Short-term water stress impacts on stomatal, mesophyll and biochemical limitations to photosynthesis differ consistently among tree species from contrasting climates

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Predicting the large-scale consequences of drought in contrasting environments requires that we understand how drought effects differ among species originating from those environments. A previous meta-analysis of published experiments suggested that the effects of drought on both stomatal and non-stomatal limitations to photosynthesis may vary consistently among species from different hydroclimates. Here, we explicitly tested this hypothesis with two short-term water stress experiments on congeneric mesic and xeric species. One experiment was run in Australia using Eucalyptus species and the second was run in Spain using Quercus species as well as two more mesic species. In each experiment, plants were grown under moist conditions in a glasshouse, then deprived of water, and gas exchange was monitored. The stomatal response was analysed with a recently developed stomatal model, whose single parameter $g_1$ represents the slope of the relationship between stomatal conductance and photosynthesis. The non-stomatal response was partitioned into effects on mesophyll conductance ($g_m$), the maximum Rubisco activity ($V_{cmax}$) and the maximum electron transport rate ($J_{max}$). We found consistency among the drought responses of $g_1$, $g_m$, $V_{cmax}$ and $J_{max}$, suggesting that drought imposes limitations on Rubisco activity and RuBP regeneration capacity concurrently with declines in stomatal and mesophyll conductance. Within each experiment, the more xeric species showed relatively high $g_1$ under moist conditions, low drought sensitivity of $g_1$, $g_m$, $V_{cmax}$ and $J_{max}$, and more negative values of the critical pre-dawn water potential at which $V_{cmax}$ declines most steeply, compared with the more mesic species. These results indicate adaptive interspecific differences in drought responses that allow xeric tree species to continue transpiration and photosynthesis for longer during periods without rain.

**Keywords**: drought sensitivity, gas exchange, hydroclimate, $J_{max}$, mesophyll conductance, model, stomatal conductance, $V_{cmax}$.

Introduction

Water stress is the principal environmental factor limiting photosynthesis, growth and primary production globally (Nemani et al. 2003), with profound consequences for ecosystems and human societies (Tezara et al. 1999). The accurate model prediction of climate change impact on the global carbon and water cycles requires a realistic representation of drought effects on major plant physiological processes, especially photosynthesis and transpiration, and the fluxes of CO₂ and water vapour between plants and the atmosphere. In particular, we need to understand how drought stress impacts vary among ecosystems. Based on the theoretical analysis by Mäkelä et al.
(1996), it is expected that mesic species that experience higher probability of rain in their natural habitats will show a steeper decline rate of the stomatal sensitivity parameter \(g_i\) with decreasing soil moisture availability, relative to xeric species. Recent experimental work suggests that vulnerability to drought is similarly high in both mesic and xeric ecosystems (Choat et al. 2012), indicating that there may be important differences among species in their sensitivity to absolute levels of soil moisture. However, such differences are not currently reflected in models. In this paper, we aimed to investigate whether there are systematic differences in drought sensitivity among species from different hydroclimates that should be represented in process-based models.

There has been a long-standing controversy on the mechanistic basis for photosynthesis \((A)\) reduction under drought. While many studies have suggested that diffusive limitations—that is, reduced stomatal conductance \(g_s\) and/or mesophyll conductance \(g_m\)—are the main cause of photosynthesis reduction (at least until severe drought sets in) (Bota et al. 2004, Flexas et al. 2004, 2006a, 2012, Grassi and Magnani, 2005, Egea et al. 2011, Galmés et al. 2013, Cano et al. 2014), others have reported that inhibition of ribulose-1,5-bisphosphate-carboxylase-oxygenase (Rubisco) activity (Kanechi et al. 1996, Castrillo et al. 2001, Parry et al. 2002, Tezara, 2002) and/or an impaired capacity for ribulose-1,5-bisphosphate (RuBP) regeneration (Tezara et al. 1999, Thimmanaik et al. 2002) play a central role. Reflecting this controversy, models also differ in how they represent drought effects on photosynthesis (Kauwe et al. 2013). Some models assume that, due to diffusive limitations, drought decreases the intercellular \(\text{CO}_2\) concentration \(C_i\) and thereby also the rate of carbon assimilation. Other models assume that, due to Rubisco inhibition, drought reduces the photosynthetic rate at a given \(C_i\). However, recent meta-analyses of experimental data have indicated the importance of including both effects in models (Egea et al. 2011, Zhou et al. 2013).

Based on a re-analysis of data from a number of published studies, Zhou et al. (2013) found that both stomatal and non-stomatal processes were drought-sensitive. Furthermore, the sensitivity of both components appeared to vary systematically, with both being related to plant functional type (PFT) and climate in similar ways. It appeared that sensitivity to soil moisture availability increased with rainfall frequency, as predicted by the Mäkelä theory. However, the experiments analysed varied widely with respect to experimental conditions, pot size and drought treatments, so it was not possible to attribute variation in drought sensitivity unambiguously to either climate of origin or PFT.

To directly test for a systematic relationship between plant drought responses and climate of origin, we conducted two independent short-term drought experiments on tree species of contrasting climatic origins in Australia and Europe. In each experiment, we used congeneric species from mesic and xeric origins, and grew them under identical experimental conditions. In Australia, we compared drought responses of four Eucalyptus species, all evergreen. In Europe, we compared drought responses between three Quercus species from different hydroclimates and two additional mesic species (Figure 1).

During the drought period, we monitored the decline in stomatal and non-stomatal limitations. We also monitored pre-dawn leaf water potential \(\Psi_{pd}\) as an indicator of soil moisture stress. To distinguish stomatal and non-stomatal limitations, we followed the model-oriented framework of Zhou et al. (2013). This framework interprets stomatal limitation as a change in the substomatal \(\text{CO}_2\) concentration \(C\) and non-stomatal limitation as a change in the response of \(A\) to \(C\). This interpretation differs from the traditional partitioning approach as described by Jones (1985), in which stomatal limitation is measured as reduced \(g_s\) rather than reduced \(C\). The frameworks differ because models (realistically) represent a coupling between \(g_s\) and \(A\), which means that any reduction in the maximum Rubisco activity \(V_{\text{cmax}}\) will drive a reduction in \(g_s\). Such a reduction in \(g_s\) is a consequence of the non-stomatal limitation, rather than a stomatal limitation per se, and hence is treated here as part of the non-stomatal limitation.

