Title
Does size matter? Atmospheric CO2 may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO2

Authors(s)
Elliott-Kingston, Caroline, Haworth, Matthew, Yearsley, Jonathan M., McElwain, Jennifer C., et al.

Publication date
2016

Publication information
Elliott-Kingston, Caroline, Matthew Haworth, Jonathan M. Yearsley, Jennifer C. McElwain, and et al. “Does Size Matter? Atmospheric CO2 May Be a Stronger Driver of Stomatal Closing Rate than Stomatal Size in Taxa That Diversified under Low CO2” 7 (2016).

Publisher
Frontiers Media

Item record/more information
http://hdl.handle.net/10197/7804

Publisher's statement
This document is Protected by copyright and was first published by Frontiers. All rights reserved. It is reproduced with permission.

Publisher's version (DOI)
10.3389/fpls.2016.01253

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd_oa)

© Some rights reserved. For more information
Does size matter? Atmospheric CO2 may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO2.

Caroline Elliott-Kingston¹, Matthew Haworth², Jon M. Yearsley³, Sven P. Batke⁴, Tracy Lawson⁴, Jennifer C. McElwain³

¹School of Agriculture and Food Science, University College Dublin, Ireland, ²Institute of Tree and Timber IVALSA, Italian National Research Council, Italy, ³School of Biology and Environmental Science, The Earth Institute, O'Brien Centre for Science - University College Dublin, Ireland, ⁴School of Biological Science, University of Essex, United Kingdom

Submitted to Journal: Frontiers in Plant Science
Specialty Section: Plant Biophysics and Modeling
Article type: Original Research Article
Manuscript ID: 197656
Received on: 16 Mar 2016
Revised on: 29 Jul 2016
Frontiers website link: www.frontiersin.org
Conflict of interest statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement
C.E-K. Primary researcher. Carried out all stomatal conductance and speed of stomatal closing measurements. Wrote the manuscript. Awarded an Irish Research Council funding grant to undertake the research.
M.H. Carried out stomatal pore length and stomatal density measurements.
J.M.Y. Created the model to work out half-time closing from raw data. Wrote the R Script for the model.
S.P.B. Provided considerable statistical help. Produced Figures 1 and 3.
T.L. Visited at beginning of project and co-designed study. Provided instructive comments on the original manuscript.
J.C.McE. Principal Investigator. Designed the study and edited the manuscript. Awarded funding from European Research Council to undertake the research.

Keywords
stomata, Half-closure time in response to darkness, stomatal size, Atmospheric CO2 concentration, time of taxa diversification

Abstract

Word count: 211
(1) One strategy for plants to optimise stomatal function is to open and close their stomata quickly in response to environmental signals. It is generally assumed that small stomata can alter aperture faster than large stomata.
(2) We tested the hypothesis that species with small stomata close faster than species with larger stomata in response to darkness by comparing rate of stomatal closure across an evolutionary range of species including ferns, cycads, conifers and angiosperms under controlled ambient conditions (380ppm CO2; 20.9% O2).
(3) The two species with fastest half-closure time and the two species with slowest half-closure time had large stomata while the remaining three species had small stomata, implying that closing rate was not correlated with stomatal size in these species. Neither was response time correlated with stomatal density, phylogeny, functional group or life strategy.
(4) Our results suggest that past atmospheric CO2 concentration during time of taxa diversification may influence stomatal response time. We show that species which last diversified under low or declining atmospheric CO2 concentration close stomata faster than species that last diversified in a high CO2 world. Low atmospheric [CO2] during taxa diversification may have placed a selection pressure on plants to accelerate stomatal closing to maintain adequate internal CO2 and optimise water use efficiency.

Funding statement

http://dx.doi.org/10.13039/501100001596, "Irish Research Council for Science, Engineering and Technology" (Embark scholarship R10679);
EU Marie Curie Excellence Grant (MEXT-CT-2006-042531);
EU Marie Curie Intra-European Fellowship (PEA-IEF-2010-275626);
ERC grant (ERC-279962-OXYEVOL).

Ethics statement

(Author are required to state the ethical considerations of their study in the manuscript including for cases where the study was exempt from ethical approval procedures.)

Did the study presented in the manuscript involve human or animal subjects: No
Does size matter? Atmospheric CO$_2$ may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO$_2$.

C. Elliott-Kingston$^{1,*}$, M. Haworth$^2$, J. M. Yearsley$^3$, S. P. Batke$^3$, T. Lawson$^4$ and J. C. McElwain$^3$

$^1$School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland
$^2$Italian National Research Council, Institute of Tree and Timber IVALSA, Rome, Latium, Italy
$^3$Earth Institute, Science Centre East, School of Biology and Environmental Science, University College Dublin, Belfield, Dublin 4, Ireland
$^4$School of Biological Science, University of Essex, Colchester CO4 3SQ, United Kingdom

*Correspondence: 1Corresponding Author

carolineelliottkingston@gmail.com

Key words: stomata, half-closure time in response to darkness, stomatal size, atmospheric CO$_2$ concentration, time of taxa diversification.

Abstract

(1) One strategy for plants to optimise stomatal function is to open and close their stomata quickly in response to environmental signals. It is generally assumed that small stomata can alter aperture faster than large stomata.

(2) We tested the hypothesis that species with small stomata close faster than species with larger stomata in response to darkness by comparing rate of stomatal closure across an evolutionary range of species including ferns, cycads, conifers and angiosperms under controlled ambient conditions (380ppm CO$_2$; 20.9% O$_2$).

(3) The two species with fastest half-closure time and the two species with slowest half-closure time had large stomata while the remaining three species had small stomata, implying that closing rate was not correlated with stomatal size in these species. Neither was response time correlated with stomatal density, phylogeny, functional group or life strategy.
Our results suggest that past atmospheric CO$_2$ concentration during time of taxa diversification may influence stomatal response time. We show that species which last diversified under low or declining atmospheric CO$_2$ concentration close stomata faster than species that last diversified in a high CO$_2$ world. Low atmospheric [CO$_2$] during taxa diversification may have placed a selection pressure on plants to accelerate stomatal closing to maintain adequate internal CO$_2$ and optimise water use efficiency.

**Introduction**

Stomata are microscopic pores on aerial surfaces of land plants, surrounded by guard cells that adjust turgor in order to regulate pore size, thus controlling gas exchange between the plant interior and atmosphere. Fossil records show that stomata evolved more than 400 million years ago (Ma) and their morphology remains largely unchanged (Edwards, Kerp, & Hass, 1998), apart from the evolution of dumbbell-shaped guard cells in grasses (Franks & Farquhar, 2007). Extant species have evolved from ancestors that originated under diverse environmental conditions; therefore a simple expectation is that stomata in extant plants will exhibit morphological and functional diversity. Stomatal conductance governs gas exchange, photosynthesis, water loss and evaporative cooling and is determined by density and size of stomata along with functional responses such as rate of aperture change. Stomatal density and size also determine maximum gas diffusion rate (Brown & Escombe, 1900; Parlange & Waggoner, 1970; Raschke, 1976; Wong et al., 1979; McElwain & Chaloner, 1995; Hetherington & Woodward, 2003; Franks & Beerling, 2009; McElwain et al. 2016). Density and size are linked and both are often correlated with atmospheric carbon dioxide concentration ([CO$_2$]$_{atm}$) (Hetherington & Woodward, 2003; McElwain et al., 2005; Franks & Beerling, 2009).

