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Field, K.J., Duckett, J.G., Cameron, D.D. et al. (1 more author) (2015) Stomatal density and aperture in non-vascular land plants are non-responsive to above-ambient atmospheric CO2 concentrations. Annals of Botany, 115 (6). 915 - 922.

https://doi.org/10.1093/aob/mcv021

Stomatal density and aperture in non-vascular land plants are non-responsive to above-ambient atmospheric CO_2 concentrations

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Short title: Stomata and CO_2 in bryophytes

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Abstract

• *Background and Aims:* Following the consensus view for unitary origin and conserved function of stomata across over 400 million years of land plant evolution, stomatal abundance has been widely used to reconstruct palaeo-atmospheric environments. However, the responsiveness of stomata in mosses and hornworts, the most basal stomate lineages of extant land plants, has received relatively little attention. We aimed to redress this imbalance and provide the first direct evidence of bryophyte stomatal responsiveness to atmospheric CO_2.

• *Methods:* We grew a selection of hornwort (*Anthoceros punctatus*, *Phaeoceros laevis*) and moss sporophytes (*Polytrichum juniperinum*, *Mnium hornum*, *Funaria hygrometrica*) with contrasting stomatal morphologies under different atmospheric CO_2 concentrations ($a[CO_2]$) representing both modern (440 ppm CO_2) and ancient (1,500 ppm CO_2) atmospheres. Upon sporophyte maturation, stomata from each bryophyte species studied were imaged, measured and quantified.

• *Key Results:* We show that densities and dimensions are unaffected by changes in $a[CO_2]$, other than a slight increase in stomatal density in *Funaria* and abnormalities in *Polytrichum* stomata under elevated $a[CO_2]$.

• *Conclusions:* The changes to stomata in *Funaria* and *Polytrichum* are attributed to differential growth of the sporophytes rather than stomata-specific responses. The absence of responses to changes in $a[CO_2]$ in bryophytes is in line with findings previously reported in other early lineages of vascular plants. Our findings strengthen the hypothesis of an incremental acquisition of stomatal regulatory processes through land plant evolution and warn considerable caution in using stomatal densities as proxies for paleo-atmospheric CO_2 concentrations.

Key words: bryophytes, carbon dioxide, evolution, hornworts, mosses, stomata

Introduction

Stomata are considered one of the crucial adaptations in the evolution of the land flora and the development of the terrestrial landscape and atmosphere on Earth. These microscopic pores on the plant epidermis first appeared in the fossil record more than 400 million years ago, some 50-60 million years after the first land plants (Edwards *et al.*, 1998). Today they are found on the sporophyte generation of all land plant groups with exceptions only in the liverworts, the earliest moss lineages and a few derived hornwort clades (Fig. 1). Exposure to high a[CO_2] has been shown to consistently result in a reduction of stomatal density (number of stomata per mm^2) and index (the ratio of stomata to epidermal cells) in the newly developed leaves of many vascular plant species (Woodward, 1987; Beerling *et al.*, 1998). Today, the general consensus view is that stomatal morphology is conserved throughout land plants (Edwards *et al.,* 1998) and that their primary function is related to the regulation of gas and water exchange with atmospheric CO_2 concentrations (a[CO_2]) being a key activator of stomatal frequency (Woodward, 1987; Woodward & Bazzaz, 1988). This is in line with Francis Darwin's observations of more than a century ago (Darwin, 1898). As such, stomatal abundance in the fossilised remains of ancient plants has been widely used as a proxy to reconstruct palaeo-atmospheric a[CO_2] (e.g. Beerling *et al.*, 1995; Beerling & Woodward, 1997; Berner, 1998; Beerling *et al.*, 2001; Beerling & Royer, 2002). However, it should be noted that, though the size of stomata in angiosperms, commonly measured as guard cell length, has been shown to be positively correlated with genome size (Beaulieu *et al.,* 2008), a similar relationship is absent in bryophytes (Table 1).

