Light and growth form interact to shape stomatal ratio among British angiosperms

Christopher D. Muir[1]

[1] Biodiversity Research Centre and Botany Department, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada

Author for correspondence:
Christopher D. Muir
Tel: +17782284851
Email: chrisdmuir@gmail.com
University of British Columbia
6270 University Blvd.
Vancouver, BC, Canada
V6T 1Z4

Short title: Shedding light on stomatal evolution

Word count:
Summary: 190
Introduction: 1179
Materials and Methods: 1176
Results: 433
Discussion: 1504
Acknowledgement: 24
3 Figures and 1 Table, 1 Supplemental Figure

Summary

- In most plants, stomata are located only on the abaxial leaf surface (hypostomy), but many plants have stomata on both surfaces (amphistomy). High light and herbaceous growth form have been hypothesized to favor amphistomy, but these hypotheses have not been rigourously tested together using phylogenetic comparative methods.
- I leveraged a large dataset including stomatal ratio, Ellenberg light indicator value, Raunkiær lifeform, and phylogenetic relationships for 372 species of British angiosperms. I used phylogenetic comparative methods to test how light and/or growth form influence stomatal ratio.
- High light and herbaceous growth form are correlated with amphistomy, as predicted, but they also interact; the effect of light is pronounced in therophytes (annuals) and perennial herbs, but muted in phanerophytes (mostly trees). Interestingly, amphistomy and stomatal density evolve together in response to light, suggesting coordinated selection on this trait combination.
- I show for the first time that light and growth form interact to shape variation in stomatal ratio; amphistomy is advantageous in high light, but mostly for herbs. These results improve our understanding of the adaptive significance of stomatal ratio as well as its use as functional trait for paleoecology and crop improvement.

Keywords

Adaptation, amphistomy, Ellenberg light indicator value, growth form, phylogenetic comparative methods, Raunkiær lifeform, stomata, stomatal ratio

Introduction

Natural selection shapes leaf anatomy in order to optimize its photosynthetic function in a given environment (Haberlandt, 1914; Givnish, 1987; Smith et al., 1997). By understanding the adaptive significance of leaf anatomical variation we can learn about natural history, find targets for crop improvement, and identify anatomical proxies for paleoclimates preserved in the fossil record (e.g. Wolfe, 1971; Royer, 2001; McElwain and Steinthorsdottir, 2017). The size, density, and distribution of stomata on a leaf vary widely and impact the flux of CO_2 and water vapour (recently reviewed in Sack and Buckley, 2016), as well as susceptibility to foliar pathogens that infect through stomata (McKown et al., 2014; Melotto et al., 2017). Hence, stomata have been especially useful in understanding plastic and evolutionary response to climate change and domestication (Woodward, 1987; Beerling and Royer, 2011; Milla et al., 2013).

While the density and size of stomata have been researched extensively (Sack and Buckley, 2016, and references therein), the adaptive significance of stomatal distribution is less well understood. Stomata are most often found only on the lower leaf surface (hypostomy) but occur on both surfaces (amphistomy) in many species (Metcalfe and Chalk, 1950; Parkhurst, 1978; Mott et al., 1984). Theory and experiments demonstrate that amphistomy increases photosynthetic rates under many

conditions. By creating a second parallel pathway for CO_2 diffusion within the mesophyll, amphistomy optimally supplies CO_2 (Parkhurst, 1978; Gutschick, 1984; Jones, 1985). Amphistomy is correlated with greater CO_2 diffusion (Beerling and Kelly, 1996) and higher photosynthetic rates (McKown et al., 2014). These observations are corroborated by experiments demonstrating that amphistomy increases maximum photosynthetic rates by up to 20% (Parkhurst and Mott, 1990). On the other hand, amphistomy can increase transpiration (Jones, 1985; Foster and Smith, 1986; Buckley et al., 2015). While transition to amphistomy is thus thought to increase transpiration, empirical studies suggest greater water-use efficiency in amphistomatous species (Bucher et al., 2017). Hence, amphistomy appears to benefit a plant's carbon use relative to water loss and should be favored when CO_2 limits photosynthetic rate. The open questions are under what ecological conditions does CO_2 supply most strongly limit photosynthetic rate (Peat and Fitter, 1994) and when is photosynthetic rate most important to fitness?