In an investigation into how morphological diversity in stomatal complexes influences stomatal function, Franks & Farquhar (2007) determined that morphological structure of the stomatal complex (guard cell shape and presence or absence of subsidiary cells) impacts mechanical opening and closing of stomata. In particular, the mechanical advantage of fully turgid subsidiary cells constrains guard cell lateral movement, limiting maximum aperture and leaf diffusive conductance. They showed that morphological and mechanical diversity ultimately translated into functional diversity. They concluded that the combination in grasses of dumbbell-shaped guard cells and the ability to quickly shuttle osmotica between subsidiary and guard cells facilitated swift alteration of turgor pressure, allowing rapid stomatal movements,
which conferred a functional advantage upon grasses (Hetherington & Woodward, 2003; Franks & Farquhar, 2007). Another aspect of morphological diversity is number and size of stomata. On a geological timescale, a trend has been suggested with recently evolved species having high densities of small stomata compared to species with fewer, larger stomata in the past (Hetherington & Woodward, 2003; Franks & Beerling, 2009). Leaves with short lifespans, built for higher rates of gas exchange, are thought to have small stomata and faster stomatal response times to offset the risks associated with large tissue water potential gradients that may result in xylem cavitation (Drake, Froend, & Franks, 2013). It has been suggested that the ability of angiosperms to sustain high stomatal conductance rates may be due to the possession of large numbers of small stomata (Hetherington & Woodward, 2003; Franks & Beerling, 2009). In addition, high densities of small stomata allow exploitation of the ‘edge effect’ as small pores have a greater proportion of edge than large pores, resulting in a shorter diffusion pathway from the pore (H. G. Jones, 1992). In contrast to angiosperms, ferns and gymnosperms tend to have large stomata in small numbers (Franks & Beerling, 2009). For the same total pore area, a leaf with few large stomata will have a lower maximum stomatal conductance than a leaf with many small stomata because of the longer diffusion pathway through the stomatal pore. Thus, Franks & Beerling (2009) have proposed that high numbers of small stomata are necessary in low CO₂ atmospheres, such as pertains today, to achieve high maximum diffusive conductance to CO₂. In addition, they suggest that small stomata respond faster than large stomata, enhancing their ability to function effectively in dynamic environments (Franks & Beerling, 2009). Robinson (1994) hypothesized that certain factors, such as declining atmospheric CO₂ and water limitation, place selection pressures on plants to develop compensating mechanisms, including improved stomatal efficiency. Since atmospheric [CO₂] has declined over the past 20 million years, Robinson (1994) suggested that the most recently evolved group, angiosperms, with faster rates of evolution, have more efficient stomata than ferns and gymnosperms. This hypothesis was tested on angiosperm and coniferous gymnosperm species; however, ferns and cycads were excluded (Robinson, 1994). In contrast to angiosperms, cycads are an ancient plant group (Jones, 2002; Nagalingum et al., 2011) with slow reproductive biology, long leaf lifespan and relatively large stomata (Haworth, Fitzgerald, & McElwain, 2011); the question remains whether their large stomata are less efficient than the smaller stomata of angiosperms in our currently low CO₂ world.

Cowan (1977) and Cowan & Farquhar (1977) hypothesised that plants display optimal stomatal behaviour, defined as maximising photosynthetic gain to water loss. It is reasonable to suppose
that different taxa have developed diverse strategies for optimisation. For example, a strategy for optimising water use efficiency (WUE) via stomatal behaviour is to open stomata rapidly to take advantage of irradiance for photosynthetic gain, and to close them again quickly when conditions become unfavourable (Lawson & Blatt, 2014), for example, under limited water availability. The rate of stomatal opening and closing response is, therefore, one method of stomatal optimisation (Katul et al., 2010; Lawson et al., 2010; Lawson & Blatt, 2014). In a study on stomatal opening and closing rate in different plant functional types, including graminoids, forbs, woody angiosperms and gymnosperms, in both wet and dry climates, graminoids were shown to have the fastest stomatal responses (Vico, Manzoni, Palmroth, & Katul, 2011). The long pore length in grass stomata combined with narrow, dumbbell-shaped guard cells means that very small changes in guard and subsidiary cell turgor cause comparatively large changes in aperture and stomatal conductance (Hetherington & Woodward, 2003). Therefore, in grasses, large stomata (in terms of stomatal pore length) are not an impediment to efficient stomatal response to changing environmental conditions. Perhaps the evolutionary trend towards higher numbers of small stomata from few, large stomata has led to the common perception that small stomata are more efficient than large stomata, and that rate of stomatal response is directly linked to stomatal size. “Small stomata can open and close more rapidly…” (Hetherington & Woodward, 2003). “Smaller stomata are capable of faster response times...” (Franks & Beerling, 2009). “…leaves with smaller and more numerous stomata exhibit faster absolute rates of response of stomatal conductance to water vapour” (Drake et al., 2013). Logically, this might be expected to be the case given that changes in osmotic potential are needed for guard cell swelling and smaller stomata have a greater surface area to volume ratio than larger stomata; changes in osmotic potential therefore affect small stomata relatively more than they affect large stomata. The assumption or perception that small stomata are faster may hold across related species within the same genus (Drake et al., 2013). However, this hypothesis has not been comprehensively tested across a range of phylogenetic groups. Here we test the hypothesis that small stomata are more efficient than large stomata with respect to rate of stomatal closure in response to a changing environmental signal, in this case, darkness. To test this hypothesis, an evolutionary range of species including one fern, four gymnosperms and two angiosperms, including one cereal grass, were grown under identical controlled ambient conditions, and rate of stomatal closure in response to darkness was measured.

Materials and Methods
A range of plants representing all major vascular plant groups was selected for determining stomatal closure rate in response to darkness. These include: *Osmunda regalis* L. (Osmundaceae), a perennial, rhizomatous, deciduous fern; *Lepidozamia peroffskyana* von Regel (Zamiaceae), an evergreen cycad; *Ginkgo biloba* L. (Ginkgoaceae), a deciduous gymnosperm tree; two broad-leaved, evergreen conifers in the order Pinales, including *Podocarpus macrophyllus* (Thunb.) D. Don (Podocarpaceae) and *Agathis australis* (D. Don) Loudon (Araucariaceae); *Solanum lycopersicon* L. (Solanaceae), a dicotyledonous, herbaceous, perennial angiosperm; and *Hordeum vulgare* L. (Poaceae), a monocotyledonous, graminaceous, annual angiosperm. All species were individually planted into 4 litre square pots (15 x 15 x 23 cm) in a growing medium comprising 80% compost (Shamrock® Multi-Purpose compost; Scotts Horticulture Ltd., Co. Kildare, Ireland), 20% vermiculite (2-5mm horticultural grade; William Sinclair Horticulture Ltd., UK) and 7kg/m$^3$ Osmocote® Exact® 16-18 months slow release fertiliser (15% N, 8% P$_2$O$_5$, 11% K$_2$O, 2.5% MgO plus trace elements; Scotts International BV, The Netherlands).

Cycad seeds were initially scarified, soaked for 24 hours in 3% potassium nitrate solution to encourage germination (Bradbeer, 1988), then placed in plastic bags containing a damp mixture of 50:50 perlite and vermiculite (2-5mm Sinclair Standard; William Sinclair Horticulture Ltd., UK). To prevent fungal infection, the seeds were sprayed fortnightly with 0.06 g l$^{-1}$ Doff Systemic Fungus Control spray (Doff, UK) containing myclobutanil. Following the first appearance of the radical, seeds were sown in seed trays containing a 80:20 mixture of compost and vermiculite and placed in well-ventilated propagators under atmospheric treatment conditions (380ppm CO$_2$; 20.9% O$_2$) in a Conviron BDW40 growth control chamber. After radicle development but just before emergence of the plumule, the seeds were planted individually into 4 litre square pots (15 x 15 x 23 cm) using the growing medium described above. *Hordeum vulgare* (barley) seeds were germinated in seed trays in the growing medium detailed above and potted up individually in the same medium 14 days after emergence of the coleoptile. After 18 months (or 3 months in the case of tomato and barley), plants were liquid fed with Osmocote® Plus Multi-Purpose Plant Food. One application feeds for up to 6 months, contains 15% N, 9% P$_2$O$_5$, 12% K$_2$O plus 9 other essential nutrients, and is suitable for all plant types and all soil conditions. All plants were grown in controlled environment chambers under identical conditions (see below).

