Using modern plant trait relationships between observed and theoretical maximum stomatal conductance and vein density to examine patterns of plant macroevolution

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**Summary**

- Understanding the drivers of geological-scale patterns in plant macroevolution is limited by a hesitancy to use measurable traits of fossils to infer palaeoecophysiological function.
- Here, scaling relationships between morphological traits including maximum theoretical stomatal conductance ($g_{\text{max}}$) and leaf vein density ($D_v$) and physiological measurements including operational stomatal conductance ($g_{\text{op}}$), saturated ($A_{\text{sat}}$) and maximum ($A_{\text{max}}$) assimilation rates were investigated for 18 extant taxa in order to improve understanding of angiosperm diversification in the Cretaceous.
- Our study demonstrated significant relationships between $g_{\text{op}}$, $g_{\text{max}}$ and $D_v$ that together can be used to estimate gas exchange and the photosynthetic capacities of fossils. We showed that acquisition of high $g_{\text{max}}$ in angiosperms conferred a competitive advantage over gymnosperms by increasing the dynamic range (plasticity) of their gas exchange and expanding their ecophysiological niche space. We suggest that species with a high $g_{\text{max}}$ ($> 1400 \text{ mmol m}^{-2} \text{s}^{-1}$) would have been capable of maintaining a high $A_{\text{max}}$ as the atmospheric CO$_2$ declined through the Cretaceous, whereas gymnosperms with a low $g_{\text{max}}$ would experience severe photosynthetic penalty.
- Expansion of the ecophysiological niche space in angiosperms, afforded by coordinated evolution of high $g_{\text{max}}$, $D_v$ and increased plasticity in $g_{\text{op}}$, adds further functional insights into the mechanisms driving angiosperm speciation.

**Introduction**

Examination of large scale ecological, ecophysiological and phytogonomic datasets in plant biology has revealed important trait relationships that are conserved across species (Wright et al., 2004; Grime, 2006; Reich et al., 2007; Kattge et al., 2011; Yang et al., 2015). These suites of correlated traits have enabled classification of extant taxa into broad ecological categories – such as plant functional types – which serve as the starting point for mapping and predicting vegetation responses to past and future global change. They have also contributed strongly to the development of palaeoclimate proxies (Yang et al., 2015). Ongoing studies of extant plant trait relationships are beginning to have a significant impact on the understanding of plant macroecological and macroevolutionary processes in the fossil plant record by providing critical insights into the palaeoecophysiology and general functional attributes of fossil taxa (Beerling & Woodward, 1997, 2001; Franks & Beerling, 2009a; Wilson & Knoll, 2010; de Boer et al., 2012; Lee et al., 2015) including those that are extinct (Wilson et al., 2008). Many trait based datasets incorporate only functional or only morphological/morphometric traits, yet integration of both data types (such as exemplified by TRY; Kattge et al., 2011) is required if any inferences on the palaeoecophysiology of fossil taxa from measured morphological attributes is to be made. Gaining insights on the functional biology of fossil taxa will permit a more nuanced assessment of plant macroevolutionary patterns from the fossil plant record.

A compelling example of how modern trait based datasets can be used to gain novel insights into the mechanisms driving plant macroevolution is the ‘vein density hypothesis’ of angiosperm evolution, which uses changes in Cretaceous fossil plant morphological traits (in this case leaf vein density) to reconstruct maximum conductive and photosynthetic capacity of angiosperms vs gymnosperms in Cretaceous fossil floras (Boyce et al., 2009; Brodribb & Field, 2010; Field et al., 2011b). According to this hypothesis angiosperms uniquely evolved the capacity to increase leaf vein density above $c. 6 \text{ mm mm}^{-2}$ in the mid Cretaceous $c. 100$ million yr ago (Mya; late Albian–early Cenomanian periods) (de Boer et al., 2012). This anatomical innovation enabled angiosperms to outcompete incumbent gymnosperms (with low vein densities) as it removed a developmental constraint on...
potential productivity. Supplying more water via a high vein density network to stomata enabled greater transpiration (Boyce et al., 2009; Brodribb & Feild, 2010) and ultimately enhanced the photosynthetic capacity (Brodribb et al., 2007; de Boer et al., 2012).

Another example of how the observed morphological traits measured in fossils are used to estimate palaeophysiology is demonstrated in the palaeo-proxy CO2 model of Franks et al. (2014). This mechanistic model uses a scaling relationship between the maximum theoretical stomatal conductance (gmax in mmol m⁻² s⁻¹), calculated from the density (SD), size and geometry of stomata when fully open (Parlange & Waggoner, 1970; Franks & Beerling, 2009b) and measured conductance values (gop) to infer stomatal conductance of fossil taxa. It is widely known that because stomata respond dynamically with the environment, the anatomical gmax is rarely observed in field conditions (Lawson & Morison, 2004; Dow et al., 2014) and that the operational stomatal conductance of a leaf, which we refer to here as gop, is usually measured at much lower values than gmax (Franks et al., 2009, 2014). Quantification of the scaling relationship between gop and gmax has, however, only been undertaken in detail for two extant angiosperm species – Eucalyptus globulus (Franks et al., 2009) and Arabidopsis thaliana (Dow et al., 2014) and it is not known whether a universal relationship exists across many species. This currently hampers a wider application to the fossil record and integration with other likely correlated functional traits such as vein density.