In the model framework, stomatal limitation is expressed in terms of the model parameter \(g_i\), which is inversely proportional to the marginal carbon cost of water \(\lambda\), and is expected to differ among PFTs, climates and species of the same genus with different geographical origins (Medlyn et al. 2011, Héroult et al. 2013, Zhou et al. 2013). Mäkelä et al. (1996) indicated that \(1/\lambda\) should be expected to decline exponentially with decreasing soil water moisture, and the rate of decline with soil moisture should increase with the probability of rain. To quantify the role of stomatal limitation, we analysed the effect of water stress on \(g_i\). To quantify the role of non-stomatal limitation, we analysed the effect of water stress on \(g_m\), \(V_{\text{cmax}}\), and the maximum electron transport rate \(J_{\text{max}}\) (the primes denote apparent values, calculated in the standard way and thus implicitly assuming infinite mesophyll conductance, \(g_m\)). We also explored the effect of water stress on \(V_{\text{cmax}}\) and \(J_{\text{max}}\), i.e., estimates of the ‘true’ values accounting for effects of finite and variable \(g_m\).

Our two major hypotheses were as follows: (i) drought should impose concurrent limitations on \(g_i\), \(g_m\), \(V_{\text{cmax}}\) and \(J_{\text{max}}\); and (ii) \(g_i\), \(g_m\), \(V_{\text{cmax}}\) and \(J_{\text{max}}\) should decline more rapidly with drought for mesic species that rarely experience drought in their native growing environment, compared with xeric species that normally endure periods of drought. Our goal was to test these hypotheses and thereby contribute to an understanding of how stomatal, mesophyll and biochemical processes are influenced by short-term drought, and how these responses differ among species originating from different hydroclimates.
Materials and methods

Choice of species and range of hydroclimates

For the experiment in Australia, four species were selected from the widely distributed Australian genus Eucalyptus. The four Eucalyptus species were all evergreen and included E. striaticalyx and E. occidentalis from southwestern Australia, Eucalyptus camaldulensis subsp. subcinerea from central Australia, and E. camaldulensis subsp. camaldulensis from southeastern Australia. For the experiment in Spain, species of contrasting climatic origins were selected from the widespread northern-hemisphere genus Quercus, including the evergreen Q. ilex L., winter-deciduous Q. pubescens L., and deciduous Quercus robur L. Two other deciduous and mesic species were also included: the riparian species Alnus glutinosa L., and Fraxinus excelsior L., which is a component of upland forests in northern Europe but becomes confined to canyons and riparian situations in the drier regions of southern Europe.

To quantitatively describe the range of hydroclimates of these species from two continents, we use the moisture index—the ratio of mean annual precipitation (mm) to mean annual

Figure 1. Responses of \( A \) (filled squares) and \( g_s \) (open squares) to \( \Psi_{pd} \) in the experiment on three contrasting Quercus species and also two mesic species in Europe (Plots 1–5), and in the experiment on four contrasting Eucalyptus species in Australia (Plots 6–9).
potential evapotranspiration (mm)—to provide an indirect bioclimatic predictor of annual plant water availability. Moisture index has been shown to be a better indicator of annual plant water availability and thus plant distribution than mean annual precipitation, which neglects differences in evaporative demand among climate zones (Gallego-Sala et al. 2010). Moisture index ranges from a minimum of zero up to values of three to six in the wettest regions. We compared the distribution range of each species against a global map of moisture index, and then attributed the approximate value of moisture index to each species (Table 1), from which species were ranked as more ‘xeric’ versus more ‘mesic’ within two genera of species and two other strongly mesic species. Eucalyptus camaldulensis subsp. camaldulensis and E. camaldulensis subsp. subcinerea were considered to be mesic species within the genus Eucalyptus because they occur along river systems.

**Plant material, growth conditions and drought treatments**

For the Australian experiment, seeds of Eucalyptus species were obtained from the Australian Tree Seed Centre in Canberra and germinated in May 2012. At 3 months, the seedlings were transplanted into 90-l pots containing 80 kg of loamy soil (collected from the Robertson area in New South Wales, Australia), evenly mixed with slow-release fertilizer. The plants were grown in the open air with regular watering and full sunshine for 3 months to allow natural establishment. At 6 months of age (December 2012), the plants were placed in a glasshouse at Macquarie University under a 25 versus 18 °C diurnal temperature cycle and maintained under moist conditions (100% field capacity). Measurements on E. striaticalyx were conducted after 3 months in April 2013; measurements on the other Eucalyptus species (including chlorophyll fluorescence, which was not available for E. striaticalyx) were conducted after 5 months in June 2013.

In the European experiment, 2-year-old saplings of Quercus species, A. glutinosa and F. excelsior were purchased from a plant nursery in Barcelona and transplanted into 18-l pots in a glasshouse at the University of Barcelona in June 2013. They were maintained under moist conditions (100% field capacity) and ambient temperature for 2 months (the diurnal temperature cycle was 24 versus 15 °C in June and 28 versus 19 °C in July).

In both experiments, all species were subjected to short-term drought by ceasing watering. Measurements were conducted daily during the drying-down process, until stomatal conductance was close to zero. The number of days taken for the drying-down process is shown in Table 1 for each species.

Due to the availability of materials, the two experimental platforms in Sydney and Barcelona differed in several ways, including in soil texture, pot size and root volume. Therefore, to robustly test the systematic relationship between drought response and climate of origin, we focus on contrasts between xeric and mesic species within each experiment. However, some limited comparisons can also be made across experiments because we represent plant water availability by $\Psi_{pd}$, which can be argued to measure plant water stress independently of growth conditions.

