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Title	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
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Publication date	2016
Publication information	Elliott-Kingston, Caroline, Matthew Haworth, Jonathan M. Yearsley, Jennifer C. McElwain, and et al. "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 " 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

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

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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

(Authors 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 CO₂ may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO₂.

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Abstract

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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 ([CO₂]_{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% P₂O₅, 11% K₂O, 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 CO₂; 20.9% O₂) 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% P₂O₅, 12% K₂O 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 3.7m²) with atmospheric control of [CO₂] at ambient (380ppm) and [O₂] 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 CO₂ (BOC UK, Surrey, England) and was continuously monitored with a PP-systems WMA-4 IRGA (Amesbury, Massachusetts, USA); injection of CO₂ 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$; 0600–0900 hours, light intensity increased from 300 to 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$; 0900–1700 hours, PPFD maintained at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$; 1700–2000 hours, light intensity decreased from 600 to 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$; 2000–2100 hours, light intensity decreased from 300 to 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$), 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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

g_s 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 0.09mm^2 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 mm^2) 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 β 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 β values of 0.2, 0.4, 0.5, 0.6, 0.8 and 1.0. Theoretical maximum stomatal conductance ($g_{s\max}$) was then calculated using the morphological measurements of fully open stomata and the following diffusion equation (Parlange & Waggoner, 1970; Franks & Beerling, 2009):

$$g_{s\max} = \frac{\frac{dw}{v} \cdot SD \cdot pa_{\max}}{pd + \frac{\pi}{2} \sqrt{\frac{pa_{\max}}{\pi}}} \quad \text{Eq. 1}$$

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 is stomatal density (m⁻²); pa_{\max} is maximum stomatal pore area (m²) 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-[CO₂])

Best estimates of origination date and last diversification date for each of the seven taxa were gathered from the literature. Atmospheric CO₂ concentration ([CO₂]_{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 [CO₂]_{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 [CO₂]_{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:

$$g_s(t) = g_s(\infty) + (g_s(0) - g_s(\infty)) \cdot \exp(-\exp(A) \cdot t) \quad \text{Eq. 2}$$

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 CO₂ 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) ($\text{mmol m}^{-2} \text{s}^{-1}$) 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

min; R^2 fit = 0.99); *Agathis australis* (median half-closure time: 15.02 min; mean 13.47 ± 3.18 min; R^2 fit = 0.91); 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 μ m; 18.8 ± 4.2 μ m; and 15.4 ± 3.5 μ m 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 μ m) 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 μ m) (Figure 2; Table 1).

Mean differences in stomatal density (mm^2) and stomatal pore length (μ m) 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 (μ m) 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 $\text{mmol m}^{-2} \text{s}^{-1}$) 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 $\beta = 0.5$ (linear model: DF = 1, 5, F = 0.069, $R^2 = -0.18$, $p > 0.05$).

Correlations between \log_e (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: $R^2 = 0.07$, $p > 0.05$; GEOCARB III: $R^2 = 0.08$, $p > 0.05$).

Correlations between \log_e (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) (Figure 3; Table 1) were tested. The correlations showed evidence for a relationship (COPSE: $DF = 6, 18$, $F = 4.45$, $R^2 = 0.52$, $p < 0.05$; GEOCARB III: $DF = 6, 18$, $F = 5.71$, $R^2 = 0.55$, $p < 0.05$). 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 \log_e (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 (g_s) 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 $\text{mmol m}^{-2} \text{s}^{-1}$ compared to *L. peroffskyana*, which was only 61 $\text{mmol m}^{-2} \text{s}^{-1}$ (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 53 $\text{mmol m}^{-2} \text{s}^{-1}$ (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 ($r^2 = 0.01$) nor stomatal density ($r^2 = 0.02$). 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 CO₂ concentration on stomatal closure rate

We explored the possibility that the concentration of atmospheric CO₂ ([CO₂]_{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 CO₂ tended to develop increased stomatal efficiency”. The difficulty in ascertaining exactly when taxa originated and last diversified, along with accurate determination of atmospheric [CO₂] during those times, limits the accuracy with which the impact of past [CO₂] 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 [CO₂]. Half-closure time was not found to be correlated with estimated concentration of CO₂ in the atmosphere when the taxa originated but correlation between half-closure time and estimated [CO₂]_{atm} during the time of taxa diversification was observed (Figure 3); species whose ancestors underwent their last major diversification event in low or declining [CO₂]_{atm} closed their stomata faster in response to darkness than species whose ancestors last diversified under high [CO₂]_{atm}. Therefore, we suggest that the concentration of CO₂ 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 CO₂ 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 CO₂ composition (Table 1). In contrast, the two slowest stomatal responders, *Osmunda regalis* and *Ginkgo biloba*, diversified much earlier in a high CO₂ 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 CO₂ 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). Solanaceae 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;

