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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

Co-ordination of physiological and morphological responses of stomata to elevated [CO_2] in vascular plants

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Abstract Plant stomata display a wide range of short-term behavioural and long-term morphological responses to atmospheric carbon dioxide concentration ([CO_2]). The diversity of responses suggests that plants may have different strategies for controlling gas exchange, yet it is not known whether these strategies are co-ordinated in some way. Here, we test the hypothesis that there is co-ordination of physiological (via aperture change) and morphological (via stomatal density change) control of gas exchange by plants. We examined the response of stomatal conductance (G_s) to instantaneous changes in external [CO_2] (C_a) in an evolutionary cross-section of vascular plants grown in atmospheres of elevated [CO_2] (1,500 ppm) and sub-ambient [O_2] (13.0 %) compared to control conditions (380 ppm CO_2, 20.9 % O_2). We found that active control of stomatal aperture to [CO_2] above current ambient levels was not restricted to angiosperms, occurring in the gymnosperms *Lepidozamia peroffskyana* and *Nageia nagi*. The angiosperm species analysed appeared to possess a greater respiratory demand for stomatal movement than gymnosperm species displaying active stomatal control. Those species with little or no control of stomatal aperture (termed passive) to C_a were more likely to exhibit a reduction in stomatal density than species with active stomatal control when grown in atmospheres of elevated [CO_2]. The relationship between the degree of stomatal aperture control to C_a above ambient and the extent of any reduction in stomatal density may suggest the co-ordination of physiological and morphological responses of stomata to [CO_2] in the optimisation of water use efficiency. This trade-off between stomatal control strategies may have developed due to selective pressures exerted by the costs associated with passive and active stomatal control.

Keywords Carbon dioxide · Conifer · Angiosperm evolution · Stomatal conductance · Stomatal density · Stomatal evolution

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Introduction

Stomata are the pores on a leaf surface that allow plants to balance CO_2 uptake for photosynthesis against water loss through transpiration. A reduction in stomatal conductance (G_s) is commonly observed in response to an increase in the atmospheric concentration of carbon dioxide ([CO_2]) to enhance plant water use efficiency (Woodward 1987). This stomatal control is achieved by the regulation of stomatal aperture through changes in guard cell turgor, and by alteration of stomatal density through modification of stomatal initiation and leaf expansion during leaf development. Active stomatal control is considered to represent physiological control of stomatal aperture via active guard

cell ion transport, regulated by plant signalling mechanisms such as abscisic acid, that permit rapid stomatal movements. Passive stomatal behaviour does not involve physiological control, instead guard cell turgor passively reflects leaf water status (Doi and Shimazaki 2008; Brodribb and McAdam 2011; Ruszala et al. 2011). Species with passive stomatal control are unresponsive to the plant stress hormone abscisic acid, and do not exhibit rapid stomatal movements (Brodribb and McAdam 2011; McAdam et al. 2011). These components of stomatal control are likely to have played a critical role in plant evolution and the interaction of plants with their atmospheric environment over earth history (Robinson 1994; Hetherington and Woodward 2003; Franks and Beerling 2009; Berry et al. 2010; Haworth et al. 2011b), and in the response of vegetation to current climate change (Drake et al. 1997; de Boer et al. 2011; Lammertsma et al. 2011; Franks et al. 2012).

A diverse range of physiological and morphological stomatal responses to [CO_2] are observed in controlled environment (e.g. Woodward and Kelly 1995; Beerling et al. 1998a; Hirano et al. 2012), free air carbon enrichment (e.g. Ainsworth and Rogers 2007; Bernacchi et al. 2007) and herbarium studies (e.g. Kouwenberg et al. 2003; Miller-Rushing et al. 2009; Haworth et al. 2010). It has been suggested that the stomata of more recently derived angiosperms exhibit different physiological responses to environmental stimuli such as [CO_2], light quality or water vapour pressure deficit than more ancient groups such as conifers, ferns and lycophytes (Doi et al. 2006; Doi and Shimazaki 2008; Brodribb et al. 2009; Brodribb and McAdam 2011; McAdam et al. 2011; McAdam and Brodribb 2012). In an analysis of major plant clades, angiosperms, conifers, ferns and lycophytes all exhibited increases in G_s in response to [CO_2] reduced below current ambient ($\sim$380 ppm); however, only angiosperms reduced stomatal conductance when [CO_2] was increased above ambient (Brodribb et al. 2009). Moreover, the presence of the plant drought stress hormone abscisic acid was found to increase stomatal sensitivity to [CO_2] in the angiosperm *Senecio minimus*, but not in two conifer species, *Callitris rhomboidea* and *Pinus radiata* (McAdam et al. 2011). This divergence in physiological responses between more recently derived angiosperms and plant groups with more ancient lineages has led to the suggestion of an evolutionary transition from passive to active metabolic stomatal control (Brodribb and McAdam 2011; McAdam and Brodribb 2012). However, evidence of abscisic acid and [CO_2] sensitivity in the stomatal aperture response of the ancient lycophyte *Selaginella uncinata* (Ruszala et al. 2011) and moss *Physcomitrella patens* (Chater et al. 2011) do not support this interpretation.

The relationship between stomatal density and/or stomatal index (a ratio of the number of stomata to epidermal cells) and the atmospheric [CO_2] in which a leaf developed also differs between plant species, in direction, strength and the range of [CO_2] over which stomatal initiation is modified (Woodward 1987; Kürschner et al. 1997; Royer et al. 2001; Kouwenberg et al. 2003; Haworth et al. 2011c; Hirano et al. 2012). Many angiosperm species exhibit a "ceiling of response" at 350–400 ppm [CO_2], above which stomatal density and index no longer respond (Woodward 1987; Kürschner et al. 1997, 2008, Bettarini et al. 1998), whereas many conifers with ancient evolutionary origins often continue to reduce stomatal initiation at [CO_2] levels above current ambient (Kouwenberg et al. 2003; Haworth et al. 2010, 2011a; Grein et al. 2011). A similar stomatal density and stomatal index response to [CO_2] above 400 ppm is observed in *Ginkgo biloba* (Beerling et al. 1998a; Royer et al. 2001); however, atmospheric [CO_2] does not influence stomatal initiation in Cycadaceae (Haworth et al. 2011c). The lower ceiling of response observed in angiosperms (Woodward 1987; Kürschner et al. 1997) relative to conifers (Kouwenberg et al. 2003; Grein et al. 2011) may be associated with greater stomatal aperture control of angiosperms at [CO_2] above 400 ppm (Brodribb et al. 2009), possibly indicating a degree of co-ordination between physiological and morphological control of stomatal conductance in response to [CO_2] (Haworth et al. 2011b).

