E. Gimeno1,2,3,*, Noelia Saavedra1,4, Jérôme Ogée1, Belinda E. Medlyn5,6, and Lisa Wingate1

1 INRA, UMR ISPA, 33140 Villenave d’Ornon, France
2 Basque Centre for Climate Change (BC3), 48940 Leioa, Spain
3 IKERBASQUE, Basque Foundation for Science, 48008 Bilbao, Spain
4 Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, Skogsmarksgränd 17, 907 36 Umeå, Sweden
5 Hawkesbury Institute for the Environment, Western Sydney University, Locked bag 1797, Penrith, NSW 2751, Australia

* Correspondence: teresa.gimeno@bc3research.org

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Abstract

The primary function of stomata is to minimize plant water loss while maintaining CO₂ assimilation. Stomatal water loss incurs an indirect cost to photosynthesis in the form of non-stomatal limitations (NSL) via reduced carboxylation capacity (CAP) and/or mesophyll conductance (MES). Two optimal formulations for stomatal conductance (gs) arise from the assumption of each type of NSL. In reality, both NSL could coexist, but one may prevail for a given leaf ontogenetic stage or plant functional type, depending on leaf morphology. We tested the suitability of two gs formulations (CAP versus MES) on species from six plant functional types (C₄ crop, C₃ grass, fern, conifer, evergreen, and deciduous angiosperm trees). MES and CAP parameters (the latter proportional to the marginal water cost to carbon gain) decreased with water availability only in deciduous angiosperm trees, while there were no clear differences between leaf ontogenetic stages. Both CAP and MES formulations fit our data in most cases, particularly under low water availability. For ferns, stomata appeared to operate optimally only when subjected to water stress. Overall, the CAP formulation provided a better fit across all species, suggesting that sub-daily stomatal responses minimize NSL by reducing carboxylation capacity predominantly, regardless of leaf morphology and ontogenetic stage.

Keywords: Drought, fern, mesophyll conductance, ontogeny, optimization, photosynthesis, plant functional type, stomatal conductance, transpiration, water use efficiency.

Introduction

Climate change is predicted to decrease water availability and increase drought risk in many regions around the world (Sheffield and Wood, 2007; Dai and Zhao, 2017). Water availability is one of the main factors regulating vegetation carbon and water fluxes and drought impacts on the vegetation, reducing productivity, changing species distribution, or...
Leaves that are likely to experience drought during their lifespan exhibit morphological traits that contribute to maintain $\Psi_{leaf}$ and physiological activity under water stress (Mediavilla and Escudero, 2003; Flexas et al., 2006); one such trait is high leaf mass per area (LMA). High-LMA leaves often have multiple layers of cells with thick walls and tortuous mesophyll interspaces (Niinemets et al., 2009; Onoda et al., 2017). These traits protect the photosynthetic machinery from dehydration (Gimeno et al., 2010; Limousin et al., 2013) but they are also associated with low mesophyll diffusivity (Niinemets et al., 2009). $g_{m}$ can be the most important limitation to photosynthesis in evergreen trees and shrubs (Flexas et al., 2012; Peguero-Pina et al., 2016) and also in many ferns (Carriquia et al., 2015; Tosens et al., 2016). Ferns have more rudimentary stomata, in the sense that they are insensitive to abscisic acid, CO$_2$, and blue light (Brodribb and McAdam, 2011) and they appear to respond only to changes in vapour pressure deficit (Martins et al., 2016). Franks and Britton-Harper (2016) observed that stomata of some ferns can close in response to elevated CO$_2$, but these responses have not been tested under the framework of optimization theory.

In addition to drought tolerance and leaf morphology, longevity and construction costs can also influence stomatal behaviour. Lin et al. (2015) evaluated the convergence across plant functional types (PFTs) in their ability to operate stomata optimally, and found that PFTs with low marginal water use per unit of C gain had water-transport systems with greater construction costs, except for species with a C$_4$ pathway, for which optimization theory predicts the lowest marginal water cost. In addition to an associated cost to water transport, leaf construction costs could also influence stomatal regulation. Leaf construction costs depend on leaf lifespan and turnover: generally, the longer the lifespan, the slower the return of nutrient and dry mass investment (Wright et al., 2004). Leaf development is a crucial stage for all PFTs and in deciduous species, it can occur over a significant fraction of their lifespan. Yet, only mature fully expanded leaves are targeted for the vast majority of physiological measurements (with some exceptions, e.g. Field and Mooney, 1983; Medlyn et al., 2002; Locke and Ort, 2014; Macinnis-Ng et al., 2017), and hence DGVM assume constant stomatal behaviour throughout leaf ontogeny (Keenan et al., 2013). During leaf construction, respiratory costs are high, so that net photosynthesis per unit of water transpired is usually low (Rajaona et al., 2013; Jensen et al., 2015). Additionally, during development, the internal leaf anatomy experiences modifications including cell multiplication and expansion, increase in the number of chloroplasts, thickening of the cell wall, and formation of the intercellular air spaces (Niinemets et al., 2009; Kuusk et al., 2018). Collectively, these modifications should result in greater carboxylation capacity and the ability to maintain greater $\Psi_{leaf}$ under drought stress, but also lower $g_{m}$ (Asaamaa et al., 2005; Grassi and Magnani, 2005; Barbour et al., 2016). Hence, in developing leaves the photosynthetic costs of stomatal opening likely result from a decrease in carboxylation capacity, rather than in $g_{m}$.