The stomatal response to a[CO_2] is developmental, involving long-distance signalling from mature to new leaves (Lake *et al.*, 2001; Lake *et al.,* 2002). This serves to maximise water use efficiency under high a[CO_2] and to ensure optimal photosynthesis (Brodribb & McAdam, 2013). Some groups of land plants however, including cycads, other gymnosperms and ferns have recently been found to be unresponsive to such changes in a[CO_2] (Brodribb *et al.*, 2009; Haworth *et al.*, 2011). To date, similar studies examining stomatal responses to a[CO_2], including those relevant to plant evolutionary timescales (e.g. Berner, 2006), across a suite of non-vascular plants are missing. However it is widely assumed that stomata have evolved once and their functioning and regulation were conserved from mosses through to angiosperms (Franks & Beerling, 2009). Underpinning these tenets of structural and

functional congruence for the early evolution of the stomatal 'toolkit' some 400 million years ago, are recent demonstrations in the moss *Physcomitrella* (Chater *et al.*, 2011) and the lycopod *Selaginella* (Ruszala *et al.,* 2011) of the same mechanisms actively regulating stomatal movements as those found in angiosperms, particularly pore closure responses to the plant hormone abscisic acid (ABA). This hormone is also associated with desiccation tolerance in several plant groups, including the mosses (Bopp & Werner, 1993; Mayaba *et al.*, 2001; Stark *et al*., 2007) and has been shown to initiate stomatal closure under elevated a[CO_2] (Chater *et al.*, 2014). The discovery that numerous stomatal genes, including those determining density, are common to both vascular plants and mosses (Chater *et al.*, 2011) further supports the hypothesis that the first stomata to evolve more than 400 million years ago in non-vascular plants were analogous to their modern angiosperm counterparts both in function and in their active regulation via ABA-mediated opening and closing. Counter to this, based on their findings that stomata in a group of six ferns and a lycophyte do not respond to ABA by closure of stomatal pores, Brodribb & McAdam raised the hypothesis that "early-diverging clades of vascular land plants may preserve an ancestral stomatal behaviour that predates much of the complexity present in angiosperm stomatal responses" (Brodribb & McAdam, 2011). Their findings support this, suggesting that ABA-mediated "active" control of stomata is likely to have evolved after the divergence of the ferns and lycophytes.

However, a crucial missing piece in the jigsaw of stomatal evolution and function is the responsiveness of bryophyte stomata to a[CO_2]. In the only previous studies on the effects of a[CO_2] on stomata in non-vascular plants, Chater *et al.* (2011) recorded larger apertures in the mosses *Physcomitrella* and *Funaria* grown in the absence of CO_2. Baars & Edwards (2008) reported a decrease in stomatal size and density in *Leptobryum pyriforme,* but absolute number of stomata per capsule remained the same at 10 times ambient a[CO_2]. These results were interpreted as general growth responses with stomatal numbers pre-programmed in the closed determinate development of moss sporophytes and highlight the need for further studies on a range of taxa (Baars & Edwards, 2008). Such developmental constraints are absent in hornworts, where stomata are produced continuously from derivatives of the sporophyte meristem (Pressel *et al.,* 2014). The responsiveness of hornwort stomata to a[CO_2] potentially holds particular significance given their placement as sister group to all vascular land plants in a study by Wickett *et al.* (2014) and contrary

to their position as sister to vascular plants in other phylogenies (Qiu *et al*., 2006, 2007; Liu *et al*., 2014). It must therefore be underlined that considerable uncertainty still remains as to the precise relationships between the bryophyte groups at the foot of the land plant tree.

Previous studies have suggested hornwort stomata close in response to environmental stimuli and exogenous application of ABA (Hartung *et al*., 1987; Bopp & Werner, 1993; Hartung, 2010). Conversely, it has also been reported that application of ABA does not elicit stomatal closure in hornworts (Lucas & Renzaglia, 2002). As such, function and CO_2 responsiveness of stomata in hornworts, in addition to mosses, and their significance in the evolution of active control of stomata in land plants, remains unclear. In the present study, we redress this imbalance and challenge the widely-accepted dogma that the responsiveness of stomata to a[CO_2] in terms of density and opening is conserved across the land plant phylogeny through careful experimentation and cytological observation. Specifically, we address the following questions:

(1) Are stomatal numbers on moss and hornwort sporophytes affected in the same way by elevated a[CO_2] representative of atmospheric concentrations in the Palaeozoic (Berner, 2006) as those in angiosperms?

(2) Do guard cell lengths and apertures in bryophytes change when subjected to representative Palaeozoic a[CO_2] throughout development?