**Pre-dawn leaf water potential measurement**

$\Psi_{pd}$ was measured using a pressure chamber (PMS 1000, PMS Instruments, Corvallis, OR, USA). All measurements were completed before sunrise. Two leaves per sapling were sampled. When the observed difference between the two leaves was $>0.2$ MPa, a third leaf was measured. $\Psi_{pd}$ is the best measure of water availability to the plant since it integrates soil water potential over the root zone (Schulze and Hall 1982). $\Psi_{pd}$ is independent of differences in rooting depth and soil water access, and is not influenced by daytime transpiration, while daytime leaf water potential depends strongly on transpiration as well as on soil water status. $\Psi_{pd}$ is also independent of soil texture, unlike volumetric soil moisture content, enabling us to compare species from two experimental platforms using different soil types. We take the $\Psi_{pd}$ threshold for gas exchange measurement ($\Psi_0$) as one parameter defining the variation of water-use strategies among species.

**Photosynthetic parameters based on CO₂ response curves**

Leaf gas exchange measurements were performed on current-year fully-exposed sun-exposed leaves, using a portable photosynthesis system (LI-6400, Li-Cor, Inc., Lincoln, NE, USA) equipped with a LI-6400-40 Leaf Chamber Fluorometer (except E. striaticalyx). Before each measurement, the leaf was acclimated in the chamber for 20–30 min to achieve stable gas exchange, with leaf temperature maintained at 25 °C, reference CO₂ concentration at 400 µmol CO₂ mol⁻¹ air, and a saturating photosynthetic photon flux density (Q) of 1800 µmol photon m⁻² s⁻¹ for Eucalyptus species and

### Table 1. The value of moisture index of the habitat in which the species occur, and the duration of drying-down process in number of days.

<table>
<thead>
<tr>
<th>Species</th>
<th>Moisture index</th>
<th>Days of drying-down process</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. striaticalyx</td>
<td>0.15</td>
<td>42</td>
</tr>
<tr>
<td>E. occidentalis</td>
<td>0.35</td>
<td>26</td>
</tr>
<tr>
<td>E. camaldulensis subsp.</td>
<td>0.19</td>
<td>19</td>
</tr>
<tr>
<td>subcinerea</td>
<td>0.19</td>
<td>19</td>
</tr>
<tr>
<td>E. camaldulensis subsp.</td>
<td>0.46</td>
<td>19</td>
</tr>
<tr>
<td>camaldulensis</td>
<td>0.46</td>
<td>19</td>
</tr>
<tr>
<td>Q. ilex L.</td>
<td>0.88</td>
<td>8</td>
</tr>
<tr>
<td>Q. pubescens L.</td>
<td>1.0</td>
<td>12</td>
</tr>
<tr>
<td>Q. robur L.</td>
<td>1.37</td>
<td>5</td>
</tr>
<tr>
<td>A. glutinosa L.</td>
<td>1.25</td>
<td>4</td>
</tr>
<tr>
<td>F. excelsior L.</td>
<td>1.31</td>
<td>4</td>
</tr>
</tbody>
</table>
1200 μmol photon m⁻² s⁻¹ for other species. The vapour pressure deficit (D) was held as constant as possible during the measurement. After the leaf acclimated to the cuvette environment, light-saturated net CO₂ assimilation rate (A) and stomatal conductance (gₛ) were measured. The A–Cᵢ curve measurement was then conducted with the cuvette reference CO₂ concentration set as follows: 300, 200, 150, 100, 50, 400, 400, 600, 800, 1000, 1400 and 2000 μmol CO₂ mol⁻¹ air. The leaf was allowed to equilibrate for at least 3 min at each Cᵢ step before logging data. After completing the A–Cᵢ curve measurements, the light was switched off for 3 min of darkness and then leaf respiration rate was measured at the ambient CO₂ concentration. A and Cᵢ values at each step were corrected for CO₂ diffusion leaks with a diffusion correction term (k) of 0.445 μmol m⁻² s⁻¹, following the manufacturer’s recommendation (Li-Cor, Inc.). Values of Vₘₜₐₓ ’ and Jₘₜₐₓ ’ were quantified from A–Cᵢ curves using the leaf photosynthesis model by Farquhar et al. (1980), based on a least-squares fitting method in the ‘R’ environment (R Development Core Team 2010). The curve fitting routine was that introduced by Domingues et al. (2010), with enzyme kinetic constants from von Caemmerer (2000).

**Chlorophyll fluorescence measurement**

At each step of the A–Cᵢ curve measurement, steady-state fluorescence (Fₛ) and maximum fluorescence (Fₘₛ) were measured during a light-saturating pulse, thus enabling the calculation of the photochemical efficiency of PSII (Φₚₛₛ) as Φₚₛₛ = (Fₘₛ ‑ Fₛ)/Fₘₛ. The rate of photosynthetic electron transport from fluorescence (Jₑᵗₚᵣ) was then calculated following Kraus and Edwards (1992), as Jₑᵗₚᵣ = 0.5 Φₚₛₛ α Q, where 0.5 is a factor accounting for the light distribution between the two photosystems and α is the leaf absorptance which is assumed to be 0.85–0.88 in the calculations of LI-6400.

**Mesophyll conductance and the ‘true’ values of Vₘₜₐₓ ’ and Jₘₜₐₓ ’**

Two possible classes of causes accounting for a reduction in Vₘₜₐₓ ’ during the drying-down process were investigated: reductions in the internal mesophyll diffusion conductance from the sub-stomatal chamber to the chloroplasts (gₘ), and biochemical reductions in Vₘₜₐₓ. Mesophyll conductance gₘ was quantified following the variable electron transport rate method by Harley et al. (1992):

\[
gₘ = \frac{A}{Cᵢ - (Γ⁻[Jₑᵗₚᵣ + 8(A + Rₛ)]/[Vₑᵗₚᵣ - 4(A + Rₛ)])},
\]

where the Γ⁻ value was taken from Bernacchi et al. (2002), and the rate of non-photorespiratory respiration continuing in the light (Rₛ) was taken as half of the rate of respiration measured in the dark (Ninemets et al. 2005). Thereafter, gₘ was quantified for every step of the A–Cᵢ curves, and then used to calculate the CO₂ concentration at the chloroplast (Cₜₚ) as follows:

\[
Cₜₚ = Cᵢ - A/gₘ.
\]