There is close developmental and physiological coordination of water supply via veins and water loss via stomata at the leaf level (Sack et al., 2003; McElwain, 2011; Brodribb et al., 2013) yet the role of stomatal evolution as a potential driver or accessory to vein density evolution has not been systematically investigated. Furthermore, there are no modern trait datasets that incorporate Dv, gmax, gop and photosynthetic traits across species. The expectation is that the maximum stomatal conductance should follow a similar evolutionary trajectory in angiosperms and gymnosperms as vein density (Boyce et al., 2009). A primary objective of this study therefore was to investigate the role of stomatal evolution in the ecological and evolutionary success of angiosperms compared with gymnosperms by undertaking a comparative study on plasticity in gop in relation to the theoretical maximum stomatal conductance (gmax) and vein density (Dv). Specifically we ask: is there a universal scaling relationship between gop and gmax; did an increase in maximum gas exchange capacity, facilitated by high gmax and Dv, enable angiosperms to increase plasticity in their day-to-day operational range of stomatal conductance (gop) compared with gymnosperms; and what are the likely evolutionary implications of a greatly expanded gop range in terms of ecological competition, resource use and assimilation rates in a high CO2 world of the Cretaceous (c. 2000 ppm) compared with that of today (c. 400 ppm)?

**Materials and Methods**

**Plant growth conditions**

*Laurus nobilis* L., *Drimys winteri* J.R. Forst. & G. Forst., *Osmunda regalis* L., *Agathis australis* (D. Don) Loudon, *Nageia nagi* Thunb. O. Kunze, *Lepidozamia hopei* Regel, *Ginkgo biloba* L. and *Passionaria caerulea* L. were grown in 51 square pots in a production glasshouse (Cambridge HOK) at UCD Rosemount Environmental Research Station, Dublin, Ireland under ambient Dublin light. Growth light intensity at canopy level (minimum 92 μmol m⁻² s⁻¹; maximum 386 μmol m⁻² s⁻¹; mean 221 μmol m⁻² s⁻¹), glasshouse ambient air temperature (minimum 22°C; maximum 31°C; mean 25.8°C) and relative humidity (minimum 42%; maximum 81%; mean 61.4%) were recorded hourly during the measurement period (27 June 2011–15 July 2011). *Protea eximia* (Knight) Fourc., *Punica granatum* L., *Grevia suberlindi* Hook & Harv., *Colocasia esculenta* (L.) Schott, *Pelargonium ‘Robert Fish’, Citrus × sinensis* (L.) Osbeck, *Ceratonia siliqua* L., *Olea europea* L., *Manihot esculenta* Crantz and *Ricinus communis* L. were growing in the Mediterranean Collections of the Curvilinear Range glasshouse of the National Botanic Gardens Glasnevin, Ireland under ambient Dublin light. Light intensity at canopy level (304 ± 179 μmol m⁻² s⁻¹), glasshouse air temperature (minimum 19°C; maximum 31°C; mean 22°C) and relative humidity (minimum 47%; maximum 87%; mean 67%) were recorded hourly during the measurement period (17 June 2012–21 June 2012). All species were optimally watered and fed.

**Leaf gas exchange measurements**

The gop measurements were taken over a 13 d period in 2011 using a PP Systems CIRAS 2 portable infra-red gas analyser (IRGA) equipped with a PP systems PLC 6 (U) leaf cuvette fitted with a rice plate (1.75 cm²) and a 5 d period in July 2012 using a SC-1 leaf porometer (Decagon Devices, Pullman, WA, USA). Species growing at UCD Rosemount were measured with the IRGA set to the following: CO₂ (400 μmol mol⁻¹); light intensities were set to ambient; humidity was set to 60%; air flow to 150 μmol m⁻² s⁻¹ and vapour pressure deficit (VPD) c. 1 kPa. Species growing in the Mediterranean collections were measured using the SC-1 leaf porometer. Each species is represented by measurements from two individuals per species, one to three leaves per individual, two to six measurements per leaf over the course of the day. This protocol was repeated for each leaf over a 5–13 d period resulting in a mean gop based on between 42 and 72 individual measurements per species. This protocol, which we refer to here as the ‘variance protocol’ was carried out in order to capture the fullest range of variance in gop for each species, growing in optimal soil water and nutrient conditions and under the same prevailing climate and light regime. In this respect the chosen methodology was nonconventional because typical protocols used to measure gop attempt to minimize variance by standardizing the light and VPD conditions at the time of measurement and allowing a substantial time for gop to stabilize to the new standardized measurement conditions. To ensure that our ‘variance protocol’ measured in 2011 and 2012 produced a robust mean gop value for each species we repeated the entire experiment in October 2014 with a PP Systems CIRAS 2 portable IRGA following a standardized protocol as follows: the maximum operational stomatal conductance gop(max) for each species was
measured at saturating light intensity (determined from preliminary light curves for each species), a leaf temperature of 25°C and VPD c. 1 kPa. The intact leaves were left to equilibrate under optimal conditions for a minimum of 20 min until the increasing stomatal conductance reached a plateau. The \( g_{\text{op(max)}} \) of each leaf was then calculated as the average of three recordings taken upon full induction of stomatal opening.

**Morphological trait measurements and calculation of \( g_{\text{max}} \)**

Following completion of all the physiological measurements the leaves on which the \( g_{\text{op}} \) measurements were taken were removed from the plants in order to calculate the theoretical \( g_{\text{max}} \) using the following diffusion equation (Parlange & Waggoner, 1970; Franks & Beerling, 2009b);

\[
g_{\text{max}} = \frac{dw \cdot \text{SD} \cdot p_d \text{max}}{pd + \frac{3}{2} \sqrt{p_d \text{max}^2 / \pi}}
\]

where \( dw \) = diffusivity of water vapour at 25°C (0.0000249 m² s⁻¹) and \( v \) = molar volume of air (0.0224 m³ mol⁻¹) are both constants, SD = stomatal density (m⁻²), \( p_d \text{max} \) is maximum stomatal pore area (m²) calculated as an ellipse using stomatal pore length (m) as the long axis and l/2 as the short axis; \( pd \) = stomatal pore depth (m) considered to be equivalent to the width of an inflated, fully turgid guard cell (Franks & Beerling, 2009a, 2009b).