Here, we tested the formulations proposed by Dewar et al. (2018) to determine when carboxylation capacity (the CAP formulation) or $g_{m}$ (the MES formulation) are the predominant...
NSL to photosynthesis. We expected developing leaves to have a greater marginal water cost per unit of C gain than mature leaves. We also hypothesized that in developing leaves the carboxylation capacity should be more sensitive to \( \Psi_{\text{leaf}} \) and the CAP formulation should fit better. Regarding the effect of leaf morphological differences among species from various PFTs, we hypothesized that in leaves with lower LMA the photosynthetic machinery would be more sensitive to \( \Psi_{\text{leaf}} \) and the CAP formulation would be more suitable, while in species where \( g_{\text{m}} \) constitutes the main limitation to photosynthesis, such as in evergreen species with greater LMA and ferns, the MES formulation could fit better. On the other hand, in leaves with low maximum realized \( g_{\text{m}} \), fluctuations in \( g_{\text{m}} \) would have a marginal effect and NSL should arise from limited carboxylation capacity. We address these hypotheses within the theoretical framework of Dewar et al. (2018) by comparing stomatal behaviour between mature and developing leaves of species from six PFTs (including a fern) maintained under two watering regimes.

**Materials and methods**

**Plant material and experimental design**

We selected seven species economically or ecologically relevant to the ecosystems in the south-west of France and representative of six PFTs: a fern, common bracken (**Pteridium aquilinum** L. Kuhn); an evergreen conifer, maritime pine (**Pinus pinaster** Ait.); two deciduous angiosperm temperate trees, pedunculate oak (**Quercus robur** L.) and silver birch (**Betula pendula** Roth); a C₃ grass, purple moor grass (**Molinia caerulea** (L.) Moench); an evergreen angiosperm tree, cider gum (**Eucalyptus gunnii** Hook.f.); and a C₄ crop, maize (**Zea mays** L.).

Saplings of **P. pinaster**, **Q. robur**, **B. pendula**, and **E. gunnii** were grown from seeds obtained from nearby plantations in an open-air nursery at INRA Pierroton (Cestas, France; annual precipitation 977 mm, annual mean temperature 13 °C). Plants of **M. caerulea** and **P. aquilinum** were grown from tussocks (~15 cm diameter) and overwintering rhizomes, respectively, collected in February 2015 from a local forest (Le Bray experimental site; Wingate et al., 2010). Plants of **Z. mays** (variety DKC 5784) were grown from seeds sown in May 2015. All species were grown in 3.4 l square pots. The soil substrate consisted of a 4:2:1 (v/v) mix of bark:peat:soil (typical sandy soil from the Le Bray experimental site; Wingate et al., 2010). Plants of **Z. mays** were made with a Scholander-type pressure chamber (SARL SAM PRECIS, Gradignan, France).

**Morphological and physiological measurements**

LMA was calculated from one mature and one young developing leaf collected from five individuals per species in mid-June 2015. Mature fully expanded leaves were sampled from the middle of the plant (in height), and young developing leaves, not fully expanded, from the upper third. In August 2015, there were no significant differences among species \((F=1.9, P=0.11)\) and plants in the LW treatment had significantly lower \( \Psi_{pd} \) \((F=15.9, P<0.01)\).

**Table 1.** Mean ±SE (n=5–7) predawn leaf water potential (\( \Psi_{pd} \)) for the study species in the trial experiment and in the August 2015 campaign under the well-watered (WW) and low water availability (LW) regimes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Trial experiment</th>
<th>August 2015</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max ( \Psi_{pd} ) (MPa)</td>
<td>Min ( \Psi_{pd} ) (MPa)</td>
</tr>
<tr>
<td>B. pendula</td>
<td>-0.56±0.1</td>
<td>-2.31±0.25</td>
</tr>
<tr>
<td>E. gunnii</td>
<td>-0.58±0.28</td>
<td>-2.03±0.3</td>
</tr>
<tr>
<td>M. caerulea</td>
<td>-0.46±0.04</td>
<td>-2.24±0.22</td>
</tr>
<tr>
<td>P. pinaster</td>
<td>-0.32±0.04</td>
<td>-1.19±0.11</td>
</tr>
<tr>
<td>P. aquilinum</td>
<td>-0.47±0.13</td>
<td>-1.24±0.06</td>
</tr>
<tr>
<td>Q. robur</td>
<td>-0.46±0.21</td>
<td>-2.44±0.2</td>
</tr>
<tr>
<td>Z. mays</td>
<td>-0.28±0.03</td>
<td>-1.94±0.26</td>
</tr>
</tbody>
</table>
For *P. aquilinum*, we selected a pinna (leaflet) close to the base of the frond as a mature pinna, and the most distal as a young pinna. Sampled leaves were photographed and dried at 70 °C for 48 h. Leaf area was calculated using ImageJ software for image analysis (Rasband, 2009). Leaf thickness was measured on the same leaves.