The estimated error of gₘ thus calculated is relatively low when Cᵢ is between 100 and 300 ppm, but outside this Cᵢ range the results can be unreliable (Harley et al. 1992). Also, this estimate of gₘ can strongly decline at high Cᵢ, where photosynthesis is not limited by Rubisco activity but by RuBP regeneration or triose phosphate utilization (Flexas et al. 2007), potentially leading to overestimated Cₜₚ and underestimated Jₘₜₐₓ values from the latter part of the A–Cᵢ curve. Accordingly, we estimated Vₘₜₐₓ using the mean value of gₘ calculated for Cᵢ between 100 and 300 ppm (also see the near 1 : 1 relationship between gₘ values at ambient CO₂ concentration and mean gₘ values in the Cᵢ range between 100 and 300 ppm, Figure S6 available as Supplementary Data at Tree Physiology Online). We estimated Jₘₜₐₓ using variable gₘ values at each Cᵢ step to account for the variation of gₘ at high Cᵢ. In this way, A–Cᵢ curves were converted to A–Cᵢ curves, from which Vₘₜₐₓ and Jₘₜₐₓ values were quantified by fitting the leaf photosynthesis model of Farquhar et al. (1980) with the enzymatic kinetic constants from von Caemmerer (2000). To ensure reliable characterization of drought responses of the maximum rate of electron transport, we used the maximum Jₑᵗₚᵣ derived from chlorophyll fluorescence as an independent estimate of Jₘₜₐₓ and compared its drought response with that of Jₘₜₐₓ fitted from A–Cᵢ curves.

**Analytical model for stomatal limitation**

Medlyn et al. (2011) showed that the optimal stomatal theory results in a simple theoretical model of very similar form to widely used empirical stomatal models (Ball et al. 1987, Collatz et al. 1991, Leuning 1995, Arathoon et al. 2002):

\[
gₛ = gₒ + 1.6 \left(1 + \frac{gₒ}{\sqrt{D}}\right) \frac{A}{Cₛ},
\]

where Cₛ is the atmospheric CO₂ concentration at the leaf surface (μmol mol⁻¹) and gₒ is the leaf water vapour conductance when photosynthesis is zero (mol H₂O m⁻² s⁻¹). An alternative derivation of the same expression and further empirical support were provided by Prentice et al. (2014). The derivation of the model by Medlyn et al. (2011) provides an interpretation for the single model parameter gₒ (kPa⁻¹), as being inversely proportional to the marginal carbon cost of water:

\[
gₒ \propto \frac{Γ₋}{λ},
\]

where Λ is the CO₂ compensation point in the absence of mitochondrial respiration (μmol mol⁻¹), and λ is the marginal
water-use efficiency ($\frac{\partial A/\partial E}{mol C \ mol^{-1} H_2O}$) (Medlyn et al. 2011). $D$ has a strong influence on $g_b$ but it is not integrated directly into the leaf photosynthesis model by Farquhar et al. (1980). The $g_b$ parameter encompasses stomatal responsiveness to $D$ and coupling to $A$, and hence it establishes the connection of stomatal behaviour with plant hydraulic functioning (Medlyn et al. 2011, Hérout et al. 2013).

We estimated $g_b$ for each pre-dawn leaf water potential from measurements of $A$, $g_b$, $C_a$ and $D$ by rearranging Eq. (3). The parameter $g_b$ is not part of the optimization. In the analysis, $g_0$ was estimated to be 0.001 mol m$^{-2}$ s$^{-1}$.

We used the following equation to relate $g_b$ to pre-dawn leaf water potential based on the data:

$$g_b = g_{b*}\exp(b_1(\Psi_{pd} + 0.3)),\quad(5)$$

where $g_{b*}$ and $b_1$ were fitted parameters: $g_{b*}$ was the $g_b$ value at $\Psi_{pd} = -0.3$ MPa and $b_1$ represented the sensitivity of $g_b$ to $\Psi_{pd}$. Species adopting different water-use strategies were predicted to differ in their estimated $g_b$ values under moist conditions ($g_{b*}$), and their $g_b$ sensitivity to water stress ($b_1$).

### Analytical model for non-stomatal limitation

An exponential response curve of $g_m$ to $\Psi_{pd}$ was also fitted to each set of observations:

$$g_m = g_{m*}\exp(b_2(\Psi_{pd} + 0.3)),\quad(6)$$

where $g_{m*}$ and $b_2$ are fitted parameters: $g_{m*}$ is the $g_m$ value at $\Psi_{pd} = -0.3$ MPa and $b_2$ represents the sensitivity of $g_m$ to $\Psi_{pd}$. Species adopting different water-use strategies were expected to differ in their $g_m$ sensitivity to water stress ($b_2$).

The response of $V_{cmax'}$ and $J_{max'}$ to water stress was quantified using the logistic function (Tuzet et al. 2003):

$$f(\Psi_{pd}) = K\frac{[1 + \exp(S(\Psi_{pd} - \Psi_{pd}))]}{[1 + \exp(S(\Psi_{pd} - \Psi_{pd}^*)]}\quad(7)$$

We also quantified all parameters defining the drought response of $V_{max}$. The function $f(\Psi_{pd})$ accounts for the relative effect of water stress on $V_{max}$, $V_{cmax'}$ and $J_{max'}$. The form of this function allows a relatively flat response of $V_{cmax}$, $V_{cmax'}$ and $J_{max'}$ under moist conditions, followed by a steep decline, with a flattening again (towards zero) under the driest conditions. $K$ is the value of $f(\Psi_{pd})$ under moist conditions. $S$ is a sensitivity parameter indicating the steepness of the decline, while $\Psi_{pd}$ is a reference value indicating the water potential at which $K$ decreases to half of its maximum value. Species adopting different water-use strategies might be expected to differ in their estimated $V_{cmax}$, $V_{cmax'}$ and $J_{max'}$ values under moist conditions ($V_{cmax}$, $V_{cmax'}$ and $J_{max'}$, estimated as the maximum $V_{cmax}$, $V_{cmax'}$ and $J_{max'}$ values at $\Psi_{pd}=0$, respectively), and in the sensitivity of $V_{cmax}$, $V_{cmax'}$ and $J_{max}$ to water stress ($S_{v'}$, $S_{v''}$ and $S_{v''}$, respectively) and referent water potential ($\Psi_{v'}$, $\Psi_{v''}$ and $\Psi_{v'''}$, respectively).

