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Selection pressures on stomatal evolution

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Contents

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Summary

Fossil evidence shows that stomata have occurred in sporophytes and (briefly) gametophytes of embryophytes during the last 400 m yr. Cladistic analyses with hornworts basal are consistent with a unique origin of stomata, although cladograms with hornworts as the deepest branching embryophytes require loss of stomata early in the evolution of liverworts. Functional considerations suggest that stomata evolved from pores in the epidermis of plant organs which were at least three cell layers thick and had intercellular gas spaces and a cuticle; an endohydric conducting system would not have been necessary for low-growing rhizophytes, especially in early Palaeozoic CO_2-rich atmospheres. The 'prestomatal state' (pores) would have permitted higher photosynthetic rates per unit ground area. Functional stomata, and endohydry, permit the evolution of homoiohydry and the loss of vegetative desiccation tolerance and plants > 1 m tall. Stomatal functioning would then have involved maintenance of hydration, and restricting the occurrence of xylem embolism, under relatively desiccating conditions at the expense of limiting carbon acquisition. The time scale of environmental fluctuations over which stomatal responses can maximize carbon gain per unit water loss varies among taxa and life forms.

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1. Introduction

The topic of selection pressure on stomatal evolution can, and will, be considered at a number of different levels. Perhaps the most fundamental question relates to the origin of stomata: what were the selection pressures which favoured the evolution of stomata, what was the environmental and biological context of the evolution of stomata, and did the stomata have a single evolutionary origin (i.e. are monophyletic)?

Once (or more than once) stomata has evolved, there are possibilities of further evolution of stomatal occurrence and function as the environment changes (to some extent as a result of the activities of stomata-bearing plants) and plant life forms diversify. Among these changes are, of course, the stomatal loss, which relates not only to stomatal function but also to the monophyly (or otherwise) of stomata.

Stomatal evolution has been considered by many authors. Among these are: Church (1919), Whitehouse (1952), Corner (1964), Meidner & Mansfield (1968), Chaloner (1970), Raven (1977, 1984a, 1993, 1995, 1996, 1999a,b), Ziegler (1987), Edwards (1993, 2000), Edwards & Axe (1992), Graham (1993), Edwards *et al.* (1996), Willmer & Fricker (1996), Edwards *et al.* (1998) and Woodward (1998). The account which follows draws upon these sources, but takes some different perspectives on the evolution of stomata.

A major theme in discussion of the evolution of stomata is the role of stomata in homoiohydry. Homoiohydric plants are able to exert substantial control over its degree of hydration despite varying soil water supply and varying evaporative demand by the atmosphere. The alternative condition is poikilohydry. Poikilohydric plants have little control over its degree of hydration with varying soil water supply and varying evaporative demand by the atmosphere. Ancestral embryophytes were poikilohydric.

II. Monophyly of stomata?

The presence of structurally defined stomata in the epidermis of above-ground organs of sporophytes appears to be the ancestral condition for vascular plants (Kenrick & Crane, 1997). The situation becomes more complex when extant embryophytes other than vascular plants (i.e. hornworts, liverworts and mosses) are considered, where the matrotrophic (Graham & Wilcox, 2000a,b; Proctor, 1977) sporophyte phase of the life cycle often has structurally defined stomata in the mosses and hornworts, and never has stomata in the liverworts (Kenrick & Crane, 1997). Furthermore, there are doubts as to the functionality of structurally defined stomata in some bryophyte sporophytes, including all hornworts (Paton & Pearce, 1957). However, response of stomata to light, dark and abscisic acid is known for the moss *Funaria hygrometrica*, at least from the 5th to the 10th day of capsule expansion, although functionality declines in mature capsules (Garner & Paolillo, 1973).

Further complexity is introduced when studies of early fossil embryophytes are considered (Edwards *et al.*, 1998). The earliest well-authenticated stomata are from the Pøídolí series (*c.* 410 Ma ago), uppermost Silurian (Edwards *et al.*, 1998). However, the extinct lycophyte *Baragwanathia* is known from older Silurian deposits (Ludlow, *c.* 413 Ma ago); Edwards *et al.* (1998), and by analogy with all extant lycophytes of similar life form we would expect *Baragwanathia* to have stomata. The state of preservation of fossil material, or the techniques used, may not be appropriate to detect stomata (e.g. in *Baragwanathia*). A further problem with the interpretation of unbranched axes bearing stomata and a sporangium is the difficulty of distinguishing a fragment of a polysporangiophyte (branched sporophyte bearing many sporangia) from a bryophyte or bryophyte-like organism with an unbranched sporophyte bearing a single sporangium (Kenrick & Crane, 1997; Edwards *et al.*, 1998).

A further complication is the occurrence of stomata in the very well-preserved gametophytes from the Lower Devonian (Pragian) Rhynie Chert. These gametophytes were apparently a phase in the life cycle of a range of stomata-bearing extinct polysporangiophyte sporophytes which are not all vascular plants as defined by the occurrence of xylem (Kenrick & Crane, 1997; Edwards *et al.*, 1998). No other fossil or extant gametophytes of embryophytes are known to have stomata.

Such observations on extant and extinct plants have been used, with many other characters, in the cladistic analysis of Kenrick & Crane (1997). A stomatophyte clade (hornworts, mosses and polysporangiophytes), implying monophyly of structurally defined stomata, was recognised as one of a set of equally parsimonious trees that included alternative topologies supporting a liverwort-moss-polysporangiophyte clade or a liverwort-hornwort clade (Kenrick & Crane, 1997). The analysis of introns in mitochondrial genomes by Qiu *et al.* (1998) identifies liverworts as the earliest land plants, supporting monophyly of stomata. However, Renzaglia *et al.* (2000) find support in their three cladistic analyses, using ultrastructural, morphological and developmental and molecular genetic characters, for hornworts as the earliest embryophytes, that is, not supporting a stomatophyte clade. While some early (lower Silurian) spores resemble extant liverwort (sphaerocarpalean) dyads (Taylor, 1995), supporting the occurrence of liverworts in the lower Silurian, the embryophyte spore record goes back at least to the Lower Ordovician with a diversity of spore types in the Ordovician (Edwards, 2000; Wellman & Gray, 2000; Raven & Edwards, 2001), so the spores cannot unequivocally be used to show that liverworts were the earliest embryophytes. An example of the difficulties of assigning early embryophyte fossils to extant taxa is the description by Habgood (2000) of a Lower Devonian (Lochkovian) axis with stomata bearing a terminal sporangium containing dyads, similar to those found in earlier dispersed spore assemblages (Taylor, 1995; Wellman & Gray, 2000).

The discussion above shows that the available evidence is not conclusive as to the monophyly of stomata. If liverworts are the most ancient embryophytes (Qiu *et al.*, 1998) then monophyly is likely, while having hornworts as the most ancient embryophytes (Renzaglia *et al.*, 2000) requires that stomata have been lost in liverworts if the monophyly of stomata is to be supported. It is clear that the stomatal loss has occurred among embryophytes at taxonomic levels lower than the superdivision Marchantiomorpha (with the single division Marchantiophyta), the taxon to which Kenrick & Crane (1997) assign the liverworts. The loss of stomata in taxa at lower taxonomic levels is considered below. We shall assume in further discussion that stomata are probably monophyletic.

Table 1 Water potentials at which various processes occur in photolithotrophs

Process	Water potential (ψ_w)/MPa
Complete closure of stomata in flowering plants acclimated to water deficit	−1.9 to −5.3
50% loss of xylem conducting capacity due to embolism in conifers	−3.0 to −6.5
50% loss of xylem conducting capacity due to embolism in flowering plant trees	−4.0 to −6.5
Lowest water potential at which photosynthesis at cell level can occur at the maximum rate in the flowering plant *Avicennia marina* (mangrove)	−4.0
Lowest water potential at which measurable net photosynthesis occurs in terrestrial bryophytes	−20.0
Lowest water potential for survival of the most desiccation-tolerant of desiccation-intolerant (nonresurrection) vascular plants	−22.0
Lowest water potential for survival of the least tolerant of desiccation-tolerant (resurrection) vascular plants	−217.0
Lowest water potential at which growth occurs in terrestrial green algal lichens	−38.0
Lowest water potential at which growth occurs in terrestrial green algae	−48.0
Lowest water potential at which growth occurs in aquatic green algae	−22.0

Adapted from Raven (1993) with data on water potentials for survival of all vascular plants from Gaff (1981) converted from relative humidity to ψ_w following Nobel (1999). See also Walter & Stadelmann (1968).