We conducted two gas-exchange campaigns, each over the course of 3 or 4 sunny and cloudless days with comparable conditions within campaigns. In the first campaign (18, 22, and 24 June 2015), we measured five well-watered individuals of each species, and during the second campaign (5, 6, 20, and 21 August 2015), we measured three or four individuals of each species and water treatment (well-watered and low-watered). On each day, we measured gas exchange starting at 08:00 h (local time) every 2–2.5 h on the same set of plants, to track net photosynthesis (*A*<sub>net</sub>) and *g*<sub>sc</sub> along a gradually increasing gradient of *D*<sub>w</sub> (Fig. 1). Plants measured each day were kept outside in an open area next to the glasshouse, exposed to full sun, from the first until the last round of measurements. A mature fully expanded leaf, and in June also a young developing leaf, was measured on each individual at each measurement round. Gas-exchange measurements were performed with an open-flow portable photosynthesis system (IRGA, LI-6400, LI-COR, Lincoln, NE, USA) with a standard leaf chamber fluorometer head (LI-6400–40). We used a portable photosynthesis system (IRGA, LI-6400, LI-COR, Lincoln, NE, USA) with a standard leaf chamber fluorometer head (LI-6400–40). We measured *A*<sub>net</sub>, *g*<sub>sc</sub>, leaf thickness, and stomatal opening, Dewar et al. (2018) predict that *g*<sub>sc</sub> (stomatal conductance to CO<sub>2</sub> in mol m<sup>−2</sup> s<sup>−1</sup>) should follow:

\[ g_{sc} = g_0 + \frac{\xi}{\sqrt{D_{w}}} \frac{A_{net}}{C_a - \Gamma^*} \]  

(1)

where *D*<sub>w</sub> (mmol mol<sup>−1</sup>) is the leaf-to-air vapour pressure deficit, *A*<sub>net</sub> (µmol m<sup>−2</sup> s<sup>−1</sup>) is net photosynthesis, *C<sub>a</sub>* (µmol mol<sup>−1</sup>) is the ambient CO<sub>2</sub> concentration, *Γ* (µmol mol<sup>−1</sup>) is the CO<sub>2</sub> photorespiratory compensation point according to Bernacchi et al. (2001), and *ξ* (mmol<sup>0.5</sup> mol<sup>−0.5</sup>) is a fitting parameter proportional to the square root of *λ* (Cowan and Farquhar, 1977) and equivalent to the *g*<sub>i</sub> parameter in Medlyn et al. (2011). The intercept *g*<sub>0</sub> (mol m<sup>−2</sup> s<sup>−1</sup>) is another fitting parameter that was added to the model to represent suboptimal water losses through leaf cuticles or leaky stomata (Medlyn et al., 2011). Parameters *ξ* and *g*<sub>0</sub> were estimated from non-linear least-square regressions using the ‘nls’ function in R (R Development Core Team, 2017). The non-linear nature of the CAP formulation does not allow a straightforward comparison among species and between treatments. Therefore, to assess differences in *ξ* among species, between leaf ages (June campaign) and between watering treatments (August campaign), we compared the 95% confidence intervals (CI) for the fitted parameter (*ξ*, Gimeno et al., 2016). Our estimates of *ξ* (in mmol<sup>0.5</sup> mol<sup>−0.5</sup>) and calculated for *g*<sub>i</sub> can be related to the *g*<sub>i</sub> estimates of Lin et al. (2015, in kPa<sup>0.5</sup> and calculated for *g* sub water) according to *g*<sub>i</sub> = *ξ* / (1/6√*P*), where *P* is atmospheric pressure and the factor 1.6 accounts for the ratio of the molecular diffusion coefficients for CO<sub>2</sub> and water.

In addition, according to the CAP formulation, Dewar et al. (2018) also predict that parameter *ξ* should vary according to:
\[ \xi \sqrt{V_{\text{max}}} = \sqrt{\frac{K_{d} |\Psi| k_{m} + \Gamma^{*}}{1.6}} \]  

(2)

where \( V_{\text{max}} \) is the maximum carboxylation capacity in the absence of NSI, \( k_{m} \) is the Michaelis constant for CO2 (710 \text{ mol}^{-1} \text{ mol}^{-1}; \text{Bernacchi et al., 2001}), and \( \Psi \) is the critical leaf water potential when photosynthetic capacity is zero (2 \text{ MPa}; \text{Dewar et al., 2018}). Noting that \text{Dewar et al. (2018)} neglect mitochondrial respiration, \( V_{\text{max}} = 0 \) at \( \Psi_{\text{leaf}} = \Psi \), also translates into \( A_{\text{net}} = 0 \). We compared our estimates of \( \xi \sqrt{V_{\text{max}}} \) with the predicted values according to equation 2 for a range of predawn \( \Psi \) (\( \Psi_{\text{leaf}} \) from 0 to –2 \text{ MPa}). To do so, we estimated temperature-corrected \( V_{\text{max}} \) (\( \bar{V}_{\text{max}} \)) with the one-point method (\text{De Kauwe et al., 2016}) from mid-morning gas-exchange measurements (see Supplementary Protocol S1 at \text{JXB online}). The soil-to-leaf hydraulic conductance (\( K_{d} \)) was estimated from equations 5b and 5c in \text{Dewar et al. (2018)} using a range of fixed values of the root-to-leaf hydraulic conductance (\( K_{rl} = 2, 5, 12, \text{and 50 mmol m}^{-2} \text{s}^{-1} \text{ MPa}^{-1}; \text{Dewar et al., 2018} \)), and a soil-to-root conductance estimated from retention curve parameters typical of an organic soil (\text{Ogée and Brunet, 2001}) and a bulk density of 0.25 g cm\(^{-3}\).