### Statistical analyses

The nonlinear least-squares package nls() in R was used to find initial values (least-squares estimates) of the parameters of the exponential functions for $g_b$ and $g_m$ responses ($g_{b*}$, $b_1$, $g_{m*}$ and $b_2$); the alternative nonlinear least-squares package nls2() was used to find initial values of the parameters of the logistic functions ($V_{cmax}$ and $V_{cmax'}$, $S_{v'}$ and $S_{v''}$, $\Psi_{v'}$ and $\Psi_{v''}$, $J_{max'}$, $S_{v'}$ and $S_{v''}$; Table 2). These initial values were then input into the maximum likelihood estimation package bbrlme() to yield best estimates and standard errors for each parameter. The package glm() was used to conduct multi-comparison analysis on response curves of each parameter across contrasting species. The best estimates for all parameters were input into a principal components analysis (PCA) to investigate

### Table 2. Values of 14 traits defining drought responses of stomatal and non-stomatal components across nine species. The 14 traits are: $\Psi_{v'}$ (the $\Psi_{pd}$ threshold for gas exchange measurement), $b_1$ (sensitivity of $g_b$), $g_{b*}$ ($g_b$ estimated at $\Psi_{pd} = 0.3$ MPa), $b_2$ (sensitivity of $g_m$), $g_{m*}$ ($g_m$ estimated at $\Psi_{pd} = 0.3$ MPa), $V_{cmax}$ ($V_{cmax}$ estimated at $\Psi_{pd} = 0$), $S_{v'}$ (sensitivity of $V_{cmax}$), $\Psi_{v'}$, $V_{cmax}$ ($V_{cmax}$ estimated at $\Psi_{pd} = 0$), $S_{v''}$ (sensitivity of $V_{cmax'}$), $\Psi_{v''}$, $J_{max'}$ ($J_{max}$ estimated at $\Psi_{pd} = 0$), $S_{v''}$ (sensitivity of $J_{max'}$). $\Psi_{v'''}$ NA means the value is not applicable.

<table>
<thead>
<tr>
<th>Species</th>
<th>$b_1$</th>
<th>$g_{b*}$</th>
<th>$V_{cmax}$</th>
<th>$S_{v'}$</th>
<th>$\Psi_{v'}$</th>
<th>$V_{cmax}$</th>
<th>$S_{v''}$</th>
<th>$\Psi_{v''}$</th>
<th>$J_{max'}$</th>
<th>$S_{v''}$</th>
<th>$\Psi_{v'''}$</th>
</tr>
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<tbody>
<tr>
<td>E. striatulax</td>
<td>0.55</td>
<td>4.41</td>
<td>119.15</td>
<td>0.81</td>
<td>-5.53</td>
<td>-5.88</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>181.85</td>
</tr>
<tr>
<td>E. occidentalis</td>
<td>0.97</td>
<td>3.68</td>
<td>107.05</td>
<td>1.82</td>
<td>-2.28</td>
<td>-3.05</td>
<td>0.55</td>
<td>0.32</td>
<td>133.99</td>
<td>1.94</td>
<td>-2.69</td>
</tr>
<tr>
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<td>0.31</td>
<td>3.54</td>
<td>113.3</td>
<td>0.65</td>
<td>-1.28</td>
<td>-3.23</td>
<td>0.51</td>
<td>0.26</td>
<td>126.31</td>
<td>0.63</td>
<td>-2.16</td>
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<tr>
<td>subcinerina</td>
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<tr>
<td>E. camaldulensis subsp.</td>
<td>0.84</td>
<td>3.04</td>
<td>106.18</td>
<td>1.9</td>
<td>-1.85</td>
<td>-2.23</td>
<td>0.5</td>
<td>0.28</td>
<td>126.81</td>
<td>2.71</td>
<td>-1.93</td>
</tr>
<tr>
<td>cadinaldulensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q. ilex L.</td>
<td>1.9</td>
<td>3.77</td>
<td>110.71</td>
<td>1.97</td>
<td>-1.59</td>
<td>-1.65</td>
<td>0.89</td>
<td>0.31</td>
<td>113.14</td>
<td>4.68</td>
<td>-1.69</td>
</tr>
<tr>
<td>Q. pubescens L.</td>
<td>0.68</td>
<td>3.54</td>
<td>66.71</td>
<td>2.6</td>
<td>-1.68</td>
<td>-1.8</td>
<td>0.86</td>
<td>0.1</td>
<td>81.92</td>
<td>3.46</td>
<td>-1.36</td>
</tr>
<tr>
<td>Q. robur L.</td>
<td>1.96</td>
<td>1.69</td>
<td>117.35</td>
<td>6.68</td>
<td>-0.84</td>
<td>-1</td>
<td>1.96</td>
<td>0.31</td>
<td>165.65</td>
<td>3.85</td>
<td>-0.54</td>
</tr>
<tr>
<td>A. glutinosa L.</td>
<td>2.87</td>
<td>2.19</td>
<td>106.69</td>
<td>4.67</td>
<td>-0.58</td>
<td>-0.85</td>
<td>1.73</td>
<td>0.23</td>
<td>109.71</td>
<td>52.8</td>
<td>-0.81</td>
</tr>
<tr>
<td>F. excelsior L.</td>
<td>2.36</td>
<td>3.28</td>
<td>134.68</td>
<td>6.4</td>
<td>-0.69</td>
<td>-0.93</td>
<td>3.86</td>
<td>0.51</td>
<td>140.65</td>
<td>113.9</td>
<td>-0.69</td>
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</table>
the correlations among the key traits defining the drought responses of $g_{1}$, $V_{cmax}'$ and $J_{max}'$.