III. Roles of stomata in extant plants

This is a brief indication of the proposed roles of stomata in extant plants. The underlying theme is the role of stomata in homoiohydry (Raven, 1977; Edwards *et al.*, 1996; Edwards *et al.*, 1998; Woodward, 1998). Homoiohydry involves stomata operating in organisms which also have cuticle, intercellular gas spaces, an endohydric water conducting system, and a means of taking water from the soil (Raven, 1977). Plants with all of these attributes permit the fixation of carbon dioxide with limited transpiratory water loss per unit carbon fixed under fluctuating conditions of water supply and of atmospheric evaporative demand (Cowan, 1977, 1986; Wong *et al.*, 1979; Farquhar *et al.*, 1989). Such plants also have the capacity to slow the loss of water when water supply is restricted and/or evaporative demand is high, thus permitting vegetative desiccation intolerance and limiting loss of xylem function, albeit at the expense of restricting carbon fixation.

1. 'Optimizing' carbon fixation per unit water lost under conditions of water supply and potential loss which do not bring the plant close to lethal overall dehydration or catastrophic embolism

Cowan (1977, 1986) has developed models of optimization of carbon gain per unit water loss under conditions of varying evaporative demand by the atmosphere and competition for water from the soil among plants in a population or community. These models, in which stomatal behaviour is a key element, accord quite well with observation (Cowan, 1977, 1986), and such regulation of carbon gain relative to water loss seems to be a major role of stomata in today's atmosphere.

2. Maintenance of vegetative hydration when soil water supply is limited and/or evaporative demand by the atmosphere is high

This is the classic role of stomata as part of the mechanism which prevents, or delays, dehydration of the sporophytes of vascular plants under conditions in which water supply is less than evaporative demand. The homoiohydric apparatus includes the cuticle and the endohydric water transport system as well as the intercellular gas space system. Closure of stomata limits water loss and hence slows the rate at which the plant approaches the lethal water potential. The great majority of homoiohydric plants are desiccation-intolerant, that is, cannot survive tissue water potentials lower than *c.* −10 MPa or, as an extreme, −22 MPa in the vegetative state (Table 1). This desiccation-intolerance seems to be correlated with a well authenticated vertical extent of the plant of > 1 m. While many shorter vascular plant sporophytes are desiccation-tolerant, all of the vegetatively desiccation-tolerant vascular plant sporophytes are < 1 m tall.

3. Prevention of catastrophic xylem embolism when water supply is limited and/or evaporative demand by the atmosphere is high

The negative potential of the xylem contents at which xylem embolism occurs by direct embolization (gas entry through flaws in the xylem wall) or, less likely, by cavitation or implosion to cause embolism is higher (less negative) than that at which vegetative cell death occurs in plants which are vegetatively desiccation-intolerant (Raven, 1993, 1995; Table 1). The loss of conductive function of a single xylem element under low ψ_w conditions means that the remaining elements are operating at an even more negative ψ_w, thereby increasing the likelihood of embolism of the remaining functional conducting elements. It has been suggested that the limitation on the height of desiccation-tolerant vascular plants might be limited by the size of plants in which conducting elements (xylem and phloem) could be re-activated after complete desiccation, although this suggestion does not go into mechanistic details (Raven, 1999a, b). At all events, avoidance of complete loss of conductive function in the xylem involves stomatal closure at plant ψ_w values which are less negative than the values involved in avoiding lethal whole-plant desiccation (Jones & Sutherland, 1991).

This role of stomata, and the two previous roles which were mentioned above, all operate in the same direction and can all be regulated (in principle) with the same set of control mechanisms.

However, the following two possible roles of stomata run to some extent counter to the previous three roles in that they could lead to more water loss per unit carbon gain than the optimization hypothesis (and, perhaps, restricting damage to protoplasm and the onset of embolism) suggests.

4. Cooling of leaves when boundary layer conductances are low

The arguments and counter-arguments are very well explained by Woodward (1998), and the précis here depends heavily on Woodward's discussion. The cooling argument relates to conditions at the canopy scale in which the convective boundary layer rather than the stomata is the major controller of transpiration. This is likely to be the case particularly for dwarf vegetation in which the total boundary layer conductance may, according to some models, be two orders of magnitude lower than the boundary layer conductance of a very tall canopy. In this situation the effect of stomata on transpiration in the dwarf canopy would only be significant at very low conductances, while in the tall canopy the dominant control of the rate of transpiration is via stomata. Since, according to these arguments, stomata would not be significant regulators of transpiration at stomatal conductances much in excess of total boundary conductance, it could be argued that dwarf canopies would have low stomatal densities and conductances. However, Woodward (1998) points out that there is no significant difference between dwarf and tall species in the range of stomatal densities and of maximum leaf conductances between the two life forms.

Woodward (1998) considers the possibility that the higher stomatal density and maximum leaf conductance for dwarf canopies than would be predicted on the basis of regulating transpirational water loss could be related to the need for transpirational cooling in dwarf canopies. There is no evidence for high temperature tolerance in most dwarf plants species of the type found in succulents with a low stomatal conductance especially in the light. Woodward (1998) then considers evidence that the convective boundary layer might exert less control on canopy transpiration than had previously been suggested, due to dry air entrainment from the bulk atmosphere into the convective boundary layer.

These arguments suggest that stomatal control of transpiration is important on the canopy scale even for dwarf vegetation, and that considerations of leaf cooling are not dominant in the evolution of stomatal function.

5. Transport of nutrients from the roots to the shoots in the transpiration stream

It is clear that the transpiration stream is very important in transporting soil-derived nutrients to the shoot in terrestrial vascular plants (Church, 1919; Smith, 1991). However, any relationship between the rate of transpiration and the flux of nutrients to the shoot (both measured as quantity moved to the shoot per plant per day) is not readily perceived for many nutrients (Tanner & Beevers, 2001), although silicic acid transport (measured as deposition of silica in the shoots) can, in some plants, be a reasonable proxy for the quantity of water transpired (Datnoff *et al.*, 2001). A low transpiration rate (e.g. in Crassulacean Acid Metabolism plants) means that a longer time is taken for a given nutrient solute molecule to move from the soil to the shoot for a plant of a given height than is the case for a more rapidly transpiring plant (Raven, 1986b). A low transpiration rate means that, for a given plant size, composition and growth rate, more nutrients will be in transit from root to shoot than is the case for a plant with a higher transpiration rate, and there may also be problems of solubility of some solutes in the more concentrated xylem sap (Raven, 1986b). However, such considerations do not seem to influence significantly the nutrition and growth of slowly transpiring species (Raven, 1986b; Woodward, 1998). Furthermore, it must be reiterated that Church's view that transpiration was not necessary for acquisition of carbon dioxide from the atmosphere was untenable even in the context of what was known in 1919 (Brown & Escombe, 1900, 1905; Edwards *et al.*, 1996), so that rapid delivery of nutrients to the shoot was very unlikely to have been the major role of transpiration of stomata in early land plants (cf. Church, 1919).

The discussion so far deals solely with the delivery of nutrients to the shoot as a whole. The evolution of the shoot system in vascular plants has involved an increasing separation of the main sites of photosynthesis and transpiration (mature photosynthetic tissues) and the main sites of growth (stem apices, young leaves, reproductive organs) which are often only weakly, or non-, photosynthetic and rely on organic carbon translocated to them in the phloem from the photosynthetic tissues (Raven *et al.*, 1994; Raven, 1996b; Gray *et al.*, 2000; Beerling, Osborne & Chaloner, 2001; Pryor *et al.*, 2001). Most of the nutrient elements delivered to the transpirational termini in the transpiration stream can be transported from the nongrowing photosynthetic structure (with no net demand for nutrients) to the growing regions in the phloem, with the organic carbon produced in the photosynthetic structures. However, some elements (e.g. Ca) are essentially phloem-immobile (Raven, 1996b), and whatever evolutionary consideration led to the spatial and temporal separation of 'photosynthesizing' and 'growing' parts of the shoot (Raven *et al.*, 1994) led to problems with Ca delivery to growing parts of the shoot. This is especially the case if considerations of whole-organism carbon fixation per unit water lost in transpiration mean that the growing parts of the shoot usually with no net carbon fixation in the light, have very little transpiration and so have minimal delivery of (e.g. Ca) in the xylem (Raven, 1986b; Raven *et al.*, 1994).