Materials and Methods

Plant materials and growth

Wild plants from the same population of three mosses (*Polytrichum juniperinum*, *Mnium hornum*, *Funaria hygrometrica*) with young sporophytes prior to capsule expansion and two hornworts (*Anthoceros puctatus*, *Phaeoceros laevis*) with sporophytes protruding just 1-2 mm above the tops of the involucres, were collected in southern England between early January and late March 2014. Extensive observations on the hornwort populations for studies on stomatal differentiation (Pressel *et al.,* 2014) indicate that there is little or no variation between clones. The three mosses were selected to include: 1) species with sufficient numbers of stomata/sporophyte to allow a 10-15% or more change in number to be readily

detected (*Physcomitrella* with only 14 stomata is unsuitable), 2) *Polytrichum,* the sister group to all other stomata-producing mosses, 3) stomata with apertures of different sizes and shapes including free-floating apertures (i.e. the single guard cells in *Funaria;* see Fig. 1) and 4) contrasting stomatal configurations: superficial versus sunken and whether or not they are covered by a calyptra (see Table 1). Vouchers of all the specimens are housed in the Natural History Museum, London.

Wild-collected plants were transferred into seed trays filled with inert acid-washed silica sand within controlled growth environment chambers (BDR16, Conviron, Canada) prior to sporophyte development. Plants were maintained under the following conditions throughout development of the sporophyte: 50 μmol m^{-2} s^{-1} (representing half light-saturating conditions for non-vascular plants; Nobel, 1999; Fletcher *et al.,* 2006), 70% relative humidity, 15 °C : 12 °C day : night temperatures and a 12-h day length. Atmospheric CO_2 concentrations within the growth chambers were monitored using CARBOCAP GMP343 CO_2 sensors (Vaisala, UK) and maintained through gaseous CO_2 addition. Experimental plants were grown either at 440 ppm a[CO_2] (*Funaria hygrometrica* n = 30, *Mnium hornum n* = 50, *Polytrichum juniperinum n* = 50, *Phaeoceros laevis n* = 95, *Anthoceros punctatus n* = 30), or at 1,500 ppm a[CO_2] (*Funaria hygrometrica* n = 30, *M. hornum n* = 49, *P. juniperinum n* = 50, *P. laevis n* = 50, *A. punctatus n* = 30) and were rotated within cabinets regularly. All plants were misted daily with an artificial rainwater solution (see SI). Both cabinets and contents were alternated every two weeks to avoid pseudo-replication. The three mosses were harvested after their capsules had become fully expanded with late stage sporogenous cells to mature spores, whilst the hornworts were harvested after their sporophytes had reached at least 2 cm in length.

Stomatal measurements

Sporophytes of all experimental plants, except those of *Mnium*, were cut longitudinally, mounted in water on slides with the external surfaces uppermost. In *Mnium* it is impossible to obtain accurate measurements from surface views as many of the deeply sunken stomata are obscured by the overarching epidermal cells; in this species the sporophytes were cut into quarters longitudinally, the spongy photosynthetic tissues scraped away and measurements taken from the inside view (Fig. 3f, g). Stomata were imaged with a Zeiss Axioscop 2 microscope equipped with an AxioCam MRc digital camera and numbers, aperture width and length and guard

cell length were measured using the autocalibrated Axiovision Microscope Software. For mosses, every stoma/sporophyte was measured; for hornworts, every stoma from the first 1cm of the sporophytes immediately above the top of the involucres was measured (see Pressel *et al.* 2014) ensuring that all the stomata measured had developed well after the start of the elevated a[CO_2] treatment. Stomata of a selection of wild collected plants were also imaged by cryo-scanning electron microscopy using the method of Duckett *et al*. (2009).

Statistics

Effects of plant species and $[CO_2]_a$ on stomatal abundance and aperture were tested using two-way ANOVA with post-hoc Tukey testing where indicated. Data were checked for normality and homogeneity of variance prior to ANOVA. Student's T-tests were performed where indicated in text. All statistics were carried out using Minitab v 12.21 (Minitab Inc., USA).