A 1 cm² leaf disc was taken for each leaf from the exact position of the \( g_{\text{op}} \) measurements and positive leaf impressions were taken from the abaxial leaf surface using dental impressions with the cuticle preparations were rinsed in water, dehydrated using ethanol, cleared in a mixture of hydrogen peroxide and heated at 65°C and stained with safranin and mounted onto a slide containing glycerol gelatin.

Resulting measurements of vein length in mm mm⁻² area were made for each species in Auto-Montage (v.5.03).

Estimating saturated assimilation rate (\( \tau_{A_{\text{sat}}} \)) from \( g_{\text{max}} \)

Instantaneous measurements of the assimilation rate (\( A; \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)) at light intensities (PAR) of 700 μmol m⁻² s⁻¹ and ambient CO₂ (400 μmol mol⁻¹) were recorded alongside \( g_{\text{op}} \) measurements for a subset of eight species (L. nobilis, D. winteri, O. regalis, A. australis, N. nagi, L. hopei, L. peroffskyana, G. biloba) growing at UCD Rosemount Environmental research station using a CIRAS 2 portable IRGA (see \( g_{\text{op}} \) methodology earlier) over an 11 d period in 2011 (27 June 2011–15 July 2011). A logarithmic trend line was fitted to \( A \) and \( g_{\text{op}} \) for each of the eight species and used to estimate the theoretical saturated assimilation rates (\( \tau_{A_{\text{sat}}} \)) from \( g_{\text{max}} \) (see later Table 2). The reliability of the estimated \( \tau_{A_{\text{sat}}} \) values was assessed by comparison with a new dataset of mean (of 10–12 replicates per species) \( A_{\text{sat}} \) and \( A_{\text{max}} \) measurements of the same eight species taken in 2013. The \( A_{\text{sat}} \) measurements were all performed at 400 ppm CO₂ and a saturating light of 1000 μmol m⁻² s⁻¹ and all \( A_{\text{max}} \) measurements were performed at 2000 ppm CO₂ and saturating light of 1000 μmol m⁻² s⁻¹ using a PP Systems CIRAS 2 portable infrared gas analyser. The flow rates were set at 150 μmol and all leaves were left in the chamber for c. 3 min (or until full photosynthetic induction). All measurements were carried out between 09:30 and 12:30 h.

**Results and discussion**

Scaling relationship between \( g_{\text{max}} \) and \( g_{\text{op}} \)

The best fit scaling relationship between \( g_{\text{max}} \) and \( g_{\text{op}} \) for 12 angiosperm, five gymnosperm and one fern species investigated in this study was found to be \( g_{\text{op}} = 0.25 \cdot g_{\text{max}} \left( r^2 = 0.5446, P = 0.00039 \right) \) (Fig. 1a) which is in good agreement with the previously reported results from single species studies such as Eucalyptus \( g_{\text{op}} \) c. 0.2 \( g_{\text{max}} \) (Franks et al., 2009) and Arabidopsis \( g_{\text{op}} = 0.2 \cdot g_{\text{max}} \); Dow et al., 2014) but lower than some purely experimental based reports \( g_{\text{op}} = 0.5 \cdot g_{\text{max}} \); Dow, et al., 2014). The results of this study are the most comprehensive, to date, in terms of the number of species investigated and confirm that, on average, plants operate at a stomatal conductance that is only a fraction (c. 25%) of their theoretical maximum anatomical limits when growing in field and glasshouse conditions (Fig. 1a, Supporting Information Fig. S1).

Despite the apparent convergence in the ratio of \( g_{\text{op}} : g_{\text{max}} \) from three independent studies (Franks et al., 2009; Dow et al., 2014; this work) considerable variability exists at the individual species level which cannot be easily explained by biogeography, habitat, ecology or partitioned by phylogenetic group (e.g. angiosperm vs gymnosperm; Table S1, Fig. S2). For example, stomatal conductivities of the tropical crops R. communis (castor oil), Ceratonia siliqua (carob) and M. esculenta (cassava) occasionally reach 80% of their respective \( g_{\text{max}} \), but another important crop C. esculenta (taro) operates further under its theoretical \( g_{\text{max}} \) than \( G. \) biloba, a ‘living fossil’ gymnosperm (Fig. S2). At the opposite end of the scale, N. nagi, a broad-leafed temperate conifer from Japan and two woody Magnoliids from contrasting climates, L. nobilis
(Mediterranean) and D. winteri (temperate rain forest) all conduct at <20% of their theoretical potential ($g_{\text{op}} : g_{\text{max}}$, Fig. S2). The species-level scaling relationship between $g_{\text{max}}$ and $g_{\text{op}}$ could also not be predicted based on stomatal density ($R^2 = 0.0166$), stomatal pore length ($R^2 = 0.0862$) or pore depth ($R^2 = 0.2927$) (Table 1). However, there was a tendency among all species belonging to more recently derived lineages to utilize a much greater proportion of the theoretical $g_{\text{max}}$ than species with deep phylogenetic origins (Table S2). This pattern, although not statistically significant ($g_{\text{op}} : g_{\text{max}}$ vs generic stem age: $R^2 = 0.1662$, Table S2) suggested that perhaps older lineages were constrained by some other aspect of their morphology/anatomy, such as vein density than more recently derived species. It is also possible that aspects of stomatal complex morphology, anatomy and/or chemistry (e.g. guard cell lignification, presence/absence of stomatal ledges and subsidiary cells) not considered here may also play a role in the range of interspecies variability observed between $g_{\text{op}} : g_{\text{max}}$ (Franks & Farquhar, 2007).