There are plausible means of delivering more nutrients in the xylem per unit of transpiration to the nongrowing organs than to the major, nongrowing, transpirational termini in photosynthetic organs. An example is that xylem loading in the root directs more nutrients per unit water to xylem elements supplying growing regions than those supplying transpiration termini in nongrowing regions (Raven, 1986b; Raven *et al.*, 1994). This mechanism has the potential difficulty that the xylem elements supplying growing leaves are the same as those which subsequently supply mature leaves, so some signal is needed to alter xylem loading characteristics in the root. A similar need for long-distance signalling is needed if the enrichment of xylem streams heading for growing organs are enriched in nutrients relative to those supplying mature photosynthetic structures by xylem-to-xylem (*via* shoot transfer cells) fluxes of nutrients (Raven, 1986b; Raven *et al.*, 1994). Clearly any such mechanisms are not adequate to supply adequate amounts of Ca to reproductive structures of some plants, at least under horticultural conditions: witness the occurrence of Blossom End Rot of *Lycopersicon* fruits and Bitter Pit of *Malus* fruits, both related to inadequate Ca supply to the fruit (Raven, 1986b).

This rather lengthy discussion of the role of stomata, and of the transpiration stream, in nutrient supply suggests that the rate of transpiration has little influence on the supply of soil-derived nutrients to the shoot as a whole, at least for wild plants under natural conditions. The cases in which nutrient (e.g. Ca) deficiency can be found in parts of the shoot which have a limited rate of transpiration arise as a result of shoot differentiation processes which evolved subsequent to the origins of stomata. This tends to dissociate the occurrence on different parts of stomata in extant plants from a major role in nutrient supply via the transpiration stream.

IV. Ecophysiology of ancestrally astomatous terrestrial plants

1. Background

The absence of stomata means that a photosynthetic organism cannot be homoiohydric. Even in the presence of a cuticle, an endohydric conducting apparatus, an intracellular gas space system, and structures which can take up liquid water from the soil, homoiohydry cannot occur if there are no functional stomata (Raven, 1977, 1984a). In discussing the ecophysiology of ancestrally astomatous terrestrial plants we are, then, dealing with poikilohydric organisms, that is, organisms which cannot significantly (on a daily or weekly time scale) delay desiccation when soil water is relatively unavailable and the atmosphere is desiccating. Such organisms may be desiccation-intolerant in the vegetative phase; in this case the organisms must complete the vegetative part of their life cycle without encountering significant soil drying. Poikilohydric phototrophs in less reliably wet habitats must be desiccation-tolerant in the vegetative phase.

2. Terrestrial algae

Starting with the consideration of extant algae (Table 1), many such organisms are too small to either possess or (as far as can be seen) benefit from the occurrence of stomata. Raven (1999a,b) showed that stomata or their analogues cannot occur in unicellular organisms, or organisms consisting of a uniseriate filament or unistratose thallus. The minimum structural requirement for functional stomata is two cell layers with a gas space between them which, when the stomata are closed, is isolated from the bulk air (Raven, 1999a,b). The stomata in each of these cell layers would, to be functional, occur in parallel with cuticle plus wax on the outer, atmospheric, surface of the organism. Despite the occurrence of some terrestrial algae which fulfil the minimum structural complexity requirement for the presence of stomata (Raven, 1977, 1984a) terrestrial algae nevertheless lack both intercellular gas spaces and stomata. Unlike the lichens considered below, dehydration of fully hydrated algal thalli does not give rise to intercellular gas spaces, thus removing an essential prerequisite for stomatal evolution. Raven (1993, 1995) considers the impact of increasing CO_2 concentration on the photosynthesis of mats of terrestrial algae (and cyanobacteria). The higher CO_2 concentration in the Silurian and Lower Devonian (Berner, 1998; Moulton, West & Berner, 2000; Berner & Kothavala, 2001) would have increased the CO_2 fixation rate on a ground area basis of algal mats from approx. 2 to 6 µmol

Table 2 The leaf (thallus) area indices, and ratio of internal area of gas–cell wall interface of photosynthetic cells to the projected external area of photosynthetic structures in lichens, ectohydric, non-ventilated of gametophytes of bryophytes and endohydric, ventilated of gametophytes of bryophytes. Adapted from Green and Lange (1994); see Raven (1995)

Organism	Leaf (thallus) area index	Ratio of internal area of gas cell wall interface to projected external area of photosynthetic structures
Foliose lichens (ventilated)	1	*c.* 2–3[a]
Fructicose lichens (ventilated)	≤ 19	*c.* 2–3[a]
Ectohydric nonventilated thalloid liverworts and hornworts	1	1[b]
Ectohydric nonventilated leafy liverworts and mosses	≤ 129	2[c]
Endohydric, ventilated thalloid marchantiaceous liverworts	1	*c.* 9[d]
Endohydric, ventilated polytrichaceous mosses	≤ 18	*c.* 9[e]

[a] Surface area of photobiont relative to projected thallus area. [b] Value refers to upper surface of thallus where gas exchange with the atmosphere occurs. [c] Leaves of ectohydric mosses and leafy liverworts are generally only once cell layer thick except at the midrib (nerve). Gas exchange occurs at both leaf surfaces. [d] Ratio of internal area of photosynthetic cells to thallus area. [e] Ratio of area of exposed to the gas phase of photosynthetic cells in leaf lamellae to leaf projected area.

CO$_2$ m^{-2} s^{-1} (Raven, 1993). All algae are poikilohydric; most terrestrial algae are desiccation-tolerant (Raven, 1993, 1995).

3. Lichens

Lichens are also ancestrally astomatous. In lichens the fungal component is the main determinant of the structure of the lichen. In many cases the intercellular spaces between fungal hyphae, and between the fungal hyphae and the photobionts, are filled with water when the thallus is fully hydrated. However, when the rate of water loss by evaporation exceeds the rate of water uptake from the soil the intercellular spaces become air-filled (Table 2), sometime paralleling a decrease in thallus volume. The intercellular gas space system of lichens is less precisely controlled than is that of embryophytes (see below; Raven, 1986b, 1996; Green & Lange, 1994), and the variable thallus water content has two opposing effects on photosynthesis. Starting from a completely hydrated thallus (when the water content is perhaps 8 times the d. wt; Lange *et al.*, 1996; Woodward, 1998) the photosynthetic rate at the present atmospheric CO$_2$ mole fraction (360 µmol mol^{-1} total gas) is low, with CO$_2$ fixation limited by inorganic C diffusion through the hydrated intercellular spaces. As water is lost the photosynthetic rate increases; this results as water in intracellular spaces is replaced by a gas phase in which the diffusion coefficient for CO$_2$ is 10 thousand times that in water. For *Peltigera neckeri* the increase in CO$_2$ fixation rate increases as water falls from approx. five times to four times the dry matter content. Further water loss involves removal of water from the inside of the cells, with a decreased biochemical capacity for CO$_2$ fixation. The steady decline in CO$_2$ fixation rate with decreasing water content results in zero net photosynthesis at a water content of about half the d. wt of the thallus (Lange *et al.*, 1996).