Prasad *et al.*, 2005; Jacobs *et al.*, 1999) 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 CO₂ 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 CO₂ 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 [CO₂] 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 CO₂ 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 [CO₂] 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 CO₂ world to species with more recent stem lineages from a low CO₂ 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 CO₂ 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 CO₂ 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 (Convion, 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).

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Figure 1. Change in stomatal conductance (g_s) ($\text{mmol m}^{-2} \text{s}^{-1}$) 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 CO₂ concentration at time of taxa diversification into low, declining or high CO₂ groups. For COPSE model (Bergman *et al.* (2004) *Am. J. Sci.*), low CO₂ (280-439 ppm) includes *Hordeum vulgare*, *Lepidozamia peroffskyana* and *Solanum lycopersicon*; declining CO₂ (346-825 ppm) includes *Podocarpus macrophyllus* and *Agathis australis*; high CO₂ (876-1443 ppm) includes *Osmunda regalis* and *Ginkgo biloba* (see Table 1). For GEOCARB III model (Berner and Kothavala (2001) *Am. J. Sci.*), low CO₂ (300-420 ppm) includes *Hordeum vulgare*, *Lepidozamia peroffskyana*, *Podocarpus macrophyllus* and *Solanum lycopersicon*; declining CO₂ (300-630 ppm) includes *Agathis australis*; high CO₂ (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) ($\text{mmol m}^{-2} \text{s}^{-1}$) under illumination to minimum g_s in the dark; estimated time of taxa diversification (millions of years ago); [CO₂] (ppm) at time of taxa diversification; mean maximum g_s under illumination to mean minimum g_s in the dark ($\text{mmol m}^{-2} \text{s}^{-1}$); mean reduction in g_s ($\text{mmol m}^{-2} \text{s}^{-1}$) (%) from maximum to minimum; mean stomatal pore length (μm); mean stomatal density (mm^2); and mean theoretical maximum conductance ($g_{s\text{max}}$) ($\text{mmol m}^{-2} \text{s}^{-1}$) 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).

Table 1. Median and mean stomatal half-closure time (min) from maximum stomatal conductance (g_s) ($\text{mmol m}^{-2} \text{s}^{-1}$) under illumination to minimum g_s in the dark; estimated time of taxa diversification (millions of years ago); $[\text{CO}_2]$ (ppm) at time of taxa diversification; mean maximum g_s under illumination to mean minimum g_s in the dark ($\text{mmol m}^{-2} \text{s}^{-1}$); mean reduction in g_s ($\text{mmol m}^{-2} \text{s}^{-1}$) (%) from maximum to minimum; mean stomatal pore length (μm); mean stomatal density (mm^2); and mean theoretical maximum conductance ($g_{s\text{max}}$) ($\text{mmol m}^{-2} \text{s}^{-1}$) 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).