To investigate the potential underlying reasons for interspecies variability, $g_{\text{op}} : g_{\text{max}}$ ratios were plotted against saturating assimilation ($A_{\text{sat}}$) rates for all 18 species investigated. A significant positive relationship was observed ($r^2 = 0.36652$, $P = 0.0077$) suggesting that species with innately higher net photosynthetic rates use more of their maximum anatomical potential in terms of gas exchange than species with lower $A_{\text{sat}}$ values (Fig. 1b). This is an important finding in the context of palaeoecophysiological studies as it provides further constraints on our capacity to estimate gas exchange rates from fossil stomatal anatomical data. It also has a bearing on palaeo-CO$_2$ estimates that are based in large part on accurate estimates of the fossil plant $g_{\text{op}}$ such as that of Franks et al. (2014). Based on the analysis presented in Fig. 1(b), a $g_{\text{op}} : g_{\text{max}}$ ratio of >0.25 should be assigned to fossil taxa with high photosynthetic rates (which can be inferred from high vein densities ($D_v$) (Boyce & Zwieniecki, 2012) or short vein to stomatal distances (Brodribb et al., 2007). By contrast $g_{\text{op}} : g_{\text{max}}$ ratios of <0.25 should be assigned to fossil taxa if other anatomical data indicate that they had low photosynthetic rates.

Coordination of stomatal and vein density evolution

This study is the first detailed examination of the relationships between $g_{\text{op}}$, $g_{\text{max}}$ and $D_v$. A strong positive correlation was observed between vein density ($D_v$) and $g_{\text{op}}$ ($R^2 = 0.8314$; $y = 14.379x^2 - 65.238x + 139.79$) (Fig. 2, Table 1) which indicates close coordination of stomatal and vein density evolution in agreement with previous studies (Sack et al., 2003; Brodribb et al., 2007; Boyce et al., 2009; Feild et al., 2011b). We also demonstrate a significant positive correlation between vein density and theoretical $g_{\text{max}}$ ($R^2 = 0.741$; Fig. 2) suggesting that the evolution of high vein density angiosperms during the Cretaceous period may have facilitated a rise in their theoretical maximum stomatal conductances. This would have allowed an increase in the average operational conductivities of angiosperms with $D_v$ over 4 mm mm$^{-2}$ (Fig. 2). More importantly, the results also show that the margin of difference between $g_{\text{max}}$ and $g_{\text{op}}$ increases.
with $D_v$ (Fig. 53). This suggests that the evolution of high vein densities also dramatically increased the range of $g_{op}$ in angiosperms providing them with significantly greater plasticity and flexibility to maximize CO$_2$ uptake and water loss when conditions were optimal (Fig. 2). Our analysis shows that species with vein densities of $\leq 4.0$ mm mm$^{-2}$ have mean $g_{op}$ values c. 400 mmol m$^{-2}$ s$^{-1}$ below their respective theoretical $g_{max}$ values, whereas the majority of species with $D_v$ of 6 mm mm$^{-2}$ and greater are typically operating between 600 and 1000 mmol m$^{-2}$ s$^{-1}$ below their theoretical maximum potential ($g_{max}$) (Fig. 2). Our study therefore supports the 'vein density hypothesis' (Boyce et al., 2009; Brodribb & Feild, 2010; Feild et al., 2011a) that proposes a transformative influence of high vein density on conductive capacity of angiosperms compared with gymnosperms but goes further to show that the coordination of high vein density and high $g_{max}$ also dramatically increased the dynamic operational conductance range of angiosperms (as indicated by wide interquartile $g_{op}$ ranges in Fig. 2b) compared with other seed plant groups. Indeed the examination here of scaling relationships between $g_{max}$, $g_{op}$ and $D_v$ (Fig. 2) helps to explain the apparent coordinated surge in both $D_v$ (Boyce et al., 2009; Feild et al., 2011a) and modelled $g_{op}$ (Fig. 7 in Franks & Beerling, 2009a,b) c. 100 Mya in the mid Cretaceous, the latter of which Franks & Beerling attribute to falling atmospheric CO$_2$.

The advantage of a significantly expanded $g_{op}$ range in angiosperms is that it likely conferred greater ecophysiological plasticity allowing species with a high vein density and high $g_{max}$ to operate within a much wider 'ecophysiological niche space'. This in turn may have provided an opportunity for a population to segregate resource use by stomatal conductance. The plasticity of behaviour would only be possible in high $D_v$ and high $g_{max}$ species because species with low $D_v$ and low $g_{max}$, as illustrated in Fig. 2(b), have very constrained and overlapping $g_{op}$ ranges. Following this reasoning it could be argued that on evolutionary timescales increasing vein densities in angiosperms not only expanded the capacity for increased uptake of CO$_2$ in exchange for water (Boyce et al., 2009; Brodribb & Feild, 2010; Feild et al., 2011a), but also expanded the breadth of ecophysiological niche space on which selection could act. Stomatal control of $g_{op}$ in a patchy landscape may therefore have enabled different individuals within the same species, or different species within the same community, to share rather than compete for available resources (N, P, H$_2$O) by controlling how close or far away the species operated from maximum theoretical ($g_{max}$) limits. Increased landscape complexity and 'patchiness' in terms of available niches has been suggested for the Cretaceous period (Coiffard et al., 2012). This potential mechanism for angiosperm speciation is demonstrated conceptually in Fig. 2(b) where the number of theoretical 'stomatal conductance niches' are shown to increase with increasing $g_{max}$ and $D_v$. By contrast, all species with a low $D_v$ have narrow and overlapping $g_{op}$ ranges. Support for the concept of increasing ecological complementarity via an expansion of stomatal conductance niches comes from stable carbon and oxygen isotopic studies (Moreno-Gutiérrez et al., 2012). These show evidence of consistent segregation of 'ecophysiological niche space' among coexisting species to maximize community level plant water use efficiency (Moreno-Gutiérrez et al., 2012). The concept of wide plasticity in angiosperms compared with gymnosperms has been demonstrated for many traits such as genome size (Leitch & Leitch, 2013), post-disturbance regeneration time (Midgley & Bond, 1991), pollination to fertilization interval (Cernusak et al., 2009). We suggest that plasticity of operational stomatal conductance is an additional example of behavioural flexibility that angiosperms may have capitalized on. We acknowledge, however, that when considering the role of