Lange *et al.* (1996) performed a similar experiment with high CO$_2$ (2400 µmol CO$_2$ mol^{-1} total gas). Here the effects of water loss below approx. 3.5 times the d. wt closely resembles that of the experiment at 360 µmol mol^{-1} total gas. However, at higher water contents the CO$_2$ fixation rate is essentially independent of CO$_2$ partial pressure up to eight times the dry matter content. The presence of much higher CO$_2$ in the atmosphere than is present today essentially overcomes the limitation on CO$_2$ fixation by diffusion through water in the intercellular spaces. In the context of lichen evolution any lichens which existed in the high CO$_2$ conditions of the Silurian and early Devonian would have had a CO$_2$ fixation rate which was independent of water content between 3.5 and 8 times the dry matter content. Fossil evidence for lichens in the Palaeozoic seems to be restricted to a zygomycete plus cyanobacterium lichen from the Rhynie Chert, a symbiosis which has no extant equivalent in terms of an ectosymbiosis (Taylor *et al.*, 1997). However, recent molecular phylogenetic work (Lutzoni *et al.*, 2001) suggests that the lichen habit arose earlier in the evolution of ascomycetes than was previously thought. Since ascomycetes have probably existed since the Silurian (Sherwood-Pike & Gray, 1985), there could have been lichens with a recognizably modern aspect in the Silurian and Early Devonian, with negligible limitation of photosynthesis by CO$_2$ diffusion up to full thallus hydration at the high atmospheric CO$_2$ mole fraction. It would not have been until the lower CO$_2$ mole fractions in the Upper Devonian (Berner, 1998; Moulton *et al.*, 2000; Berner & Kothalava, 2001) that the restriction on CO$_2$ supply by diffusion in the aqueous phase through the highly hydrated thallus would have become very significant, and could have led to selective advantage for a homoiohydric lichen with stabilized intercellular gas spaces, stomata, cuticle and a mechanism of water uptake from the soil if not an endohydric water conduction system. Raven (1986a) considers what precedents there are in lichens for the evolution of the homoiohydric habit. The necessary cell wall hydrophobicity could come from the more recently discovered hydrophobins (proteins causing hydrophobicity of the cell wall of aerial portions of fungi: Raven, 1996). Hydrophobins could form the basis of a system of stable intercellular gas spaces and of a water-repellent and water-resistant outer cuticle. Raven (1986a) points out that the rapid generation and loss of high turgor pressures which are essential aspects of stomatal regulation, have precedence in the behaviour of carnivorous fungi. However, there seems to be no evidence of any stomata-like behaviour of pores on the surface of lichens (Raven, 1986a).

4. Gametophyte phase of bryophytes

The sporophyte phase of bryophytes, in the case of many hornworts and mosses, has functional stomata, yet there are no reports of stomata on the gametophyte phase of these organisms. Stomata in extant polysporangiophytes are invariably associated with the occurrence of other attributes of homoiohydry (cuticle, intercellular gas spaces, endohydric conducting system), and one or more of these characters are found in a number of bryophyte gametophytes. Raven (1977, 1984a, 1993) points out that cuticle occurs on many bryophyte gametophytes, and that its function is more likely to relate to water repellence (limiting the occurrence of liquid water on the plant surface) than water resistance (limiting water permeability of the plant surface; this would also limit carbon dioxide permeability and hence photosynthesis). Limitation of the occurrence of surface water layer would increase the conductance to the plant surface to CO_2 provided that the water repellency of the cuticle did not necessarily increase water (and CO_2) resistance to a greater extent than eliminating surface water decreases the resistance. However, elimination of surface water also eliminates the ectohydric option for water supply to evaporating surface and to the growing shoot. Thus, plants more than a few cm high which have a hydrophobic outer surface can only supply water to their photosynthesising, growing aerial portions *via* a differentiated endohydric system (Raven, 1977, 1984a, 1993, 1995, 1999a,b; Green & Lange, 1994; Woodward, 1998). Some thalloid liverworts (those in the order Marchantiales) with intercellular gas spaces, pores on the upper surface and cuticle on the upper surface are only hundreds of µm thick and have no differentiated endohydric system related to vertical movement of liquid water (Raven, 1993). However, these organisms are clearly endohydric (Raven, 1993; Green & Lange, 1994).

These gas spaces have a different developmental origin from those of embryophyte sporophytes, and their gas exchange with the atmosphere involves complex multicellular pores which are not functional as stomata (Raven, 1993, 1996). Despite their different evolutionary origin these gas spaces and pores function as do intercellular gas spaces and open stomata in the sporophyte phase of embryophytes in allowing CO_2 diffusion in the gas phase to a larger area of gas–water interface than occurs at the surface of the plants (Raven, 1993, 1995, 1996). P. E. Northing & J. A. Raven (unpublished) have shown a positive correlation between the light- and CO_2-saturated (and the CO_2-limited) rate of photosynthesis and the calculated pore conductance among species and collections of marchantiaceous liverworts. The pore apparatus also functions to limit water entry to the intercellular gas space system from any surface water that accumulates (cuticle notwithstanding) on the thallus.

Raven (1993, 1995) has suggested that the increased CO_2 fixation rate per unit ground area found for thalli such as those of nonleafy liverworts could have been a selective advantage of intercellular gas spaces and pores in nonhomoiohydric plants lacking an endohydric conducting system. Such a thallus structure, with a nonwettable surface, could overcome the problems noted in the extant atmosphere for lichens with no means other than thallus drying of producing intercellular gas spaces (see above). However, as was discussed in the consideration of lichens, the advantage of intercellular gas spaces and surface nonwettability would have been smaller in the Silurian or Lower Devonian, although all of the higher values for light- and CO_2-saturated rates in thalloid (foliose) liverworts investigated by P. E. Northing & J. A. Raven (unpublished) were for thalli with intercellular gas spaces and pores.

The foliose thallus with intercellular gas spaces and pores of the Marchantiales gives a clear example of the kind of evolutionary background in which stomata could have evolved. The alternative mode of increasing photosynthetic rate on a unit ground area in extant bryophyte gametophytes is the leafy shoot, found in all mosses and many liverworts. The 'leaves' of bryophyte gametophytes are typically one cell thick except in the midrib ('nerve'). While these leaves result in at least a 20-fold (counting both sides of the leaf) increase in gas–water interface for CO_2 exchange compared with an unventilated foliose or crustose thallus oppressed to the ground, there is little scope for the development of stomata in such structures. Raven (1993, 1995) has suggested that a stomata-like (but stomata-less) mechanism permitting homoiohydry could evolve in the Polytrichales (e.g. *Polytrichum*, and the tallest known moss, *Dawsonia*). Here, although the leaves are several (about seven) cells thick there is no sign of intercellular space development. However, each longitudinal file of upper epidermal cell bears a lamella, one cell wide, about six cells high and parallel to the long axis of the leaf (Sarafis, 1971; van Zanten, 1974). The lamellae have gas spaces between them. The uppermost cells in a lamella contain fewer chloroplasts per cell, and are wider than, the rest of the lamella cells. The rest of the lamella cells contain most of the chlorophyll in the leaf as a whole (Sarafis, 1971; van Zanten, 1974). The possible functioning of this apparatus in a homoiohydric mechanism (Raven, 1993, 1995) would involve the lamellae being parallel to each other when the plant was at relatively high water potentials, with the uppermost cells in adjacent lamellae separated by a gap of several µm permitting diffusive gas exchange between the atmosphere and the photosynthetic cells in the lamella. It is then hypothesized that signals analogous to those leading to stomatal closure could act on the hydrostatic skeleton of the leaf, possibly increasing the turgor and hence volume of the uppermost row of cells in a lamella to decrease the gap between them. More plausibly (in view of signals indicating a potential excess of water loss over water supply), a decrease in turgor in the upper cells of the leaf blade relative to the lower cells, would bring the lamellae closer to together and decrease the gap for gas exchange between the uppermost rows of cells. There is little evidence as to the functioning of the leaves of members of the Polytrichales as water supply decreases relative to evaporative demands, although some of the responses to water deficiency do appear to restrict the loss of water (Sarafis, 1971; van Zanten, 1974) and the appression of lamellae during water loss has been observed (J. Duckett, pers. comm.). However, this suggestion is worth considering in widening the search for stomata-like mechanisms in almost homoiohydric plants: such mechanisms could be based on an external ('evagination') as well as an internal ('invagination') amplification of the area of gas–water interface relative to the gross external area of the organism. Certainly the polytrichalean gametophytes have other attributes of homoiohydry, that is, an endohydric conducting system in the 'stem' and a waxy layer on the cuticle of the 'shoot' epidermal surfaces which abut on the bulk air phase and which act in water repellency (Proctor, 1979a,b), as well as an extensive below-ground system of 'rhizomes' and rhizoids.

5. Free-living photosynthetic gametophytes of extant polysporangiophytes

None of the photosynthetically competent free-living gametophytes of extant pteridophytes are as complex as the most complex gametophytes of extant bryophytes. None of the extant pteridophyte gametophytes known have stomata, nor do they have intercellular gas spaces, an endohydric conducting system or a well-developed cuticle (Raven, 1977). Accordingly, this phase in the life cycle of free-sporing homosporous pteridophytes is poikilohydric, as is the gametophyte phase of bryophytes (Raven, 1977). Most of these organisms are desiccation tolerant in the vegetative phase (Raven, 1977).