In addition to fluctuations in [CO_2], levels of atmospheric oxygen ([O_2]) have also varied throughout earth history (Berner 2006, 2009; Belcher et al. 2010). Ribulose-1,5-bisphosphate carboxylase-oxygenase displays an affinity for both CO_2 and O_2 as part of the competing processes of photosynthesis and photorespiration (Mizi-orko and Llorimer 1983). The level of [O_2] may therefore possibly affect stomatal initiation through changes in the photosynthetic availability of CO_2 expressed by the atmospheric CO_2:O_2 ratio (Beerling and Woodward 1997; Beerling et al. 1998b). Oxygen may also influence stomatal function via the respiratory costs associated with stomatal opening and closing (Mawson 1993; Srivastava et al. 1995). The expansion of angiosperms during the Late Cretaceous and Tertiary has been associated with falling levels of atmospheric [CO_2] (McElwain et al. 2004; Heimhofer et al. 2005). However, this period in earth history also coincides with rising levels of atmospheric [O_2], possibly reducing the respiratory costs associated with more functional stomata, and thus favouring plants with more effective stomatal control (Haworth et al. 2011b), and accounting for the apparent shift towards active stomatal control in more recently derived plant groups (Brodribb and McAdam 2011).

This study intends to test the hypothesis that vascular plants show a co-ordination of physiological (via stomatal aperture) and morphological (via changes in stomatal

Table 1 Levels of atmospheric $[CO_2]$ and $[O_2]$ used during growth treatments experienced by plant species in this study and CO_2:O_2 ratio of respective atmospheres

Treatment	$[O_2]$ (%)	$[CO_2]$ (ppm)	CO_2:O_2 ratio
Control	20.9	380	0.0018
Low $[O_2]$	13.0	380	0.0029
High $[CO_2]$	20.9	1,500	0.0072
Low $[O_2]$/high $[CO_2]$	13.0	1,500	0.0115

initiation) control of leaf gas exchange in response to $[CO_2]$ and $[O_2]$ (Table 1). Specifically, we aim to investigate: (1) stomatal sensitivity to fluctuations in external atmospheric $[CO_2]$ concentration (C_a) across a range of plants with divergent evolutionary lineages; (2) the effect of growth at elevated $[CO_2]$ and sub-ambient $[O_2]$ on stomatal sensitivity to C_a; (3) the stomatal density, index and pore length responses to growth at elevated $[CO_2]$ and sub-ambient $[O_2]$; and (4) possible co-ordination of stomatal functional and morphological responses to $[CO_2]$ in the control of leaf gas exchange.

Materials and methods

Controlled environment experiments

Lepidozamia peroffskyana (cycad), *Hordeum vulgare* (angiosperm) and *Solanum lycopersicum* (angiosperm) grown from seed, 6-month old specimens of *Osmunda regalis* (fern) and 2-year-old specimens of *Ginkgo biloba* (Ginkgoaceae), *Nageia nagi* (conifer), *Podocarpus macrophyllus* (conifer) and *Agathis australis* (conifer) were potted in 4-l square pots (15 × 15 × 23 cm) with 80 % compost (2 kg m^{-3} 15:10:20 N:P:K; Bord na Móna, Newbridge, County Kildare, Ireland), 20 % vermiculite and 2.5 g l^{-1} slow release Osmocote fertilizer (15 % N, 10 % P_2O_5, 10 % K_2O, 2 % MgO, plus trace elements; Scotts, Marysville, OH, USA). Plants were grown in four Conviron BDW-40 (Winnipeg, Manitoba, Canada) walk-in growth chambers in UCD's PÉAC facility at Thornfield (see Table 1 for atmospheric growth conditions). Plants were grown under experimental atmospheric conditions for 18 months with the exception of *H. vulgare* and *S. lycopersicum* that were grown for 3 months. To avoid chamber effects, plants were rotated between chambers every 3 months (Hirano et al. 2012). Atmospheric concentration of $[CO_2]$ within the chambers was monitored by a PP-systems WMA-4 IRGA (PP-Systems, Amesbury, MA, USA) and supplemented by compressed CO_2 to increase $[CO_2]$ above ambient (BOC, Guildford, Surrey, UK). Atmospheric oxygen level was monitored by a PP-systems OP-1 Oxygen Sensor. To reduce $[O_2]$, the nitrogen level in the chambers was supplemented via a compressed air line from a nitrogen generator (Dalco Engineering, Dunshaughlin, County Meath, Ireland). All other growth conditions remained constant, with plants experiencing 16 h of light per day in a simulated day/night program (0500–0600 hours, dawn; 0600–0900 hours, light intensity rises from 300 to 600 μmol m^{-2} s^{-1}; 0900–1700 hours, midday light intensity of 600 μmol m^{-2} s^{-1}; 1700–2000 hours, light intensity decreases 600 to 300 μmol m^{-2} s^{-1}; 2000–2100 hours, dusk), 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 and receiving 60 ml of water each day. In order to avoid mutual shading plants were randomised within areas of identical canopy height within the growth chambers (Hammer and Hopper 1997; Sager and McFarlane 1997). After full leaf development and expansion, the uppermost leaves receiving full irradiance and not affected by self-shading were used for stomatal $[CO_2]$ sensitivity analysis through analysis of G_s response to instantaneous step changes in C_a, and then destructively sampled for stomatal counts.

Measurement of stomatal conductance sensitivity to $[CO_2]$

Stomatal conductance (G_s) measurements were conducted on a minimum of three replicates per species from each atmospheric treatment. Plants were removed from the growth chamber and measurements were recorded in a well-ventilated room maintained at a constant temperature of 25 °C under ambient levels of $[CO_2]$ and $[O_2]$. Timings of day/night programs on the plant growth chambers were staggered to allow the maximum number of plants to be analysed at the optimal time of the day/night program for photosynthetic activity, and thus avoid the influence of circadian stomatal behaviour; particularly where stomata close at midday or during the early afternoon when temperatures rise and leaf water potentials decrease. Stomatal conductance responses to fluctuations in external $[CO_2]$ concentration (C_a) measurements were taken between 0900 and 1100 hours using a PP-Systems Ciras-2 attached to a PLC6(U) leaf cuvette and LED light unit (PP-Systems) under saturating light intensity calculated from PAR (photosynthesis response curves) (Parsons et al. 1998). Temperature within the cuvette was maintained at 25 °C. Leaves were allowed to stabilise within the cuvette for approximately 20 min at 380 ppm $[CO_2]$, before step changes in C_a (200, 400, 750, 1,000 and 2,000 ppm $[CO_2]$) occurred. At each C_a value G_s was allowed to stabilise and then recorded after G_s had remained stable for ~10 min. Vapour pressure deficit in the leaf cuvette was maintained constant throughout each C_a step change analysis at 1.3 ± 0.1 kPa. This protocol was used to examine the