When decreased mesophyll conductance (MES) underlies the cost of stomatal opening, \text{Dewar et al. (2018)} predict:

\[ g_{sc} = g_{0} + \sqrt{\frac{E_{\text{max}} A_{\text{net}}}{1.6D_{w}(C_{i} - \Gamma^{*})}} \]  

(3)

where

\[ E_{\text{max}} = K_{d}(|\Psi| - \Psi_{s}) \]  

(4)

and \( \Psi_{s} \) represents here the critical leaf water potential that leads also to \( A_{\text{net}} = 0 \) but through a reduction of \( g_{sc} \) rather than \( V_{\text{max}} \). We estimated \( E_{\text{max}} \) from the slope of the linear relationship between \( g_{sc} \) and \( \sqrt{\frac{A_{\text{net}}}{\text{S} \sqrt{C_{i} - \Gamma^{*}}}} \) (hereafter the MES index) for each species, leaf age, and watering treatment. We used analyses of homogeneity of slopes to test for the effects of species, leaf age (June campaign), and water stress (August campaign) on \( E_{\text{max}} \). Additionally, we compared our estimates of \( E_{\text{max}} \) with the predicted value according to equation 4 for the same range of \( \Psi_{s} \) (0 to –2 \text{ MPa}) and assuming \( \Psi = \Psi_{s} \) and \( K_{d} \), calculated from four \( K_{rl} = 2, 5, 12, \text{and 50 mmol m}^{-2} \text{s}^{-1} \text{ MPa}^{-1}; \text{Dewar et al., 2018} \) as explained above.

We fitted equations 1 and 3 to our gas-exchange data and used the likelihood ratio test, the \text{Akaike Information Criterion (AIC}, with \text{DAIC}>10 indicating an improvement in the model fit), and root mean square error (RMSE) from the relationship between observed and predicted \( g_{sc} \), to compare between formulations for each species, leaf age, and watering treatment. We assessed differences among species and between leaf ages on morphological parameters (LMA and leaf thickness, June campaign) and among species and between watering treatments on \( \Psi_{pd} \) (August campaign) with two-way ANOVA. We tested for the overall effect of species and leaf age (June campaign) or watering treatment (August campaign) on \( A_{\text{net}} \) and \( g_{sc} \) with linear mixed models including plant and round of measurements as random factors (\text{Zuur et al., 2009}). We tested for the effect of leaf age (June campaign) and watering (August campaign) on \( \xi \) and \( E_{\text{max}} \), with one-way ANOVA. Finally, we explored the relationship between \( \xi \) and \( E_{\text{max}} \) and LMA and leaf thickness, for different species and leaf ages (only the June campaign), using linear regression. All analyses were performed in R v3.4.3 (\text{R Development Core Team, 2017}).

\textbf{Results}

The seven study species exhibited morphological trait values representative of their corresponding PFT (\( F = 60.1 \) for LMA and 38.6 for thickness, \( P < 0.001; \text{Supplementary Fig. S4} \)). In all species, LMA and leaf thickness were significantly lower in developing leaves than mature leaves (\( F = 11.1, P = 0.002 \) for LMA and \( F = 16.2, P < 0.001 \) for leaf thickness; \text{Supplementary Fig. S4}).

Over the course of the gas-exchange campaigns, \( D_{w} \) increased gradually from 8 to 42 mmol mol\(^{-1}\). For all species and in both campaigns, we measured \( A_{\text{net}} \) and \( g_{sc} \) under a \( D_{w} \) range of at least 12–35 mmol mol\(^{-1}\) (Fig. 1). We measured maximum \( A_{\text{net}} \) and \( g_{sc} \) on the first round of measurements, when \( D_{w} \) was minimal. In general, as \( D_{w} \) increased, \( A_{\text{net}} \) and \( g_{sc} \), and estimated carboxylation capacity (\( \bar{V}_{\text{max}} \)) decreased from mid-morning to midday measurements (Supplementary Fig. S1). The ratio of intercellular to ambient CO2 concentrations (\( C_{i}/C_{a} \)) was between 0.6 and 0.8 for the \( C_{3} \) species and remained relatively constant within species along an increasing \( D_{w} \) gradient in the June campaign (Supplementary Fig. S2). We measured the lowest \( C_{i}/C_{a} \) in the \( C_{4} \) crop (\( Z. \text{mays} \)) under well-watered conditions (Supplementary Fig. S2). In August, under low water availability, \( C_{i}/C_{a} \) gradually increased with \( D_{w} \) in \( P. \text{pinaster} \) and \( Z. \text{mays} \) (Supplementary Fig. S2). There were significant differences among species (\( P < 0.05 \), except for \( g_{sc} \) in June, \( P = 0.07 \)) in \( g_{sc} \) and \( A_{\text{net}} \) the \( C_{4} \) crop (\( Z. \text{mays} \) had significantly higher \( A_{\text{net}} \) and lower \( g_{sc} \) than the other species (Fig. 1)). In June, developing leaves had significantly higher \( g_{sc} \) and lower \( A_{\text{net}} \) than mature fully expanded leaves, although the leaf age effect was species specific, with maximum differences in the \( C_{4} \) species for \( A_{\text{net}} \) and in the fern for \( g_{sc} \) (Fig. 1). In addition, developing leaves had lower \( \bar{V}_{\text{max}} \) (Supplementary Fig. S1). The low-water treatment reduced \( \Psi_{pd} \) \( g_{sc} \), \( A_{\text{net}} \), and \( \bar{V}_{\text{max}} \) (Table 1, Fig. 1, Supplementary Fig. S1). Again, the treatment effect was species specific: the reduction in both \( g_{sc} \) and \( A_{\text{net}} \) under low-water treatment was least pronounced in the two evergreen trees (Fig. 1), to the point that \( \Psi_{pd} \) of \( P. \text{pinaster} \) was only marginally affected (Table 1).

