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Hornwort stomata do not respond actively to exogenous and environmental cues

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• **Backgrounds and Aims** Because stomata in bryophytes occur on sporangia, they are subject to different developmental and evolutionary constraints from those on leaves of tracheophytes. No conclusive experimental evidence exists on the responses of hornwort stomata to exogenous stimulation.
• **Methods** Responses of hornwort stomata to abscisic acid (ABA), desiccation, darkness and plasmolysis were compared with those in tracheophyte leaves. Potassium ion concentrations in the guard cells and adjacent cells were analysed by X-ray microanalysis, and the ontogeny of the sporophytic intercellular spaces was compared with those of tracheophytes by cryo-scanning electron microscopy.
• **Key Results** The apertures in hornwort stomata open early in development and thereafter remain open. In hornworts, the experimental treatments, based on measurements of >9000 stomata, produced only a slight reduction in aperture dimensions after desiccation and plasmolysis, and no changes following ABA treatments and darkness. In tracheophytes, all these treatments resulted in complete stomatal closure. Potassium concentrations are similar in hornwort guard cells and epidermal cells under all treatments at all times. The small changes in hornwort stomatal dimensions in response to desiccation and plasmolysis are probably mechanical and/or stress responses of all the epidermal and spongy chlorophyllose cells, affecting the guard cells. In contrast to their nascent gas-filled counterparts across tracheophytes, sporophytic intercellular spaces in hornworts are initially liquid filled.
• **Conclusions** Our experiments demonstrate a lack of physiological regulation of opening and closing of stomata in hornworts compared with tracheophytes, and support accumulating developmental and structural evidence that stomata in hornworts are primarily involved in sporophyte desiccation and spore discharge rather than the regulation of photosynthesis-related gaseous exchange. Our results run counter to the notion of the early acquisition of active control of stomatal movements in bryophytes as proposed from previous experiments on mosses.

Key words: Abscisic acid, dehiscence, evolution of land plants, potassium flux, plasmolysis, spore discharge, stomata, sporangia, X-ray microanalysis.

INTRODUCTION

Stomata are a key innovation that enabled freshwater algae to colonize Earth's land masses >500 million years ago (Morris *et al.*, 2018). A long-held hypothesis posits that stomata evolved once in the common ancestor of land plants and that their role and regulation are conserved across all land plant lineages (Raven, 2002). However, this major assumption has been based on scarce or no structural and functional evidence for phylogenetically key groups at the base of the land plant tree: bryophytes (hornworts, mosses), the most basal clade, and pteridophytes (lycophytes, ferns), the sister group to the seed plants. Recent studies focusing on these groups have presented conflicting evidence either supporting the evolutionary theory of early acquisition of active stomatal control (Chater *et al.*, 2011, 2013, 2017; Ruszala *et al.*, 2011; Xu *et al.*, 2016; Hõrak *et al.*, 2017; Merilo *et al.*, 2017) or rejecting it in favour of the alternative hypothesis of gradual acquisition of active control mechanisms (Brodribb *et al.*, 2009; Brodribb and McAdam, 2011, 2013, 2017; Pressel *et al.*, 2014; Field *et al.*, 2015;

Villarreal and Renzaglia, 2015; Sussmilch *et al.*, 2017; Duckett and Pressel, 2017a; Merced and Renzaglia, 2017; Renzaglia *et al.*, 2017). Following recent demonstrations of key stomatal developmental stages unique to bryophytes (Pressel *et al.*, 2014; Villarreal and Renzaglia, 2015; Merced and Renzaglia, 2017), here we investigate stomatal behaviour in hornworts – the bryophyte clade currently considered sister to the vascular plants (Liu *et al.*, 2014; Qiu *et al.*, 2006, 2007).

Stomata occur in all embryophyte groups except liverworts. Unlike tracheophytes where stomata abound on vegetative organs, especially leaves, of the sporophyte, those in bryophytes are restricted to the sporangia. As monosporangiates, bryophytes are characterized by the occurrence of a single sporangium on a matrotrophic sporophyte (Ligrone *et al.*, 1993). Undoubtedly, stomata on these sporangia have experienced a suite of evolutionary constraints independent from those in tracheophytes, potentially leading to fundamental differences in structure and function across these groups. The hornwort sporophyte is unparalleled in any living or extinct group in that it is an elongating sporangium with asynchronous meiosis. Such a

system requires precise co-ordination of all portions of the sporophyte to ensure protection of all stages, from meristematic cells to dehiscence of mature spores. Much like the apical meristem of vegetative organs, it affords a dynamic experimental system in which to examine spatial and temporal changes in a static structure. It is perplexing therefore that, in spite of the multitude of physiological studies on tracheophyte stomata, no conclusive analysis has been conducted on the regulation of stomatal function in hornworts.

Conflicting reports exist on regulation of movement in hornwort stomata. Responses to environmental stimuli and exogenous abscisic acid (ABA) were reported by Bopp and Werner (1993), Hartung (2010) and Hartung *et al.* (1987, 1994). However, Lucas and Renzaglia (2002) found no evidence for stomatal closure in response to ABA or darkness, with some response to desiccation, in line with previous studies by Paton and Pierce (1957). Staining in the guard cells (GCs) with Fast Violet B was associated with pore opening, thus suggesting organic acid accumulation (e.g. malate), but cobaltous nitrate staining for potassium produced equivocal results between open and closed stomata. Lucas and Renzaglia (2002) concluded that hornwort stomata open only once, through osmotic changes, thereafter remaining open with a dual role in providing a passageway for photosynthesis-based gaseous exchange and facilitating sporophyte dehydration.

Consequent on a recent study of early stomatal ontogeny in hornworts, Pressel *et al.* (2014) came to the same conclusion about function (Lucas and Renzaglia, 2002), a view echoed most recently by Villarreal and Renzaglia (2015), Merced and Renzaglia (2017) and Renzaglia *et al.* (2017). An unexpected discovery by Pressel *et al.* (2014) was that in vascular plants, the schizogenous intercellular spaces associated with stomata are gas filled from their inception whereas those in hornworts are initially fluid filled. Opening of the stomatal pores is a prerequisite for drying the spaces and replacing the liquid with gas. The importance of this process and the ultimate drying of the sporophyte for spore release would seem to preclude any requirement for regulation of water loss through stomatal movements. Further additional pieces in the jigsaw of stomatal function and evolution are that asymmetric stomata along the sporophyte sutures in hornworts, originally considered abnormal (Pressel *et al.*, 2014), are in fact an integral component in the dehiscence mechanism (Villarreal and Renzaglia, 2015) and that, in hornworts and mosses, apertures and numbers do not respond to different atmospheric CO_2 concentrations (Field *et al.*, 2015). In contrast, in vascular plants, species with passive stomatal control have reduced stomatal densities when grown in raised CO_2 (Brodribb *et al.*, 2009; Brodribb and McAdam, 2011, 2013, 2017), whereas those with active regulation respond by aperture reductions (Haworth *et al.*, 2015; Lake *et al.*, 2016). It should also be noted that, whilst some authors strongly promote early acquisition of active control on the basis of changes in stomatal number and density in response to both ABA and CO_2 in ferns and angiosperms under different laboratory growth conditions (Xu *et al.*, 2016; Hõrak *et al.*, 2017; Merilo *et al.*, 2017), more broadly based and critical evaluations by others make a strong case for gradual evolution (Sussmilch *et al.*, 2017; Brodribb and McAdam, 2017).