Results

Stomatal abundance

We observed no differences in stomatal abundance on sporophytes of the mosses *Mnium hornum* and *Polytrichum juniperinum* or in the hornworts *Phaeoceros laevis* and *Anthoceros puncutatus* when grown under 440 ppm a[CO_2] or 1,500 ppm [CO_2] (Fig. 2a). There was a small increase in stomatal abundance of *Funaria hygrometrica* sporophytes that underwent development at 1,500 ppm a[CO_2] compared to those at ambient a[CO_2] [two-sample t (27 df) = -4.17, P = 0.0003] (Fig. 2a).

Stomatal aperture

The stomatal aperture of *P. juniperinum* was significantly larger in sporophytes that underwent development at 1,500 ppm a[CO_2] compared to those that matured under 440 ppm a[CO_2] [two-sample t (72 df) = -4.31, P = 0.0001] (Fig. 2b). There were no differences in stomatal aperture of any of the other moss or hornwort species examined (Fig. 2b).

Guard cell length

There were no significant differences in guard cell length between a[CO_2] in the mosses *M. hornum* and *F. hygrometrica* or the hornworts *P. laevis* and *A. punctatus* (Fig. 2c). Guard cell length was reduced in in *P. juniperinum* sporophytes that underwent development at 1,500 ppm a[CO_2].

Discussion

Our results unequivocally demonstrate that stomata on the sporophytes of several extant species of mosses and hornworts are non-responsive to changes in a[CO_2] in terms of stomatal numbers, guard cell length and of stomatal aperture dimensions (Fig. 2, Table 2).

The small numerical increase in stomatal abundance of *Funaria* sporophytes that underwent development at 1,500 ppm a[CO_2] (Fig. 2a) is contrary to the reduction in stomatal abundance that would be expected if bryophyte stomata were analogous to angiosperms in their a[CO_2] response. The increase in stomatal abundance in *Funaria* is opposite to the small decrease reported previously in *Leptobryum* (Baars & Edwards, 2008), although in this moss the lower stomatal abundance was elicited by a ten-fold increase in ambient a[CO_2] and was due to an increase in capsule length with the overall number of stomata per capsule in fact remaining unaltered (Baars & Edwards, 2008). The significantly larger apertures in *Polyrichum juniperinum* sporophytes grown under elevated a[CO_2] (Fig. 2b) are also contrary to expectation and, together with the misaligned and abnormal stomata recorded in this moss under 1,500 ppm a[CO_2] (Fig. 3l-q) are almost certainly the result of slightly altered sporophyte development, as also seen in *Leptobryum* (Baars & Edwards, 2008). Whereas under 440 ppm a[CO_2] individual sporophytes of *Polytrichum juniperinum usually* have from 1-3 stomata with 3 or 4 guard cells (Fig. 3h-k), under 1,500 ppm a[CO_2] we found many more occurring as groups and up to 25% with abnormal guard cells. The teratologies included misplaced, extra and incomplete walls dividing the guard cells (Fig. 3l-q). These malformations recall the asymmetrical stomata along the dehiscence grooves in hornworts, attributed to differential cell expansion (Pressel *et al.,* 2014). Indeed, CO_2-induced changes in epidermal cell growth patterns, perhaps as a consequence of increased carbon assimilation, are the simplest explanation for the stomatal abnormalities observed in *Polytrichum* under elevated a[CO_2].

Our results are in line with those of some previous studies on lycophytes and ferns showing that these basal groups of vascular plants lack aperture closure responses to a[CO_2] (Brodribb & McAdam, 2013). These findings and the recent demonstration that angiosperms are the only group of land plants that utilise calcium-based signalling pathways led Brodribb and McAdam (2011) to argue for an incremental acquisition of stomatal regulatory processes. This is contrary to the perhaps more widely-held view based on a large body of physiological and molecular evidence, that these are evolutionary ancient and that physiologically active stomatal control evolved before the divergence of the bryophytes (Brodribb & McAdam, 2011; Chater *et al.*, 2011; Ruszala *et al.,* 2011). Another possible scenario for evolution of stomatal functionality is neofunctionalisation following whole-genome replication given that ABA is also associated with desiccation tolerance in mosses (Bopp & Werner, 1993; Mayaba *et al*., 2001; Stark *et al.,* 2007).