We found that both the CAP and the MES formulations fitted our data for most species, leaf ages, and watering treatments, with a few exceptions. Neither the CAP nor the MES formulation fitted the fern (\( P. \text{aquilinum} \)) data for either mature or developing leaves in the June campaign, or for well-watered ferns in the August campaign (Supplementary Fig. S3). Similarly, neither the CAP nor the MES formulation fitted the measurements on well-watered plants of \( M. \text{caerulea} \) (the \( C_{3} \) grass) in the August campaign (Supplementary Fig. S3). In addition, the CAP formulation did not fit the measurements on the \( C_{4} \) species \( Z. \text{mays} \) for developing leaves in June and under either watering treatment in August (Supplementary Fig. S3). For the other species, leaf ages, and watering treatments, both the CAP and MES formulations fitted our observations. When both the CAP and MES formulations could explain our data, the AIC value and the RMSE, as well as the likelihood ratio test (in most cases), indicated that the CAP formulation provided a better fit than that of the MES formulation, with the exception of developing leaves in \( P. \text{pinaster} \) and \( Q. \text{rubus} \) (Table 2). Observed and predicted \( g_{sc} \) values were more closely related for the CAP formulation (lower RMSE), regardless of leaf age and watering regime (Fig. 2). For the CAP formulation, there was a significant difference between mature and developing leaves, with a smaller RMSE for mature leaves.
<table>
<thead>
<tr>
<th>Species</th>
<th>Campaign</th>
<th>Leaf age</th>
<th>Treatment</th>
<th>CAP</th>
<th>MES</th>
<th>AIC</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>$g_0$ (mmol m$^{-2}$ s$^{-1}$)</td>
<td>$\xi$ (mmol m$^{-0.5}$ mol$^{-0.5}$)</td>
<td>$g_1$ (kPa$^{0.5}$)</td>
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<td>Z. mays</td>
<td>June 2015</td>
<td>Mature</td>
<td>Well-watered</td>
<td>1.3±11.4</td>
<td>2±0.7</td>
<td>0.4±0.1</td>
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<td>B. pendula</td>
<td></td>
<td></td>
<td></td>
<td>4.1±7.7</td>
<td>7.7±1.9</td>
<td>1.5±0.4</td>
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<tr>
<td>Q. robur</td>
<td></td>
<td></td>
<td></td>
<td>1.5±4.7</td>
<td>6.2±1</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>E. gunnii</td>
<td></td>
<td></td>
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<td>3.2±7.8</td>
<td>5.7±1.1</td>
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<td>90.6±39.9</td>
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<td>Z. mays</td>
<td>August 2015</td>
<td>Mature</td>
<td>Well-watered</td>
<td>7.8±8</td>
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<tr>
<td>B. pendula</td>
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<td>8.1±3.3</td>
<td>3.8±0.9</td>
<td>0.8±0.2</td>
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<td>39.8±31.2</td>
<td>2±15.5</td>
<td>0.4±3.1</td>
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<td>7.5±3.4</td>
<td>4.1±0.8</td>
<td>0.8±0.2</td>
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<td></td>
<td></td>
<td>24.2±15.7</td>
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<td>0.7±0.7</td>
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<td>Mature</td>
<td>Low-watered</td>
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<td>−0.2±0.1</td>
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<td></td>
<td>4.7±1</td>
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<td>0.4±0.1</td>
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<tr>
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<td></td>
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<td>5.3±2</td>
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<tr>
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<tr>
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<td>12.2±2</td>
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<td>2.8±1.1</td>
<td>2±0.7</td>
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</table>

Significant ($P<0.05$) results ($\chi^2$) and coefficients significantly different from 0 are in bold.
compared with developing leaves (Table 2). There were no significant differences in the fit between low-watered and well-watered plants (Fig. 2).

Comparison of estimated ξ (CAP formulation) values with their 95% CI showed that there were some differences among species. Deciduous angiosperm trees had the highest ξ values and the C₄ crop (maize) the lowest (Table 2). Under well-watered conditions and in mature leaves, estimated ξ values for the four tree species were similar (Fig. 3A). We did not find clear differences in ξ values between mature and developing leaves in any species (Fig. 3A), although in *M. caerulea* ξ tended to be higher in developing than mature leaves. The low-water treatment decreased ξ in the deciduous angiosperm trees (oak and birch; Fig. 3B). For the MES formulation, analyses of homogeneity of slopes showed similar results to the comparison of the 95% CI for ξ values. Species differed significantly in their slopes (√*E*ₘₐₓ) in both measurement campaigns (Table 3). Deciduous angiosperm trees had the highest slope, and the C₄ crop the lowest, whereas all tree species had comparable slopes for mature leaves under well-watered conditions (Table 2). Leaf age did not have a significant effect on *E*ₘₐₓ and the water-stress treatment decreased *E*ₘₐₓ in angiosperm deciduous trees (Tables 2 and 3), although the relationship between *g*ₑ and the MES index did not appear to be linear for the majority of plants under the low-water treatment (Supplementary Fig. S2).