There are few studies of moss stomatal structure and physiology (Sack and Paolillo, 1983*a*, *b*, *c*, 1985; Duckett *et al.*, 2009; Merced and Renzaglia 2013, 2014; Merced, 2015). In *Funaria hygrometrica* and the closely related model moss *Physcomitrella patens*, the GCs are slightly sensitive to ABA, the fungal toxin fusicoccin that stimulates proton pumping across plant cell membranes, darkness and CO_2 concentrations (Chater *et al.*, 2011). Expanding green capsules of *Funaria* have only a short period of up to 5 d during which stomata are responsive to environmental stimuli (Garner and Paolillo, 1973), following which they remain open if hydrated. Merced and Renzaglia (2014) demonstrated that in *Funaria*, GC walls are thinner, contain higher pectin concentration and are flexible during this period when pores first form. With capsule maturation, GC walls thicken, contain reduced pectin and become less flexible. Stomatal function and homology have been called into question in *Sphagnum*, the sister group to other mosses (Newton *et al.*, 2000; Cox *et al.*, 2004; Shaw and Renzaglia, 2004). In *Sphagnum*, the paired GC-like structures adorning the capsule walls never open into intercellular spaces and there is no evidence of potassium fluxes between these and the adjacent epidermal cells (Beerling and Franks, 2009; Duckett *et al.*, 2009). The conclusion from these and a more recent ultrastructural and immunocytochemical study of wall architecture (Merced, 2015) is that these structures in *Sphagnum* facilitate capsule desiccation leading to spore discharge, but their relationship to true stomata remains an open question. Echoing this notion, Chater *et al.* (2016) state that delayed dehiscence in stomata-less mutants of *Physcomitrella* implies that stomata might have promoted dehiscence in the first complex land-plant sporophytes, though this conclusion is difficult to reconcile with the fact that *Physcomitrella* sporophytes are cleistogamous (McDaniel *et al.*, 2009).

Most recently it has been reported that hornwort stomata are short lived, with the GCs dying prior to drying of other sporophytic cells (Merced and Renzaglia, 2017; Renzaglia *et al.*, 2017). These stomata subsequently collapse and remain locked with the apertures wide open over the sub-stomatal cavity. GC collapse precedes the final dehydration of the sporogenous tissue, which is surrounded by pectin-containing mucilage. Thus, as in mosses, hornwort stomata have a short window of potential physiological response to their environment in the green lower portions of the sporophyte.

Against the background of new information on stomatal and intercellular space development in hornworts, we report here an extensive experimental programme to determine whether or not: (1) aperture dimensions, immediately after initial opening, are subsequently capable of changing in response to the same external factors that affect mosses and vascular plants (i.e. ABA, desiccation, darkness and plasmolysis); and (2) opening is associated with a potassium flux into the GCs.

MATERIALS AND METHODS

Nomenclature of hornworts follows Duff *et al.* (2007) and Glenny (1998).

Electron microscopy observations were made on a range of freshly collected hornwort species: *Anthoceros punctatus* L. [UK]; *Dendroceros javanicus* (Nees) Nees [Peninsular Malaysia]; *Folioceros fuciformis* (Mont.) D.C. [Bharadwaj]; *Notothylas levieri* Schiffn. Ex Steph. [India]; *Phaeoceros carolinianus* (Michx.) Prosk. [USA]; *P. laevis* (L.) Prosk. [Wakehurst Place, Sussex, UK]; and *Phaeomegaceros fimbriatus* (Gottsche)

Duff *et al.* [Panama]. Voucher specimens are in the herbarium at the Natural History Museum, London.

For all the experimental treatments to measure stomatal responses to exogenous and environmental cues (exogenous ABA, desiccation and darkness), we used wild-collected UK plants of *A. punctatus* and *P. laevis*. We also used the same plants to study the effects of loss of turgor pressure in hornwort GCs by plasmolysing these in 1.0 M sucrose. Since plasmolysis in seed plants results in closure of stomata due to loss of turgidity in GCs, open apertures following plasmolysis in hornwort stomata would be indicative of mechanical maintenance of open pores simply via cell wall properties.

The experiments on the wild plants were conducted over a period of 1–3 d after collection, i.e. as quickly as was feasible to record the large numbers of apertures needed. During this time, plants were maintained on their nutrient-poor native substrates in a growth cabinet with a constant irradiance of 100 µmol m^{-2} s^{-1} at 18 °C. We used constant illumination to compensate for the higher irradiances experienced by the plants in nature (typically up to 600–900 µmol m^{-2} s^{-1}).

As well as rigorous hornwort controls and, as a further check on our procedures, during our hornwort experiments we also observed the effects of ABA and sucrose on angiosperms (arabidopsis, *Lactuca* and *Hedera*) kept in the same growth cabinets. Unlike the hornworts, their stomata closed completely.

Because hornwort stomata open at or just above the involucres (Pressel *et al.*, 2014) and moss stomata are considered to be responsive to stimuli for only a few days after pore formation (Chater *et al.*, 2011; Merced and Renzaglia, 2014), plus the recent demonstration that apertures become fixed open on older parts of sporophytes (Renzaglia *et al.*, 2017), we restricted observations to stomata in the first 1 cm of the sporophytes above involucres. As a further precaution to minimize the risk of measuring possible dying or dead stomata, we selected sporophytes at least 3 cm long with the change in colour of the developing spores to yellow or black in *Phaeoceros* and *Anthoceros*, respectively, usually >2.5cm above the involucres. For the ABA treatments and desiccation, measurements were taken from ten sporophytes; for darkness and plasmolysis they were taken from five sporophytes.

Sporophytes were excised from the subjacent thalli level with the bases of the involucres and sliced longitudinally at right angles to the dehiscence grooves. The two halves were mounted in water, epidermis upwards, and digital images were taken of all the stomata using a Zeiss Axioscop 2 microscope equipped with an AxioCam MRc digital camera. Aperture dimensions (width, length and surface area – calculated with the equation for an ellipse) were measured using the associated Axiovision software. For the desiccation treatment, sporophytes were mounted in immersion oil to avoid rehydration. Control (hydrated) sporophytes were also mounted in immersion oil to discern any possible effect of the oil on aperture dimensions. There was no evidence that any of our manipulations led to the death of the GCs as the plasmolysed sporophytes in sucrose rapidly recovered when placed in water.

For the exogenous ABA treatment, we followed the protocol of Hartung (1983) and Hartung *et al.* (1987). Intact sporophytes and others sectioned longitudinally to optimize uptake were exposed to a range of ABA concentrations (100, 10 and 1 µM),

dissolved either in water (unbuffered) or in a medium containing 10 mM KCl in 10 mM MES/NaOH (buffered) (Hartung *et al.*, 1987) for 1, 2, 4 and 24 h in the controlled-environment cabinet. For the buffered treatment, sporophytes were placed in the medium for 1 h prior to addition of ABA as per Hartung *et al.* (1987). Control treatments were kept in water or in the same medium for corresponding lengths of time. The buffered and unbuffered ABA treatments, for intact or sectioned sporophytes, and irrespective of ABA concentration used and length of exposure, all gave similar results. Therefore, here we report only the results for the intact sporophytes in unbuffered 100 µM after 4 h exposure. The same ABA treatments were repeated using leaves of *Lactuca sativa* L, *Arabidopsis thaliana* (L.) Heyn. and *Hedera helix* L. as vascular plant controls.

For the desiccation treatment, sporophyte-bearing thalli were allowed to dry under natural conditions – after approx. 72 h, the dried thalli bearing flaccid sporophytes had lost >50 % of their original water (Fig. 1D) and were used for imaging and aperture measurements.

Possible effects of darkness on aperture dimensions were studied on sporophytes kept in the dark for 15–24 h, and measurements were taken straight away, within 5 min of mounting these. While exposure to darkness was longer than that experienced by plants under natural conditions, the additional time reflects experimental time constraints and the fact that we had to record a large number of stomata. Sporophyte plasmolysis was brought about by submersion of intact sporophytes in 1.0 M sucrose for 15 min and compared with the leaves of a range of identically treated vascular plants (*L. sativa*, O*smunda regalis* L. and *Scolopendrium vulgare* Sm.).