**Controlled growth chambers**
Six plants of each species were grown in two Conviron (Winnipeg, Manitoba, Canada) BDW-40 walk-in growth rooms (internal chamber size 3.7m$^2$) with atmospheric control of [CO$_2$] at ambient (380ppm) and [O$_2$] at ambient (20.9%) in the Programme for Experimental Atmospheres and Climate (PÉAC) facility at Rosemount Environmental Research Station, University College Dublin. Carbon dioxide concentration was maintained at 380 ppm by injection of compressed CO$_2$ (BOC UK, Surrey, England) and was continuously monitored with a PP-systems WMA-4 IRGA (Amesbury, Massachusetts, USA); injection of CO$_2$ gas was controlled by opening and closing a solenoid valve. Oxygen concentration was monitored and maintained at 20.9% by a PP-systems OP-1 Oxygen Sensor. All other growth conditions remained constant, with 16 h. day length (0500–0600 hours, light intensity rose from 0 to 300 µmol m$^{-2}$ s$^{-1}$; 0600–0900 hours, light intensity increased from 300 to 600 µmol m$^{-2}$ s$^{-1}$; 0900–1700 hours, PPFD maintained at 600 µmol m$^{-2}$ s$^{-1}$; 1700–2000 hours, light intensity decreased from 600 to 300 µmol m$^{-2}$ s$^{-1}$; 2000–2100 hours, light intensity decreased from 300 to 0 µmol m$^{-2}$ s$^{-1}$), temperature regime (nighttime temperature of 18°C rising to a midday peak of 28°C), relative humidity of 80 %, downward ventilation to ensure mixing of atmospheric gases; with each plant receiving 30 ml of water per day in the first year of growth, and 60 ml thereafter, except for ferns, which received 60 ml of water day$^{-1}$ in the first year and 120 ml day$^{-1}$ thereafter. In order to avoid mutual shading, plants were randomised within areas of identical canopy height in the growth chambers (Hammer & Hopper, 1997; Sager & McFarlane, 1997). *O. regalis, L. peroffskyana, G. biloba, P. macrophyllus* and *A. australis* were grown for a minimum of eighteen months before analysis. *S. lycopersicon* and *H. vulgare* were grown for a minimum of three months before analysis. To avoid chamber effects, plants were rotated between chambers every three months (Hirano, Hongo, & Koike, 2012).

**Measuring rate of stomatal closure in response to darkness**

Rate of stomatal closure in response to darkness (0 µmol m$^{-2}$ s$^{-1}$ Photosynthetic Photon Flux Density (PPFD)) was measured using a PP-Systems CIRAS-2 portable photosynthesis system (Amesbury, Massachusetts, USA) from saturating light intensity calculated from photosynthesis response curves (Parsons, Weyers, Lawson, & Godber, 1998) to 0 µmol m$^{-2}$ s$^{-1}$ PPFD in a single step decrease in PPFD. Measurements were performed on intact, mature, fully expanded leaves on three replicates of each species between 9am and 11am each day. Within the leaf cuvette, temperature was set to 25°C and water vapour pressure deficit (VPD) was maintained at 1.0 ± 0.2 kPa. Cuticular conductance was assumed to be negligible. After
had reached steady state, irradiance was removed in the leaf cuvette chamber. To ensure no light leaked into the chamber from external sources, the room lights were also extinguished. Measurements of stomatal conductance \( (g_s) \) were recorded every ten seconds for ninety minutes (min), during which time all species reduced \( g_s \) to a minimum value. The half-closure time (min) was calculated; this was defined as the time taken for \( g_s \) to reach 50% of the difference between the first and final values. The first \( g_s \) value was taken 1 to 12 minutes, depending on species, after lights were extinguished to exclude the fluctuation in \( g_s \) that occurs due to a change in energy balance in the CIRAS-2 when it recalculates \( g_s \) in darkness (as distinct from full light previously). The technical nature of the fluctuation is caused by temperature recalculation in the CIRAS-2 and is an artefact of the machine. The rate at which stomatal conductance declined can be quantified by the value of the half-closure time of the stomata: the shorter the time of half-closure, the faster the rate.

**Stomatal morphology measurements**

Following completion of stomatal conductance \( (g_s) \) measurements, the leaves on which \( g_s \) measurements were recorded were removed from the plants. Leaf impressions were taken from the abaxial leaf surface using dental impression material (Coltene PRESIDENT light body) and nail varnish ‘positives’ were mounted onto glass slides (Weyers & Johansen, 1985). In the case of *Hordeum vulgare*, leaf impressions were taken from both the abaxial and adaxial leaf surfaces. Five photomicrographs per leaf impression were recorded at x200 magnification using a Leica (DMLB) epifluorescent microscope. Stomatal density was counted on each photomicrograph using AcQuis (version 4.0.1.10- Syncroscopy Ltd., Cambridge, UK) by placing a 0.09mm² grid on the image (half-way down the leaf between midrib and leaf edge) and counting the number of stomata within the box and those touching two of the border lines and the corner where they intersect, on five micrographs for each of three leaves per plant and on three plants, giving a total of 45 counts. Mean stomatal density (number of stomata per mm²) for the abaxial surfaces of all hypostomatous species was recorded. For amphistomatic *H. vulgare*, the average of both surfaces was recorded as one measurement. Stomatal pore length (SPL) (µm) and guard cell width measurements (µm) were taken for five to twenty open stomata per photomicrograph using the hand tool in Acquis.

Stomatal geometry was calculated from guard cell width, stomatal pore depth, pore length and density of stomata when fully open \( (g_{max}) \) (Table 1). Maximum stomatal pore area \( (m^2) \) when the guard cells were fully turgid was calculated as an ellipse using stomatal pore length (m)
multiplied by the width of the guard cell pair with maximum aperture defined as a fraction $\beta$
of the stomatal pore; in the case of a circular pore with diameter equal to pore length, $\beta = 1.0$
while in long narrow stomata $\beta = 0.2$. Maximum aperture was calculated with $\beta$ values of 0.2,
0.4, 0.5, 0.6, 0.8 and 1.0. Theoretical maximum stomatal conductance ($g_{\text{max}}$) was then
calculated using the morphological measurements of fully open stomata and the following
diffusion equation (Parlange & Waggoner, 1970; Franks & Beerling, 2009):

$$g_{\text{max}} = \frac{dw \cdot SD \cdot p_{a_{\text{max}}}}{pd + \frac{\pi}{2} \sqrt{p_{a_{\text{max}}}}}$$

Eq. 1

where $dw =$ diffusivity of water vapour at 25°C (0.0000249 m$^2$ s$^{-1}$) and $v =$ molar volume of
air (0.0224 m$^3$ mol$^{-1}$) are both constants; $SD$ is stomatal density (m$^{-2}$); $p_{a_{\text{max}}}$ is maximum
stomatal pore area (m$^2$) calculated as an ellipse using stomatal pore length (l in m) as the long
axis and $\frac{1}{2}$ as the short axis; and $pd$ is stomatal pore depth (m) considered to be equivalent to
the width of an inflated, fully turgid guard cell (Franks & Beerling, 2009).

Palaeo-carbon dioxide concentration (palaeo-[CO$_2$])

Best estimates of origination date and last diversification date for each of the seven taxa were
gathered from the literature. Atmospheric CO$_2$ concentration ([CO$_2$]$_{\text{atm}}$) over Phanerozoic time
was taken from Bergman, Lenton and Watson (2004) COPSE model and from Berner and
Kothavala (2001) GEOCARB III model. The relationship between estimated [CO$_2$]$_{\text{atm}}$ at the
time of each taxa’s origination date and last known diversification date was tested against the
log$_e$ of each species’ half-closure time to determine whether [CO$_2$]$_{\text{atm}}$ was correlated with rate
of stomatal closing.

Statistical Analysis

The decrease of stomatal conductance ($g_s$) (mmol m$^{-2}$ s$^{-1}$) over time ($t$, minutes) was fitted to
the following exponential decay curve:

$$g_s(t) = g_s(\infty) + (g_s(0) - g_s(\infty)) \cdot \exp(-\exp(A) \cdot t)$$

Eq. 2

where $g_s(0)$ is the stomatal conductance at time $t=0$, $g_s(\infty)$ is the long-term residual stomatal
conductance and $A$ is a parameter related to the half-closure time response, $t_{1/2}$, by log$_e(t_{1/2}) =$
log$_e$($\log_e(2)) - A$. The fit was performed for each replicate of each of the seven species using
generalized non-linear least squares with an error structure that allowed for first-order
autoregressive temporal autocorrelation (implemented using the nlme package in R version
3.1.1) (R Core Team, 2014); as shown in Figure 1. Each fit gave best-estimates and standard errors for $g_s(0)$, $g_s(\infty)$ and $A$. From the fitted values of $A$, the half-closure time response was calculated for each replicate and the median, maximum and minimum half-closure time (min) calculated across replicates for a species. The half-closure time response is defined as the time taken for the stomatal conductance to decrease to half of its value at time $t$. For exponential decay, this half-time is a constant, independent of the initial stomatal conductance. ANOVA with Tukey HSD post-hoc analysis was used to test for differences between species in the $\log_e$(half-closure times). It was only possible to perform a between-species variance analysis, as the low number of replicates did not permit satisfactory analysis of the variability within species. Differences between species in the mean stomatal density (SD), stomatal pore length (SPL) and half-closure time were analyzed using a One-Way ANOVA with Tukey HSD pairwise comparison. Data were $\log_e$(SD) and square root (SPL) transformed prior to analysis. Generalized linear mixed-effects models (GLMM) were implemented using the lmer package in R to describe the relationship between the response variable, $\log_e$(median half-closure time) and the fixed variables, stomatal density, stomatal pore length, plant functional type, shade tolerance, drought tolerance and climate, as defined by Vico et al. (2011). Species was treated as a random variable. ANOVA and Akaike information criterion (AIC) were used to identify the model with the best fit. Linear models (LM) were used to test for correlations between $\log_e$(half-closure time) and estimated atmospheric CO$_2$ concentration at time of taxa origination and diversification. Moreover, LM were also used to test the correlations between $\log_e$(half-closure time), SD and SPL.