| Species                  | SD mm$^{-2}$ | Pore length µm | Pore depth µm | Maximum $g_{op} \cdot g_{max}$ mmol m$^{-2}$ s$^{-1}$ | $g_{max}$ mmol m$^{-2}$ s$^{-1}$ | Average $g_{op}$ mmol m$^{-2}$ s$^{-1}$ | Maximum $g_{op}$ mmol m$^{-2}$ s$^{-1}$ | Average $g_{op}: g_{max}$ | $D_v$ mm mm$^{-2}$ |
|-------------------------|-------------|----------------|--------------|-------------------------------------------------|-------------------------------|----------------------------------------|-------------------------------|--------------------------|------------------|
| Lepidozamia hopei       | 26.2 ± 1.8  | 14.12 ± 5.9    | 0.398        | 374                                             | 66 ± 29.8                     | 149                                     | 0.18                          | 0.93                     |                  |
| Nagea nagi              | 15.9 ± 0.8  | 11.20 ± 1.44   | 0.146        | 526                                             | 31 ± 20.9                     | 77                                      | 0.06                          | 1.02                     |                  |
| Agathis australis       | 19.4 ± 0.8  | 15.09 ± 1.52   | 0.308        | 554                                             | 64 ± 31.5                     | 171                                     | 0.11                          | 1.13                     |                  |
| L. peroffskyana         | 27.7 ± 1.4  | 9.65 ± 6.33    | 0.334        | 491                                             | 65 ± 41.5                     | 164                                     | 0.13                          | 1.33                     |                  |
| Ginkgo biloba           | 19.9 ± 1.8  | 10.57 ± 2.83   | 0.692        | 374                                             | 117 ± 68.3                    | 259                                     | 0.31                          | 1.37                     |                  |
| Osmunda regalis         | 32.7 ± 4.0  | 17.02 ± 3.2    | 0.375        | 698                                             | 132 ± 66.0                    | 262                                     | 0.19                          | 3.27                     |                  |
| Drimys winteri          | 18.3 ± 2.9  | 10.19 ± 1.05   | 0.293        | 863                                             | 130 ± 44.1                    | 253                                     | 0.15                          | 4.87                     |                  |
| Protea eximia           | 14.1 ± 2.3  | 8.9 ± 1.0      | 0.590        | 361                                             | 129 ± 115.2                   | 213                                     | 0.36                          | 2.6                      |                  |
| Punica granatum         | 7.5 ± 1.1   | 3.8 ± 0.5      | 0.765        | 226                                             | 81 ± 43.0                     | 173                                     | 0.36                          | 3.0                      |                  |
| Greyia sutherlandii     | 17.7 ± 3.3  | 8.8 ± 0.9      | 0.714        | 559                                             | 186 ± 95.9                    | 399                                     | 0.33                          | 3.2                      |                  |
| Laurus nobilis          | 19.2 ± 1.9  | 9.14 ± 1.19    | 0.164        | 1529                                            | 105 ± 88.7                    | 250                                     | 0.07                          | 5.71                     |                  |
| Colocasia esculenta     | 13.3 ± 2.4  | 4.4 ± 1.3      | 0.530        | 487                                             | 71 ± 58.1                     | 258                                     | 0.15                          | 4.4                      |                  |
| Pelargonium rf          | 15.7 ± 2.7  | 5.3 ± 0.7      | 0.966        | 443                                             | 191 ± 89.1                    | 428                                     | 0.43                          | 4.6                      |                  |
| Citrus sinensis         | 6.3 ± 1.0   | 6.4 ± 1.0      | 0.364        | 626                                             | 113 ± 45.3                    | 228                                     | 0.18                          | 5.1                      |                  |
| Ceratonia siliqua       | 12.9 ± 1.8  | 3.8 ± 0.6      | 0.834        | 1292                                            | 354 ± 194.2                   | 1077                                    | 0.27                          | 6.2                      |                  |
| Olea europea            | 6.2 ± 0.7   | 2.7 ± 0.4      | 0.481        | 865                                             | 195 ± 110.1                   | 416                                     | 0.23                          | 6.2                      |                  |
| Manihot esculenta       | 16.7 ± 1.8  | 8.0 ± 1.3      | 0.811        | 1164                                            | 452 ± 180.4                   | 944                                     | 0.39                          | 6.7                      |                  |
| Ricinus communis        | 15.1 ± 1.8  | 5.3 ± 0.8      | 0.984        | 1736                                            | 677 ± 350.0                   | 1708                                    | 0.39                          | 8.7                      |                  |

$g_{op}$, operational stomatal conductance; $g_{max}$, maximum theoretical stomatal conductance; $D_v$, leaf vein density.
stomata in ecological interactions among species the speed of stomatal opening/closing response and regulation of stomata by environmental factors and at the signalling level (Hetherington & Woodward, 2003; Franks & Farquhar, 2007; Brodribb et al., 2009; Brodribb & McAdam, 2011; Lawson et al., 2011; Lawson & Blatt, 2014; McAdam & Brodribb, 2015) are likely equally important as wide plasticity in stomatal pore area and $g_{\text{op}}$ in species with high vein densities (Fig. 2a) was achieved by multiple combinations of different stomatal density and pore geometries and was not exclusively driven by high densities of small stomata as previously predicted (Franks et al., 2009; de Boer et al., 2012). The lack of a significant relationship between the stomatal pore area and $g_{\text{op}}$ may be due to the small sample size of just 18 species.