The protocol for transmission electron microscopy (TEM) follows Ligrone and Renzaglia (1990), and the cryo-scanning electron microscopy (cryo-SEM) protocol follows Duckett *et al.* (2009). For comparisons on intercellular space ontogeny in hornworts, we used representatives of all the major tracheophyte lineages: *Huperzia selago* (L.) Bernh. ex Schrank & Mart. (lycophyte), *Scolopendrium vulgare* (monilophyte), *Ginkgo biloba* L., *Podocarpus nivalis* Hook., *Pinus mugo* Turra (gymnosperms) and *H. helix* (angiosperm).

Element X-ray spectra were obtained for both GCs of at least five stomata plus adjacent epidermal cells from the following: (1) stomata inside involucres; (2) exposed stomata below and (3) above the dehiscence point; (4) exposed stomata in plants that had been allowed to dry out for 72 h; (5) exposed stomata on plants kept in the dark for 15–24 h (these were exposed to light for only 5 min, the time between mounting the specimens and freezing); and (6) plants treated with 1, 10 or 100 µM ABA for 1–24 h. Thallus epidermal cells provided controls for potassium concentrations in somatic cells. Spectra were obtained from pits in the cells produced by ion milling and directly from the lumina of fractured cells. Since both procedures produced closely similar results, these are not separated in Table 1.

For comparison with hornworts, element spectra sets were obtained from five open stomata and adjacent epidermal cells immediately following removal of leaves from well-watered arabidopsis plants. Other detached leaves were allowed to dry out in the laboratory and five further sets of spectra were collected as soon as the stomata closed (after approx. 1 h and 10 % water loss).
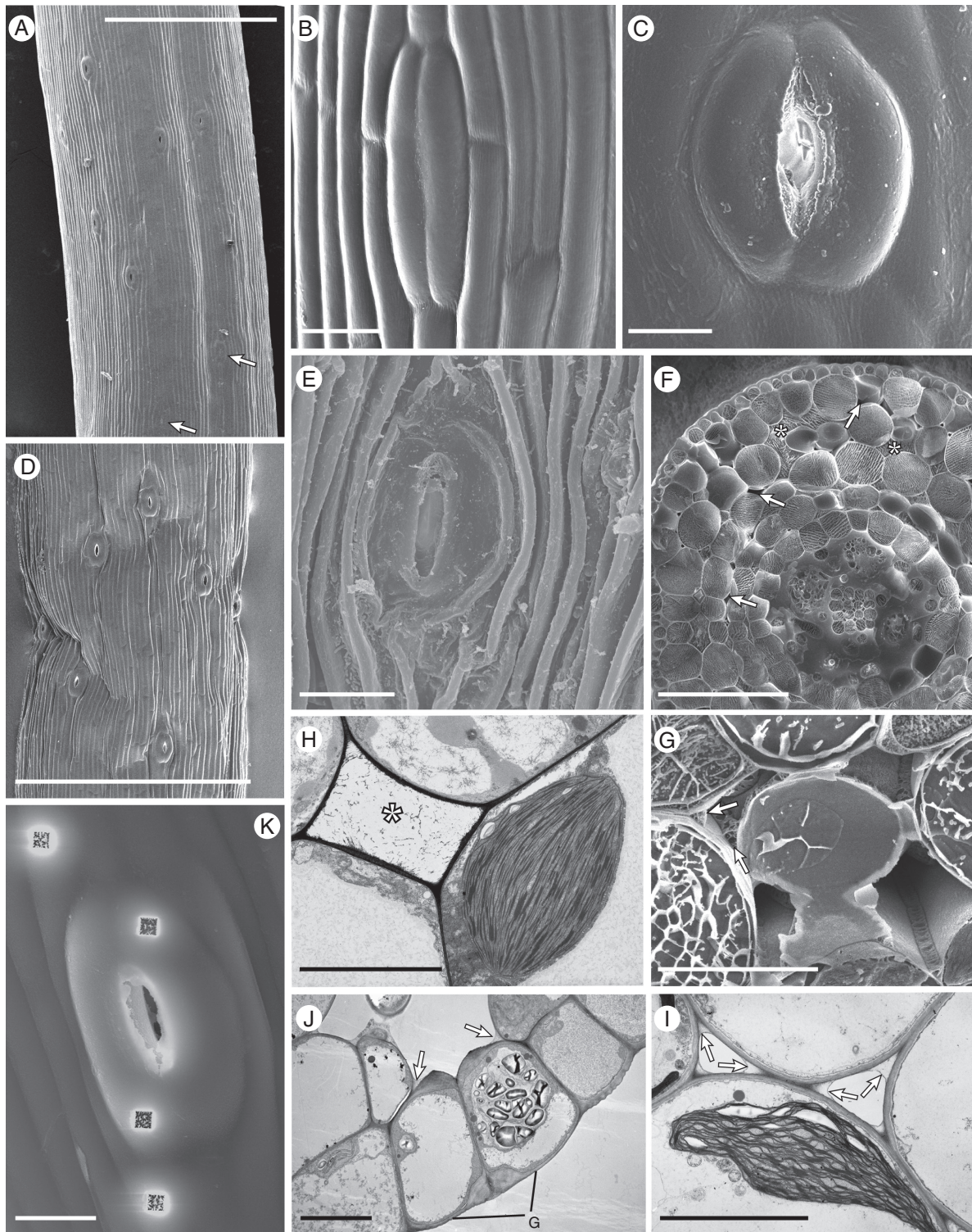
FIG. 1. Cryo-scanning (A–G, K) and transmission (H–J) electron micrographs of *Anthoceros punctatus* (A–F), *Phaeoceros laevis* (G, J, K), *Phaeomegaceros fimbriatus* (H) and *Folioceros fuciformis* (I). (A) Portion of sporophyte just above the involucre with young stomata at the bottom (arrowed) and stomata with fully open apertures above. (B) Closed stoma with smooth walls. (C) Fully opened stoma. (D) Open stomata in a dehydrated sporophyte. (E) Stoma in the wall of a sporophyte well beyond the dehiscence point. (F) Cryo-fractured sporophyte with a mixture of liquid- (*) and gas-filled (arrowed) intercellular spaces. Note the liquid investing the developing spores and elaters. (G) Above the level where the stomata open the liquid is replaced by air, with liquid dried down to a thin layer lining the cell junctions (arrowed). (H, I) Liquid is also visible by TEM: (H) liquid-filled intercellular space (*) and (I) dried out liquid along the cell junctions in the assimilatory tissue (arrowed). (J) Section through stomatal guard cells; note the thickened inner and outer walls of the guard cells (G), the large starch-filled plastid and the dried down liquid in the corners of the intercellular spaces (arrowed). (K) An open stoma after ion milling. Scale bars: (A, D) 500 µm; (F) 100 µm; (B, C, E, J, K) 20 µm; (H, I) 10 µm.

TABLE 1. *Means from 8–9 readings of the percentage mass of potassium from X-ray microanalysis*

Species	Inside involucre	Exposed	Splitting	Desiccated	Dark	4 h of 100 μM ABA
Phaeoceros laevis						
Guard cells	2.1 ± 0.18	0.9 ± 0.07	3.8 ± 0.42	1.6 ± 0.12	2.4 ± 0.16	2.4 ± 0.20
Stomatal condition	Closed	Open	Open	Open	Open	Open
Epidermis	1.7 ± 0.12	1.3 ± 0.10	3.9 ± 0.31	1.5 ± 0.09	2.1 ± 0.18	2.0 ± 0.21
Ratio of K in guard cells to that in epidermal cells	1.24	0.69	0.97	1.06	1.14	1.2
Guard cell K relative to epidermis	Higher	Lower	Same	Same	Higher	Same
Thallus control	0.98 ± 0.07					
Anthoceros punctatus						
Guard cells	1.2 ± 0.06	0.9 ± 0.05	1.3 ± 0.05	1.1 ± 0.06	1.0 ± 0.04	6.0 ± 0.82
Stomatal condition	Closed	Open	Open	Open	Open	Open
Epidermis	1.0 ± 0.02	1.1 ± 0.02	1.3 ± 0.04	1.2 ± 0.04	0.6 ± 0.03	5.8 ± 0.74
Ratio of K in guard cells to that in epidermal cells	1.2	0.82	1.0	0.92	1.67	1.03
Guard cell K relative to epidermis	Higher	Lower	Same	Same	Higher	Same
*Arabidopsis**		Turgid		Wilted		
Guard cells		3.36 ± 0.36		1.56 ± 0.12		
Stomatal condition	Open			Closed		
Epidermis		3.24 ± 0.45		3.91 ± 0.64		
Ratio of K guard cells to epidermal cells		1.04		0.40		
Ratio of K in guard cells to that in epidermal cells		Same		Lower		

*From Duckett *et al.* (2009).