Analyses of homogeneity of slopes for *E*ₘₐₓ and comparisons of the 95% CIs for ξ values were consistent with results of one-way ANOVAs. Under low water availability, *E*ₘₐₓ decreased (Fig. 4, *F*=8, *P*=0.015), but the species mean ξ values did not decrease significantly (Fig. 3, *F*=2.2, *P*=0.16). Leaf age did not have a significant effect on *E*ₘₐₓ (*F*=0.5, *P*=0.511) or ξ (*F*=0.4, *P*=0.561). We did not find any significant correlation between ξ and *E*ₘₐₓ with LMA or leaf thickness (*F*≤3 and *P*≥0.1 for all analyses; Supplementary Fig. S5).

When we plotted ξ√*V*ₐₓ against *Ψ*ₚd for well-watered and low-watered plants (Fig. 4A), we found that estimates for
deciduous angiosperm trees varied greatly between watering treatments and fell close to the values predicted by the CAP formulation (equation 2) for high $K_{\text{st}}$ ($50 \text{ mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$).

In contrast, for all other C$_3$ species, $\xi \sqrt{V_{\text{max}}} \text{Ca}$ did not appear to decrease notably from well-watered to low-watered plants, and our estimates fell within the CAP model predictions for lower $K_{\text{st}}$ ($2$–$12 \text{ mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$). The plot of $E_{\text{max}}$ against $\Psi_{\text{ad}}$ showed a similar pattern (Fig. 4B), but with a larger spread. Our $E_{\text{max}}$ estimates for deciduous angiosperm trees fell closer to the theoretical estimates (equation 4) predicted for higher $K_{\text{st}}$.

**Discussion**

In this study we compared two analytical solutions to predict $g_m$, based on optimization theory, assuming that the water costs of stomata opening originate from NSL to photosynthesis caused by either a reduction in carboxylation capacity (the CAP formulation) or in $g_m$ (the MES formulation; Dewar *et al.*, 2018). We expected the CAP formulation to fit better for developing leaves and for PFTs with low-LMA leaves. Our results showed that the CAP formulation fitted better to our measurements not only for low-LMA leaves, but for all species, leaf ages, and watering regimes.

One single formulation to predict stomatal behaviour across plant functional types

We had hypothesized that the suitability of the two formulations proposed by Dewar *et al.* (2018) would vary across species from PFTs depending on leaf morphology. Here, the variation in LMA observed among species from contrasting PFTs was consistent with previous classifications (Poorter *et al.*, 2009). Anatomical traits encompassing low $g_m$ are usually associated with high LMA, and thus the limitation to photosynthesis imposed by $g_m$ should increase with LMA (Flexas *et al.*, 2008; Niinemets *et al.*, 2009). We expected a better fit of the MES formulation for evergreen species (which have high LMA; Verömann-Jurgenson *et al.*, 2017). However, this was not the case. Instead, the CAP formulation provided a better fit for all species, including the conifer and evergreen angiosperm trees, especially under conditions of reduced water availability (Fig. 4). This result is consistent with the midday depression observed in carboxylation capacity for all species, leaf ages, and watering levels (Supplementary Fig. S1). A better fit of the CAP over the MES formulation does not imply that $g_m$ is not limiting photosynthesis, but rather that decreased carboxylation capacity in response to diurnal changes in $\Psi_{\text{leaf}}$ can impose a stronger constraint on carbon gain over the timescale at which stomata operate (Franks *et al.*, 2017). This result supports our alternative expectation that in evergreen leaves, with low maximum $g_m$ (Niinemets *et al.*, 2009; Flexas *et al.*, 2012), marginal reductions in $g_m$ in response to daily oscillations in $\Psi_{\text{leaf}}$ are unlikely to impose further NSL to photosynthesis over short (sub-daily) timescales. Nevertheless, the extent of our results is limited by the lack of a quantitative analysis of the relative limitations to photosynthesis (Grassi and Magnani, 2005).

Our estimates of $\xi$ (parameter of the CAP formulation) for our study species were generally lower than but comparable to those of Lin *et al.* (2015) for their corresponding PFT. In agreement with previous classifications, we found that $\xi$ was lowest for the C$_4$ species (maize) and higher for the deciduous and herbaceous angiosperm species (Lin *et al.*, 2015; Miner *et al.*, 2017). In addition, our $\xi$ estimates for tree saplings and potted plants were comparable to previously reported values for congeneric and closely related species grown under controlled conditions (Héroult *et al.*, 2013; Zhou *et al.*, 2013, 2014). For the crop with a C$_4$ pathway (maize), we found that the CAP formulation provided a better fit (especially for mature leaves) in the June campaign, but in the August campaign it did not provide a reasonable fit. Our measurements of $A_{\text{net}}$ and $g_m$ in this latter campaign were lower than those from the June campaign and some were nearing the limit of detection of our porometer (Fig. 1). It is possible that during this second campaign, maize plants were close to the senescent

![Image](https://academic.oup.com/jxb/article-abstract/70/5/1639/5306169 by Western Sydney University Library user on 25 March 2019)
stage (Jordan-Meille and Pellerin, 2004), and a coordinated age-related decline in both $g_{sc}$ and $g_{m}$ could have influenced our measurements (Barbour et al., 2016).