Even more problematic for the notion of stomatal functional continuity across land plants is the lack of stomata in the basal moss lineages (Fig. 1). These are absent in Takakiopsida and Andreaeopsida (here, as in liverworts, dehiscence is via splitting of lidless capsules) and the paired cells adorning the capsules in *Sphagnum* are now regarded as pseudostomata since they are enclosed by the calyptra until maturation of the sporophytes and lack both open pores and subjacent intercellular spaces (Duckett *et al.,* 2009). Their primary role appears to be facilitation of capsule desiccation leading to spore discharge rather than regulation of gaseous exchange.

Whereas in vascular plants stomatal densities and numbers make sense in terms of their regulatory role, the same is not true of mosses where numbers (and absences) differ enormously even between closely related genera with very similar ecologies (Table 1, Fig. 3a-d, h). Equally perplexing is the absence of any relationship in bryophytes between stomatal dimensions and genome sizes. Thus, hornworts have some of the largest stomata and the smallest genome sizes. Those of *Mnium hornum* are larger than in *Plagiomnium cuspidatum* despite a smaller genome size and the same is true between *Funaria* and *Physcomitrella* (Voglmayr, 2000; Renzaglia *et al.,* 1995; Rensing *et al.,* 2008) and the two *Polytrichum* species (Table 1). Similarly there are wide disparities between closely related taxa in pore shapes, the presence or absence of subsidiary cells and stomatal orientation (Table 1, Fig. 3a-h).

A further question mark over an active regulatory role and a significant contribution of CO_2 ingress through the pores to sporophyte nutrition in mosses is that

304 they open only when the sporophytes have almost reached their full dimensions, i.e.
305 the bulk of their carbon must have been acquired either from the parent gametophytes
306 via the placenta (Ligrone *et al*., 1993) or directly through the epidermal cells. Added
307 to this are further complications;- 1) the stomata in many mosses are either covered
308 by the calyptra until sporophyte maturation or are tightly enveloped by perichaetial
309 leaves (e.g. *Physcomitrella*); 2) unlike the exponential water loss from drying out
310 gametophytes moss sporophytes lose water very slowly whether or not they possess
311 stomata; 3) though Chater *et al.* (2011) state that the stomata in *Physcomitrella* and
312 *Funaria* close in response to various stimuli their data actually show only small
313 changes in aperture dimensions unlike the complete closures seen in vascular plants.
314 That stomatal responsiveness to environmental cues in these two mosses is restricted
315 to the developmental stage when green capsules are expanding (Chater *et al.,* 2011) is
316 in line with a recent study showing that, in *Funaria*, guard cell walls are thin and
317 flexible soon after pore formation and that a decrease in pectin content coupled with
318 changes in wall architecture during development renders mature stomata immobile
319 (Merced & Renzaglia, 2014). However, the discovery that the intercellular spaces in
320 moss and hornwort sporophytes, unlike those in vascular plants, are liquid-filled until
321 well after the stomata open (Pressel *et al.,* 2014) (see also Fig. 3e) casts serious doubt
322 on any role of young stomata in active regulation of gaseous exchange. It should also
323 be underlined that there has never been an unequivocal demonstration of reversible
324 aperture changes in peristomate mosses and the possible presence of potassium fluxes
325 between the guard cells and their neighbours has yet to be investigated. Taking all
326 these factors into account sporophyte desiccation rather than gas regulation seems the
327 more likely primary role for moss stomata. Indeed their location in most mosses
328 around the base of the capsule seems more fitted for removal of water ascending the
329 setae than provision of CO_2.
330 Many of these arguments are equally applicable to hornworts, following their
331 wide acceptance as the sister group to vascular plants (Wickett *et al*., 2014). Any case
332 for stomatal structural and functional continuity needs to explain the plastid-
333 determined division of the guard mother cells, inelastic guard cell walls and initially
334 liquid-filled sporophytic intercellular spaces in hornworts (Pressel *et al*., 2014). It
335 should also be underlined that a new configuration of the land plant tree based on
336 phylotranscriptomics (Wicketts *et al*., 2014) places hornworts at the base of the land
337 plant tree, although this placement is currently under debate. Implicit in this new

phylogeny, very different from liverworts sister to all other land plants (Chang & Graham, 2011; Qiu *et al.,* 1998; Gao *et al.,* 2010) and liverworts, mosses, and hornworts as successive sister groups to vascular plants (Liu *et al.,* 2014; Qiu *et al.,* 1998; 2006; 2007), is the loss of stomata in liverworts and their reacquisition in mosses.