Statistical analysis

Because in our experiments we start from the proposition that experimental treatments have no effect when compared with the controls (reference value) and since 'absence of evidence is not evidence of absence', here we take a different approach from the usual statistical analyses, e.g. Student's *t*-tests, to avoid using the null hypothesis significance test (NHST) and its well-known defects, including giving only a binary result.

Instead we define a zone of indifference (ZoI) on either side of the reference value, and plot $(T – R)/R$, and its 95 % confidence interval (CI_{95}); where R is the reference mean, and T the treatment mean. This plot (see Fig. 5) has a central *y*-axis value of 0, with the symmetrical ZoI lines above and below set at 0.1 and 0.2, because we judge that a difference of up to ±10 % of the control indicates a small treatment effect that we suggest is, in the circumstances, of little biological importance since it would correspond to a change in aperture width of less than about 1 and 2 μm against a mean of about 8–16 μm for *Anthoceros* and *Phaeoceros*, respectively (see Figs 4 and 5). We suggest that a 20 % difference (ZoI ± 0.2) is, again in the circumstances, of some but small biological importance.

RESULTS

Stomatal architecture and ontogeny of intercellular spaces

In sporophytes of this length and stage of maturation, the first few (1–5) stomata immediately above the involucre are recently formed and unopened (Fig. 1A, B; Supplementary Data Fig. S1a). Further up the sporophyte, stomata open (Supplementary Data Fig. S1b, c) with full aperture dimensions in stomata 1 mm above the involucre (Fig. 1A, C; Supplementary data Fig. S1d, e); see also Pressel *et al.* (2014).

Whereas the outer walls in GCs of unopened stomata (Fig. 1B) are smooth, additional wall materials are progressively deposited around pores and over GCs with maturation (Supplementary Data Fig. S1f). Above the point of sporophyte dehiscence, the desiccated epidermal cells are deeply ridged and GCs remain broad in surface view with apertures remaining wide open (Fig. 1E) (see also Villarreal and Renzaglia, 2015; Renzaglia *et al.*, 2017).

Cryo-sections of sporophytes within involucres of the genera with stomata we examined (*Anthoceros*, *Folioceros*, *Phaeoceros* and *Phaeomegaceros*) invariably show liquid-filled intercellular spaces (data not shown, but see Pressel *et al.*, 2014, Fig. 7A). In TEM micrographs, liquid is also visible as a finely granular network (Fig. 1H). Above the involucres, the liquid is gradually replaced by gas (Fig. 1F) and the assimilatory cell chloroplasts become positioned next to the gas-filled spaces (Fig. 1I). This gradual drying process is illustrated by the presence higher up the sporophytes of liquid that has dried down to a thin layer lining the cell junctions (Fig. 1G, I).

In sporophytes of hornworts that lack stomata such as *Nothoceros* (Fig. 2A, B), *Dendroceros* and *Notothylas* (data not shown), intercellular spaces are absent in the assimilatory tissues (Fig. 2A), which dry down and collapse post-dehiscence (Fig. 2B).

In contrast to hornworts, the intercellular spaces in the leaves from representatives of all major tracheophyte groups (lycophytes, ferns, gymnosperms and angiosperms) are gas filled from the outset, as illustrated here in *H. helix* (Fig. 2C–E). In all stages of development in the array of species we examined, thin pectic strands (Carr *et al.*, 1980) extend across intercellular spaces with no remnants of liquid lining the cell junctions around spaces (Fig. 2D).

X-ray microanalysis

Fig. 1K shows a cryo-SEM image of ion beam milling prior to X-ray microanalysis in an open stoma and adjacent epidermal
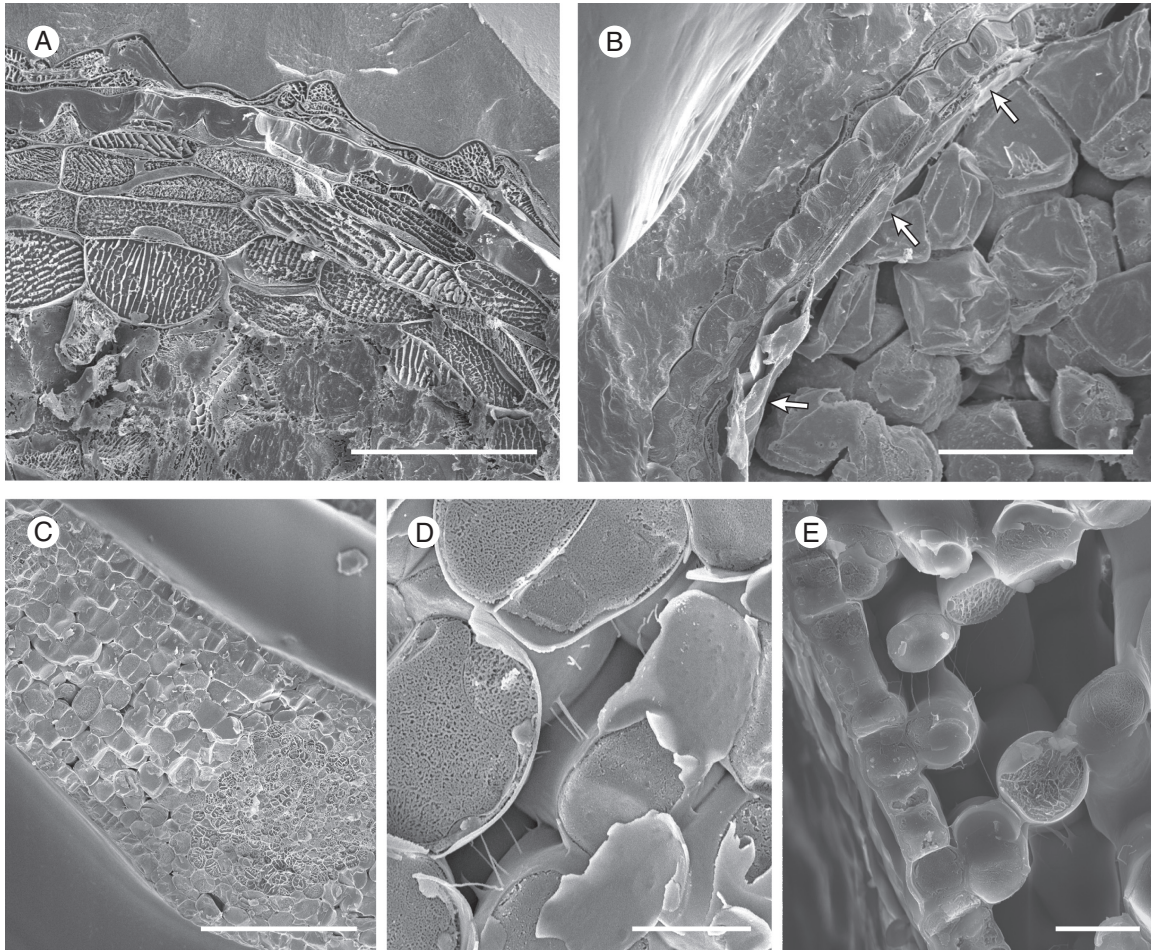
FIG. 2. Cryo-scanning electron micrographs of *Notothylas levieri* (A, B) and *Hedera helix* (C–E). (A, B) Cryo-fractured sporophytes of the estomate hornwort species *Notothylas levieri*, young (A) and mature (B); note the complete absence of intercellular spaces in the assimilatory layers (A) which collapse and dry out at maturity (B, arrowed). (C–E) Cryo-fractured young leaves. (C) General aspect of a leaf approximately one-tenth its final size with nascent intercellular spaces. (D) Detail of an intercellular space from (C). (E) Spongy mesophyll and lower epidermis in a leaf approximately a quarter of its final size. Scale bars: (C) 100 μm; (A, B) 50 μm; (E) 20 μm; (D) 10 μm.

cells. The milling holes are precisely positioned to penetrate into the vacuoles of the guard and epidermal cells (Fig. 1J).