**Results**

We quantified the stomatal limitation to photosynthesis by analysing the effect of water stress on $g_{1}$, and the non-stomatal limitation to photosynthesis by analysing the effect of water stress on $g_{m}$, $V_{cmax}'$ and $J_{max}'$. We also analysed the effect of water stress on $V_{cmax}$. The data consistently showed a decline in $g_{1}$, $g_{m}$, $V_{cmax}$, $V_{cmax}'$ and $J_{max}'$, indicating a progressive increase in both stomatal and non-stomatal limitation with increasing water stress (Figures 2–6; Figures S1–5 available as Supplementary Data at *Tree Physiology* Online).

**Response of $g_{1}$ to water stress**

Estimated parameter values for each species are given in Table 2. Species varied considerably in their $g_{1}$ response curves under water stress (Figure 2, Table 2; Figure S1 available as Supplementary Data at *Tree Physiology* Online). The $g_{1}$ response curves differed significantly in both their estimated $g_{1}$ values under moist conditions (parameter $g_{1}^\ast$, $P<0.001$), and the rates at which $g_{1}$ declined with water stress (parameter $b_{1}$, $P=0.014$). Within the European experiment, species typically from mesic habitats in Europe (Q. robur, A. glutinosa and F. excelsior) had lower $g_{1}^\ast$ values and a higher decline rate $b_{1}$ with water stress than Q. ilex, and Q. pubescens (Figure 2, Table 2; Figure S1, Tables S1 and S2 available as Supplementary Data at *Tree Physiology* Online). Within the genus *Eucalyptus* and genus *Quercus* in each experiment, respectively, species of different climatic origin differed greatly in their $g_{1}$ responses to drought.

**Response of $V_{cmax}'$ to water stress**

The logistic function of $V_{cmax}'$ decline with decreasing $\Psi_{pd}$ was fitted to observations of each species with Eq. (7). The fitted values of $V_{cmax}'^\ast$, $S_{fV}'$ and $\Psi_{fV}'$ are shown in Table 2.

**Response of $J_{max}'$ to water stress**

The exponential function of $J_{max}'$ decline with decreasing $\Psi_{pd}$ was fitted to observations of each species with Eq. (6). The fitted values of $J_{max}'^\ast$, $S_{fJ}'$ and $\Psi_{fJ}'$ are shown in Table 2.

**Response of $g_{m}$ to water stress**

Figure 3. Responses of $V_{cmax}'$ to $\Psi_{pd}$ in the first experiment on four contrasting *Eucalyptus* species in Australia (solid lines), and in the second experiment on three contrasting *Quercus* species and two other species in Europe (dash lines). The logistic function of $V_{cmax}'$ decline with decreasing $\Psi_{pd}$ was fitted to observations of each species with Eq. (7). The fitted values of $V_{cmax}'^\ast$, $S_{fV}'$ and $\Psi_{fV}'$ are shown in Table 2.

Figure 4. Responses of $g_{m}$ to $\Psi_{pd}$ in the first experiment on three contrasting *Eucalyptus* species in Australia (solid lines), and in the second experiment on three contrasting *Quercus* species and two other species in Europe (dash lines). There was no fluorometer measurement on *E. striatulata* to estimate $g_{m}$. The exponential function of $g_{m}$ decline with decreasing $\Psi_{pd}$ was fitted to observations of each species with Eq. (6). The fitted $b_{2}$ and $g_{m}^\ast$ values are shown in Table 2.

**Response of $J_{max}'$ to water stress**

Figure 5. Responses of $J_{max}'$ to $\Psi_{pd}$ in the first experiment on four contrasting *Eucalyptus* species in Australia (solid lines), and in the second experiment on three contrasting *Quercus* species and two other species in Europe (dash lines). The exponential function of $J_{max}'$ decline with decreasing $\Psi_{pd}$ was fitted to observations of each species with Eq. (5). The fitted $b_{1}$ and $g_{1}^\ast$ values are shown in Table 2.

**Response of $g_{1}$ to water stress**

Figure 2. Response of $g_{1}$ to $\Psi_{pd}$ in the first experiment on four contrasting *Eucalyptus* species in Australia (solid lines), and in the second experiment on three contrasting *Quercus* species and two other species in Europe (dash lines). The exponential function of $g_{1}$ decline with decreasing $\Psi_{pd}$ was fitted to observations of each species with Eq. (5). The fitted $b_{1}$ and $g_{1}^\ast$ values are shown in Table 2.
Similarly within the Australian experiment, *E. striaticalyx* from desert habitats showed a lower $g_1$ decline rate than that of *E. camaldulensis* subsp. *camaldulensis* and *E. occidentalis*.

**Response of $g_m$ to water stress**

All species measured (except *E. striaticalyx* without fluorometer measurements to estimate $g_m$) showed a decline of $g_m$ as water availability declined, while there were great differences among species of different climate of origin in their $g_m$ response curves under water stress (Figure 4, Table 2; Figure S3 available as Supplementary Data at *Tree Physiology* Online). The $g_m$ response curves differed greatly in their estimated $g_m$ values under moist conditions (parameter $g_m^*$, $P<0.001$), and in their rates at which $g_m$ declined with water stress (parameter $b_2$, $P<0.001$). Within the European experiment, the decline rate of $g_m$ with drought varied considerably among species of different climate of origin, as *Q. ilex* and *Q. pubescens* from more xeric habitats in Europe showed a lower $g_m$ decline rate with water stress than that of species typically from more mesic habitats (*Q. robur, A. glutinosa* and *F. excelsior*) while declining slowly with drought in species from more xeric habitats (*Q. ilex, Q. pubescens*) (Figure 4, Table 2; Figure S3, Tables S3 and S4 available as Supplementary Data at *Tree Physiology* Online).