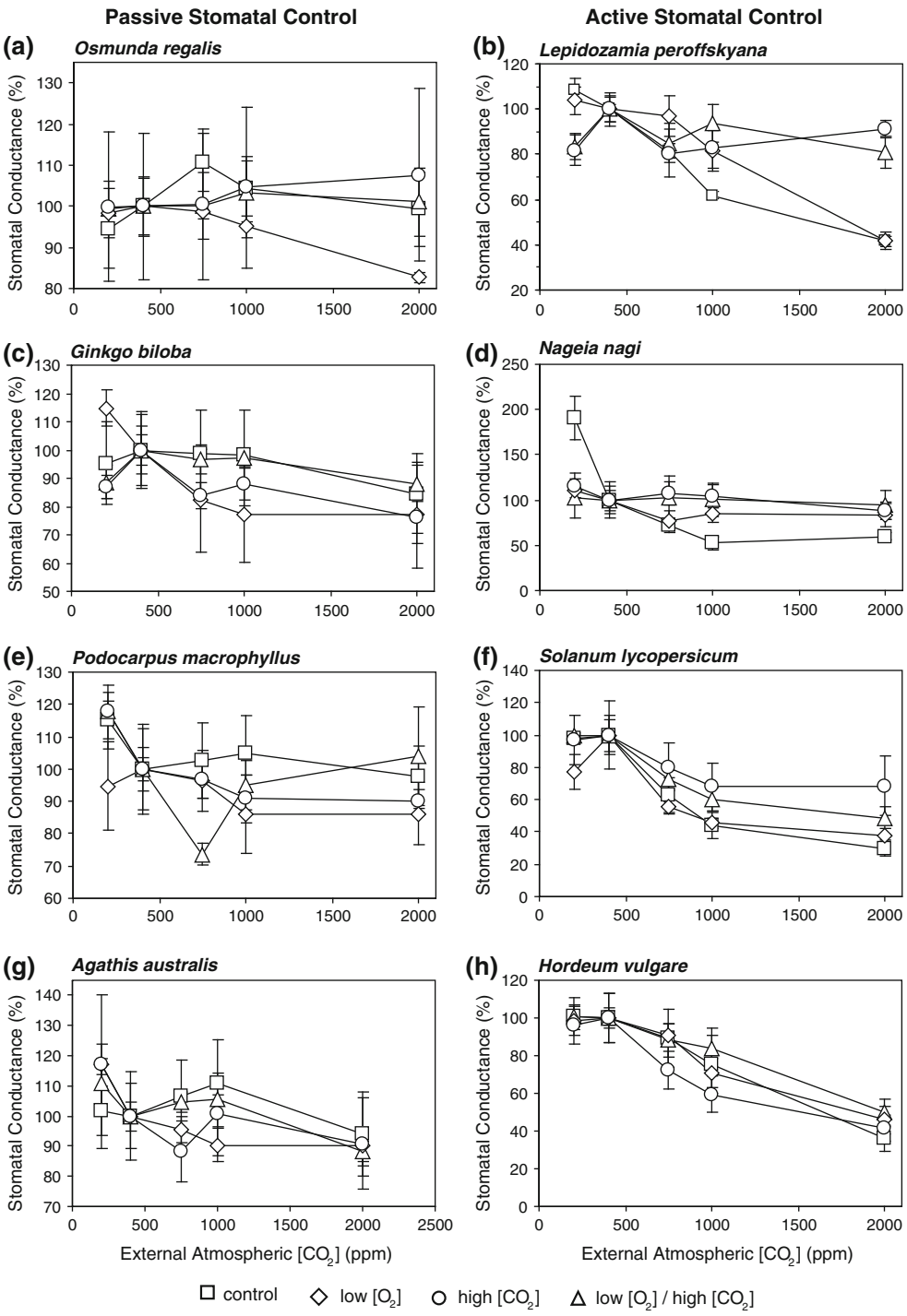

Fig. 1 Relative stomatal conductance response to an increase of external atmospheric [CO_2] (200, 400, 750, 1,000, 2,000 ppm [CO_2]) of an evolutionary cross-section of plants grown in atmospheres of elevated [CO_2] and sub-ambient [O_2] in comparison to control atmospheric conditions (see Table 1): control (*open squares*); low [O_2] (*open diamonds*); high [CO_2] (*open circles*); and combined low [O_2]/high [CO_2] (*open triangles*). *Error bars* one standard error either side of the mean

extent to which G_s was actively controlled by changes in guard cell turgor, whereby a change in G_s was used to infer "active stomatal control", and no change in G_s to infer "passive stomatal control" (see Fig. 1; Table 2).

Stomatal density and index counts

Conifer, *G. biloba* and *L. peroffskyana* leaf cuticles were macerated using a 50:50 solution of glacial acetic acid and 30 % H_2O_2 at 70 °C, stained using safranin-O solution and mounted in glycerol on glass slides. *Osmunda regalis*, *S. lycopersicum* and *H. vulgare* leaf impressions were taken using dental impression gel (Coltène President Light Body Material), and nail varnish "positives" mounted onto glass slides (Weyers and Lawson 1985). Cuticle images were taken under transmitted light using a Leica DM2500 microscope attached to a Leica DFC300FX camera (Leica Microsystems, Wetzlar, Germany) and Syncroscopy

Table 2 One-way ANOVA results for stomatal conductance of plant species grown in atmospheres of elevated [CO_2] and sub-ambient [O_2] when exposed to sub-ambient and super-ambient levels of C_a (see Fig. 1)

Species	Sub-ambient C_a (200, 400 ppm [CO_2])								Super-ambient C_a (400, 750, 1,000, 2,000 ppm [CO_2])							
	Control		Low [O_2]		High [CO_2]		Low [O_2]/High [CO_2]		Control		Low [O_2]		High [CO_2]		Low [O_2]/High [CO_2]	
	$F_{1,4}$	P	$F_{1,4}$	P	$F_{1,4}$	P	$F_{1,4}$	P	$F_{3,8}$	P	$F_{3,8}$	P	$F_{3,8}$	P	$F_{3,8}$	P
Osmunda regalis	0.050	0.833	0.189	0.686	0.001	0.974	0.000	0.998	0.114	0.950	5.351	0.026	0.009	0.999	0.009	0.999
Lepidozamia peroffskyana	0.306	0.610	0.041	0.849	1.088	0.356	1.906	0.240	10.409	0.004	98.058	1.2×10^{-6}	0.348	0.792	0.393	0.761
Ginkgo biloba	0.014	0.912	0.271	0.630	0.536	0.505	0.476	0.528	0.061	0.979	0.105	0.955	0.251	0.859	0.427	0.739
Nageia nagi	0.496	0.520	0.136	0.731	0.001	0.974	0.200	0.678	6.086	0.018	4.671	0.036	0.008	0.999	0.121	0.945
Podocarpus macrophyllus	0.267	0.624	0.019	0.898	1.687	0.264	8.517	0.043	0.021	0.996	0.099	0.958	1.289	0.343	0.479	0.706
Agathis australis	0.003	0.962	1.237	0.292	0.387	0.568	0.095	0.773	0.085	0.967	0.232	0.873	2.305	0.153	0.054	0.982
Solanum lycopersicum	0.011	0.921	0.234	0.654	0.000	0.988	0.006	0.941	7.944	0.009	4.156	0.048	13.115	0.002	5.977	0.019
Hordeum vulgare	0.000	0.983	0.010	0.926	0.001	0.977	0.012	0.917	4.671	0.036	7.564	0.010	1.790	0.227	78.738	2.8×10^{-6}