Given these major issues about developmental, functional and evolutionary continuity between bryophyte and vascular plant stomata, our failure to detect any responses to elevated a[CO_2] is not surprising. This pattern of stomatal non-reponsiveness to a[CO_2] is likely to extend into other early non-vascular and vascular land plant lineages. Our findings lend further support to the hypothesis that active stomatal regulation and a[CO_2] responsiveness occurred later in the evolution of land plants (Brodribb & McAdam, 2011). As such, our data warn considerable caution in using stomatal densities as proxies for past paleo-atmospheric CO_2 concentrations where extant counterparts are not available.

Figure legends

Figure 1. Single phylogram scenario illustrating key land plant lineages (bold text) and moss genera and the appearance of stomata in modern plants. Dashed lines indicate absence of stomata and solid black lines illustrate their presence. This phylogram with liverworts basal (Qiu *et al.,* 2006, 2007; Liu *et al.,* 2014) indicates a single origin of stomata and multiple losses whereas an alternative topology with hornworts basal implies multiple origins (Wickett *et al.,* 2014; Haig, 2013).

Figure 2(a) Stomatal abundance on sporophyte generations of plants studied grown under 440 ppm a[CO_2] (grey bars) and a replicated Palaeozoic a[CO_2] of 1,500 ppm (white bars). Error bars show ± 1 S.E., different letters denote statistical significance where $P < 0.05$ (ANOVA, post-hoc Tukey test) $n = 50$ (*M. h.*), 50 (*P. j.*), 15 (*F. h.*), 50 (*P. l.*), 30 (*A. p.*) **(b)** Mean aperture of stomatal pores on 5 individual sporophytes of each non-vascular species studied. Sporophytes have all undergone complete development at 440 ppm a[CO_2] (grey bars) or 1,500 ppm a[CO_2] (white bars). ± 1 S.E., $n = 5$. Different letters indicate where $P < 0.05$ (ANOVA, post-hoc Tukey test) **(c)** Mean length of guard cells measured on 5 individual sporophytes of each species of bryophyte.

Figure 3. Light (d, f, g, i-q) and cryo-scanning electron micrographs (a-c, h, r) of moss and hornwort stomata. (a, b) *Physcomitrella patens*: 12-14 stomata slightly

372 irregularly spaced (e.g. the paired stomata in b) and randomly orientated around the
373 capsule base; pores round and subsidiary cells absent. (c, d) In the closely related
374 *Funaria hygrometrica* the numerous stomata are axially-orientated and regularly
375 spaced. Also note the radial arrangement of the epidermal cells around the long-pored
376 stomata (d), cf. hornworts (r). (e-g) *Mnium hornum* stomata sunken in deep pits.
377 Note the liquid-filled subtending intercellular spaces (*) in (e). Stomata are often
378 irregularly spaced (see the paired stomata in (h)) and have small round pores (f, g). (h-
379 q) *Polytrichum juniperinum* (h-k, grown at 440 ppm a[CO_2]; l-q grown at 1,500 ppm
380 a[CO_2]). Note the predominately axially arranged long-pored stomata frequently
381 occurring in multiple groups (h-k). Abnormalities occur on almost all sporophytes and
382 these increase under elevated CO_2 as does the size of some of the apertures (l-q). (j) A
383 pair of stomata with a shared pore. (m-p) Stomata with abnormal pores. (o) Stoma
384 with massive aperture. (p) Stoma with 4 guard cells. (r) Sporophyte of the hornwort
385 *Anthoceros puctuatus* . Note the regularly spaced axial stomata lacking subsidiary
386 cells. Scale bars: (c, h, r) 200µm; (a) 100µm; (d-g, i-q) 50µm; (b) 20µm

Literature Cited

Baars C, Edwards D. 2008. Effects of elevated atmospheric CO_2 on spore capsules of the moss *Leptobryum pyriforme*. *Journal of Bryology* **30:** 36-40.

Bainard JD, Villarreal JC. 2013. Genome size increases in recently diverged hornwort clades. *Genome* **56:** 431-435.

Beerling DJ, Birks HH, Woodward FI. 1995. Rapid late-glacial atmospheric CO_2 changes reconstructed from the stomatal density record of fossil leaves. *Journal of Quaternary Science* **10:** 379-384.