**Response of $V_{cmax}'$ to water stress**

All species showed a decline of $V_{cmax}'$ as water availability declined, while species varied greatly in their response curves of $V_{cmax}'$ with water stress (Figure 3, Table 2; Figure S2 available as Supplementary Data at *Tree Physiology* Online). Considerable difference existed among species under water stress in $V_{cmax}'$ (the water potential at which $V_{cmax}'$ decreases to half of its maximum value), $S_{Vcmax}'$ (the steepness of the decline), and $K (V_{cmax}')$ (Figure 3, Table 2; Figure S2, Tables S5, S6 and S7 available as Supplementary Data at *Tree Physiology* Online). Within the European experiment, species typically from mesic habitats (*Q. robur, A. glutinosa* and *F. excelsior*) were found to have significantly less negative $V_{cmax}'$, $S_{Vcmax}'$ and $V_{cmax}'$, higher $V_{cmax}'$ sensitivity ($S_{Vcmax}'$) than species from more xeric habitats (*Q. ilex, Q. pubescens*). Similarly, within the Australian experiment, *E. striaticalyx* was found to have significantly more negative $V_{cmax}'$, and lower $S_{Vcmax}'$ than most other *Eucalyptus* species.

Within each experiment on the genera *Eucalyptus* and *Quercus*, species of contrasting climatic origins showed very different responses of $V_{cmax}'$ to drought depending on their climate of origin. *Eucalyptus striaticalyx* showed the ability to continue active photosynthesis down to much lower soil water potential ($-5.9$ MPa) than other species. *Eucalyptus camaldulensis* subsp. *camaldulensis* from humid southeastern Australia showed a higher sensitivity of $V_{cmax}'$ to drought than other *Eucalyptus* species. *Quercus robur* from more mesic habitats in Europe showed higher sensitivity of $V_{cmax}'$ to drought and
also less negative $\Psi_{N}'$ and $\Psi_0$ than other *Quercus* species from more xeric habitats (Figure 3, Table 2; Figure S2, Tables S5 and S6 available as Supplementary Data at Tree Physiology Online).

Response of $V_{\text{c,max}}$ to water stress

All species measured (except *E. striaticalyx* without fluorometer measurement to estimate $g_m$ and $V_{\text{c,max}}$) showed a pattern of decline in $V_{\text{c,max}}$ consistent with the response of $V_{\text{c,max}}'$ (Figure 5; Figure S4 available as Supplementary Data at Tree Physiology Online). *Alnus glutinosa* and *F. excelsior*, with only a small number of $V_{\text{c,max}}$ data points, showed large variance in its estimated $S_{\text{IV}}$ and *F. excelsior* also showed a large variance of $V_{\text{c,max}}$* (Table 2, Figure 5; Figure S4, Tables S8, S9 and S10 available as Supplementary Data at Tree Physiology Online). Other species differed considerably in their $V_{\text{c,max}}$ response curves. Within the European experiment, *Q. pubescens* from more xeric habitats had significantly more negative $\Psi_{\text{IV}}$ than *F. excelsior* from more mesic habitats. (Table S8 available as Supplementary Data at Tree Physiology Online). Within the Australian experiment, *E. occidentalis* from more xeric habitats showed significantly more negative $\Psi_{\text{IV}}$ than *E. camaldulensis* subsp. *camaldulensis* from more mesic habitats.

Response of $J_{\text{max}}$, $J_{\text{max}}'$, and maximum $J_{\text{ETR}}$ to water stress

All species showed a decline of $J_{\text{max}}'$ as water availability declined, while species varied greatly in their response curves of $J_{\text{max}}'$ with water stress (Figure 6, Table 2; Figure S5 available as Supplementary Data at Tree Physiology Online). Notable difference existed among species of different climates of origin in their $S_{\text{IV}}'$ within the European and Australian experiments (Figure 6, Table 2; Figure S5 and Table S11 available as Supplementary Data at Tree Physiology Online). However, it was not possible to find a clear drought-response pattern of $J_{\text{max}}$ and maximum $J_{\text{ETR}}$ (Figures S7 and S8 available as Supplementary Data at Tree Physiology Online).

Water relation strategies

Results showed that the decline in $V_{\text{c,max}}'$ is not explained just by the decline in $g_m$, but by the decline in both $g_m$ and $V_{\text{c,max}}$. Principal components analysis (Figure 7) showed strong dominance by the first principal component (PC1), which explained 58.5% of variance in the nine parameters included. We inferred the existence of one major gradient, characterized by the positive correlation among $b_1$, $S_{N}'$, $S_{J}'$, $\Psi_{N}'$, $\Psi_{J}'$ and $\Psi_{O}'$ and by their negative correlations with $g_1'$. Species with this combination of traits are aligned to the right part of Figure 7, with positive scores on PC1. Species with lower values of $b_1$, $S_{N}'$, $S_{J}'$, $\Psi_{N}'$, $\Psi_{J}'$ and $\Psi_{O}'$ are characterized by (i) a decrease of $V_{\text{c,max}}'$ and $J_{\text{max}}$ setting at a lower pre-dawn leaf water potential, (ii) a lower rate of decline in $g_1$, $V_{\text{c,max}}'$ and $J_{\text{max}}'$ under drought and (iii) higher ‘initial’ $g_1$ under moist conditions.

Discussion

There are large discrepancies in the way ecosystem models represent the drought responses of plant gas exchange, largely because of a dearth of experimental studies on the separate effects of drought on stomatal and non-stomatal processes. Although many experiments have been focused on the drought responses of plant gas exchange, few studies have explicitly characterized the concurrent drought responses of different aspects of drought response, including stomatal, mesophyll and biochemical components (Limousin et al. 2010, Martin-StPaul et al. 2012). Meanwhile, studies comparing the drought sensitivity of these components among contrasting species
from diverse hydroclimate are also very limited (Cano et al. 2014). By conducting two independent experiments on tree species from diverse hydroclimates, this study directly tested whether species from contrasting hydroclimates respond differently in each component response of gas exchange during short-term water stress. This study has suggested the existence of a general pattern of co-ordination among different aspects of the drought response and a systematic relationship between drought response and climate of origin.