Additional detail and post hoc testing using the Bonferroni method are given in supplementary data

Automontage (Syncroscopy, Cambridge, Cambridgeshire, UK). As an indicator of stomatal aperture size, the stomatal pore length (Wagner et al. 1996; Hetherington and Woodward 2003) of ~20 stomata was measured using Automontage, with the average taken to represent the treatment value for a given species. A 0.09-mm^2 grid (Poole and Kürschner 1999) was superimposed on the images for stomatal and epidermal counts using Syncroscopy AcQuis. In the controlled environment study, five stomata/epidermal cell counts were performed on each of three leaves from a plant, with the average of 15 counts taken to represent the mean stomatal density and stomatal index of an individual plant (except for *L. peroffskyana* where 9 counts were averaged). Stomata and epidermal cells were counted on 1,548 images in total, with 9 or 15 images counted per plant and then the average of three plants taken to represent the mean stomatal density or stomatal index value for a species in a given atmospheric treatment. The abaxial surface was analysed for stomatal counts in all species, with the exception of *H. vulgare* where stomatal counts were taken from the abaxial and adaxial surfaces; these were broadly similar and the mean was taken to produce an average *H. vulgare* value for Figs. 3 and 4 (individual abaxial and adaxial values are given in supplementary data tables). The percentage area of stomatous regions of the cuticle available as stomatal pore during maximal stomatal opening ($A_{\%}$) was calculated assuming elliptical stomatal pore geometry and assuming stomatal width at full stomatal opening was equivalent to 0.5 stomatal pore length (Beerling and Chaloner 1993). Relative changes in stomatal density, stomatal index and $A_{\%}$ (Δ stomatal density, Δ stomatal index and $\Delta A_{\%}$) between the control and each treatment were then calculated and plotted against relative changes in stomatal conductance (Δ stomatal conductance) sensitivity to C_a increases from 400 to 2,000 ppm [CO_2]. One-way Bonferroni method ANOVAs were performed using SPSS 20 (IBM, New York, USA) to test whether G_s, stomatal index, stomatal density and stomatal pore length values of the plants differed significantly between treatments to identify any affects of elevated [CO_2] and sub-ambient [O_2] on stomatal morphology and conductance (for full details and results of post hoc analysis, see supplementary data).

Results

As [CO_2] was increased the evolutionary cross-section of plants studied showed a diverse range of physiological responses to C_a, from no change (passive) to pronounced reductions (active) in G_s (Fig. 1; Table 2). The fern *O. regalis*, ginkgoalean *G. biloba* and conifers *P. macrophyllus* and *A. australis* exhibited passive stomatal control,