Species	Median estimated half-closure time (minutes) (min. & max. in brackets)	Mean estimated half-closure time (minutes) \pm SEM	Estimated time of taxa diversification (Millions years ago)	$[\text{CO}_2]$ (ppm) at time of taxa diversification COPSE ⁸	$[\text{CO}_2]$ (ppm) at time of taxa diversification GEOCARB III ⁹	Mean maximum to mean minimum g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)	Mean change in g_s ($\text{mmol m}^{-2} \text{s}^{-1}$) from max. to min. (% change in brackets)	Mean Stomatal Pore Length (μm) \pm SD	Mean Stomatal Density (mm^2) \pm SD	Mean theoretical maximum conductance ($g_{s\text{max}}$) ($\text{mmol m}^{-2} \text{s}^{-1}$)
<i>Hordeum vulgare</i>	4.83 (4.25,12.41)	7.16 \pm 2.63	10,000 yrs ¹	333-280 ppm (low)	300 ppm (low)	558 - 53	505 (90.5)	28.1 \pm 6.2	79.8 \pm 30.7	1347.33
<i>Lepidozamia peroffskyana</i>	6.53 (4.30,19.96)	10.26 \pm 4.89	12 – 6 Ma ²	401-363 ppm (low)	300 ppm (low)	61 - 0	61 (100.0)	35.6 \pm 5.5	33.3 \pm 7.9	519.16
<i>Podocarpus macrophyllus</i>	12.74 (11.71,29.41)	17.96 \pm 5.74	33 – 2.6 Ma ³	718-346 ppm (declining)	420-300 ppm (low)	97 - 26	71 (73.2)	14.7 \pm 2.3	145.4 \pm 24.9	476.62
<i>Agathis australis</i>	15.02 (7.35,18.05)	13.47 \pm 3.18	39 – 11 Ma ⁴	805-394 ppm (declining)	630-300 ppm (declining)	85 - 41	44 (51.8)	18.8 \pm 4.2	119.4 \pm 43.3	669.58
<i>Solanum lycopersicon</i>	16.86 (14.60,41.94)	24.47 \pm 8.76	16 Ma ⁵	439 ppm (low)	360-300 ppm (low)	377 - 103	274 (72.7)	15.4 \pm 3.5	316.8 \pm 92.4	1793.94
<i>Osmunda regalis</i>	25.27 (19.57,45.55)	30.13 \pm 7.88	100 – 66 Ma ⁶	1283-912 ppm (high)	1590-960 ppm (high)	386 - 210	176 (45.6)	29.8 \pm 6.5	56.3 \pm 16.5	621.57
<i>Ginkgo biloba</i>	78.69 (25.70,212.07)	105.49 \pm 55.45	146 – 100 Ma ⁷	1443-876 ppm (high)	2280-1590 (high)	42 - 6	36 (85.7)	24.3 \pm 5.0	76.8 \pm 20.6	689.19