**Results**

The stomatal conductance ($g_s$) (mmol m$^{-2}$ s$^{-1}$) change in response to darkness was measured in the seven species (Figure 1). From these measurements $\log_e$(stomatal half-closure time) was calculated (Figure 2). Of the species studied, the fastest responder with respect to stomatal closing response was barley, *Hordeum vulgare* (median half-closure time: 4.83 min; mean 7.16 ± 2.63 min; $R^2$ fit = 0.96) (Figure 2; Table 1), a species with comparatively large stomata (stomatal pore length (SPL): 28.1 ± 6.2 μm) (Table 1). The second fastest responder was the cycad *Lepidozamia peroffskyana* (median half-closure time: 6.53 min; mean 10.26 ± 4.89 min; $R^2$ fit = 0.98) (Figure 2; Table 1), which had the largest stomata of all species studied (SPL: 35.6 ± 5.5 μm) (Table 1). The next three species in order of decreasing rate of closure were two conifers: *Podocarpus macrophyllus* (median half-closure time: 12.74 min; mean 17.96 ± 5.74
Agathis australis (median half-closure time: 15.02 min; mean 13.47 ± 3.18 min; $R^2$ fit = 0.99); and the angiosperm Solanum lycopersicon (median half-closure time: 16.86 min; mean 24.47 ± 8.76 min; $R^2$ fit = 0.99) (Figure 2; Table 1). All three species have the smallest stomata of those measured (SPL: 14.7 ± 2.3 μm; 18.8 ± 4.2 μm; and 15.4 ± 3.5 μm respectively) (Table 1). Finally, the two slowest species to close in response to darkness had large stomata: the fern Osmunda regalis (median half-closure time: 25.27 min; mean 30.13 ± 7.88 min; $R^2$ fit = 0.95; SPL: 29.8 ± 6.5 μm) and Ginkgo biloba (median half-closure time: 78.69 min; mean 105.49 ± 55.45 min; $R^2$ fit = 0.97; SPL: 24.3 ± 5.0 μm) (Figure 2; Table 1).

Mean differences in stomatal density (mm$^2$) and stomatal pore length (μm) of all seven species were tested using ANOVA with pairwise comparison. Differences in stomatal density at alpha 0.05 were observed for one pairwise comparison, namely H. vulgare versus G. biloba (overall comparison: DF = 6, 880, F = 629.4, p<0.05). The remaining pairwise comparisons showed no differences. Differences in stomatal pore length were observed for two pairwise comparisons (O. regalis versus H. vulgare and S. lycopersicon versus P. macrophyllus) (overall comparison: DF = 6, 880, F = 344.8, p<0.05). The remaining pairwise comparisons showed no differences.

The differences in half-closure time between species were tested using ANOVA comparison (overall comparison: DF = 6, 13, F = 4.453, p<0.05). Post-hoc analysis revealed that four comparisons were different, namely G. biloba versus A. australis; G. biloba versus H. vulgare; G. biloba versus L. peroffskyana and G. biloba versus P. macrophyllus.

Generalized linear mixed models (GLMM) were used to describe the relationship between log$_e$(half-closure time) and stomatal density, stomatal pore length, plant functional type, shade tolerance, drought tolerance and climate. The best fit model following AIC comparison was log$_e$(half-closure time) as a function of species (AIC = 174.81, $R^2$ = 0.52).

Maximum stomatal aperture (μm) was calculated with β values of 0.2, 0.4, 0.5, 0.6, 0.8, 1.0; the relationship between theoretical maximum stomatal conductance ($g_{max}$ in mmol m$^{-2}$ s$^{-1}$) and log$_e$(half-closure time) was tested for all β values. No relationship was found between $g_{max}$ and rate of stomatal closing in the case of β = 0.5 (linear model: DF = 1, 5, F = 0.069, $R^2$ = -0.18, p>0.05).
Correlations between loge(half-closure time) and estimated palaeo-CO₂ concentration (ppm) at the time when taxa originated (millions of years ago (Ma)) for the COPSE model (Bergman et al., 2004) and GEOCARB III model (Berner and Kothavala, 2001) (Table 1) demonstrated no correlations between rate of closing and atmospheric CO₂ concentration at time of taxa origination (COPSE: R² = 0.07, p>0.05; GEOCARB III: R² = 0.08, p>0.05).

Correlations between loge(half-closure time) and estimated palaeo-CO₂ concentration (ppm) at the time when taxa last diversified (Ma) for the COPSE model (Bergman et al., 2004) and GEOCARB III model (Berner and Kothavala, 2001) (Table 1) were tested. The correlations showed evidence for a relationship (COPSE: DF = 6, 18, F = 4.45, R² = 0.52, p<0.05; GEOCARB III: DF= 6, 18, F = 5.71, R² = 0.55, p<0.05). For both models, species that diversified under low or declining [CO₂] (280-805 ppm) were different from species that diversified under high [CO₂] (912-2280 ppm); (overall comparison: F = 14.57, DF = 2, 39, p<0.05) in their loge(half-closure time) (Figure 3). However, no differences were found between species that diversified in low or declining atmospheric [CO₂].

**Discussion**

**Stomatal efficiency in relation to stomatal size and density**

It has been assumed in the past that small stomata respond faster in terms of opening and closing than large stomata. Rate of stomatal opening and closing response to environmental signals is an essential characteristic of stomatal efficiency, required to maintain optimum CO₂ assimilation to transpiration rate (Lawson et al., 2010; Lawson & Blatt, 2014). The evolutionary trend towards high densities of small stomata from few large stomata (Hetherington & Woodward, 2003; Franks & Beerling, 2009) is thought to represent a move towards increased efficiency in stomatal function under low or declining [CO₂] atmospheres over geological time. This is because it is believed that species with high densities of small stomata achieve greater maximum stomatal conductance due to reduced pore depth in small stomata, decreasing the distance for diffusion of gas molecules through the stomatal pore (Franks & Farquhar, 2007; Franks & Beerling, 2009). However, Monda et al. (2016) have shown that Arabidopsis thaliana ecotype Me-0, whose stomata are significantly larger than those of the wild type Columbia (Col), had higher stomatal conductance (gₛ) than Col., confirming that the longer diffusion pathway in the larger stomata did not restrict conductance. Therefore, the commonly accepted assumption that smaller stomata attain higher conductance did not hold in this case (Monda et al., 2016). In this study, we defined stomatal efficiency in
terms of half-closure time in response to darkness. Therefore, if the evolutionary trend in stomatal size and density represents a move towards more efficient stomata, it could be expected that the fastest responders in this study would be those species with the smallest stomata. In a study by Drake et al. (2013), stomatal size was found to be negatively correlated with the maximum rate of stomatal opening in response to light within the genus *Banksia*, indicating that leaves with many, small stomata exhibit faster stomatal conductance to water vapour than leaves with few, large stomata; however, that study measured five species within a single genus. So, while it has been shown that smaller stomata are faster over a range of stomatal sizes within a single genus, this finding cannot be said to apply generally across plant taxa. In contrast to the study by Drake et al. (2013) where stomatal opening in response to light was measured, our study measured stomatal closing in response to darkness. Our results, in comparison, suggest that smaller stomata are not always faster as we show that rate of stomatal closure in response to darkness is not correlated with stomatal size, measured as stomatal pore length (SPL), nor with stomatal geometry, measured as guard cell width, stomatal pore depth, pore length and density for calculation of maximum theoretical conductance in the species studied (Table 1).