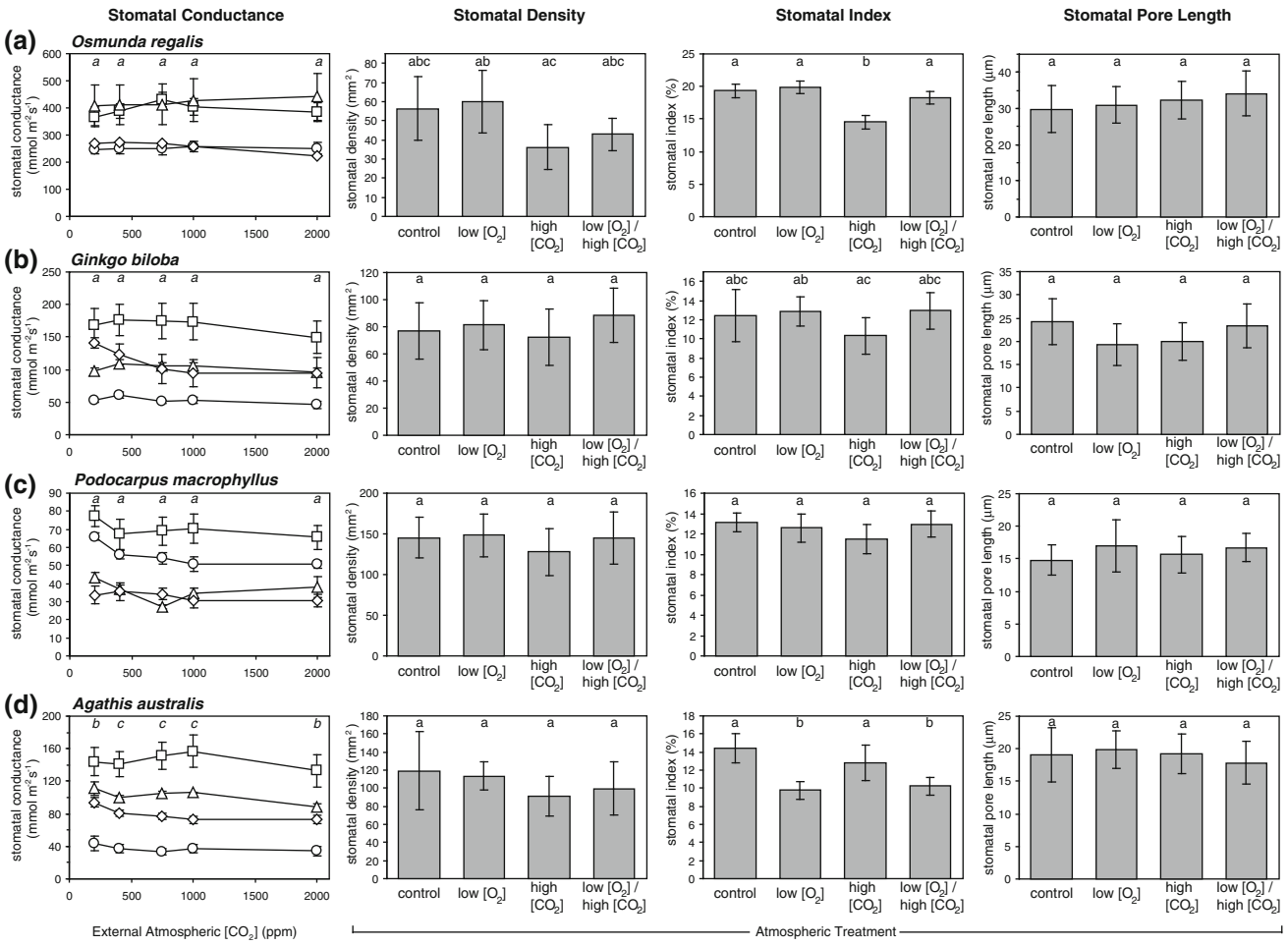

Fig. 2 Physiological and morphological responses of plant species with passive stomatal behaviour grown in atmospheres of elevated $[CO_2]$ (1,500 ppm) and sub-ambient $[O_2]$ (13.0 %), relative to control conditions of ambient $[CO_2]$ (380 ppm) and $[O_2]$ (20.9 %). *Line graphs* indicate stomatal conductance response to external atmospheric $[CO_2]$ (200, 400, 750, 1,000, 2,000 ppm CO_2) of plants grown in control (*open squares*); low $[O_2]$ (*open diamonds*); high $[CO_2]$ (*open circles*); and combined low $[O_2]$/high $[CO_2]$ (*open triangles*): *error bars* one standard error either side of the mean; *italicised letters* significant differences between treatments at each C_a level ($a \geq 0.05$; $b \leq 0.05$; $c \leq 0.01$; $d \leq 0.001$). Statistical analyses of differences in G_s of plants grown in the same atmospheric treatments in response to changes in C_a are reported in Table 2. *Histograms* indicate stomatal density, index and pore length responses of plants to growth in atmospheres of elevated $[CO_2]$ and sub-ambient $[O_2]$ relative to control atmospheres (see Table 1). *Histogram error bars* one standard deviation either side of mean, *letters* significant difference between treatments using Bonferroni method ANOVA

with no reduction in G_s to C_a above 400 ppm (Fig. 2; Table 1). In contrast, the cycad *L. peroffskyana* and conifer *N. nagi* grown in atmospheres of ambient $[CO_2]$ exhibited pronounced reductions in G_s (−58.5 and −40.2 %, respectively) as C_a was increased from 400 to 2,000 ppm $[CO_2]$. However, when grown in atmospheres of elevated $[CO_2]$, both *L. peroffskyana* and *N. nagi* no longer altered G_s in response to changes in C_a, suggesting the loss of stomatal sensitivity to $[CO_2]$ (Figs. 1e, f, 3; Table 2). Stomatal sensitivity to C_a in *G. biloba* appeared to be enhanced by growth in atmospheres of 13.0 % $[O_2]$, possibly suggesting a respiratory requirement for physiological control of stomatal aperture (Fig. 2b) as G_s sensitivity to C_a was measured under ambient $[O_2]$ (G_s reduction from C_a 400 to 2,000 ppm $[CO_2]$: control −15.5 %; low $[O_2]$ −23.0 %; high $[CO_2]$ −11.8 %, and; combined low $[O_2]$/high $[CO_2]$ −23.7 %). Active stomatal control of G_s in response to C_a is also evident in the angiosperms *S. lycopersicum* and *H. vulgare* (Figs. 1g, h, 3c, d). The atmospheric growth conditions of the two angiosperms did not affect the relative changes in G_s in response to C_a, suggesting that $[CO_2]$ sensitivity was not impaired (see supplementary data) by growth at elevated $[CO_2]$ or sub-ambient $[O_2]$ for these taxa.

Those species with passive stomatal control in response to increased C_a above ambient (*O. regalis*, *G. biloba*, *P. macrophyllus* and *A. australis*) did, however, show an effect of atmospheric conditions during growth on G_s (Fig. 2). *Osmunda regalis* displayed significantly higher G_s when grown in atmospheres of ambient $[CO_2]$ compared to