6. *Incertae sedis*

Here we consider Palaeozoic fossils which consist largely or entirely of cuticle, are believed to be parts of terrestrial photosynthetic plants, and are not obviously related to any embryophytes (Edwards, 1986; Edwards *et al.*, 1996; Edwards *et al.*, 1998). The most widespread, geographically and stratigraphically, of these is the *Nematothallus*-like cuticle, found from approx. 460 m yr to 400 m yr ago (Edwards & Rose, 1984). The thalli of *Nematothallus* was of similar size (several square centimetres) to extant thalloid liverworts. It is not clear whether the holes found in some of these cuticles functioned as do the pores of extant Marchantiales; the Nematothallus-like cuticle contained more aromatic residues than the cuticle of embryophytes (Edwards *et al.*, 1996).

7. Ancestrally astomatous plants and its relation to the functions of stomata

The bryophyte gametophyte is the best 'model' of ancestrally astomatous land plants that we have, and their functioning can be compared with the plants with stomata in the context of the five possible 'roles of stomata in extant plants' considered above. We consider two extant endohydric bryophytes, *Marchantia* and *Polytrichum*, both of which are ventilated (see above; Green & Lange, 1994).

The pores of *Marchantia* do not act as stomata and so could not optimize carbon gain per unit water loss in the way considered by Cowan (1977, 1986), since the pores only decrease gas exchange rates when the thallus water potential has fallen so low as to inhibit the biochemistry of photosynthesis. Proctor *et al.* (1992) and Raven *et al.* (1998) provide, and review, evidence on the 'instantaneous' water cost of photosynthesis for terrestrial liverworts, and their natural abundance $^{13}C : ^{12}C$ ratio which can be used to estimate how much of the limitation on CO_2 fixation in a C_3 plant relates to the diffusive conductance of the gaseous and aqueous pathway from the bulk air, and how much can be attributed to the biochemical pathways of CO_2 fixation and subsequent metabolism. This latter measure integrates carbon fixation over the organism's life, but the averaging is biased toward times of rapid carbon assimilation (since that would be when most of the carbon whose $^{13}C : ^{12}C$ is being measured would have been fixed). There are some complications in interpreting these field data, since the $^{13}C : ^{12}C$ of the source CO_2 could have been lower than that in the air (the assumed $^{13}C : ^{12}C$ of source CO_2 in the analysis) because of inputs of ^{13}C-depleted CO_2 from soil respiration. Conversely, in very dense canopies of mosses and leafy liverworts (Green & Lange, 1994; Rice *et al.*, 2001), depletion of CO_2 would increase the mean $^{13}C : ^{12}C$ of source CO_2. An illustrative example of the use of $\delta^{13}C$ values in the Marchantiales is in computing internal CO_2 concentrations is as follows.

The mean $\delta^{13}C$ values for three male and seven female thalli of *Marchantia polymorpha* collected from the University of Dundee campus in 1992 is –27.08 ± 0.28‰ (standard error of the mean) (J. A. Raven & A. M. Johnston, unpublished). Using equation 8 of Farquhar *et al.* (1989), values of a and b from their Table 1, and a bulk air $\delta^{13}C$ of –8‰ the ratio of internal to external CO_2 during steady-state photosynthesis is 0.71. This value is derived assuming negligible CO_2 transfer resistance in the liquid phase from the gas–cell wall interface to the carboxylation enzymes. Taking this ratio of internal to external CO_2 at face value it appears that the rate of photosynthesis is 29% restricted by CO_2 diffusion and 71% restricted by biochemistry when averaged over the growth period. These values are similar to those derived from gas exchange measurements (Green & Lange, 1994), including those in which the pore conductance in the Marchantiales is related to photosynthetic rates (P. E. Northing & J. A. Raven, unpublished). This indicates that marchantialean liverworts fix most of their CO_2 when the limitation of photosynthesis by diffusion of CO_2 is similar to that in C_3 vascular plants. Similar conclusions can be drawn from measurements of gas exchange yielding 'instantaneous' estimates of the ratio of transpiration to photosynthesis (Raven *et al.*, 1998).

The role in restricting water loss which is played by stomata in homoiohydric plants is not applicable to an organism to the extent that *Marchantia* is relatively desiccation-tolerant (Table 1). Their role in preventing embolism in an endohydric conducting system is also not directly relevant in the absence of such a system. Raven (1993) considers water movement through parenchymatous tissues in the context of vertical water movement through a marchantiaceous thallus, and concludes that very modest water potential differences between soil and transpiring surface (less than 15 kPa) would suffice to move water from the soil to the transpiring surface at the required rate. However, continuity of supply might be threatened for movement through water-filled intercellular spaces rather than cell walls or protoplast plus walls, under conditions in which the cell ψ_w had not become so negative as to decrease photosynthetic rates.

The other two possible 'uses' of stomata in vascular plants are temperature regulation and nutrient supply via the transpiration stream. I know of no data on the thallus temperature of marchantiaceous liverworts. The role of transpiratory water loss in marchantiaceous liverworts in their mineral nutrition is also unknown.

The situation for *Polytrichum* shows many similarities to, but some differences from, that in *Marchantia.* For the role of stomata in limiting the rate of water loss to lethally low water contents, when water supply does not keep pace with potential water loss, the relatively desiccation-tolerant *Polytrichum* (Richardson, 1981) (Table 1) can clearly survive significant water loss. As for the role of limiting embolism in the endohydric water conducting system, again it would appear that *Polytrichum* can refill its hydrome after embolism and significant whole-organism desiccation. *Polytrichum*, and even *Dawsonia*, have a smaller stature than the *c.* 1 m height limit for desiccation-tolerant land plants.

Dealing first with the water cost of carbon fixation, data on gas conductance of *Polytrichum* come from natural abundance C isotopes. The natural abundance $^{13}C : ^{12}C$ ratio in *Polytrichum* is, like that of *Marchantia*, in the range of that of C_3 vascular plants (Proctor *et al.*, 1992; Raven *et al.*, 1998) so that, as in *Marchantia*, the ratio of diffusive to total conductance is approx. 0.75. An illustrative examples is *Polytrichum commune* collected near Umeå, Sweden in 1995 which had a $\delta^{13}C$ of −29.2‰ (J. A. Raven & A. M. Johnston, unpublished). Using the method of computation used earlier for *Marchantia* the ratio of internal to external CO_2 is 0.77. This means that photosynthesis is limited to 23% by CO_2 diffusion and 77% by biochemistry (Nobel, 1977; Green & Lange, 1994; Rice *et al.*, 2001) and that the transpiratory water cost of carbon assimilation in *Polytrichum* is similar to that of C_3 vascular plants (Raven *et al.*, 1998).

The fourth possible 'use' of stomata in vascular plants is in temperature regulation in a *Bryum argenteum* turf on Ross Island in the frigid Antarctic is, in early afternoon in the Antarctic summer, 2°C warmer than the soil 2 cm below and 5°C warmer than the air 2 cm above and 12°C warmer than the bulk air (Longton, 1988). Alas, there are no parallel data for neighbouring vascular plants, so it is not clear how stomata regulate the temperature of photosynthetic tissues in this environment.

The final influence that stomata might have on nutrient transport is from below-ground to above-ground parts of *Polytrichum.* The endohydric conducting system might be expected to transport nutrients, and Grubb (1961) obtained evidence consistent with such a role. However, Richardson (1981) cites evidence (Trachtenberg & Zamski, 1978) which appears to show transport of SO_4^{2-} and Pb^{2+} in leptome rather than hydrome. However, the quantity of label in a tissue in transverse sections is not a very reliable indication of the pathway of transport; detailed kinetic studies are needed, with care taken to avoid redistribution during sample preparation from conducting tissues (in this case hydrome) which may be under tension. The role of the hydrome in movement of soil-derived nutrients needs further investigation, especially in the context of the absence of an obvious analogue of the endodermis which is almost ubiquitous in below-ground nutrient-absorbing parts of vascular plants and which allegedly has a major role in the selectivity of transfer to the xylem (Raven & Edwards, 2001). In this poorly understood system the lack of stomata is but one of many problems of interpreting nutrient transport in endohydric mosses. However, granted a similar water loss in transpiration per unit carbon fixed in *Polytrichum* as in vascular C_3 plants, it is tempting to think of nutrient supply in the transpiration stream in *Polytrichum* in a similar manner to that in vascular C_3 plants.