As far as we know, this is the first study to specifically test for optimal stomatal behaviour in a fern species. We found that, unless subjected to reduced water availability, stomata in the fern *P. aquilinum* did not operate according to optimization theory. The main NSL to photosynthesis in many ferns is $g_{m}$ (Gago et al., 2015; Tosens et al., 2016), so we expected the MES formulation to provide the best fit, but this was not the case. We suggest that in ferns, although $g_{m}$ often imposes the main limitation to photosynthesis in many ferns (Carriqui et al., 2015), at the sub–daily timescale at which stomata operate, marginal reductions in $g_{m}$ with $\Psi_{pd}$ are unlikely to incur further costs on C gain. Additionally, although carboxylation capacity can co-limit photosynthesis in some ferns (Gago et al., 2013), it is unlikely that rudimentary fern stomata are capable of exhibiting a coordinated response to minimize damage to the photosynthetic machinery (Brodribb and McAdam, 2011). Instead, collectively, our results could be interpreted as further supporting the passive hydraulic control of stomata in ferns (despite our coarse characterization of the sensitivity of $g_{sc}$ to $D_{v}$; Fig. 1), where changes in $D_{v}$ and leaf capacitance would underlie stomatal control (Brodribb and McAdam, 2011; Martins et al., 2016).

DGVM need measurable physiological traits for distinct PFTs to provide reliable estimates of vegetation–atmospheric fluxes (Miner et al., 2017). Our results support that the parameter $\xi$ (from the CAP formulation in Dewar et al., 2018, and equivalent to $g_{i}$ in Medlyn et al., 2011) is a good candidate trait to be incorporated into DGVM to predict the coupling of carbon and water fluxes under future atmospheric conditions, in combination with other fundamental physiological traits. Parameter $\xi$ (or $g_{i}$) captures well the variability across PFTs (Fig. 3) and is commonly associated with other traits, so that it could contribute to defining common plant strategies within PFTs. For example, species with high $\xi$ should exhibit low $V_{cmax}$ (Lin et al., 2015; Hasper et al., 2017; Medlyn et al., 2017), as well as high $|\Psi_{i}|$ and low $K_{s}$, and hence high resistance to cavitation. Yet, we should be cautious regarding the generalization of a static $\xi$ value for each PFT, particularly under decreasing water availability (Zhou et al., 2013; Drake et al., 2017). In addition, it remains to be tested how plasticity or endogenous regulation could impact $\xi$ estimates within and across PFTs (Peguero-Pina et al., 2016; de Dios et al., 2017; Miner and Bauerle, 2017; Wolz et al., 2017).

**Impact of water availability on optimal stomatal behaviour**

We found that under low water availability, stomata behaved according to optimization theory in all our study species. The low-watering treatment had the expected physiological effects: it reduced $A_{net}$, $g_{sc}$, $V_{cmax}$, and $\Psi_{pd}$ relative to well-watered plants (Fig. 1, Table 1), although $\Psi_{pd}$ did not decrease significantly in *P. pinaster* (Table 1), which maintained $\Psi_{pd}$ well above levels required to induce stem cavitation for this species (Bouche et al., 2016). Decreased $A_{net}$ is directly affected by stomatal closure because of diminished substrate supply, but also by NSL (Farquhar and Sharkey, 1982). Most likely, decreased $A_{net}$ under low water availability resulted from reductions in both types of NSL, that is, $g_{m}$ (Théroux-Rancourt et al., 2014;
Wang et al., 2018) and carboxylation capacity (Flexas et al., 2006; Gimeno et al., 2010). Here, we did not explicitly quantify the contributions of each NSL to reduced photosynthesis under water stress (Grassi and Magnani, 2005; Zhou et al., 2013; Drake et al., 2017) and we did not measure \( g_{\text{net}} \). Yet, we found that \( \dot{V}_{\text{cmax0}} \) decreased at midday and the CAP formulation provided the best fit under both water levels (Fig. 2). Thus, we suggest that reduced carboxylation capacity is likely to be a major driver underlying rapid (sub-daily) stomatal regulation, irrespective of water availability.

We observed some differences in stomatal behaviour among species from the six PFTs as a consequence of reduced water availability. The marginal water cost to C gain (\( \xi \) or \( g \)) was conserved under low water availability, except for the two deciduous angiosperm trees, for which \( \xi \) decreased under low water availability. Conserved \( \xi \) or \( g \), under decreasing water availability has previously been reported for species with a large drought-tolerance range, while more drought-sensitive species were capable of modulating their marginal water cost under water stress (Héroult et al., 2013; Zhou et al., 2013; Gimeno et al., 2016; Miner and Bauerle, 2017). For the fern, we found a tight coupling of \( g_{\text{sc}} \) and \( A_{\text{net}} \) according to optimization theory only under low water availability, suggesting that the hydro-passive mechanism underlying the stomatal response to increasing \( D_{\text{m}} \) might be functional only below a certain water availability threshold (Martins et al., 2016). According to the CAP formulation, Dewar et al. (2018) predict a larger decrease in \( \xi \sqrt{\dot{V}_{\text{cmax0}}} \) with \( \Psi_{\text{leaf}} \) for species with a higher root-to-leaf conductivity (\( K_{\text{d}} \)). Our results support this prediction (Fig. 4), as we found that for the two deciduous angiosperm trees, which were expected to exhibit high \( K_{\text{d}} \) (Domec et al., 2017), our estimates of \( \xi \sqrt{\dot{V}_{\text{cmax0}}} \) fell close to the CAP model predictions for the higher range of \( K_{\text{d}} \).