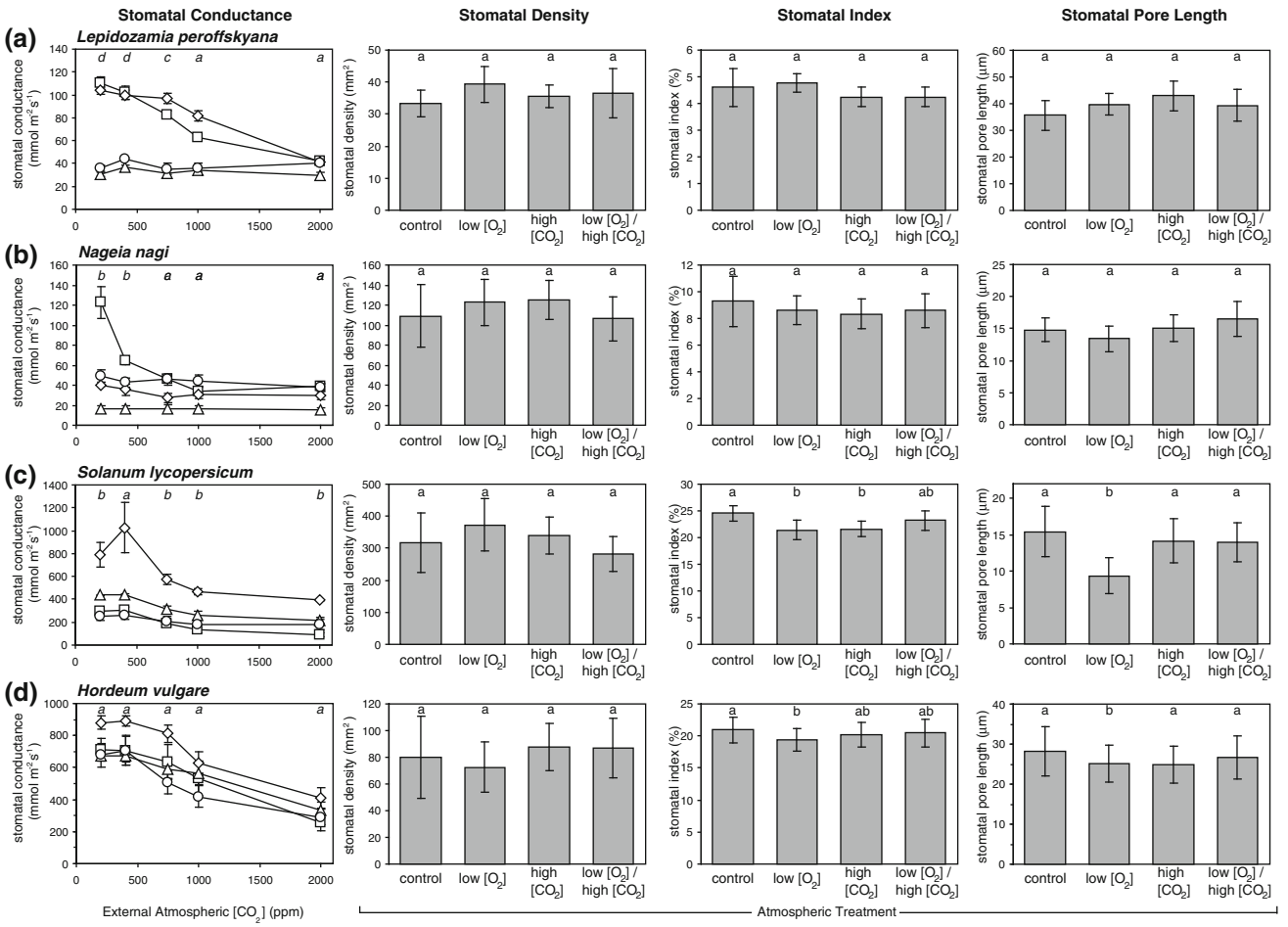

Fig. 3 Physiological and morphological responses of plant species with active stomatal behaviour grown in atmospheres of elevated $[CO_2]$ (1,500 ppm) and sub-ambient $[O_2]$ (13.0 %), relative to control conditions of ambient $[CO_2]$ (380 ppm) and $[O_2]$ (20.9 %). *Line graphs* indicate stomatal conductance response to external atmospheric $[CO_2]$ (200, 400, 750, 1,000, 2,000 ppm CO_2) of plants grown in control (*open squares*); low $[O_2]$ (*open diamonds*); high $[CO_2]$ (*open circles*); and combined low $[O_2]$/high $[CO_2]$ (*open triangles*): *error bars* one standard error either side of the mean; *italicised letters* significant difference between treatments at each C_a level ($a \geq 0.05$; $b \leq 0.05$; $c \leq 0.01$; $d \leq 0.001$). Statistical analyses of differences in G_s of plants grown in the same atmospheric treatments in response to changes in C_a are reported in Table 2. *Histograms* indicate stomatal density, index and pore length responses of plants to growth in atmospheres of elevated $[CO_2]$ and sub-ambient $[O_2]$ relative to control atmospheres (see Table 1). *Histogram error bars* one standard deviation either side of mean, *letters* significant difference between treatments using Bonferroni method ANOVA

atmospheres of elevated $[CO_2]$. When grown in high $[CO_2]$, the stomatal density values of *O. regalis* were 35.9 % lower than those in control atmospheres (Fig. 2a). *Podocarpus macrophyllus* and *A. australis* also exhibited 12.1 and 23.9 % reductions in stomatal density when $[CO_2]$ was increased to 1,500 ppm (Fig. 2c, d). In contrast, the angiosperms *S. lycopersicum* and *H. vulgare* exhibited 7.1 and 9.9 % (abaxial +10.9 %; adaxial +8.8 %) increases in stomatal density when grown in atmospheres of elevated $[CO_2]$ (Fig. 3c, d). However, *L. peroffskyana* that displayed active stomatal control to C_a when grown in 380 ppm $[CO_2]$, appeared to become passive in response to instantaneous increases in C_a following growth in elevated $[CO_2]$ (Table 1). *Lepidozamia peroffskyana* showed a reduction in G_s when grown at elevated $[CO_2]$ that was not accompanied by a reduction in stomatal density (+6.6 %) or stomatal pore length (+20.4 %). In combination, these results suggest reduced physiological stomatal function of *L. peroffskyana* grown in elevated $[CO_2]$, and perhaps that impairment of stomatal opening resulted in reduced G_s (Fig. 3a). *Solanum lycopersicum* and *H. vulgare* exhibited greater conductance rates at all C_a values when grown in atmospheres of sub-ambient $[O_2]$ and ambient $[CO_2]$, while growth at elevated $[CO_2]$ did not significantly affect G_s relative to control atmospheres (Fig. 3c, d).

The proportional change in physiological stomatal response to $[CO_2]$ above ambient (G_s change between C_a 400 to 2,000 ppm $[CO_2]$) of plants grown in low $[O_2]$, high $[CO_2]$ and combined low $[O_2]$/high $[CO_2]$ was compared in relation to plants grown in control atmospheres (Δ stomatal