To conclude, ancestrally astomatous embryophytes on land have significant physiological differences from stomata-bearing embryophytes; we shall now consider these stomata-bearing organisms.

V. Evolution of stomata

1. Background

This discussion on the evolution of stomata; and especially on the selective pressures involved, is based on discussions earlier in this article on the monophyly or polyphyly of stomata, on the roles of stomata in extant plants, and on the functioning of ancestrally astomatous plants. In considering the evolution of stomata we must be aware of the varying environmental conditions at different periods, and how these interact with the organisms with their evolutionary history of the presence of stomata (after the origin of stomata). In particular, we must bear in mind the atmospheric composition: at a given time in Earth's history this is a global constant granted the biologically induced variations in CO_2 and O_2 today in the northern hemisphere where most seasonally variable terrestrial primary productivity occurs. By contrast, the climate and edophic conditions are, and have been, globally variable. To this must be added, at least early in the evolution of stomata, the likelihood of poorly developed soils in most if not all climatic regimes, with a corresponding poor water-holding capacity (Raven & Edwards, 2001). These considerations mean that, at a given time in the past, the atmospheric CO_2 content was spatially invariant, while relative humidity, rainfall, and water retention by soil would have been spatially variable.

The temporal constancy of atmospheric CO_2 is subject to relatively short-term variations. Even ignoring current anthropogenic increases in CO_2, there were changes in atmospheric CO_2 in the glacial-interglacial cycles of the Pleistocene of from interglacial *c.* 280 µmol mol^{-1} to *c.* 180 µmol mol^{-1} at glacial maximal, with the past-glacial increase occurring over a few hundred years (Petit *et al.*, 1999). A very significant decrease in atmospheric CO_2 occurred from the Upper Silurian to the Lower Carboniferous, from at least 4 mmol CO_2 mol^{-1} to approx. 400 µmol mol^{-1} (Berner, 1998; Moulton *et al.*, 2000; Berner & Kothavala, 2001). This decrease, with very significant effects on photosynthesis, was largely bought about by the effects of the increased area of land occupied by deep-rooted plants via silicate weathering (Berner, 1998; Berner & Kothalava, 2001; Moulton *et al.*, 2000; Raven & Edwards 2001). Changes in O_2 content of the atmosphere are slower in terms of absolute concentrations of O_2 because of the higher background O_2 levels (Berner, 2001). O_2 influences CO_2 fixation at subsaturating CO_2 concentrations *via* RUBISCO oxygenase activity.

2. The origins of stomata: selection pressures

The earliest well-authenticated stomata are from approx. 410 Ma ago (Edwards *et al.*, 1998), although it is likely that there were earlier stomata which have not been preserved (see above). At this time (Upper Silurian) the atmospheric CO_2 mole fraction was at least 10 times the present value (Berner, 1998; Moulton *et al.*, 2000; Berner & Kothavala, 2001; Crowley & Berner, 2001; Raven & Edwards, 2001). This would have had significant impacts on the performance of astomatous photosynthetic organisms. As indicated above, high levels of atmospheric CO_2 can to some extent offset the inhibitory effect of intercellular water (in lichens) or surface water (or ectohydric bryophytes) on photosynthetic rates in the present atmosphere. The high CO_2 concentrations in the atmosphere can maintain high CO_2 fluxes to sites of photosynthesis despite the low diffusive conductance of CO_2 caused by the liquid water (Raven, 1993, 1995). The higher CO_2 mole fraction in the atmosphere would also have increased the quantity of carbon fixed per unit water lost in transpiration even in astomatous plants (Raven, 1993, 1995). Of course, astomatous plants could not perform any of the other functions associated with the occurrence of stomata, that is, restrict tissue water loss, limit embolism of endohydric water-conducting systems, optimize carbon gain per unit water loss under varying atmospheric and edaphic conditions, and prevent overheating and increase delivery of nutrients to particular sites in the shoot by increased transpiration.

In searching for the selective pressures which could have been involved in the evolution of stomata, it is important to note that cuticle and intercellular gas spaces are prerequisites for all of the stomatal functions indicated above. Raven (1977, 1984a, 1993, 1995, 1996) and Edwards *et al.* (1996) discuss the selective pressures influencing the evolution of these structures. Cuticle probably had early roles in defence against parasites and grazers, in reflecting UVB, and in water repellency.

Intercellular gas spaces apparently had an amplification of the surface area for gas exchange between gas (ultimately the atmosphere) and liquid phases as their earliest function, an invaginatory alternative to the evaginations of 'leaves' without intercellular spaces but sometimes (as in *Polytrichum* and *Dawsonia*) lamellae on the leaf (Raven, 1993; Green & Lange, 1994; Table 2). The role of intercellular gas spaces in increasing the area for gas exchange is connected to atmosphere by a continuous gas phase, that is, requires pores in the epidermis (as in the Marchantiales). These possible selective advantages of cuticle and of intercellular gas spaces (plus pores) in early embryophytes would have been essentially independent of life form, granted the requirement that intercellular gas spaces can only occur in relatively bulky (several cells thick) tissues and can only significantly increase the surface area for gas exchange in such tissue (Raven, 1999a,b). Relatively bulky tissue is required for the increased height which allows the organism to compete for solar photosynthetically active radiation, and to get out of the soil surface diffusion boundary layer into more vigorously mixed air which can enhance CO_2 supply and (for the sporophyte phase) aid wind dispersal of spores. Such relatively tall plants today (e.g. gametophytes of *Polytrichum* and *Dawsonia*, vascular plants sporophytes) require (and have) an endohydric conducting system (Raven, 1999a, b), but, in the case of *Polytrichum* and *Dawsonia* gametophytes, lack true intercellular gas spaces.

The possible roles (from the five interrelated functions listed above) of stomata at the time of their origin(s) depends on the assumptions made about the other attributes of the organism(s). We have already seen that cuticle and intercellular gas spaces are prerequisites, with the intercellular gas spaces connected to the atmosphere by pores in the epidermis in which stomatal functions will evolve. Did the plant(s) in which stomata evolved have an endohydric conducting xylem? If not, then avoidance of embolism in the endohydric system, and targetting of nutrients within the shoot, could not have been early functions of stomata.

We assume that stomata evolved in the sporophyte phase of embryophytes, although the fossil record only has the earliest sporophyte stomata some 15 Ma before the only known gametophyte stomata: figure 1 of Edwards *et al.* (1998). The ancestral state of the sporophyte phase of embryophytes seems to be an axial structure (Kenrick & Crane, 1997). Extant examples of the earliest-branching clades of embryophytes which have morphologically defined stomata in their (photosynthetic) sporophytes (hornworts and mosses) lack (hornworts) or sometimes have (mosses) a differentiated endohydric water conducting system in the sporophyte (Hebant, 1977; Ligrone *et al.*, 2000). However, the moss *Sphagnum* has stomata in the capsule walls but lacks *internal* dead water-conducting cells (Kenrick & Crane, 1997; J. Duckett, pers. comm.). Furthermore, the sporophytes of living mosses and liverworts are all 'parasitic' on the gametophyte phase (matrotrophy; Graham & Wilcox, 2000b), that is, depend on the gametophyte for all soil-derived resources (water, mineral nutrients) and for that fraction of the organic carbon which is not produced by sporophyte photosynthesis. The earliest-branching clade of embryophytes which currently have morphologically characterised stomata on their sporophytes is the hornworts and not the mosses, regardless of whether hornworts diverged earlier than liverworts or *vice versa* (Qiu *et al.*, 1998; Renzaglia *et al.*, 2000). No hornworts have an endohydric system in their gametophyte phase, thus making the (unlikely) possibility that prevention of embolism in the endohydric system of the gametophyte phase was an early function of the stomata in sporophytes. The absence of an endohydric conducting system would not deny a role for the earliest stomata in thermoregulation (increased transpiration at times of high temperature stress relative to other times), but does make a role in increasing the flow of soil-derived nutrients to the sporophyte as a whole, or to specific sites, less likely. This leaves roles in limiting water loss as a means of delaying desiccation in an environment with fluctuating water supply and/or evaporative demand of the atmosphere, and optimizing carbon gain per unit water lost in such fluctuating environments, as the earliest roles for stomata. It is likely that the earliest embryophytes were relatively desiccation-tolerant, so delaying water loss as a means of avoiding death by desiccation is perhaps less significant than in desiccation-intolerant vascular plants today. However, regulating water loss so that the carbon gain per unit water lost was maximized in a fluctuating environment could have been a significant early function of stomata, even in the high CO_2 conditions of the Silurian and Lower Devonian (Konrad *et al.*, 2000).