The mass-proportion of potassium, expressed as a percentage, detected by the probe in GCs of *P. laevis* and *A. punctatus* at different developmental stages and in adjacent epidermal cells, is summarized in Table 1. Thallus epidermal cells serve as controls. Typical spectra from which the numerical data were obtained are illustrated in Fig. 3. Potassium was not detected in the sporophytic intercellular liquid or on the surface of the thalli. Peaks (Fig. 3A) and mass-proportions from gametophytic cells were lower than in the sporophytes (Fig. 3B, C).

In newly opened stomata of both species, the potassium mass-proportion in GCs is lower than that in epidermal cells. Thereafter it remains the same in both cell types even when sporophytes are desiccated. Also, potassium mass-proportions of unopened stomatal GCs are higher (Fig. 3B) than those of newly opened stomata (Fig. 3B). The mass-proportions of potassium were higher in *Anthoceros* after treatment with ABA (Fig. 3D–F) and were the same whether or not the pores were open or closed. After periods in darkness, potassium mass-proportions in GCs of *Anthoceros* appeared slightly higher than

in the epidermal cells. In striking contrast, when stomata of arabidopsis close, the GC potassium mass-proportion falls significantly from parity between open GCs and epidermis in the turgid open state (see Duckett *et al.*, 2009).

Stomatal responses to environmental stimuli

Aperture dimensions (widths and lengths) for the first centimetre of sporophytes above the involucre in control plants of *A. punctatus* and *P. laevis* are shown in Supplementary Data Fig. S2.

Results from the experimental treatments are summarized in Fig. 4, and indicate that stomatal aperture dimensions in both *Anthoceros* and *Phaeoceros* do not decrease in response to exposure to exogenous ABA or darkness, while desiccation and plasmolysis appear to elicit a slight reduction, but never the complete closure observed in ABA-treated (data not shown) and plasmolysed vascular plant stomata (Supplementary Data Fig. S3). A similar decrease in size (width only) was also elicited by desiccation in hornwort sporophyte non-stomatal

epidermal cells. After 1–2 h in water, both stomata and epidermal cells regained their pre-desiccation dimensions (data not shown).

Plotting mean values of aperture sizes (width and length) of treated hornwort stomata and non-stomatal epidermal cells (width only) (CI$_{95}$) against the reference (control) value (Fig. 5) confirms that both ABA and darkness treatments have no or only a trivial effect on aperture sizes, as values fall well within a ZoI of ±10 %. Desiccation and plasmolysis, on the other hand, have an effect at ZoI ±10 %, especially on aperture width sizes and, for desiccation only, on the lumen width of non-stomatal epidermal cells. However, these values do fall within a ZoI set at ±20 %. The results for the oil treatment on *Anthoceros* indicate that mounting sporophytes in immersion oil does not affect stomatal aperture sizes when compared with the same mounted in water.

DISCUSSION

The present study reveals major physiological differences between hornwort stomata and those in both mosses and vascular plants. Hornwort stomata do not respond to external stimuli and there are no potassium fluxes between epidermal cells and GCs. Identical to hornworts, there is no evidence of potassium fluxes associated with moss stomata (Duckett and Pressel, 2017a).

Developmental data (see also Pressel *et al.*, 2014; Renzaglia *et al.*, 2017) and responses to desiccation and exogenous ABA show that in hornworts, stomatal apertures open in early development and are not subsequently actively regulated. Hornwort stomata thus appear to be different from those in the mosses *Physcomitrella* and *Funaria* where ABA, darkness and reduced CO$_2$ all decrease aperture dimensions (Chater *et al.*, 2011). These authors also demonstrate, through cross-species complementation and knockout experiments, that stomata in the moss *Physcomitrella* behave like those in seed plants. However, Merced and Renzaglia (2017) and Renzaglia *et al.* (2017), drawing attention to the frequent incidence of pore occlusion by waxes and additional wall materials in bryophytes, illustrate nearly mature capsules of *Physcomitrella* with blocked open pores and/or liquid-filled intercellular spaces.

The present physiological data, coupled with liquid-filled sporophytic intercellular spaces, point to the primary role of hornwort stomata as facilitation of sporophyte drying leading to spore discharge, a conclusion reinforced by the early death of the GCs and locking of the apertures in an open position (Renzaglia *et al.*, 2017). The same might well be true in mosses since recent observations over the entire life cycles of the sporophytes of five common mosses revealed that the stomata are invariably open regardless of the prevailing environmental conditions (Duckett and Pressel, 2017b).
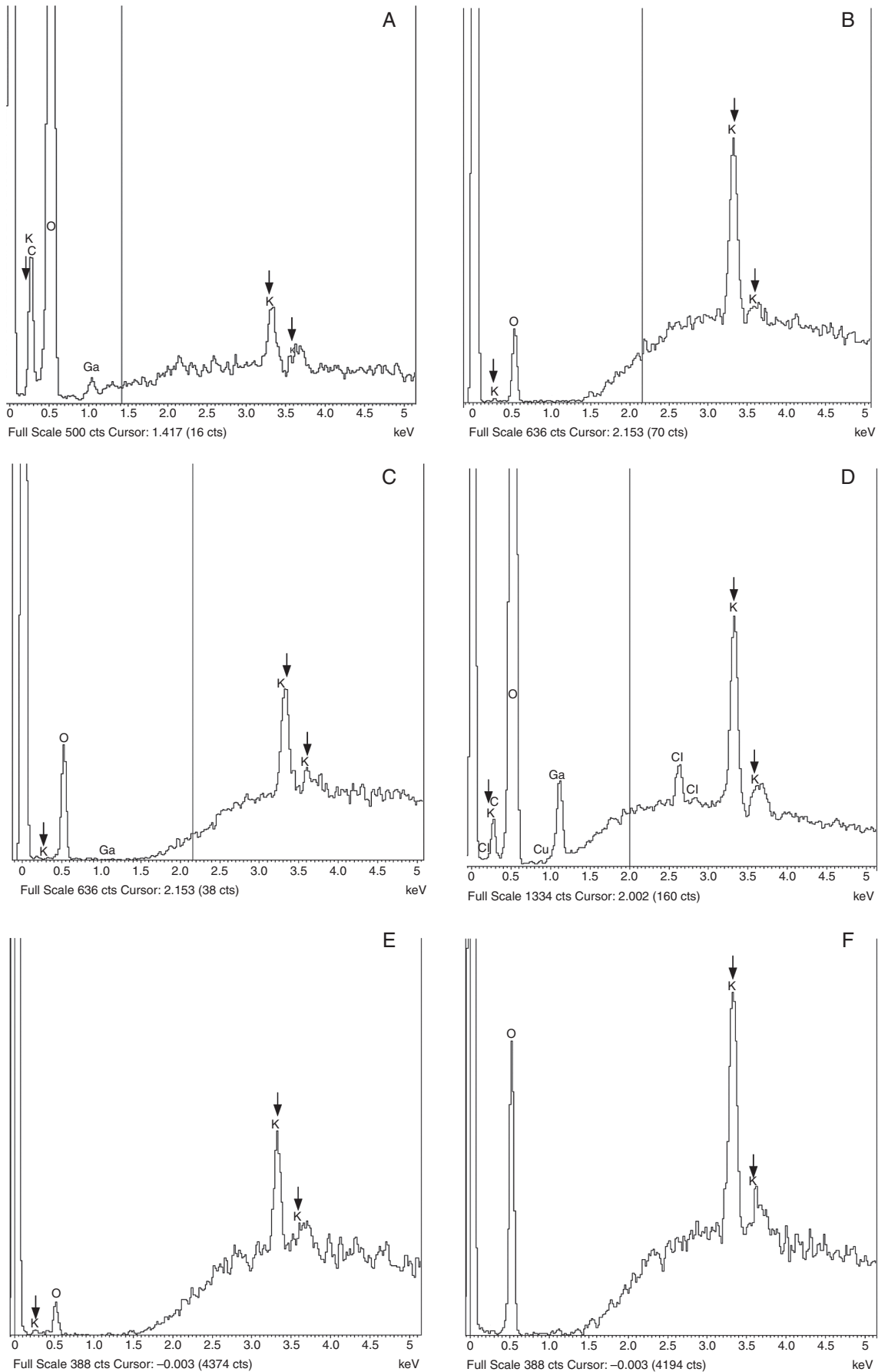
Functional considerations