For the conifer (\( P. \) pinaster), the water-stress treatment did not reduce \( \Psi_{\text{psd}} \) and our estimates of \( \xi \sqrt{\dot{V}_{\text{cmax0}}} \) from low-watered and well-watered plants overlapped (Fig. 4), a response that typifies isohydric behaviour, consistent with previous observations for this species (Ripullone et al., 2007).

**Optimal stomatal behaviour in developing leaves**

We found that optimal stomatal behaviour in developing leaves mimicked that of their mature counterparts (Fig. 3). Contrary to our expectations, we did not find lower \( A_{\text{net}} \) in developing leaves. Similar \( A_{\text{net}} \) would imply that in developing leaves respiratory costs associated with construction would be compensated for in mature leaves with greater maintenance costs (Zhou et al., 2015). Alternatively, or in addition, developing leaves would compensate for extra respiratory costs with greater carboxylation capacity (Medlyn et al., 2002; Rajaona et al., 2013; Locke and Ort, 2014), but this latter explanation is not supported by our estimates of carboxylation capacity using the one-point method (Supplementary Fig. S1) (De Kauwe et al., 2016). More importantly, the CAP formulation predicted similar coupling between \( g_{\text{sc}} \) and \( A_{\text{net}} \) with no clear differences in the marginal water cost to C gain (\( \xi \) or \( g \)) between mature and developing leaves. The CAP formulation fitted best for both leaf ages, except for the developing leaves of pine and oak, although the CAP formulation also successfully predicted \( g_{\text{sc}} \) in the developing leaves of these species. This result supports our initial expectation that the photosynthetic apparatus of developing leaves would be sensitive to diurnal oscillations in \( \Psi_{\text{leaf}} \) and their stomata would operate to minimize the likelihood of dehydration damage (Chaves et al., 2002). Similar stomatal behaviour for both leaf ages suggests that developing leaves might not be less tolerant to dehydration, although the underlying protective mechanisms could vary. For example, it has been shown that developing leaves can tolerate similar water stress to mature leaves, albeit at a greater carbon cost as they rely on the accumulation of compatible solutes (Sperdouli and Moustakas, 2014). This strategy would compensate for a lack of morphological adaptation expressed later in ontogeny. Furthermore, our results could also suggest that \( g_{\text{m}} \) limits photosynthesis in mature and developing leaves to a similar extent and that \( g_{\text{m}} \) does not necessarily decrease during leaf development. Developmental changes that result in lower \( g_{\text{m}} \), such as thicker cell walls, could be partially compensated, for example, with an increase in the chloroplast per surface area ratio (Evans et al., 2009). Overall, our results did not support that the type of formulation and the marginal water cost to C gain in the developing stage would differ from those applied to mature canopies. Instead, our results indicated that stomatal behaviour was conserved along leaf ontogeny and across PFTs, despite potentially large differences in construction costs (Wright et al., 2004; Onoda et al., 2017). So far, DGVM have ignored possible changes in stomatal behaviour with leaf ontogeny, even for deciduous forests in temperate regions (Keenan et al., 2013). Our study, together with other recent observations (Macinnis-Ng et al., 2017), suggest that the CAP formulation could serve as a homogeneous approach to predict \( g_{\text{sc}} \) under changing environmental conditions, irrespective of leaf ontogenetic stage.

**Conclusions**

We have shown that the optimal formulation assuming that water costs to carbon gain arise from reduced carboxylation capacity (CAP) provides a suitable fit for predicting stomatal behaviour irrespective of leaf ontogenetic stage, PFT, and water availability. Our results do not imply that NSL to photosynthesis are restricted to reduced carboxylation capacity. Instead, we argue that sub-daily marginal changes in \( g_{\text{sc}} \) are unlikely to be influencing the rapid stomatal responses to atmospheric drought. Our results suggest that the CAP formulation successfully captures the co-variation of \( g_{\text{sc}} \) and \( A_{\text{net}} \) with increasing \( D_{\text{m}} \), irrespective of PFT, water stress, or leaf ontogenetic stage, for the timescale at which stomata operate. Our data also support that the use of a single-parameter CAP formulation is valid for most PFTs, with the caveat that the CAP parameter (\( \xi \) or \( g \)) should not be assumed to be static in response to water availability for all PFTs, particularly for those most sensitive to drought.
Supplementary data

Supplementary data are available at JXB online.

Protocol S1. Estimated carboxylation capacity with the one-point method.

Fig. S1. Estimated carboxylation capacity.

Fig. S2. Internal to ambient CO₂ molar ratio against vapour pressure deficit.

Fig. S3. Stomatal conductance plotted against the indexes of the two formulations.

Fig. S4. Leaf mass per area and leaf thickness for all species and two leaf ages.

Fig. S5. Model slope parameters plotted against leaf mass per area and leaf thickness.

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