Of seven species under study, the two species with largest stomata, *Hordeum vulgare* (barley) and *Lepidozamia peroffskyana* (cycad) (SPL >24 µm), closed their stomata faster in response to darkness than the remaining five species (Figure 2; Table 1). While both have large stomata, their morphology is different; barley stomatal guard cells are modified into the narrow, dumbbell-shape typical of grasses and are situated level with the leaf surface; cycad kidney-shaped guard cells are broad and are sunken below the leaf surface. Dumbbell-shaped stomata have a higher diffusible area of stomatal pore than kidney-shaped stomata because they require a much smaller change in volume to produce a unit change in aperture width (Raschke, 1976) with resultant higher conductance rates (Aasamaa et al., 2001; Hetherington & Woodward, 2003; Franks & Farquhar, 2007; Franks & Beerling, 2009). Indeed, maximum stomatal conductance (g_s) observed under saturating light in *H. vulgare* was 558 mmol m^{-2} s^{-1} compared to *L. peroffskyana*, which was only 61mmol m^{-2} s^{-1} (Table 1), illustrating that maximum operational g_s and rate of closing response are not correlated. In the absence of light, g_s reduced to zero in *L. peroffskyana* indicating that all stomata were tightly closed, in contrast to *H. vulgare* where g_s decreased to a minimum of 53mmol m^{-2} s^{-1} (Table 1), confirming that stomata do not close completely in this grass in the dark, or possibly that cuticular conductance was
greater in this species. In addition, it is known that conducting at night occurs in many species
(Daley & Phillips, 2006; Caird et al., 2007; Dawson et al., 2007).

The next three species in order of decreasing rate of closure were two conifers, Podocarpus
macrophyllus and Agathis australis, followed by the angiosperm Solanum lycopersicon; these
species have the smallest stomata (SPL <19 μm) of the seven species measured (Figure 2;
Table 1). The two slowest species to close in response to darkness have large stomata,
Osmunda regalis and Ginkgo biloba (SPL >24 μm) (Figure 2; Table 1). If rate of stomatal
closure is taken as a proxy for stomatal efficiency, then small stomata are not more efficient
than larger stomata in response to removal of irradiance, at least with respect to the species
examined. Stomata optimise behaviour in order to maximise photosynthetic gain to water loss
and this optimisation can take many forms. In this study, barley is efficient in terms of response
time but may be considered inefficient in terms of water loss during the night, if night-time
conductance is considered a wasteful process, whereas the cycad is efficient in terms of both
rate and effectiveness of stomatal closure by rapidly reducing conductance through the aperture
to zero.

**Other factors that may impact stomatal efficiency**

We confirmed the notion that stomatal size (SS) and stomatal density (SD) are inversely
correlated (Hetherington & Woodward, 2003; Franks & Beerling, 2009; Franks et al., 2009).
In the present study, the two fastest and the two slowest species examined all have large stomata
and low stomatal density compared with the remaining three species, which have smaller
stomata and higher density (Table 1). Thus, half-closure time in response to darkness in these
seven species is neither correlated with stomatal size (r² = 0.01) nor stomatal density (r² = 0.02).
Since our results found that half-closure time in these species is not correlated with size or
density, we attempted to identify other factors correlated with half-closure time. It is not likely
linked to phylogeny because the two fastest stomatal responders are phylogenetically removed
from each other by millions of years. Stem group cycads, the oldest lineage of extant seed
plants, evolved in the Permian (~298 to 252 Ma) during a time of increasing global warmth
and aridity (Eyles, 2008; Tabor & Poulsen, 2008; Montañez & Poulsen, 2013). Extant crown
group cycad species result from a radiation that began approximately 12 million years ago (Ma)
during the Miocene (Nagalingum et al., 2011). Grasses evolved during the late Cretaceous/early Palaeogene (70-60 Ma), when the climate was warm and relatively wet
(Wolfe & Upchurch, 1987; Pearson et al., 2001). They subsequently radiated and diversified
in a climate of decreasing temperatures and increasing seasonally aridity (Ruddiman, 2001), occupying early grassland open habitats in South America by ~40 Ma and grassland habitats globally during the early to middle Miocene (~20-10 Ma) (Jacobs et al., 1999; Kellogg, 2001; Strömberg, 2011). The two species with the largest stomata also represent two separate plant divisions, that is, gymnosperms and angiosperms. Additionally, rate of closure is not likely linked with life strategy; L. peroffskyana is a woody, evergreen cycad, endemic to coastal and near-coastal regions of New South Wales and Queensland in Australia, where it grows in wet sclerophyll forest, littoral rainforest or open scrubby forest (Jones, 2002; Whitelock, 2002), whereas H. vulgare is an herbaceous, annual grass descended from wild barley, Hordeum vulgare subsp. spontaneum from Western Asia (Badr et al., 2000). It must also be noted that neither species is under strong selection pressure to have fast-closing stomata in response to drought as neither usually grows in water-limited environments.

Effect of atmospheric CO$_2$ concentration on stomatal closure rate

We explored the possibility that the concentration of atmospheric CO$_2$ ([CO$_2$]$_{atm}$) at the time of taxa origination and/or latest diversification event may have impacted stomatal function, bearing in mind that Robinson (1994) suggested that “plants evolving under declining CO$_2$ tended to develop increased stomatal efficiency”. The difficulty in ascertaining exactly when taxa originated and last diversified, along with accurate determination of atmospheric [CO$_2$] during those times, limits the accuracy with which the impact of past [CO$_2$] on stomatal function can be studied. Nonetheless, using current information available for origination and diversification dates for the seven taxa, along with modelled atmospheric carbon dioxide concentration at the time (Berner and Kothavala, 2001; Bergman et al., 2004), we tested for a relationship between half-closure time and [CO$_2$]. Half-closure time was not found to be correlated with estimated concentration of CO$_2$ in the atmosphere when the taxa originated but correlation between half-closure time and estimated [CO$_2$]$_{atm}$ during the time of taxa diversification was observed (Figure 3); species whose ancestors underwent their last major diversification event in low or declining [CO$_2$]$_{atm}$ closed their stomata faster in response to darkness than species whose ancestors last diversified under high [CO$_2$]$_{atm}$. Therefore, we suggest that the concentration of CO$_2$ in the atmosphere during diversification events may impact stomatal function, specifically, rate of stomatal closure.