X-ray microanalysis in hornworts demonstrates that potassium mass-proportion is lower in GCs of newly opened stomata than in the adjacent epidermal cells, and that similar potassium mass-proportions occur in GCs and epidermal cells in stomata on older regions, desiccated sporophytes and those treated with ABA. These findings eliminate the possibility that potassium fluxes are involved in stoma formation and any putative opening or closing mechanism. We view the slightly higher GC potassium mass-proportion in dark-grown specimens as simply reflecting the ability of sporophytes to grow in the dark (Ligrone and Fioretto, 1987). In the absence of potassium fluxes, it seems plausible that stoma formation is the result of uneven and specialized wall deposition in GCs coupled with mobilization of abundant starch reserves in the plastids of young GCs (Pressel *et al.*, 2014). The same arguments are equally applicable to moss stomata (Duckett and Pressel, 2017a).

Another major difference that separates hornwort stomata from those in other plant groups (Chater *et al.*, 2011) is their lack of responsiveness to the stress hormone ABA, to darkness and to exogenous/environmental cues widely known to close apertures in seed plants and to result in decreased aperture size in mosses (Chater *et al.*, 2011). Even though we followed the same protocol with far more samples, the results from our ABA treatment contrast with those reported previously by Hartung *et al.* (1987) who showed a dose-dependent ABA response in the stomata of *A. punctatus*. In trying to find an explanation for this disparity, we noticed that the two images of control and ABA-treated stomata in Hartung *et al.* (1987; see figs 5 and 6) seem to represent very different developmental stages unsuitable for comparison (compare with our Supplementary Data Fig. S1a, b and e), especially given the conspicuous difference in aperture wall thickness between the two stomata. Hartung *et al.* (1987) had no knowledge of the major ontogenetic changes subsequently described in hornwort stomata (Pressel *et al.*, 2014; Villarreal and Renzaglia, 2015; Renzaglia *et al.*, 2017) and which formed the basis for choices in the present work. There are no indications in Hartung *et al.* (1987) that they chose particular regions or ages of sporophytes to examine.

The slight reductions in aperture dimensions after desiccation and plasmolysis in hornwort stomata also contrast with the much more pronounced responses typical of vascular plant stomata to the same cues (Supplementary Data Fig. S3). The absence of closure following plasmolysis is completely at odds with the notion that stomatal movement is modulated by turgor pressure. This finding is consistent with the early development of GC walls that mechanically restrain the apertures of dead stomata in an open configuration as sporophytes dry out (Renzaglia *et al.*, 2017). Similarly, the slight reduction in stoma dimensions after desiccation is most likely to be related to physical, not physiological events, resulting from a general lateral shrinkage of all the sporophyte epidermal cells rather than a stoma-specific response (Merced and Renzaglia, 2017; Renzaglia *et al.*, 2017). The width reductions associated with plasmolysis and desiccation also indicate that the vast majority of our measurements were on living stomata. Dead stomata would not respond. It should be noted that the duration of our darkness treatment (15–24 h), a necessity of our experimental protocol, exceeds what plants experience in the wild and therefore might have caused carbon starvation in hornwort sporophytes potentially leading to stress-induced stomatal opening. However, it is extremely unlikely that 15–24 h, rather than 12 h, could have had such dramatic effects on our experimental plants; further, our results are in line with previous observations of a lack of response to darkness in hornwort stomata (Lucas and Renzaglia, 2002). Although, the dimension reductions

Full Scale 500 cts Cursor: 1.417 (16 cts) keV

Full Scale 636 cts Cursor: 2.153 (70 cts) keV

Full Scale 636 cts Cursor: 2.153 (38 cts) keV

Full Scale 1334 cts Cursor: 2.002 (160 cts) keV

Full Scale 388 cts Cursor: −0.003 (4374 cts) keV

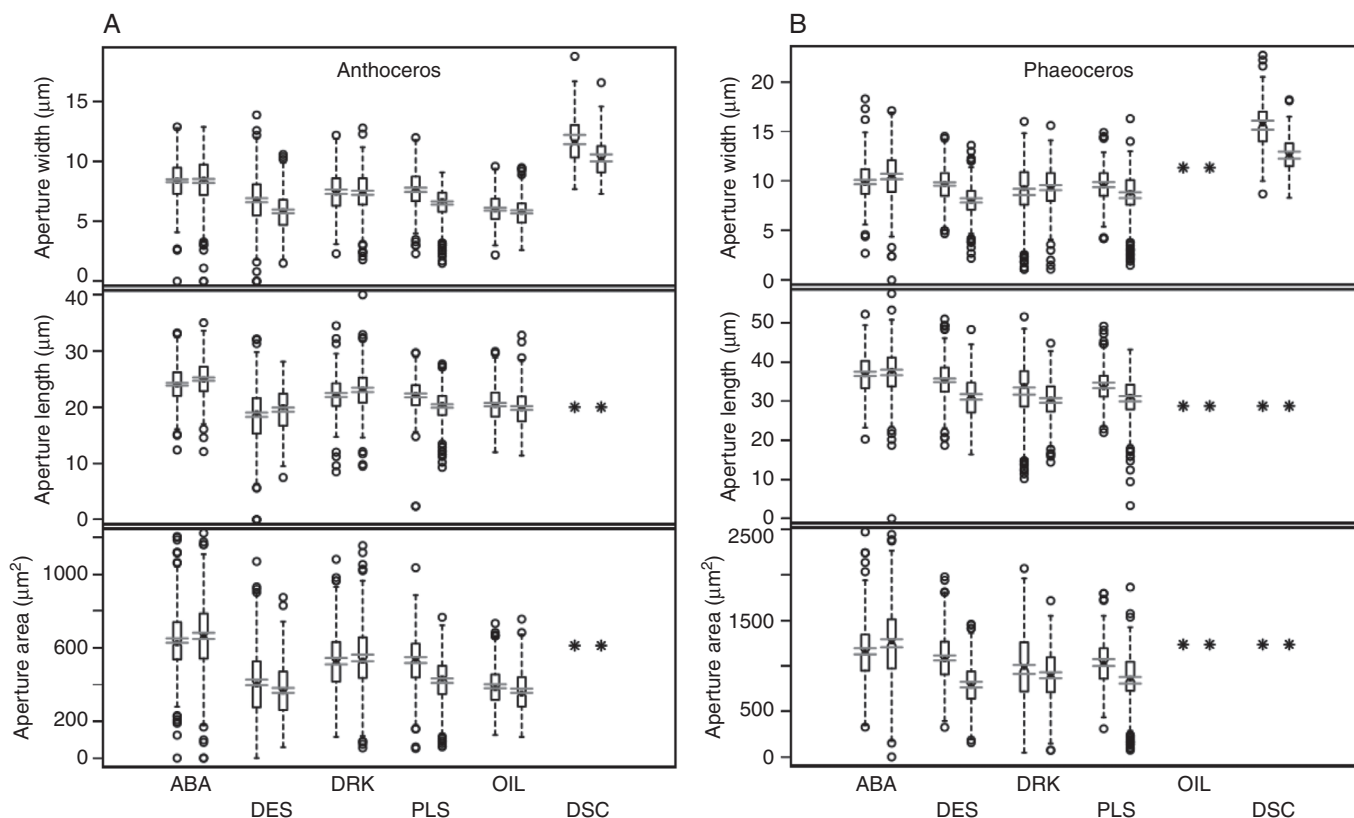Full Scale 388 cts Cursor: −0.003 (4194 cts) keV