For embryophyte sporophytes lacking a differentiated endohydric system the evolution of stomata seems most plausibly rationalized by selection pressures related to maximizing C gain per unit water lost, and (less likely) thermoregulation. In the absence of good resource costings of vegetative desiccation tolerance and of the production and operation of stomata (relative to pores not functional as stomata), it is not possible to decide if stomata (and other aspects of the homoiohydric system) have advantages in resource cost relative to vegetative desiccation tolerance (Raven, 1999b).

We now need to consider the possibility that stomata evolved in embryophytes which already had a differentiated endohydric conducting system (Raven, 1977, 1993). This possibility is not favoured by the most parsimonious interpretation of phylogenetic analyses and the functioning of extant organisms; the basal embryophytes (hornworts and hepatics) have no differentiated endohydric apparatus in their sporophytes. However, such a scenario would involve secondary loss of endohydry in bryophyte sporophytes, and perhaps a return to life-long matrotrophy by the sporophytes after an episode in which they were independent later in their life after an initial matrotrophic stage (as in extant free-sporing pteridophytes). In this case there would have been a role for stomatal function in the avoidance of embolism in the endohydric system of the sporophyte as well as the considerations noted in the last paragraph.

The occurrence of stomata on embryophyte gametophytes from the Rhynie Chert (Table 1 of Edwards *et al.*, 1998) together with other attributes of homoiohydry raises the possibility of the origin of stomata in the gametophyte phase. This possibility can be rephrased as the first expression of stomata in the gametophyte phase since, as in extant organisms, the necessary genes would be present in both phases. The paucity of known gametophyte fossil material relative to that of sporophyte material makes this possibility difficult to evaluate. On the one hand the occurrence of such features as cuticle (with or without stomata) would enhance the chances of fossilization relative to those of a noncuticularized organism. On the other hand, unless the cuticularized specimen is structurally associated with archegonia and antheridia and is not structurally associated with sporangia then it is not possible to show that the fossil was indeed a gametophyte. The possibility of stomata on gametophytes as a prepolysporangiophyte trait is not ruled out by all of the known stomata-bearing gametophytes being associated with polysporangiophyte sporophyte phases, since the deposits in which antheridia and archegonia are preserved contain no unambiguous fossils of sporophytes at the bryophyte grade of organization. The origin of stomata on gametophytes will not be further considered due to lack of evidence and because the considerations of 'stomata before endohydry' vs 'stomata after endohydry' would be similar to those for sporophytes. However, the putative advantage of a tall sporophyte for spore dispersal is replaced by the putative advantage of at least the sex organs of gametophytes being less likely to desiccate if they were not exposed to free air circulation.

3. The evolution of stomata: the ancestry of the mechanism of stomatal functioning and of regulation of stomatal index

Since this article is about selective pressures on stomatal evolution this topic is somewhat peripheral. We note that turgor regulation is a universal feature of terrestrial embryophytes (although the freshwater representatives of the Charales, a close relative to the ancestors of the embryophytes (van den Hoek *et al.*, 1995), regulate internal osmolarity, which amounts to turgor regulation in a low and constant external osmolarity) (Raven, 1977, 1984a). However, the occurrence of 'sliding scale' of turgor set points in stomatal function is apparently unknown elsewhere in walled cells.

It is also difficult to find a precedent for the asymmetry of wall structure which converts increased turgor to opening. The dominance of organic anions as turgor-generating anions (except perhaps in grass stomata) can be related to the frequent role of organic rather than inorganic anions in embryophytes (Raven, 1991, 2000; Ryan *et al.*, 2001). The regulatory mechanisms for stomatal function (responses to ABA, CO_2, relative humidity, ψ_w, blue and photosynthetically active radiation) in many cases have parallels elsewhere in plants (Raven, 1977; Leung & Giraudat, 1998; Schroeder *et al.*, 2001).

We do not know the order in which these regulatory mechanisms became involved in the functioning of stomata during evolution. Any of the environmental and internal signals listed above could plausibly have been involved in the early regulation of stomatal aperture with stomata functioning in optimising carbon gain per unit water lost even in a high CO_2 atmosphere, and in limiting the rate of water loss when water supply cannot keep pace with evaporative demand and CO_2 fixation is very limited or absent. It is likely that the relative significance of different factors in regulating stomatal function (Paterson *et al.*, 2001) would have changed with variations in atmospheric CO_2 levels in the last 410 Ma (Berner, 1998; Moulton *et al.*, 2000; Berner & Kothalava, 2001).

The genetic regulation of the presence or absence in different parts of the epidermis is not well understood, but progress is being made in understanding genetic and environmental control of stomatal indices on plant parts which normally have stomata (Gray *et al.*, 2000; Lake *et al.*, 2001). The genotypically determined stomatal index (and/or density) modulated by environmental factors (especially atmospheric CO_2 partial pressure), has been related to past CO_2 levels, and even used as a proxy for these CO_2 abundances (Woodward, 1987; McElwain & Chaloner, 1995; Berner, 1998; Edwards *et al.*, 1998; McElwain, 1998; Brownlee, 2001; Roger *et al.*, 2001).

VI. Ecophysiological implications of losses of stomata

The most obvious case of the loss of the capacity to produce morphologically defined stomata among embryophytes would be the superdivision Marchantiomorpha, assuming monophyly of stomata and the phylogeny suggested by (*inter alia*) Renzaglia *et al.* (2000) where the hornworts (superdivision Anthoceromorpha) are the earliest-branching embryophytes (Kenrick & Crane, 1997). In no other superdivision of embryophytes is there a complete absence of stomata on the sporophytes; the absence of stomata in the liverworts and the maintenance of the sporophyte in the gametophytic tissue until it is mature is correlated with the negligible capacity for photosynthesis in liverwort sporophytes (although spores are frequently green). Kenrick & Crane (1997) point out that a significant fraction of hornwort species have sporophytes without stomata. A rather smaller fraction of moss species, including all of the basal mosses in the Andreaeidae and *Takakia*, lack stomata on their sporophytes (Parihar, 1961; Murray, 1988; Smith & Davison, 1993; Kenrick & Crane, 1997). Stomata are found in the Sphagnidae although there are doubts about their functionality (Paton & Pearce, 1957; J. Duckett, pers. comm.). In the most derived mosses, the Bryidae, genera such as *Polytrichum* have species with (e.g. *P. formosum*) and without (e.g. *P. aloides*) stomata (Paton & Pearce, 1957). The absence of structurally defined stomata in certain mosses and hornworts is a result of phylogenetic loss. In the hornworts and mosses the sporophytes without functional stomata generally retain photosynthetic competence. The mosses with stomata-less capsules also generally retain the endohydric conducting system in the seta, except in genera (*Andraea*, *Sphagnum*) which Kenrick & Crane (1997) regard as having no seta. The occurrence of stomata (not necessarily functional) in hornworts and *Sphagnum* with no conducting tissue in their sporophytes recalls the hypothesis of 'stomata before endohydry' (Section VII) in the origin of stomata. By contrast, the occurrence of endohydric conducting tissue but no stomata, for example, in *Takakia* and some *Polytrichum* species, recalls the hypothesis of 'endohydry before stomata' in the origin of stomata (Section VII).