Within species there is a tendency for increased $g_{\text{op}}$ to be achieved by decreasing stomatal size and increasing density (Hetherington & Woodward, 2003; Franks et al., 2009). The present study suggests that this tendency may not apply across species as no significant correlations were observed between $g_{\text{op}}$ and stomatal pore area ($r^2 = 0.0042$) or depth ($r^2 = 0.0525$) and a complex relationship was observed with stomatal density (SD), where both operational and theoretical conductivities increased with increasing SD ($r^2 = 0.6675$, $g_{\text{max}}$ and SD; $r^2 = 0.3255$, $g_{\text{op}}$ and SD) up to a threshold of c. 250 stomata mm$^{-2}$, after which they declined sharply despite increasing SD (Fig. S4). This suggests that the observed pattern of steeply increasing $g_{\text{max}}$ and $g_{\text{op}}$ in species with high vein densities (Fig. 2a) was achieved by multiple combinations of different stomatal density and pore geometries and was not exclusively driven by high densities of small stomata as previously predicted (Franks et al., 2009; de Boer et al., 2012). The lack of a significant relationship between the stomatal pore area and $g_{\text{op}}$ may be due to the small sample size of just 18 species.

Equally, however, this may reflect different weighting of the functional roles of stomata in the species analysed as part of this study. Stomata have three primary functional roles (Raven, 2002): they optimize CO$_2$ uptake against water loss; they are involved in thermoregulation of leaves via conductive cooling and they provide protection against catastrophic embolisms. Optimization of gas exchange in environments that suffer water deficit may select for species with high densities of
small stomata (Franks et al., 2009; de Boer et al., 2012), however, the requirement for leaf cooling in hot climates with high light intensities may select for species with moderate densities of mid-sized stomata (e.g. R. communis, Table 1).

The trend of increasing disparity between $g_{\text{max}}$ and $g_{\text{op}}$ in species with a high vein density was unexpected. We have interpreted the trend in terms of a means to expand the dynamic range of ecophysiological behaviour of high vein density species. An alternative interpretation is that the ecophysiological behaviour of modern extant species is somehow a legacy of the palaeoatmospheric conditions under which they radiated. Gymnosperms as a group were ecologically dominant during the early and middle Mesozoic when atmospheric CO$_2$ concentrations were on average five times higher than today (e.g. c. 2000 µmol mol$^{-1}$). Angiosperms on the other hand underwent their greatest radiation as CO$_2$ concentrations declined through the Cretaceous period. It is widely theorized (McElwain et al., 2005; Brodribb & Feild, 2010; Feild et al., 2011a) but not universally accepted (Boyce & Zwieniecki, 2012) that the declining Cretaceous atmospheric CO$_2$ concentration from c. 2000 to c. 400 µmol mol$^{-1}$ contributed to the rise in angiosperms over gymnosperms because angiosperms uniquely developed traits that would enable them to maintain carbon gain under 'CO$_2$ starvation'. Next therefore, we quantified the direct photosynthetic advantage of increasing $g_{\text{max}}$ via an increase in stomatal density and/or stomatal pore geometry under both ambient (400 ppm) and simulated Cretaceous (2000 ppm) CO$_2$ (Fig. 3a,c).

Maximum photosynthetic capacity is regulated by coordination of $g_{\text{max}}$ and $D_v$.

Theoretical saturated assimilation rates (which we refer to here as $t_A$) were estimated for a subset of eight species (one fern, five gymnosperms and two angiosperms) based on their respective $g_{\text{max}}$ values fitted to $A$: $g_{\text{op}}$ curves collected using gas analysis in 2011 (see the Materials and Methods section). Our results suggest that although stomatal limitation on photosynthesis may be small within species (Farquhar & Sharkey, 1982; Hetherington & Woodward, 2003), comparison across species demonstrates that differences in $g_{\text{max}}$ can impose a significant constraint on the assimilation rate (Table 2). Species with low $g_{\text{max}}$ (c. 400 mmol m$^{-2}$ s$^{-1}$), for example L. hopei ($D_v$ = 0.93 mm mm$^{-2}$, $t_A$ = 7.56 µmol m$^{-2}$ s$^{-1}$) and G. biloba ($D_v$ = 3.05 mm mm$^{-2}$, $t_A$ = 10.15 µmol m$^{-2}$ s$^{-1}$), have $t_A$ values capped at c. 11 µmol m$^{-2}$ s$^{-1}$ under current ambient CO$_2$ concentrations (Fig. 3a, Table 2). Species with moderate $g_{\text{max}}$ values (c. 700 mmol m$^{-2}$ s$^{-1}$) such as A. australis ($D_v$ = 1.13 mm mm$^{-2}$, $t_A$ = 10.15 µmol m$^{-2}$ s$^{-1}$) and O. regalis ($D_v$ = 3.05 mm mm$^{-2}$, $t_A$ = 10.15 µmol m$^{-2}$ s$^{-1}$) have slightly higher assimilation rates of c. 12 µmol m$^{-2}$ s$^{-1}$, whereas species with the highest $g_{\text{max}}$ values of over 1000 mmol m$^{-2}$ s$^{-1}$ have the correspondingly highest assimilation rates (e.g. L. nobilis, $D_v$ = 5.71 mm mm$^{-2}$, $t_A$ = 18.74 µmol m$^{-2}$ s$^{-1}$ and P. caerulea, $D_v$ = 12.36 mm mm$^{-2}$, $t_A$ = 16.58 µmol m$^{-2}$ s$^{-1}$) (Fig. 3a, Table 2).