Fig. 4. Box plots of measurements (width, length and surface area) on *Anthoceros punctatus* (A) and *Phaeoceros laevis* (B) in the experimental treatments and corresponding controls. The central bar is the median; the box defines the interquartile range (IQR); dashed 'whiskers' extend 1.5 IQR beyond the box; beyond that individual measurements are circles. The two parallel horizontal lines near the median define the 95 % confidence limits on the mean. To the left are controls which were kept moist and in light; to the right are experiment treatments: ABA, 100 μM for 4 h; DES, desiccated; DRK, in the dark for 15–24 h; PLA, plasmolysed; OIL, measurements made under oil (*Anthoceros* only); DCS, non-stomatal epidermal cells desiccated (*Anthoceros* only; cell widths only). Missing items are shown by an asterisk.

following desiccation and plasmolysis were small and not *per se* significant statistically, the remote possibility that these might be significant functionally still requires close scrutiny in the context of relationships between conductance of water vapour and CO_2 which are determined by aperture sizes. The smaller the apertures the greater are the effects of changes in their sizes (Parlange and Waggoner, 1970; Brown and Escombe, 1900; Kaiser, 2009). For example, a width reduction of just 1 μm in a small 4 μm aperture width produces a 25 % reduction in conductance, whereas the corresponding figures for hornworts with larger mean aperture widths of between 6 and 10 μm (Fig. 4) are only 15 and 5 % reductions, respectively. The possibility that these last two small differences might have any real functional significance is even less likely because the stomatal densities in hornworts are an order of magnitude or more lower than those in most angiosperms (Willmer and Fricker, 1996; Field *et al.*, 2015). This observation also appears to rule out in hornworts possible interstomatal diffusion interference, an important parameter in vascular plant leaves particularly in

the context of density changes related to atmospheric CO_2 concentrations (Woodward, 1987; Beerling *et al.*, 2001; Beerling and Royer, 2002) to which bryophyte stomata are unresponsive (Field *et al.*, 2015). Interstomatal diffusion interference becomes negligible when spacing is over three times stomatal length (Parlange and Waggoner, 1970). Less than 20 % of the stomata of hornworts, even with their very large GC sizes, lie outside this range, in contrast to the much more closely spaced stomata in many mosses (see Field *et al.*, 2015; Table 1; Fig. 3).

Similar mechanical constraints in guard and subsidiary cell walls that restrict stomatal movements have also been noted in tracheophytes (Franks and Farquhar, 2007; Shtein *et al.*, 2017a). The interpretation that hornwort stomata are structures facilitating sporophyte desiccation and spore dispersal finds parallel in anthers. Stomata on young anthers may open and close, but permanent maturational opening is thought to facilitate drying out and dehiscence leading to faster pollen release (Schmid, 1976; Heslop-Harrison *et al.*, 1987; Lersten, 2004). Permanently open stomata are also a feature of nectaries, where they act as

Fig. 3. X-ray spectra from *Phaeoceros laevis* (A–D) and *Anthoceros punctatus* (E, F). Three vertical arrows indicate peaks attributable to potassium. (The peaks at 0.5 keV are gallium contamination from the ion milling.) (A) Low potassium peaks in a thallus epidermal cell (control). (B, C) The guard cell from an unopened stoma inside an involucre (B) has more pronounced potassium peaks than those in a newly opened stoma (C). (D) High potassium peaks in a guard cell of an open stoma after 4 h exposure to 100 μM ABA. (E) Guard cell in an unopened stoma. (F) High potassium peaks in a guard cell of an open stoma after 4 h exposure to 100 μM ABA.

secretory pores rather than in gas exchange (Davis *et al.*, 1986), of hydathodes, where the open pores may become occluded with granular, crystalline and waxy materials (Stevens, 1956; Takeda *et al.*, 1991), and in aquatic angiosperms where loss of function is associated with altered patterns of cellulose crystallinity (Shtein *et al.*, 2017*b*).

Evolution of stomata

The lack of stomata in four hornwort genera represents two independent losses and may be readily understood in terms of this postulated role in sporophyte desiccation (Pressel *et al.*, 2014; Renzaglia *et al.*, 2017). All four taxa fill ecological niches where drying via stomata is not adaptive. In the ephemeral *Notothylas*, the spores are usually water dispersed from highly reduced sporophytes that are, in many species, immersed in involucres throughout development (Hasegawa, 1980; Schuster, 1984; Singh, 2002). In the *Megaceros/Nothoceros/Dendrocer-os* lineage, the loss of stomata is consistent with the habitats they occupy. *Megaceros* and *Nothoceros* occur in highly humid regions with long growing seasons where they do not experience seasonal cycles of desiccation as do most temperate taxa. In the epiphytic and epiphyllous *Dendroceros*, the only desiccation-tolerant hornwort genus, cycles of rapid drying and re-wetting are the norm. Long involucres in these three genera provide additional protection of vulnerable tissue in environments where spores are dispersed over long growing seasons.