Beerling DJ, McElwain JC, Osborne CP. 1998. Stomatal responses of the 'living fossil' *Ginkgo biloba* L. to changes in atmospheric CO_2 concentrations. *Journal of Experimental Botany* **49:** 1603-1607.

Beerling DJ, Osborne CP, Chaloner WG. 2001. Evolution of leaf-form in land plants linked to atmospheric CO_2 decline in the Late Palaeozoic era. *Nature* **410:** 352-354.

Beerling DJ, Royer DL. 2002. Reading a CO_2 signal from fossil stomata. *New Phytologist* **153:** 387-397.

Beerling DJ, Woodward FI. 1997. Changes in land plant function over the Phanerozoic: Reconstructions based on the fossil record. *Botanical Journal of the Linnean Society* **124:** 137-153.

Berner RA. 1998. The carbon cycle and CO_2 over Phanerozoic time: the role of land plants. *Philosophical Transactions of the Royal Society of London Series B-Biological Science*s **353:** 75-81.

Berner RA. 2006. GEOCARBSULF: A combined model for Phanerozoic atmospheric O_2 and CO_2. *Gemochimica et Cosmochimica Acta* **70:** 5653-5664

Beaulieu JM, Leitch IJ, Patel S, Pendharkar A, & Knight CA .**2008.** Genome size is a strong predictor of cell size and stomatal density in angiosperms. *New Phytologist* **179:** 975-986.

Bopp M, Werner O. 1993. Abscisic acid and desiccation tolerance in mosses. *Botanica Acta* **106:** 103-106.

Brodribb TJ, McAdam SA. 2011. Passive origins of stomatal control in vascular plants. *Science* **331:** 582-585.

Brodribb TJ, McAdam SA. **2013.** Unique responsiveness of angiosperm stomata to elevated CO_2 explained by calcium signalling. *PLOS ONE* **8:** e82057.

Brodribb TJ, McAdam SA, Jordan GJ, Feild TS. 2009. Evolution of stomatal responsiveness to CO_2 and optimization of water-use efficiency among land plants. *New Phytologist* **183:** 839-847.

Chang Y, Graham S. 2011. Inferring the higher-order phylogeny of mosses (Bryophyta) and relatives using a large, multigene plastid data set. *American Journal of Botany* **98:** 839-849.

Chater C, Kamisugi Y, Movahedi M *et al.* 2011. Regulatory mechanism controlling stomatal behavior conserved across 400 million years of land plant evolution. *Current Biology* **21:** 1025-1029.

Chater C, Oliver J, Casson SA, Gray JE. 2014. Putting the brakes on: abscisic acid as a central environmental regulator of stomatal development. *New Phytologist* **202:** 376-391.

Darwin F. 1898. Observations on stomata. *Proceedings of the Royal Society of London* **63:** 413-417.

Duckett JG, Pressel S, P'ng KM, Renzaglia KS. 2009. Exploding a myth: the capsule dehiscence mechanism and the function of pseudostomata in *Sphagnum*. *New Phytologist* **183:** 1053-1063.

Edwards D, Kerp H, Hass H. 1998. Stomata in early land plants: an anatomical and ecophysiological approach. *Journal of Experimental Botany* **49:** 255-278.

Fletcher BJ, Brentnall SJ, Quick WP, Beerling DJ. 2006. BRYOCARB: A process-based model of thallose liverwort carbon isotope fractionation in response to CO_2, O_2, light and temperature. *Geochimica et Cosmochimica Acta* **70:** 5676-5691.

Franks PJ, Beerling DJ. 2009. Maximum leaf conductance driven by CO_2 effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences USA* **106:** 10343-10347.

Haig D. 2013. Filial mistletoes: the functional morphology of moss sporophytes. *Annals of Botany*, **111:** 337–345.

Hartung W. 2010. The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen. *Functional Plant Biology* **37:** 806-812.

Hartung W, Weiler E, Volk O. 1987. Immunochemical evidence that abscisic acid is produced by several species of Anthocerotae and Marchantiales. *Bryologist* **90:** 393-400.

Haworth M, Fitzgerald A, McElwain JC. 2011. Cycads show no stomatal-density and index response to elevated carbon dioxide and subambient oxygen. *Australian Journal of Botany* **59:** 630-639.