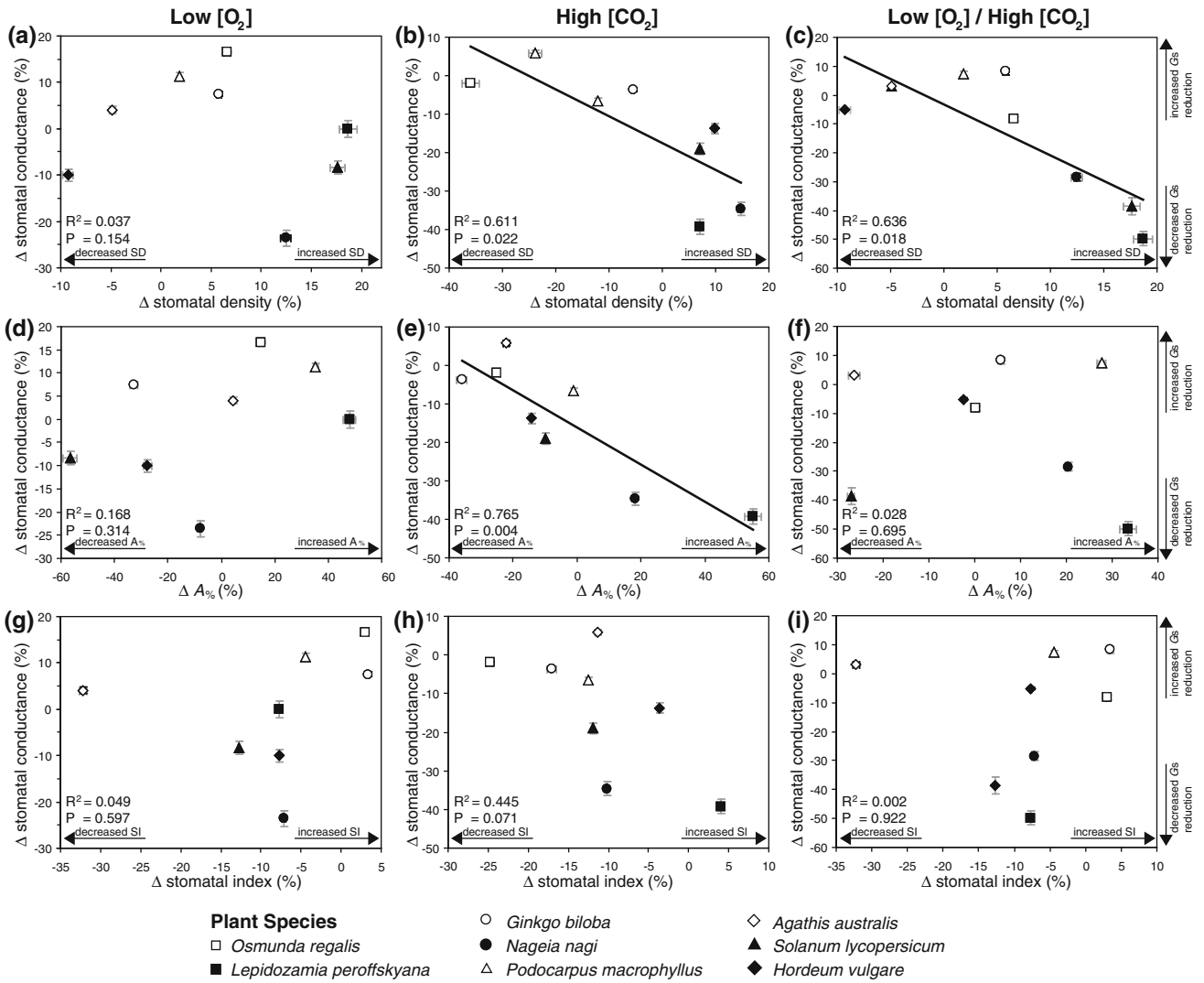

Fig. 4 Changes in stomatal morphology following long term exposure to different atmospheric treatments (Δ stomatal density; $\Delta A_{\%}$; Δ stomatal index) versus physiological response of stomata of an evolutionary cross-section of plants with passive (*open symbols*) and active (*closed symbols*) stomatal control (see Figs. 2, 3 for species' morphological and G_s response) to an instantaneous increase in C_a from 400 to 2,000 ppm CO_2 when grown in atmospheres of low [O_2], high [CO_2] and combined low [O_2]/high [CO_2] in comparison to plants grown in control atmospheres (Δ stomatal conductance): **a** Δ stomatal conductance and Δ stomatal density of plants grown in atmospheres of low [O_2]: $y = -0.2514x + 1.2146$; $R^2 = 0.0372$, regression $P = 0.154$; **b** Δ stomatal conductance and Δ stomatal density of plants grown in atmospheres of high [CO_2]: $R^2 = 0.611$, regression $P = 0.0220$; $y = -0.6954x - 17.468$); **c** Δ stomatal conductance and Δ stomatal density of plants grown in atmospheres of combined low [O_2]/high [CO_2]: $R^2 = 0.636$, regression $P = 0.0178$; $y = -1.7633x - 3.1869$); **d** Δ stomatal conductance and $\Delta A_{\%}$ of plants grown in atmospheres of low [O_2]: $R^2 = 0.168$, regression $P = 0.314$; $y = 0.1508x + 0.0971$); **e** Δ stomatal conductance and $\Delta A_{\%}$ of plants grown in atmospheres of high [CO_2]: $R^2 = 0.765$, regression $P = 0.00449$; $y = -0.4813x - 16.228$); **f** Δ stomatal conductance and $\Delta A_{\%}$ of plants grown in atmospheres of combined low [O_2]/high [CO_2]: $R^2 = 0.0275$, regression $P = 0.695$; $y = -0.1615x - 13.298$; **g** Δ stomatal conductance and Δ stomatal index of plants grown in atmospheres of low [O_2]: $R^2 = 0.0494$, regression $P = 0.597$; $y = 0.2605x + 1.8152$); **h** Δ stomatal conductance and Δ stomatal index of plants grown in atmospheres of high [CO_2]: $R^2 = 0.445$, regression $P = 0.0708$; $y = -1.2494x - 27.777$), and; **i** Δ stomatal conductance and Δ stomatal index of plants grown in atmospheres of combined low [O_2]/high [CO_2]: $R^2 = 0.00180$, regression $P = 0.922$; $y = 0.0832x - 13.261$). *Error bars* one standard error either side of mean

conductance); this was then plotted against the relative changes in morphological stomatal responses (Δ stomatal density, $\Delta A_{\%}$ and Δ stomatal index) between plants grown in control atmospheres and those grown in atmospheres of low [O_2], high [CO_2] and combined low [O_2]/high [CO_2] (Fig. 4). A strong correlation between stomatal density and G_s sensitivity to C_a was observed in plants grown in atmospheres of elevated [CO_2] ($R^2 = 0.611$; regression $P = 0.022$) and combined low [O_2]/high [CO_2] ($R^2 = 0.636$; regression $P = 0.018$), suggesting the co-ordination of physiological and morphological responses to increases in [CO_2] (Fig. 4b, c). This suggests that as [CO_2] is

increased above ambient species with active stomatal sensitivity to C_a are less likely to reduce stomatal density than species with passive stomata. Relative changes in $A_{\%}$ and G_s when grown in atmospheres of elevated [CO_2] compared to control atmospheres showed a significant correlation ($R^2 = 0.765$; regression $P = 0.045$) (Fig. 4e). However, similar relationships between shifts in $A_{\%}$ and G_s are not observed when plants are grown in atmospheres of low [O_2] ($R^2 = 0.168$; regression $P = 0.314$) and combined low [O_2]/high [CO_2] ($R^2 = 0.028$; regression $P = 0.695$) (Fig. 4d, f).

The correlations of stomatal density and $A_{\%}$ with G_s response to C_a suggest a co-ordination of stomatal functionality and morphology in the control of transpiration in response to increased [CO_2]. However, a statistically significant relationship at the 95 % confidence level between stomatal index and reduction in G_s to C_a above ambient is not observed when plants are grown in atmospheres of elevated [CO_2] ($R^2 = 0.445$; regression $P = 0.071$) (Fig. 4h), suggesting that stomatal initiation was influenced to a lesser extent than stomatal density and size, both of which directly determine limits of transpirative water loss and photosynthetic [CO_2] uptake. Growth in sub-ambient [O_2] did not appear to induce a co-ordinated function and morphological stomatal response in the plant groups studied (Fig. 4a, d, g). Tables of stomatal density, index and pore length values alongside statistical tests are presented in supplementary information.

Discussion

The plants analysed in this study exhibited a diverse range of morphological and physiological stomatal responses to [CO_2] and [O_2]. The gymnosperms *L. peroffskyana* and *N. nagi* all exhibited sensitivity to C_a above ambient, consistent with the findings of Ruszala et al. (2011) and Chater et al. (2011) that active stomatal control is not restricted to more recently derived angiosperms (cf. Brodribb et al. 2009; Brodribb and McAdam 2011; McAdam et al. 2011; McAdam and Brodribb 2012). The atmospheric growth environment of the plants also had a significant effect on stomatal function in terms of G_s response to C_a. *Lepidozamia peroffskyana* and *N. nagi* when grown in atmospheres of elevated [CO_2] no longer altered G_s to increased C_a (Figs. 1, 3a, b) possibly suggesting impairment of stomatal sensing of [CO_2] (e.g. Wheeler et al. 1999; Levine et al. 2009) or guard cell turgor modification responsible for stomatal control (e.g. Meidner 1968; Franks and Farquhar 2007). The loss of stomatal sensitivity and guard cell movement in response to increased C_a when *L. peroffskyana* and *N. nagi* were grown in atmospheres of elevated [CO_2] may also suggest that, at high [CO_2], the costs of maintaining effective stomatal function, via the operation of physiological systems for [CO_2] sensing and stomatal control, are no longer beneficial in terms of enhancing water use efficiency when compared to growth at lower ambient [CO_2] levels. However, stomatal sensitivity to C_a was unaffected in the angiosperm species (*S. lycopersicum* and *H. vulgare*) by growth in 1,500 ppm [CO_2] and 13.0 % [O_2]. This divergence in responses of the species with active stomatal control to growth [CO_2] and [O_2], suggests that these species may possess different physiological mechanisms for both [CO_2] sensing and stomatal movements (Hetherington and Woodward 2003; Franks and Farquhar 2007; Doi and Shimazaki 2008; McAdam et al. 2011).