The rapid half-closure time exhibited by the cycad, a member of an ancient plant order that has persisted over millions of years with little morphological change, was unexpected. With the
aid of DNA sequence data and fossil-calibrated phylogenies it is now known, however, that living cycad species are not relictual taxa (Treutlein & Wink, 2002; Crisp & Cook, 2011; Nagalingum et al., 2011). All extant cycad genera diversified in the last 12-6 million years (Nagalingum et al., 2011); therefore, despite their ancient origins, extant cycads last diversified with the grasses in a low CO$_2$ world. Using the same techniques, Biffin et al. (2011) have shown that despite the ancient origins of Podocarpaceae in the Triassic-Jurassic, extant species within the family are likely to be of more recent evolutionary origin (mid-to late Cenozoic). While extant Podocarp leaves can be scale-like, needle-like or broad, reconstructions of leaf morphology indicate that the ancestral state was scale-like, suggesting that modern broad leaves in Podocarps are an adaptation to compete with angiosperm radiation in shady canopies of newly-developing rainforests (Ed Biffin, Brodribb, Hill, Thomas, & Lowe, 2012). The Podocarp species included in this study, P. macrophyllus, has broad leaves analogous to angiosperms. Similarly, Crisp & Cook (2011) have concluded that conifers in the Araucariaceae family, despite their ancient origins, have a crown age estimated at only 36 Ma, while Biffin et al. (2010) have suggested the estimated age of the Agathis australis lineage to be 39–11 Ma. Thus, it appears that the cycad and conifer species in this study diversified at a similar time to angiosperms under a relatively low or declining atmospheric CO$_2$ composition (Table 1). In contrast, the two slowest stomatal responders, Osmunda regalis and Ginkgo biloba, diversified much earlier in a high CO$_2$ world (Table 1). The fern family, Osmundaceae, originated in the Permian and radiated in the Triassic (Jud, Rothwell, & Stockey, 2008). Phipps et al. (1998) established that crown group Osmundaceae has a minimum age of 220 million years, with fossil evidence of the genus Osmunda from the Late Triassic. Osmundaceous ferns diverged as early as the Carboniferous (Schneider et al., 2004) and living species began to appear no later than the Late Cretaceous (Jud et al., 2008), suggesting that some extant genera and species could be remarkably ancient. The order Ginkgoales also originated in the Permian (Royer, Hickey, & Wing, 2003) and diversified during the Jurassic and Early Cretaceous (Royer et al., 2003; Crane, 2013). The sole survivor of this order, Ginkgo biloba, has persisted through millions of years of environmental and atmospheric change but last diversified in a high CO$_2$ world. In contrast, the two angiosperm species in this study Solanum lycopersicon and Hordeum vulgare originated much later in time. Solanales originated in the mid-Cretaceous (Bremer, Friis, & Bremer, 2004). Solanales crown group divergence times vary from c.51 Ma (Paape et al., 2008) to c.40 Ma (Wikström, Savolainen, & Chase, 2001), while crown age of the genus Solanum is estimated at c.16 Ma (Paape et al., 2008). Grasses (Poaceae) originated in the latest Cretaceous to early Tertiary (Kellogg, 2001; Piperno & Sues, 2005;
and increased in abundance during the middle Tertiary (Jacobs et al., 1999). Using current knowledge on the date of diversification of the seven species studied, and estimated atmospheric composition at that time, we showed that the five species that diversified under low or declining atmospheric CO$_2$ concentration (280-805 ppm) had faster stomatal closing response times (median half-closure time 4.83-16.86 min; mean half-closure time 7.16-24.47 min) than the two species that diversified under high atmospheric CO$_2$ concentration (912-2280 ppm) (median half-closure time 25.27-78.69 min; mean half-closure time 30.13-105.49 min) (Figure 2; Figure 3; Table 1). This trend may suggest that, in these seven species at least, atmospheric [CO$_2$] during taxa diversification is a more important driver of stomatal closing rate than stomatal size, density, phylogeny or life strategy. However intriguing this idea, it must be viewed with caution as the number of species used was moderate and the sample size small for each species so overall trend in all land plants cannot be assumed from such a preliminary study. Additionally, only one cycad species was included, thus the possibility exists that fast and tight stomatal closure in Lepidozamia peroffskyana represents a species-specific response that is not typical of all cycads. It is possible that cycad species that diversified in a low CO$_2$ world were placed under selection pressure to optimise stomatal efficiency; perhaps species that could not adapt became extinct, whilst those that could adapt, survived. Nagalingum et al. (2011) have suggested that a shift from a globally warm, equatorial climate to cooler temperatures with increasing aridity and seasonality during the Late Miocene may explain the dramatic extinction of many cycad species; the reduction in atmospheric [CO$_2$] during the Miocene may have selected for cycad species with fast responding stomata while cycad species with slow stomata became extinct. Therefore, perhaps other extant cycad species also close their stomata quickly when irradiance is removed and this remains to be tested.

To our knowledge, no previous study has compared measured stomatal response rate and measured stomatal size in species with ancient stem lineages from a high CO$_2$ world to species with more recent stem lineages from a low CO$_2$ world. It is likely that several factors combine to drive optimal stomatal function and, under stressful circumstances, some factors may become more dominant in terms of driving optimality than others. We recommend further detailed studies on stomatal closing rates in a much wider phylogenetic range of species, especially those where time of diversification has been established with reasonable certainty, in order to provide more insight into this interesting topic. Vico et al. (2011) have shown that
stomatal opening and closing times are strongly correlated, with opening faster than closing. Therefore, in our future studies, we will test whether stomatal opening rate in response to light, and in particular to sun flecks, is correlated with rate of closing and with atmospheric CO$_2$ concentration at time of diversification in these same species, and will also broaden the number of species and increase replication.

**Conclusion**

Small stomata do not always close faster than large stomata when compared across a phylogenetic range of genera and plant functional groups and thus are not more efficient than large stomata if stomatal closing time is taken as a proxy for stomatal efficiency. We suggest that atmospheric concentration of CO$_2$ at the time of taxa diversification, and not stomatal size, may be a stronger driver of stomatal closing time in response to darkness in the seven species studied. We recommend that future studies testing whether small stomata are faster than large stomata should consider other adverse factors that may place a strong selection pressure on plants to optimise stomatal function. In such adverse circumstances, guard cell size may not be the most dominant driver of stomatal function.

**Acknowledgements**

We thank the following for technical assistance: Ms. Bredagh Moran, Mr. Ray O’Haire, Mr. Liam Kavanagh (UCD, Ireland); Mr. Matthew Gilroy (Conviron, UK); Mr. Aidan Blake (Aaron Refrigeration, Ireland). We gratefully acknowledge funding from: IRCSET Embark scholarship (R10679); EU Marie Curie Excellence Grant (MEXT-CT-2006-042531); EU Marie Curie Intra-European Fellowship (PEA-IEF-2010-275626); ERC grant (ERC-279962-OXYEVOL).

**References**

Aasamaa, K., Sober, A., & Rahi, M. (2001). Leaf anatomical characteristics associated with shoot hydraulic conductance, stomatal conductance and stomatal sensitivity to changes of leaf water status in temperate deciduous trees. *Australian Journal of Plant Physiology*, 28, 765–774.

Badr, A., Müller, K., Schäfer-Pregl, R., El Rabey, H., Effgen, S., Ibrahim, H. H., … Salamini, F. (2000). On the origin and domestication history of Barley (Hordeum vulgare). *Molecular Biology and Evolution*, 17(4), 499–510. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10742042

Biffin, E., Brodribb, T. J., Hill, R. S., Thomas, P., & Lowe, A. J. (2012). Leaf evolution in Southern Hemisphere conifers tracks the angiosperm ecological radiation. *Proceedings in review*
Biffin, E., Conran, J. G., & Lowe, A. J. (2011). Podocarp evolution: A molecular phylogenetic perspective. *Smithsonian Contributions to Botany*, (95), 1–20. http://doi.org/10.5479/si.0081024X.95.1

Biffin, E., Hill, R. S., & Lowe, A. J. (2010). Did Kauri (Agathis: Araucariaceae) really survive the Oligocene drowning of New Zealand? *Systematic Biology*, 59(5), 594–602. http://doi.org/10.1093/sysbio/syq030

Bradbeer, J. W. (1988). *Seed dormancy and germination*. Glasgow: Blackie and Son Ltd.

BROWN, H. T., & ESCOMBE, F. (1900). Static diffusion of gases and liquids in relation to the assimilation of carbon and translocation in plants. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 193(January), 223–291. http://doi.org/10.1098/rstb.1900.0014

Caird, M. A., Richards, J. H., & Donovan, L. A. (2007). Nighttime stomatal conductance and transpiration in C3 and C4 plants. *Plant Physiology*, 143(January), 4–10. http://doi.org/10.1104/pp.106.092940

Drake, P. L., Froend, R. H., & Franks, P. J. (2013). Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *Journal of Experimental Botany*, 64(2), 495–505. http://doi.org/10.1093/jxb/ers347

Edwards, D., Kerp, H., & Hass, H. (1998). Stomata in early land plants: an anatomical and ecophysiological approach. *Journal of Experimental Botany*, 49(March), 255–278. http://doi.org/10.1093/jxb/49.Special_Issue.255

Eyles, N. (2008). Glacio-epochs and the supercontinent cycle after ~3.0 Ga: Tectonic boundary conditions for glaciation. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 258, 89–129. http://doi.org/10.1016/j.palaeo.2007.09.021

Franks, P. J., & Beerling, D. J. (2009). Maximum leaf conductance driven by CO2 effects on
stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences of the United States of America*, 106(25), 10343–7.

http://doi.org/10.1073/pnas.0904209106

Franks, P. J., Drake, P. L., & Beerling, D. J. (2009). Plasticity in maximum stomatal conductance constrained by negative correlation between stomatal size and density: An analysis using Eucalyptus globulus. *Plant, Cell and Environment*, 32, 1737–1748.

http://doi.org/10.1111/j.1365-3040.2009.002031.x

Franks, P. J., & Farquhar, G. D. (2007). The mechanical diversity of stomata and its significance in gas-exchange control. *Plant Physiology*, 143(January), 78–87.

http://doi.org/10.1104/pp.106.089367

Hammer, P. A., & Hopper, D. A. (1997). Experimental design. In R. W. Langhans & T. W. Tibbits (Eds.), *Plant growth chamber handbook* (pp. 177–187). Ames, Iowa: Iowa State University.