The interspecies comparison in Fig. 3, highlighted by the inset figure (Fig. 3b), illustrates that assimilation rates can be enhanced by one of two possible routes: (1) by increasing $g_{\text{max}}$ and holding vein density ($D_v$) constant (Fig. 3b); or (2) by maintaining the same $g_{\text{max}}$ and increasing $D_v$. The stomatal density and geometry are highly responsive to atmospheric CO$_2$ concentration on timescales of weeks to decades (Wagner et al., 1996; Haworth et al., 2013) but vein densities are much less responsive to atmospheric CO$_2$ concentration on time-scales of weeks to decades (Wagner et al., 1996; Haworth et al., 2013) but vein densities are much less responsive
to CO₂, despite being highly responsive to many other environmental factors (Uhl & Mosbrugger, 1999). This implies that increases in assimilation rate via developmental and/or morphological processes (underlying biochemistry is not considered here but may play a role) could be rapidly achieved within an individual plant or population by increasing $g_{\text{m}}$ but could only be achieved on evolutionary timescales by increasing vein density.

It also highlights how ‘transformative’ (Boyce et al., 2009) increasing the vein density was for angiosperms compared with gymnosperms because although species with a low $D_v$ can incrementally enhance assimilation rates with relatively small changes in $D_v$ or $g_{\text{m}}$, a doubling of assimilation rates via morphological/developmental change can only be achieved by increasing $D_v$ above 5 mm mm⁻² (Fig. 3 inset). It is argued that both $D_v$ and $g_{\text{m}}$ are coordinated at the leaf level by evolutionary controls on cell size (Brodribb et al., 2013) which imply that simple modification to cell size, perhaps through whole genome duplication (which is common among angiosperms but not gymnosperms; Van de Peer et al., 2009) would offer a means of doubling $D_v$, and $g_{\text{m}}$ in a coordinated way. The comparison here of Passiflora and Laurus (Fig. 3a) also illustrates that once a $D_v$ threshold of 5 has been passed, subtle changes in $g_{\text{m}}$ can have an equally important control on the assimilation rate, as do changes in $D_v$ under current ambient CO₂. This observation was predicted by the model of de Boer et al. (2012).

Our data show an unequivocal advantage of angiosperm species with high $D_v$ and/or high $g_{\text{m}}$ at modern ambient CO₂ concentrations of c. 400 μmol mol⁻¹ (Fig. 3a). In order to test further the hypothesis that high $g_{\text{m}}$ species in with a high vein density conferred an advantage to angiosperms under declining Cretaceous atmospheric CO₂ concentration, we also examined whether the photosynthetic advantage of high $D_v$ and $g_{\text{m}}$ could be lost under elevated CO₂ conditions of 2000 μmol mol⁻¹, similar to those of the early Cretaceous when gymnosperms were the dominant ecological element in the majority of world biomes. Maximum ($A_{\text{max}}$) and saturating ($A_{\text{sat}}$) assimilation rates were measured for the same subset of eight species (one fern, five gymnosperms and two angiosperms) under saturating light (1000 μmol m⁻² s⁻¹) at 2000 μmol mol⁻¹ and 400 μmol mol⁻¹ CO₂, respectively (Tables 2, S2). The results show that an elevated atmospheric CO₂ concentration only modestly raises $A_{\text{sat}}$ above $A_{\text{max}}$ in the studied angiosperms (by 113 ± 18%) but had a profound effect on gymnosperms, raising the $A_{\text{max}}$ by 173 ± 46% above $A_{\text{sat}}$ (Fig. 3c, Table 2). This is consistent with the modelled differences between evergreens and deciduous taxa where species with robust leaves and high mesophyll resistance showed a significant increase in $A$ and water use efficiency under elevated CO₂ (Niinemets et al., 2011). These results indicate that the photosynthetic advantage conferred by high $D_v$ and high $g_{\text{m}}$ under modern ambient atmospheric CO₂ levels, was likely to be completely lost in the elevated [CO₂] world of the early Cretaceous (Fig. 3c).

Elevated CO₂ reduces the diffusional limitation of stomata on assimilation rates in any species with low $g_{\text{m}}$ values. This is best demonstrated by the substantial rise (by 173%) in $A_{\text{max}}$ values of the gymnosperms studied here when exposed to elevated CO₂. Based on our analysis it is likely that early Cretaceous gymnosperm species with low $g_{\text{m}}$ and low $D_v$, similar to the cycad, ginkgo and conifers examined in this study, would have possessed assimilation rates as high as or higher than extant angiosperms (Fig. 3c). By contrast, when the same species were subjected to CO₂ levels more similar to those of the late Cretaceous (c. 400 μmol mol⁻¹; Barclay et al., 2010), a severe diffusional penalty was imposed. These results on modern extant taxa predict that palaeospecies with $g_{\text{m}}$ values at or below a threshold level of c. 1400 mmol mm⁻² s⁻¹ (Fig. 3d) would not have been able to maintain assimilation rates equivalent to species possessing high $g_{\text{m}}$ values under Cretaceous atmospheric CO₂ decline. In a palaeoecoscape where other environmental conditions were optimal for growth lower assimilation rates would therefore undermine the competitive ability of low $g_{\text{m}}$ species. A detailed site-specific analysis of fossil leaf $g_{\text{m}}$ values in Cretaceous angiosperms vs gymnosperms is now required to examine when and if angiosperms crossed this critical $g_{\text{m}}$ threshold of c. 1400 mmol mm⁻² s⁻¹ and whether it coincided with the first occurrence of leaves with vein densities > 5 mm mm⁻² as would be predicted if both evolved in coordination. As atmospheric