The absence of any experimental evidence for regulation of aperture movements and liquid-filled subjacent intercellular spaces supports the conclusion that the primary role of hornwort stomata is sporophyte desiccation. This conclusion points to a rethink of the possible course of stomatal evolution in land plants. Although the positions of mosses sister to hornworts and hornworts sister to vascular plants (Qiu *et al.*, 2006, 2007; Liu *et al.*, 2014) are consistent with a single origin for stomata (Raven, 2002), this hypothesis infers that the absence of stomatal regulation, as shown in the present study,
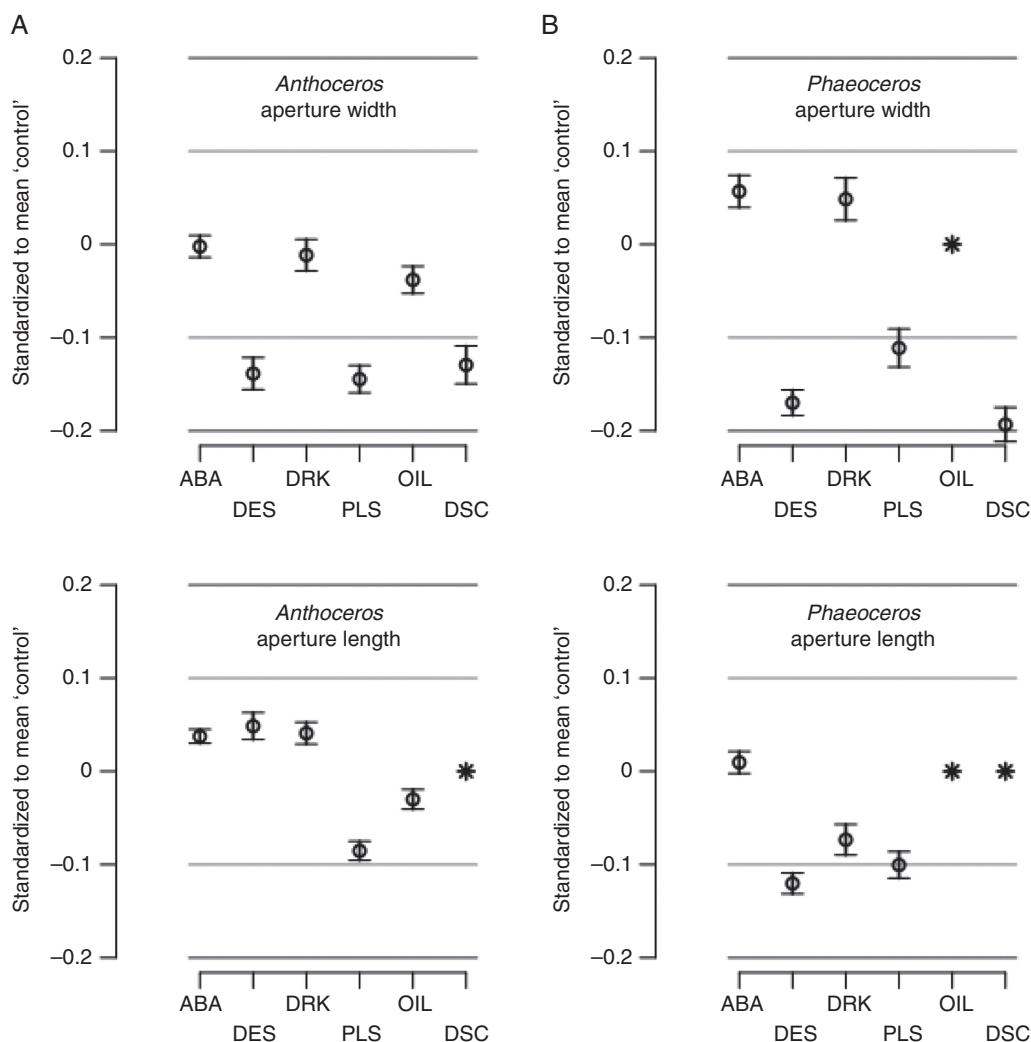

Fig. 5. *Anthoceros punctatus* (A) and *Phaeoceros laevis* (B). The 95 % confidence interval (CI_{95}) of differences in means of 'experimental' treatment and 'control', expressed as a proportion of the 'control' mean, i.e. $(x – c)/c$. This standardized measure allows comparison of different measures. The 'controls' were kept moist and in light. Treatments: ABA, 100 μM for 4 h; DES, desiccated; DRK, in the dark for 15–24 h; PLA, plasmolysed; OIL, measurements made under oil (*Anthoceros* only); DCS, non-stomatal epidermal cells desiccated (*Anthoceros* only; cell widths only). Missing items are shown by an asterisk.

was lost in hornworts following an earlier acquisition in mosses (Chater *et al.*, 2011). On the other hand, phylogenetic analyses placing hornworts sister to other land plants (Cox *et al.*, 2014; Wickett *et al.*, 2014) are in line with the evolution of the stomatal regulatory molecular toolkit described by Chater *et al.* (2011, 2013, 2016, 2017) after the split of hornworts from the remaining land plants. A recent analysis of possible stomatal gains and losses (Rensing, 2018) shows the most parsimonious configuration to be one in which stomata were independently gained during land plant evolution, with a mosses-liverworts clade as sister to all other land plants and hornworts sister to the vascular plants (Puttick et al., 2018). All other configurations involve more losses and/or gains. When the hornwort genome becomes available, thus enabling the rapid identification of stomatal genes, we predict that a host of both stomatal patterning and identity genes will be shared across stomatophytes. However, the function of those genes will require further genetic and experimental analyses.

Pertinent to these evolutionary considerations are contrasting views on stomatal physiology between seed plants and early divergent tracheophytes (Brodribb and McAdam, 2011, 2012, 2017; Chater *et al.*, 2011; McAdam and Brodribb, 2011; Ruszala *et al.*, 2011). In seed plants, control of water balance is by active metabolic regulation of stomatal aperture via ion-pumping processes and closure induced by ABA. On the basis of molecular data and physiological experiments showing small changes in aperture and stomatal conductance, Ruszala *et al.* (2011) conclude that stomatal responses in *Selaginella* to both CO_2 and the physiologically active enantiomer of ABA are similar to those in arabidopsis. They also report differences in potassium mass-proportion between GCs of light- and dark-grown *Selaginella*, although the cobaltinitrite staining method they used produced largely qualitative data. From their experiments, they argue that physiologically active stomatal control originated as least as far back as the origin of the lycophytes approximately 420 million years ago. From *Physcomitrella* studies, Chater *et al*. (2011, 2013) posit the same for mosses; they contend that mosses possess the same core regulatory components as flowering plants in GC ABA signalling, and that these originated in a common ancestor of bryophytes and tracheophytes. Phylogenomic studies of stomatal genes that include *Physcomitrella* support the single origin of GCs across land plants as *Physcomitrella* possesses GC patterning and development genes found in arabidopsis (Bergmann and Sack, 2007; Peterson *et al.*, 2010; Rychel and Peterson, 2010; Caine *et al.*, 2016; Chater *et al.*, 2017).

In contrast, Brodribb and McAdam's (2011) finding of only a 15 % reduction in stomatal apertures in the presence of ABA in a lycophyte and further extensive studies on pteridophytes (reviewed by Brodribb and McAdam, 2017; Sussmilch *et al.*, 2017) points to a lack of or very limited active control of water loss. These authors suggested that early land plants possessed passive stomatal control with the absence of an active ion-pumping mechanism, much like that demonstrated here in hornworts, and the gradual acquisition of active control mechanisms occurred as plants diversified. However, differently from hornworts, desiccation and plasmolysis did cause rapid stomatal closure in some ferns and lycophytes. The general lack of stomatal response in hornworts clearly conforms to the notion of gradual acquisition of stomatal regulation

(Brodribb and McAdam, 2017; Sussmilch *et al.*, 2017). Added to the present results is the demonstration that stomatal density and aperture in both hornworts and mosses do not respond to atmospheric CO_2 concentrations (Field *et al.*, 2015). The single study (Chater *et al.*, 2011) based on actual measurements of stomata rather than analysis of stoma-related genes provides the only direct evidence for the early acquisition of active regulation. That study showed small responses rather than complete closure in two mosses with unusual pores in single GCs (*Funaria* and *Physcomitrella*). Merced and Renzaglia (2017) highlight that studies on *Physcomitrella* stomata are challenging; 'they might not reflect the reality of the great majority of mosses because of its reduced cleistocarpic capsule with few stomata, small apertures and poorly developed intercellular spaces.' It is difficult to envisage how absence of stomata might delay the dehiscence of cleistocarpous capsules with this suite of characters (Chater *et al.*, 2016). Physiological and genetic studies including aperture measurements now need to be replicated in additional mosses with structurally elaborate capsules that contain well-developed apophyses and a system of intercellular spaces leading to regularly spaced stomata with two guard cells. No matter how many more stomata-related genes are discovered to be common across land plants, future considerations of structural and functional continuity must now take into account the recent demonstration of the multiple evolution of intercellular spaces in bryophytes (Duckett and Pressel, 2017a). Whereas this integral component of the stomatal apparatus is present at the foot of the hornwort tree (*Leiosporoceros*), it is strikingly absent from the basal moss clades, *Sphagnum*, *Takakia* and *Andreaea*.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: light micrographs of living sporophytes illustrating the ontogenetic changes in stomatal apertures in *Anthoceros punctatus*. Figure S2: *Anthoceros punctatus* and *Phaeoceros laevis* scatter plots of stomatal width and length for the four experimental treatents and corresponding controls. Figure S3: effects of plasmolysis on *Phaeoceros laevis*, *Lactuca sativa*, *Osmunda regalis* and *Scolopendrium vulgare* stomata.

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