Haworth, M., Fitzgerald, A., & McElwain, J. C. (2011). Cycads show no stomatal-density and index response to elevated carbon dioxide and subambient oxygen. *Australian Journal of Botany*, 59, 629–638. http://doi.org/10.1071/BT11009

Hetherington, A. M., & Woodward, F. I. (2003). The role of stomata in sensing and driving environmental change. *Nature*, 424(August), 901–908.

Hirano, A., Hongo, I., & Koike, T. (2012). Morphological and physiological responses of Siebold’s beech (Fagus crenata) seedlings grown under CO2 concentrations ranging from pre-industrial to expected future levels. *Landscape and Ecological Engineering*, 8, 59–67. http://doi.org/10.1007/s11355-011-0149-0

Jacobs, B. F., Kingston, J. D., & Jacobs, L. L. (1999). The origin of grass-dominated ecosystems. *Annals of the Missouri Botanical Garden*, 86(2), 590–643.

Jones, D. L. (2002). *Cycads of the world, ancient plants in today’s landscape*. Washington DC, USA: Smithsonian Institution Press.

Jones, H. G. (1992). *Plants and microclimate: a quantitative approach to environmental plant physiology*. Cambridge, UK: Cambridge University Press.

Jud, N. A., Rothwell, G. W., & Stockey, R. A. (2008). Todea from the Lower Cretaceous of western North America: implications for the phylogeny, systematics, and evolution of modern Osmundaceae. *American Journal of Botany*, 95(3), 330–339.

Katul, G., Manzoni, S., Palmroth, S., & Oren, R. (2010). A stomatal optimization theory to describe the effects of atmospheric CO2 on leaf photosynthesis and transpiration. *Annals of Botany*, 105, 431–442. http://doi.org/10.1093/aob/mcp292

Kellogg, E. A. (2001). Evolutionary history of the grasses. *Plant Physiology*, 125(March), 1198–1205.

Lawson, T., & Blatt, M. R. (2014). Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiology*, 164(April), 1556–1570.

http://doi.org/10.1104/pp.114.237107

Lawson, T., VonCaemmerer, S., & Baroli, I. (2010). Photosynthesis and stomatal behaviour. *Progress in Botany*, 72, 265–304.

McElwain, J. C., & Chaloner, W. G. (1995). Stomatal density and index of fossil plants track atmospheric carbon dioxide in the Palaeozoic. *Annals of Botany*.

McElwain, J. C., Wade-Murphy, J., & Hesselbo, S. P. (2005). Changes in carbon dioxide
during an oceanic anoxic event linked to intrusion into Gondwana coals. *Nature*, 435(7041), 479–82. http://doi.org/10.1038/nature03618

Monda, K., Araki, H., Kuhara, S., Ishigaki, G., Akashi, R., Negi, J., … Iba, K. (2016). Enhanced stomatal conductance by a spontaneous Arabidopsis tetraploid, Me-0, results from increased stomatal size and greater stomatal aperture. *Plant Physiology*, 170(March), 1435–1444. http://doi.org/10.1104/pp.15.01450

Montañez, I. P., & Poulsen, C. J. (2013). The Late Paleozoic ice age: an evolving paradigm. *Annual Review of Earth and Planetary Sciences*, 41, 629–656. http://doi.org/doi:10.1146/annurev.earth.031208.100118

Nagalingum, N. S., Marshall, C. R., Quental, T. B., Rai, H. S., Little, D. P., & Mathews, S. (2011). Recent synchronous radiation of a living fossil. *Science*, 334(6057), 796–9. http://doi.org/10.1126/science.1209926

Paape, T., Igic, B., Smith, S. D., Olmstead, R., Bohs, L., & Kohn, J. R. (2008). A 15-Myr-old genetic bottleneck. *Molecular Biology and Evolution*, 25(4), 655–663. http://doi.org/10.1093/molbev/msn016

R Core Team. (2014). R: A language and environment for statistical computing. Retrieved from http://www.r-project.org/

Raschke, K. (1976). How stomata resolve the dilemma of opposing priorities. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 273(927), 551–560. http://doi.org/10.1098/rstb.1976.0031

Robinson, J. M. (1994). Speculations on carbon dioxide starvation, Late Tertiary evolution of stomatal regulation and floristic modernization. *Plant, Cell and Environment*, 17, 345–354. http://doi.org/10.1111/j.1365-3040.1994.tb00303.x

Royer, D. L., Hickey, L. J., & Wing, S. L. (2003). Ecological conservatism in the “living fossil” Ginkgo. *Paleobiology*, 29(1), 84–104. http://doi.org/10.1666/0094-8373(2003)029<0084:ECITLF>2.0.CO;2

Ruddiman, W. F. (2001). *Earth’s Climate*. New York: W H Freeman.

Sager, J. C., & McFarlane, J. C. (1997). Radiation. In R. W. Langhans & T. W. Tibbits
Schneider, H., Schuettpelz, E., Pryer, K. M., Cranfill, R., Magallon, S., & Lupia, R. (2004). Ferns diversified in the shadow of angiosperms. *Nature*, 428, 553–557.

Strömberg, C. A. E. (2011). Evolution of grasses and grassland ecosystems. *Annual Review of Earth and Planetary Sciences*, 39, 517–544. http://doi.org/10.1146/annurev-earth-040809-152402

Tabor, N. J., & Poulsen, C. J. (2008). Palaeoclimate across the Late Pennsylvanian-Early Permian tropical palaeolatitudes: A review of climate indicators, their distribution, and relation to palaeophysiographic climate factors. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 268, 293–310. http://doi.org/10.1016/j.palaeo.2008.03.052

Treutlein, J., & Wink, M. (2002). Molecular phylogeny of cycads inferred from rbcL sequences. *Naturwissenschaften*, 89, 221–225. http://doi.org/10.1007/s00114-002-0308-0

Vico, G., Manzoni, S., Palmroth, S., & Katul, G. (2011). Effects of stomatal delays on the economics of leaf gas exchange under intermittent light regimes. *New Phytologist*, 192, 640–652. http://doi.org/10.1111/j.1469-8137.2011.03847.x

Weyers, J. D. B., & Johansen, L. G. (1985). Accurate estimation of stomatal aperature from silicone rubber impressions. *New Phytologist*, 101, 109–115.

Whitelock, L. M. (2002). *The cycads*. Oregon, USA: Timber Press Inc.

Wikström, N., Savolainen, V., & Chase, M. W. (2001). Evolution of the angiosperms: calibrating the family tree. *Proceedings. Biological Sciences / The Royal Society*, 268, 2211–2220. http://doi.org/10.1098/rspb.2001.1782

Wolfe, J. A., & Upchurch, G. R. J. (1987). North American nonmarine climates and vegetation during the Late Cretaceous. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 61, 33–77. http://doi.org/10.1016/0031-0182(87)90040-X

Wong, S. C., Cowan, I. R., & Farquhar, G. D. (1979). Stomatal conductance correlates with photosynthetic capacity. *Nature*. http://doi.org/10.1038/282424a0

**Figure 1.** Change in stomatal conductance ($g_s$) (mmol m$^{-2}$ s$^{-1}$) over time (minutes) in response to darkness in an evolutionary range of species grown at 380 ppm CO$_2$ and 20.9% O$_2$ fitted to an exponential decay curve. The fit was performed for each replicate of seven species. Species listed from fastest to slowest median half-closure time. Light microscope images of stomata x630.