Oxygen plays an important role in stomatal movements, as stomatal opening and closing are energetically expensive processes. Mitochondria within the guard cells provide the energy for transport of potassium ions across the cell membrane (Walker and Zelitch 1963; Willmer and Mansfield 1970; Raghavendra 1981). In *Gossypium barbadense*, increased stomatal conductance rates occur alongside elevated guard cell respiration (Srivastava et al. 1995). The two angiosperms analysed within this study both exhibited enhanced G_s rates when grown under sub-ambient [O_2] and ambient [CO_2] (Fig. 3c, d). As G_s response to C_a was measured under ambient [O_2], this enhanced capacity for G_s may represent an elevated respiratory capacity (Walker and Zelitch 1963) incurred through growth in atmospheres of 13.0 % [O_2] prompting greater stomatal opening at 20.9 % [O_2]. A similar pattern of increased G_s caused by growth in atmospheres of low [O_2] was not apparent in the gymnosperm species (with the possible exception of *L. peroffskyana* at C_a 750 and 1,000 ppm CO_2) and fern *O. regalis*, possibly suggesting differential mechanisms for stomatal movements such as guard cell chloroplast photosynthesis (Doi et al. 2006; Doi and Shimazaki 2008) or a lower respiratory requirement for guard cell turgor control in these species (Srivastava et al. 1995). The apparent greater respiratory demand for stomatal control in the two angiosperm species may indicate that rising [O_2] in the mid- to Late Cretaceous favoured species with greater guard cell respiratory demands and therefore played a role in the expansion and diversification of the angiosperms (Haworth et al. 2011b). In contrast, in the gymnosperm species analysed, the greatest G_s rates were observed in plants grown in control atmospheres of ambient [CO_2] and [O_2] at ambient C_a levels of 400 ppm [CO_2], suggesting that the CO_2:O_2 ratio of the growth atmosphere may play a more significant role in determining gymnosperm capacity for G_s.

Those species with passive stomatal control to increased C_a were more likely to exhibit reductions in stomatal density and $A_{\%}$ when grown in atmospheres of elevated

[CO_2] than species with active stomatal control in response to C_a (Fig. 4). This suggests that the morphological and physiological response of stomata to [CO_2] are linked, with plants with active stomatal control less likely to alter stomatal density to elevated [CO_2] than plants with passive stomatal control (Fig. 4b). The co-ordination of stomatal sensitivity and morphology to increased [CO_2] may represent a trade-off between different strategies of stomatal control, determined by the selective pressures exerted by their associated costs and benefits (Haworth et al. 2011b). Active stomatal control to C_a requires investment in mechanisms for sensing of [CO_2], co-ordination of signals and resulting stomatal movements (Heath 1950; Hetherington and Woodward 2003; Hu et al. 2010). Plant species with highly sensitive stomata to short-term stimuli, such as members of the Cycadaceae (Fig. 3a), may not alter stomatal initiation, and thus modify stomatal density and/or stomatal index, to changes in their atmospheric environment (Marler and Willis 1997; Haworth et al. 2011c). Therefore, uptake of CO_2 is not limited by stomatal number or size during periods when conditions are favourable to photosynthesis, or constrained in their ability to respond to any future shifts in atmospheric composition via prior modification of stomatal density. Conversely, species with passive stomatal control do not have to invest in physiological mechanisms to sense short-term fluctuations in C_a, signalling or control stomatal movements (Brodribb et al. 2009; Hu et al. 2010). Nevertheless, this may result in limitations to photosynthetic capacity during periods that may be favourable to photosynthesis or to a lower tolerance of water stress (Brodribb et al. 2009; McAdam et al. 2011). The two conifers with passive stomatal control in response to C_a (*A. australis* and *P. macrophyllus*) both possess stomatal wax plugs, possibly suggesting that the wax plugs restrict stomatal closure (Feild et al. 1998). However, the stomatal complexes of the fern *O. regalis* that also exhibited passive stomatal control were not occluded by stomatal wax plugs. An inverse relationship between stomatal pore length or size and stomatal density has been observed in the gas exchange responses of plants in response to increased [CO_2] (Franks et al. 2012; Roth-Nebelsick et al. 2012). However, a similar pattern was not observed in the species analysed in this study (Figs. 2, 3).

The co-ordination of stomatal C_a sensitivity with morphological response to growth at elevated [CO_2] suggests an apparent evolutionary trade-off in the control of G_s (Fig. 4b, e) determined by the respective evolutionary costs of each stomatal control strategy (Haworth et al. 2011b). Selective pressures exerted by the respective costs of active and passive stomatal control may have induced the co-ordination of physiological and morphological stomatal responses to elevated [CO_2] apparent in this dataset, suggesting an evolutionary trade-off between stomatal control strategies in the optimisation of water use efficiency. This relationship between stomatal C_a sensitivity and the degree of stomatal density response to [CO_2] may provide an explanation for the diversity of reported stomatal density responses to atmospheres enriched in [CO_2] (e.g. Woodward 1987; Kürschner et al. 1997; Beerling et al. 1998a; Bettarini et al. 1998; Reid et al. 2003; Ainsworth and Rogers 2007; Haworth et al. 2010; Hirano et al. 2012). Shifts in stomatal function and morphological response to [CO_2] are likely to affect transpiration rates at local to global scales and to influence ecological composition under rising atmospheric [CO_2] (Lammertsma et al. 2011; Franks et al. 2012). Nevertheless, further work is required to establish any relationship between the degree of stomatal density response and stomatal C_a sensitivity in plants grown across a range of [CO_2] levels. Additionally, the stomatal density and/or index values of fossil plants are increasingly used as indicators of the palaeo-atmospheric level of [CO_2] in which the leaf developed (e.g. Royer et al. 2001; Haworth et al. 2005; Kürschner et al. 2008; Passalia 2009; Smith et al. 2010; Grein et al. 2011; Stults et al. 2011). Future stomatal palaeo-[CO_2] reconstructions should be undertaken within the context of the likely stomatal C_a sensitivity of the fossil plant species based upon the stomatal control mechanisms employed by nearest living relative or analogue species.

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