### Table 2 Estimated and measured assimilation rates

| Species                        | $g_{\text{m}}$ mmol m⁻² s⁻¹ | Measured $A_{\text{sat}}$ at 400 ppm μmol⁻¹ mm⁻² s⁻¹ | Measured $A_{\text{max}}$ at 2000 ppm μmol⁻¹ mm⁻² s⁻¹ | Est. $A_{\text{max}}$ at 400 ppm μmol⁻¹ mm⁻² s⁻¹ | Model to estimate $A_{\text{max}}$ from $g_{\text{m}}$ |
|-------------------------------|-----------------------------|-----------------------------------------------|--------------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Lepidozamia hopei (G)        | 374                         | 7.63 ± 1.06                                  | 17.20 ± 4.00                                    | 7.56                                          | $A_{\text{max}} = 1.9624 \log_{10} g_{\text{m}} - 4.06$ (r² = 0.4475; P < 0.001) |
| Lepidozamia peroffskiana (G) | 491                         | 4.73 ± 1.91                                  | 8.87 ± 0.98                                     | 11.17                                         | $A_{\text{max}} = 3.1225 \log_{10} g_{\text{m}} - 8.18$ (r² = 0.8111; P < 0.001) |
| Nagia nagi (G)               | 526                         | 5.24 ± 1.02                                  | 19.34 ± 2.00                                    | 9.11                                          | $A_{\text{max}} = 2.4814 \log_{10} g_{\text{m}} - 6.44$ (r² = 0.793; P < 0.001) |
| Agathis australis (G)         | 554                         | 11.5 ± 1.59                                  | 20.80 ± 3.70                                    | 11.55                                         | $A_{\text{max}} = 3.1225 \log_{10} g_{\text{m}} - 8.18$ (r² = 0.6587; P < 0.001) |
| Osmunda regalis (F)          | 669                         | 7.79 ± 1.44                                  | 12.00 ± 2.00                                    | 12.36                                         | $A_{\text{max}} = 3.9086 \log_{10} g_{\text{m}} - 13.07$ (r² = 0.7003; P < 0.001) |
| Gingko biloba (G)            | 374                         | 4.44 ± 1.80                                  | 17.60 ± 3.30                                    | 10.15                                         | $A_{\text{max}} = 3.7973 \log_{10} g_{\text{m}} - 12.35$ (r² = 0.7109; P < 0.001) |
| Laurus nobilis (A)           | 1529                        | 9.37 ± 1.86                                  | 18.29 ± 0.26                                    | 18.74                                         | $A_{\text{max}} = 4.2584 \log_{10} g_{\text{m}} - 12.48$ (r² = 0.9055; P < 0.001) |
| Passiflora caerulea (A)       | 963                         | 9.20 ± 1.90                                  | 21.25 ± 3.29                                    | 16.58                                         | $A_{\text{max}} = 4.4852 \log_{10} g_{\text{m}} - 14.24$ (r² = 0.7092; P < 0.001) |

$g_{\text{m}}$, maximum theoretical stomatal conductance; $A_{\text{sat}}$, saturated assimilation rate; $A_{\text{max}}$, maximum assimilation rate. n = 10–12; errors, ± SD; G, gymnosperm; A, angiosperm; F, fern.
CO2 levels continue to rise over the next century the competitive landscape of gymnosperms and angiosperms may shift again to a level playing field where diffusional limitation on assimilation is reduced for species with low densities of stomata and veins.

Conclusions

Despite extensive field surveys of $g_{op}$ across different plant species and environmental gradients (Körner, 1994; Schulze et al., 1994; Lin et al., 2015) understanding of the scaling relationship between $g_{op}$ and $g_{\text{max}}$ is limited to just a handful of species (Beerling et al., 1998; Franks et al., 2009; Dow et al., 2014). Further understanding of this relationship would enable anatomical traits to be linked to function, providing a means of tracking palaeo-physiological responses over geological time. We have demonstrated that, on average, species growing in glasshouse conditions conduct H2O and CO2 through stomatal pores at c. 25% of their theoretical maximum limits ($g_{op} = 0.25 \ g_{\text{max}}$) determined by stomatal geometry and density (Fig. 1). Wide variability in mean $g_{op} \cdot g_{\text{max}}$ is, however, apparent and appears to be tightly correlated with $A_{\text{sat}}$.

A strong positive correlation was observed between vein density ($D_v$) and $g_{op}$ ($r^2 = 0.8314$), and between vein density and theoretical $g_{\text{max}}$ ($r^2 = 0.741$), both of which indicate close coordination of stomatal and vein density evolution. Our study elaborates on the 'vein density hypothesis' (Boyce et al., 2009; Brodribb & Feild, 2010; Feild et al., 2011a) by proposing that the coordinated evolution of high vein density and high $g_{\text{max}}$ in angiosperms dramatically increased their range of dynamic operational conductance compared with gymnosperm ancestors. This likely conferred greater ecophysiological plasticity to angiosperms allowing species with a high $g_{\text{max}}$ and $D_v$ to operate within a much wider ecophysiological niche space, which in turn provided an opportunity for a population to segregate resource use by stomatal conductance. Our study also supports the ‘Cretaceous carbon starvation hypothesis’: we have demonstrated that the evolution of unique traits such as high $D_v$ and $g_{\text{max}}$ in angiosperms conferred them with a competitive advantage over gymnosperms by facilitating higher assimilation rates as atmospheric CO2 declined, but also by greatly expanding the ecophysiological niche space in which they could operate.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 A comparison of the linear relationships between average $g_{op}$ and anatomical $g_{\text{max}}$ when $g_{op}$ is measured using the ‘variance protocol’ with a porometer vs the standardized protocol using an IRGA.

Fig. S2 Range of scaling relationships observed between mean $g_{op}$ and theoretical $g_{\text{max}}$ and maximum $g_{op}$ and $g_{\text{max}}$ for gymnosperms, a fern and angiosperms.

Fig. S3 Graph showing that the divergence between $g_{\text{max}}$ and $g_{op}$ increases with increasing $D_c$.

Fig. S4 Graph illustrating relationship between maximum theoretical stomatal conductance ($g_{\text{max}}$) and stomatal density (SD) and between operational stomatal conductance ($g_{op}$) and SD.

Table S1 Estimated stem and crown ages of species lineages studied.

Table S2 Species investigated and number of replicates in repeat analysis dataset October 2015 $g_{op(\text{max})}$

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