**Figure 2.** $\log_e$(median stomatal half-closure time) of seven species. Hv=*Hordeum vulgare* (graminaceous angiosperm); Lp=*Lepidozamia peroffskyana* (cycad); Pm=*Podocarpus macrophyllus* (conifer); Aa=*Agathis australis* (conifer); Sl=*Solanum lycopersicon* (angiosperm); Or=*Osmunda regalis* (fern); Gb=*Ginkgo biloba* (ginkgophyte). The fastest species to close stomata in response to darkness was *Hordeum vulgare*; the slowest was *Ginkgo biloba*. 
**Figure 3.** $\log_e$(median stomatal half-closure time) of seven species, grouped by estimated atmospheric CO$_2$ concentration at time of taxa diversification into low, declining or high CO$_2$ groups. For COPSE model (Bergman et al. (2004) *Am. J. Sci.*), low CO$_2$ (280-439 ppm) includes *Hordeum vulgare*, *Lepidozamia peroffskyana* and *Solanum lycopersicon*; declining CO$_2$ (346-825 ppm) includes *Podocarpus macrophyllus* and *Agathis australis*; high CO$_2$ (876-1443 ppm) includes *Osmunda regalis* and *Ginkgo biloba* (see Table 1). For GEOCARB III model (Berner and Kothavala (2001) *Am. J. Sci.*), low CO$_2$ (300-420 ppm) includes *Hordeum vulgare*, *Lepidozamia peroffskyana*, *Podocarpus macrophyllus* and *Solanum lycopersicon*; declining CO$_2$ (300-630 ppm) includes *Agathis australis*; high CO$_2$ (960-2280 ppm) includes *Osmunda regalis* and *Ginkgo biloba* (see Table 1).

**Table 1.** Median and mean stomatal half-closure time (min) from maximum stomatal conductance ($g_s$) (mmol m$^{-2}$ s$^{-1}$) under illumination to minimum $g_s$ in the dark; estimated time of taxa diversification (millions of years ago); [CO$_2$] (ppm) at time of taxa diversification; mean maximum $g_s$ under illumination to mean minimum $g_s$ in the dark (mmol m$^{-2}$ s$^{-1}$); mean reduction in $g_s$ (mmol m$^{-2}$ s$^{-1}$) (%) from maximum to minimum; mean stomatal pore length (µm); mean stomatal density (mm$^2$); and mean theoretical maximum conductance ($g_{s, max}$) (mmol m$^{-2}$ s$^{-1}$) for seven species grown under controlled ambient atmosphere (380 ppm CO$_2$; 20.9% O$_2$). Species listed from fastest to slowest median stomatal half-closure time (min).
Table 1. Median and mean stomatal half-closure time (min) from maximum stomatal conductance (gₛ) (mmol m⁻² s⁻¹) under illumination to minimum gₛ in the dark; estimated time of taxa diversification (millions of years ago); [CO₂] (ppm) at time of taxa diversification; mean maximum gₛ under illumination to mean minimum gₛ in the dark (mmol m⁻² s⁻¹); mean reduction in gₛ (mmol m⁻² s⁻¹) (%) from maximum to minimum; mean stomatal pore length (µm); mean stomatal density (mm²); and theoretical maximum conductance (gₛmax) (mmol m⁻² s⁻¹) for seven species grown under controlled ambient atmosphere (380 ppm CO₂; 20.9% O₂). Species listed from fastest to slowest median stomatal half-closure time (min).

| Species           | Median estimated half-closure time (min) & max. in brackets | Mean estimated half-closure time (minutes) ± SEM | Estimated time of taxa diversification (Millions years ago) | [CO₂] (ppm) at time of taxa diversification COPSE | [CO₂] (ppm) at time of taxa diversification GEOCARB III | Mean maximum to mean minimum gₛ (mmol m⁻² s⁻¹) | Mean change in gₛ (mmol m⁻² s⁻¹) from max. to min. (% change in brackets) | Mean Stomatal Pore Length (µm) ± SD | Mean Stomatal Density (mm²) ± SD | Mean theoretical maximum conductance (gₛmax) (mmol m⁻² s⁻¹) |
|-------------------|-------------------------------------------------------------|--------------------------------------------------|----------------------------------------------------------|-----------------------------------------------|--------------------------------------------------|-----------------------------------------------|---------------------------------------------------------------------------------|---------------------------------|---------------------------------|---------------------------------------------------------------|
| *Hordeum vulgare* | 4.83 (4.25,12.41)                                           | 7.16 ± 2.63                                      | 10,000 yrs¹                                               | 333-280 ppm (low)                             | 300 ppm (low)                                   | 558 - 53                                       | 505 (90.5)                                                                      | 28.1 ± 6.2                      | 79.8 ± 30.7                     | 1347.33                                                                        |
| *Lepidozamia peroffskyana* | 6.53 (4.30,19.96)                                          | 10.26 ± 4.89                                    | 12 – 6 Ma²                                               | 401-363 ppm (low)                             | 300 ppm (low)                                   | 61 - 0                                         | 61 (100.0)                                                                     | 35.6 ± 5.5                      | 33.3 ± 7.9                      | 519.16                                                                        |
| *Podocarpus macrophyllus* | 12.74 (11.7,29.41)                                         | 17.96 ± 5.74                                    | 33 – 2.6 Ma³                                              | 718-346 ppm (declining)                       | 420-300 ppm (declining)                         | 97 - 26                                        | 71 (73.2)                                                                      | 14.7 ± 2.3                      | 145.4 ± 24.9                    | 476.62                                                                        |
| *Agathis australis* | 15.02 (7.35,18.05)                                          | 13.47 ± 3.18                                    | 39 –11 Ma⁴                                              | 805-394 ppm (declining)                       | 630-300 ppm (declining)                         | 85 - 41                                        | 44 (51.8)                                                                      | 18.8 ± 4.2                      | 119.4 ± 43.3                    | 669.58                                                                        |
| *Solonum lycopersicon* | 16.86 (14.6,41.94)                                         | 24.47 ± 8.76                                    | 16 Ma⁵                                                   | 439 ppm (low)                                 | 360-300 ppm (low)                               | 377 - 103                                      | 274 (72.7)                                                                     | 15.4 ± 3.5                      | 316.8 ± 92.4                    | 1793.94                                                                        |
| *Osmunda regalis* | 25.27 (19.5,45.55)                                          | 30.13 ± 7.88                                    | 100 – 66 Ma⁶                                             | 1283-912 ppm (high)                           | 1590-960 ppm (high)                             | 386 - 210                                      | 176 (45.6)                                                                     | 29.8 ± 6.5                      | 56.3 ± 16.5                     | 621.57                                                                        |
| *Ginkgo biloba*    | 78.69 (25.7,212.07)                                         | 105.49 ± 55.45                                  | 146 – 100 Ma⁷                                            | 1443-876 ppm (high)                           | 2280-1590 ppm (high)                            | 42 - 6                                         | 36 (85.7)                                                                      | 24.3 ± 5.0                      | 76.8 ± 20.6                     | 689.19                                                                        |

¹Badr et al. 2000 Mol. Biol. Evol. 17(4): 499-510. ²Nagalingum et al. 2011 Science 334:796-799. ³Biffin et al. 2011 Smithsonian Contributions to Botany 95. ⁴Biffin et al. 2010 Syst. Biol. 59(5):594-602. ⁵Bremer et al. 2004 Syst. Biol. 53(3):496-505. ⁶Jud et al. 2008 Am. J. Bot. 95:330-339. ⁷Peter Crane 2013 Ginkgo: The Tree That Time Forgot. Yale University Press. ⁸Bergman, N.M., Lenton, T.M., Watson, A.J. (2004) COPSE: A new model of biogeochemical cycling over Phanerozoic time. Am. J. Sci. 304:397-437. ⁹Berner, R.A. and Kothavala, Z. (2001) GEOCARB III: A revised model of atmospheric CO₂ over Phanerozoic time. Am. J. Sci. 301:182-204.
Figure 1. Change in stomatal conductance ($g_s$) (mmol m$^{-2}$ s$^{-1}$) over time (minutes) in response to darkness in an evolutionary range of species grown at 380 ppm CO$_2$ and 20% O$_2$ fitted to an exponential decay curve. The fit was performed for each replicate of seven species. Species listed from fastest to slowest median half-closure time. Light microscope images of stomata at 10X.
Figure 2. The graph shows the stomatal half-closure time (min) for different species. The species are labeled as Hv, Lp, Pm, At, Si, Cr, and Gb. Each species is represented by a different color and data points with error bars indicating variability.