The occurrence of matrotrophy in bryophyte sporophytes (Graham & Wilcox 2000a,b; Proctor, 1977) means that the extent of photosynthesis by the sporophyte relative to the organic carbon derived from the gametophyte should be considered in the case of loss of stomata in bryophyte sporophytes. By analogy with tracheophyte sporophytes lacking functional stomata the situation at the bryophyte grade of organization is more comparable to those tracheophyte sporophytes which rely almost entirely on organic carbon sources other than their own photosynthesis than to those which acquire inorganic carbon for photosynthesis from a bulk water on sediment phase (see below). As is pointed out by Raven *et al.* (1987), the sporophyte phase of aquatic bryophytes only functions in reproduction when the mature capsule is exposed to air during, for example, seasonal lowering of water level or by seta elongation. An example is the very hydrophobic sporophytes of the otherwise submerged moss *Hygrohypnum* (J. Duckett, pers. comm.).

Among terrestrial tracheophytes the absence of functional stomata from the above-ground as well as below-ground parts of the shoot of the sporophyte phase is related to the plant obtaining most or all of its organic carbon from a source other than its own photosynthesis. Examples are nonphotosynthetic flowering plants which are parasitic on other flowering plants and which solely or mainly access the host plant's phloem, and 'saprophytic' flowering plants which are mycoparasites, that is, access soil organic carbon via their mycorrhizal fungi. An example of this latter category is the bird's nest orchid *Neottia nidus-avis*, in which the stomata are nonfunctional because the guard cells are fused closed (Ziegler, 1987).

Another major category of plants with no stomata, or stomata which do not respond to the normal signals, are many emergent, interfacial or submerged aquatic plants. Dealing first with emergent aquatics, aerial leaves of these plants frequently show less stomatal response to signals such as ABA; an example of such a plant is *Gunnera*. There is also evidence for little or no increase in ABA as a result of water stress in amphibious plants (Dörffling *et al.*, 1977). The hydrophytes examined also showed little or no delay in stomatal re-opening after recovery from reduced shoot water content, a response of mesophytes which is correlated with increased ABA levels (Dörffling *et al.*, 1977).

In aquatics with interfacial photosynthetic structures the stomata are commonly always open. For the rhizophytic *Nymphaea alba* and *Nuphar lutea* this seems to be a result of the absence of a substantial cavity (Ziegler, 1987), while the epipleustophyte *Lemna minor* has dead guard cells (Ziegler, 1987).

Another category among the aquatics is those which are fully submersed for most of all of their life, and which have no stomata, or at least stomata which are permanently closed (Sculthorpe, 1967). Intermediate between the emergent or interfacial plants and the fully submersed plants are amphibious plants. Amphibious plants are those which can grow (if not reproduce) with all of their leaves either under water or exposed to air.

In some cases there is suppression of stomatal development or function in amphibious vascular plants when they are grown submerged, or in atmospheres of very high humidity (e.g. several plants of the isoetid life form, such as *Litorella uniflora*, and many species of *Isoetes*: Raven *et al.*, 1988). In these cases the production of stomata is correlated with the change from Crassulacean Acid Metabolism (CAM) in astomatous leaves to C_3 metabolism in leaves with stomata (Raven *et al.*, 1998). However, *Lobelia dortmanna* and some montane amphibious species of *Isoetes* (e.g. *I. andicola*) never produce stomata on their leaves even when they are growing emersed (Raven *et al.*, 1988; Pédersen & Sand-Jensen, 1992; Keeley, 1998; Woodward, 1998). In the montane *Isoetes* spp. all of the carbon fixation involves CAM, while *Lobelia dortmanna* (also with an isoetid life form) is a C_3 plant (Keeley *et al.*, 1984; Raven *et al.*, 1988; Winter & Smith, 1996; Keeley, 1998). A common feature of the plants with the isoetid life form is a major role for the roots rather than the shoots in the net uptake of carbon dioxide in photosynthesis when there are no functional stomata on the leaves. Dominant root uptake of carbon dioxide occurs for the astomatous leaves of some of the montane *Isoetes* species (e.g. *I. andicola*, *I. andina*, *I. triquetra*) and of *Lobelia dortmanna* even when they are growing immersed (Keeley, 1998; Pédersen & Sand-Jensen, 1992). Pédersen & Sand-Jensen (1992) have suggested that the isoetid morphology and predominant root exchange of carbon dioxide (and oxygen) evolved on land before *Lobelia dortmanna* became a predominantly submerged organism. It is of interest that *L. dortmanna* retains insect pollination of its immersed flowers, and that stomata develop on the inflorescence as is the case for (vegetatively) fully submersed flowering plants which retain insect pollination, for example, *Ranunculus penicillatus* ssp. *pseudofluitans* (Raven *et al.*, 1987, 1988).

Regardless of the timing of the evolution of permanently astomatous leaves in *L. dortmanna* and certain montane *Isoetes* spp., and total reliance on root-derived CO_2 for photosynthesis, that is, before or after they reverted to the amphibious habit, certain likely, or possible roles of stomata can no longer occur.

One such role is that of evaporative cooling. Keeley *et al.* (1994) showed that leaves of emersed *Isoetes andicola* exceed air temperature by about 10°C when the (natural) photosynthetically active radiation was about 1200 μmol photon m^{-2} s^{-1}. Woodward (1998) comments on the extent to which a thermal threshold could be reached for *I. andicola* leaves on warmer and brighter days, and suggests that this might not occur for immersed leaves of *L. dortmanna* which grows at lower altitudes but higher latitudes.

Another possible role for water vapour loss through stomata is in increasing the water (and hence solute) flux up the xylem (Church, 1919). This clearly cannot occur for the immersed astomatous leaves of isoetids, however, what data are available (Keeley *et al.*, 1994; Woodward, 1998) suggest that *I. andicola* does not show any signs of N shortage in their leaves. For submersed aquatic plants transpiration is not an option, but 'Munch recycling' of water up the xylem balancing what moves out of leaves in the phloem and fluxes of water up the xylem to supply the water requirements of growth could both occur (Smith, 1991; Tanner & Beevers, 2001). Of course, in submersed leaves without the thick and essentially water-impermeable cuticle of *L. dortmanna* or montane *Isoetes* spp. the 'Munch water' and the water needed for shoot growth could be supplied by direct uptake from the shoot environment thus obviating the need for water flux up the xylem for these two processes.

These points notwithstanding, water fluxes along the xylem (from root to shoot) have been observed in several submerged water plants, including *L. dortmanna* (Pédersen & Sand-Jensen, 1993; Pédersen, 1993). These fluxes have been attributed to root pressure plus guttation as well as the 'Munch recycling' and water use in growth which were mentioned above. Pédersen & Sand-Jensen (1997) showed that such non-transient water flow was adequate for nitrogen supply to leaves of submerged specimens of *Mentha aquatica*. Raven

(1984b) pointed out that a root pressure plus guttation mechanism could even work for seagrasses, although Pédersen & Sand-Jensen (1993) failed to find a xylem water flow in the only seagrass (*Zostera marina*) that they tested.

A final point in considering the loss of stomata in most submerged, and in some amphibious, plants is that many 'terrestrial' plants naturally spend time under water with little or no change to leaf morphology and anatomy. Sand-Jensen & Frost-Christensen (1998, 1999) have investigated the photosynthesis of a range of riparian, amphibious and obligately submerged plants from lowland streams. They showed that leaves grown in water have 1.8–4.6 times higher CO_2 conductance values (and hence photosynthetic rate at a limiting CO_2 concentrations) than did leaves grown in air when both were measured in water. When measured in air the CO_2 conductance of aerial leaves was 1.3–1.6 times higher than that of aquatic leaves. All of these plants appear to have wettable leaves so that there was not an air film on the surface of leaves when submerged in water. This contrasts with the situation in *Oryza saliva* where there is an air layer over the submerged leaves and, presumably, stomatal functionality (Raskin & Kende, 1985).

VII. Conclusions

The origin of stomata in embryophytes in the Silurian (possibly earlier) has been considered in relation to five groups of selection pressures related to the ecophysiological importance of stomata in extant plants. Of these, the most likely selection pressures active in the early Palaeozoic under high CO_2 conditions are the optimization of carbon gain per unit water lost in transpiration, and limiting the rate of water loss when under restricted water supply and/or high evaporative demand thus slowing approach to a lethally low water content. Rather less likely is limiting 'runaway embolism' in the endohydric conducting system (which may not have been present in the organisms in which stomata evolved). Even less likely are roles in increasing the transpiratory water loss as part of thermoregulation, or as a means of increasing the flux of soil-derived nutrients to above-ground parts.

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