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Figure 1.TIFF

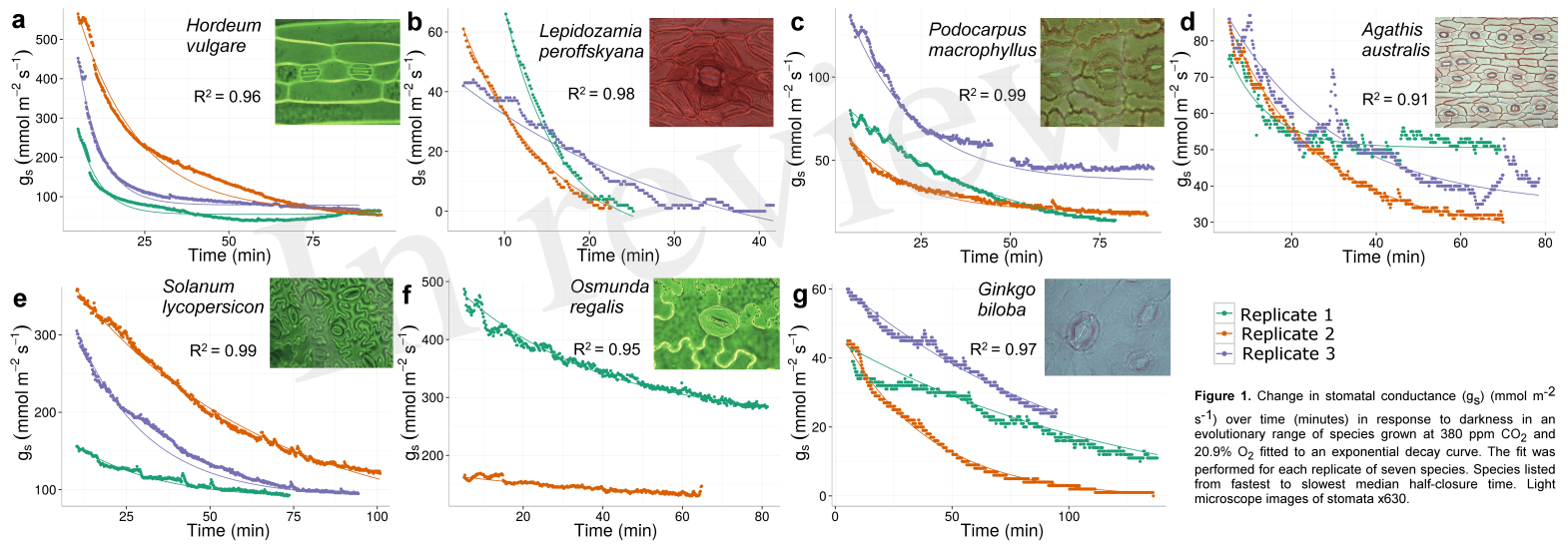


Figure 2.TIFF

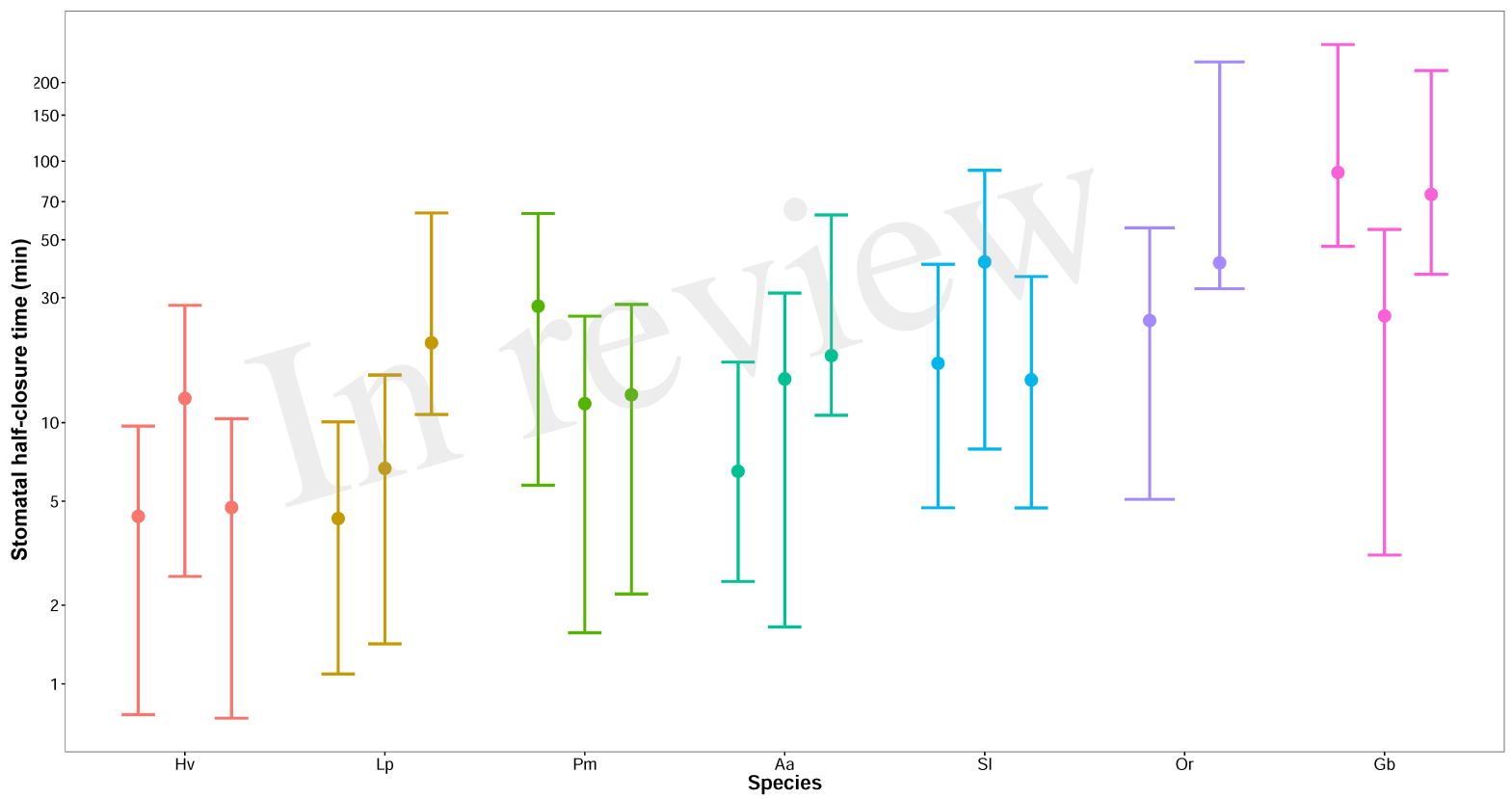


Figure 3.TIF