**Concurrent limitation on \( g_{\text{m}} \), \( V_{\text{cmax}} \) and \( J_{\text{max}} \)**

Our finding of coordinated drought responses of \( g_{\text{m}} \), \( V_{\text{cmax}} \) and \( J_{\text{max}} \) supports our first hypothesis, i.e., that there should be a coordinated response of both the stomatal, mesophyll and biochemical components of drought limitation on photosynthesis. The drought limitation would be greatly underestimated if its effects on \( g_{\text{m}} \), \( V_{\text{cmax}} \) and \( J_{\text{max}} \) were not taken into account. Meanwhile, the close correlation found among the responses of \( g_{\text{m}} \), \( V_{\text{cmax}} \) and \( J_{\text{max}} \) suggests that the observed response of \( V_{\text{cmax}} \) is composed of both a diffusive (\( g_{\text{m}} \)) and a biochemical \( (V_{\text{cmax}}) \) component. This study highlights the concurrent contribution of \( g_{\text{m}} \), \( V_{\text{cmax}} \) to the decline of \( V_{\text{cmax}} \).

**Variation of responses between species of contrasting hydroclimatic origins**

The remarkable variation among species of contrasting climatic origins in the drought responses of \( g_{\text{m}} \), \( V_{\text{cmax}} \) and \( J_{\text{max}} \) supports our second hypothesis, i.e., there should be a systematic relationship between drought response and climate of origin. The species from drier climates show a slower decline of \( g_{\text{m}} \), \( V_{\text{cmax}} \) photosynthetic capacity and RuBP regeneration capacity, and can attain lower pre-dawn leaf water potentials before the decrease of photosynthetic capacity sets in. However, when placed under moist conditions, the species from more xeric climates tend to have relatively higher \( g_{\text{m}} \) values.

Kelly and Medlyn (2013) reported that *Eucalyptus populnea* (from a xeric climate) had higher \( g_{\text{m}} \) and lower \( b_{\text{c}} \) than *Eucalyptus pilularis* (from a mesic climate) with the decrease of pre-dawn leaf water potential. The result is consistent with the theory of stomatal behaviour by Cowan (1977) and Cowan and Farquhar (1977), which suggests that species from drier climates are expected to have more conservative stomatal behaviour and more efficient use of water per unit carbon gain than species adapted to wetter climates. Our data also support the prediction by Mäkelä et al. (1996) that the increase of marginal carbon cost of water—the decline of \( g_{\text{m}} \) in time should be low in places where the frequency of rainfall is lower.

The lower sensitivity of the \( g_{\text{m}}/A \) relationship for xeric drought-tolerant species than for mesic species may relate to species-specific structural and hydraulic attributes (Héroult et al. 2013), and trade-offs between transpiration and vulnerability to hydraulic failure (Beringer and Hari 1993), which could be enforced by strong natural selection. Natural selection would be expected to enforce different structural and hydraulic characteristics and stomatal behaviour for species from climates with contrasting precipitation regimes (Brodribb et al. 2005, Franks 2006). The \( V_{\text{pd}} \) threshold for gas exchange measurement on *Quercus* species in this study is less negative than that reported in some field studies (e.g., Arend et al. 2012). However, the deciduous *Q. pubescens* and *Q. robur* in this study showed behaviour consistent with that reported by Günthardt-Goerg et al. (2013), who found a threshold of −1.3 MPa for the development of visible leaf stress symptoms.

The role of mesophyll limitation highlighted in our analysis is consistent with recent studies reporting that the mesophyll limitation can contribute as much as the stomatal limitation to the reduction of \( A \) under water stress (e.g., Flexas et al. 2012). This study also shows that \( g_{\text{m}} \) can respond as quickly as \( g_{\text{s}} \) under soil water deficit, which may be due to the involvement of aquaporins and/or carbonic anhydrase in the regulation of \( g_{\text{m}} \) (Flexas et al. 2012).

The large variation among species in \( V_{\text{cmax}} \) and \( S_{\text{c}} \) highlights the importance of climatic origin in determining the water potential at which a substantial decrease of Rubisco activity sets in. This finding adds new information to previous studies that have related this threshold to the severity and/or duration of the stress imposed (Flexas et al. 2006b, Galmés et al. 2011). The drought-induced decrease of Rubisco activity could be due to the deactivation of carboxylation sites, or a decrease of Rubisco content (Wilson et al. 2000, Xu and Baldocchi 2003, Grassi et al. 2005, Misson et al. 2006). Notable differences in the drought response patterns of \( V_{\text{cmax}} \) between xeric and mesic species may relate to the geographical isolation, which could have favoured selection of distinctive properties for Rubisco. The habitat conditions of both high temperature and low internal \( CO_2 \) concentrations—enforced by the need to conserve water and avoid hydraulic failure—appear to have imposed a selection pressure for a higher Rubisco specificity factor (Galmés et al. 2005), which implies higher photosynthetic capacity (Gulias et al. 2003). Delgado et al. (1995) and Kent and Tomany (1995) hypothesized that in hot environments subject to water stress, stomatal closure and low \( CO_2 \) concentrations at the carboxylation site may have increased the selection pressure on Rubisco for improved specificity. Similarly, Galmés et al. (2005) showed that significant variability in the Rubisco specificity factor exists among closely related \( C_3 \) higher plants, and that this variability is related to environmental pressures associated with hot and dry environments.

**Implications for ecosystem models**

Currently, ecosystem models treat all species and/or PFTs as having similar stomatal and/or non-stomatal drought responses. This study supports theory and emergent evidence suggesting that both stomatal and non-stomatal component processes are
affected, implying that both effects should be represented in models (e.g., Egea et al. 2011, Zhou et al. 2013). By investigating the systematic differences in drought sensitivity among species from different hydroclimates, this study contributes to the information base required for the representation of stomatal and non-stomatal drought effects on photosynthesis in process-based models, and highlights the fact that reliable modelling of drought effects will not be possible unless adaptive differences between the responses of species from different hydroclimates are taken into account.

Supplementary data
Supplementary data for this article are available at Tree Physiology Online.

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Conflict of interest
None declared.

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