Gao L, SU YJ, Wang T. 2010. Plastid genome sequencing, comparative genomics, and phylogenomics: current status and prospects. *Journal of Systematics and Evolution* **48:** 77-93.

Lake JA, Quick WP, Beerling DJ, Woodward FI. 2001. Plant development: Signals from mature to new leaves. *Nature* **411:** 154-154.

Lake JA, Woodward FI, Quick WP. 2002. Long-distance CO_2 signalling in plants. *Journal of Experimental Botany* **53:** 183-193.

Ligrone R, Duckett, JG, Renzaglia KS. 1993. The gametophyte-sporophyte junction in land plants. *Advances in Botanical Research* **19:** 231-318.

Liu Y, Cox CJ, Wang W, Goffinet B. 2014. Mitochondrial phylogenomics of early land plants: Mitigating the effects of saturation, compositional heterogeneity, and codon-usage bias. *Systematic biology*, **63:** 862-878.

Lucas JR, Renzaglia KS. 2002. Structure and function of hornwort stomata. *Microscopy and Microanalysis* **8:** 1090-1091.

Mayaba N, Beckett RP, Csintalan Z, Tuba Z. 2001. ABA increases the desiccation tolerance of photosynthesis in the afromontane understorey moss *Atrichum androgynum*. *Annals of Botany* **8:** 1093-1100.

Merced A, Renzaglia K. 2014. Developmental changes in guard cell wall structure and pectin composition in the moss *Funaria*: implications for function and evolution of stomata. *Annals of Botany* **114:** 1001-1010.

Nobel, PS. 1999.*Physicochemical and environmental plant physiology*. Academic Press, London.

Pressel S, Goral T, Duckett JG. 2014. Stomatal differentiation and abnormal stomata in hornworts. *Journal of Bryology* **36:** 87-103.

Qiu, YL, Cho Y, Cox JC. & Palmer JD. 1998. The gain of three mitochondrial introns identifies liverworts as the earliest land plants. *Nature* **394:** 671-674.

Qiu Y-L, Li L, Wang B *et al*. 2006. The deepest divergences in land plants inferred from phylogenomic evidence. *Proceedings of the National Academy of Sciences USA* **103:** 15511-15516.

Qiu YL, Li L, Wang B *et al*. 2007. A nonflowering land plant phylogeny inferred from nucleotide sequences of seven chloroplast, mitochondrial, and nuclear genes. *International Journal of Plant Sciences* **168:** 691-708.

Rensing SA, Lang D, Zimmer AD *et al.* 2008. The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* **319:** 64-69.

Renzaglia KS, Rasch EM, & Pike LM. 1995. Estimates of nuclear DNA content in bryophyte sperm cells: phylogenetic considerations. *American Journal of Botany* **82:** 18-25.

Ruszala EM, Beerling DJ, Franks PJ. *et al.* 2011. Land plants acquired active stomatal control early in their evolutionary history. *Current Biology* **21:** 1030-1035.

Stark LR, Oliver MJ, Mishler BD, McLetchie DN. 2007. Generational differences in response to desiccation stress in the desert moss *Tortula inermis*. *Annals of Botany* **99:** 53–60.

Temsch EM, Greilhuber J, Krisai R. 1998. Genome size in *Sphagnum* (peat moss). *Botanica Acta* 1**11:** 325-330.

Voglmayr H. 2000. Nuclear DNA amounts in mosses (Musci). *Annals of Botany* **85:** 531-546.

Wickett NJ, Mirarab S, Nguyen N, *et al*. 2014. Phylotranscriptomic analysis of the origin and early diversification of land plants. *Proceedings of the National Academy of Sciences USA***111:** E4859-E4868.

Woodward F, Bazzaz F. 1988. The responses of stomatal density to CO_2 partial pressure. *Journal of Experimental Botany* **39:** 1771-1781.

Woodward FI. 1987. Stomatal numbers are sensitive to increases in CO_2 from pre-industrial levels. *Nature* **327:** 617-618.

Acknowledgments

We thank Dr Kate Allinson for technical assistance. We also thank the editor and three anonymous reviewers for their helpful comments on this manuscript.

Funding Information

This work was supported by a Royal Society University Research Fellowship to DDC and a Leverhulme Emeritus Fellowship to JGD.