The leading, nonmutually exclusive hypotheses are that 1) open habitats favour amphistomy because CO_2 diffusion most strongly limits photosynthetic rate under high light and 2) herbaceous growth form favours amphistomy because traits that maximize photosynthetic rate are often under stronger selection in herbs. Salisbury (1927) first noted that amphistomy is most common in herbaceous plants from open habitats (i.e., with high light) of the British flora. These observations have been replicated in other studies (Mott et al., 1984; Peat and Fitter, 1994; Jordan et al., 2014; Muir, 2015) and may support physiological and ecological hypotheses that CO_2 most strongly limits photosynthesis in high light and/or photosynthesis contributes most to fitness in herbaceous plants. Under high light, CO_2 can strongly limit maximum photosynthetic rates, especially in thick leaves (Jones, 1985). Hence, having

stomata on both surfaces relieves this limitation by adding a second parallel pathway for CO_2 diffusion. Parkhurst (1978) argued that greater leaf thickness *per se* selected for amphistomy, but there is little evidence for correlations between leaf thickness and stomatal ratio independent of light (Mott et al., 1984; Gibson, 1996; Muir, 2015). Amphistomy is correlated with open habitat in warm desert plants of western North America (Mott et al., 1984; Gibson, 1996), among the Proteaceae (Jordan et al., 2014), and in continental European herbs (Bucher et al., 2017).

Stomatal ratio is also associated with growth form. In the British flora, Salisbury (1927) found that trees and shrubs are nearly always hypostomatous, whereas herbs from open habitats are amphistomatous. This pattern holds when data are averaged by family to coarsely control for phylogenetic nonindependence (Peat and Fitter, 1994) or when using alternative classification schemes, such as Raunkiær life form (Peat and Fitter, 1994). Across plants from $\sim$ 90 families worldwide, growth form is the strongest predictor of stomatal ratio when multiple factors are estimated simultaneously and controlling for phylogenetic nonindependence (Muir, 2015). These patterns are consistent with other data indicating that many herbaceous plants are under strong selection for high maximum photosynthetic rates (Bazzaz, 1979; Körner et al., 1989; Wullschleger, 1993).

Although previous comparative studies have tested whether open habitat and growth form influence stomatal ratio, we do not know if these effects are independent of one another. Open habitat and growth form may not be independent because open habitats generally consist of more short-statured, herbaceous plants. Some authors have attempted to disentangle light and growth form by contrasting herbs from open and understory habitats (Salisbury, 1927). However, this is problematic if phylogenetic relationships are not controlled for, because shade species may share

traits simply because they are more closely related to each other than they are to high light species. Finally, open habitat and growth form may also interact with one another. For example, amphistomy may only be favored when CO_2 strongly limits photosynthetic rate (e.g. in high light) *and* photosynthetic rate strongly limits fitness (e.g. in herbs).

To better understand the adaptive significance of stomatal ratio, I asked three main questions:

1. Are light habitat and growth form correlated?
2. Do light habitat and growth form influence stomatal ratio additively, or do their effects interact?
3. Is evolution of stomatal ratio mediated by changes in stomatal density on the adaxial (upper) surface, abaxial (lower) surface, or both?

The final question is important for addressing whether amphistomy is part of a coordinated syndrome of traits that promote higher photosynthetic rate, as both the light and growth form hypotheses assume. If evolved increases in stomatal ratio are mediated by shifting abaxial stomata to the adaxial surface, holding total stomatal density constant, then the overall increase in CO_2 diffusion would be small. In contrast, if amphistomy evolves by increasing adaxial stomatal density while holding abaxial density constant, then *total* stomatal density must increase as well. Evolutionary coordination of amphistomy and high stomatal density would reinforce one another, increasing CO_2 supply to chloroplasts more than changes in either trait would in isolation.

To address these questions, I reanalyzed existing data on stomatal ratio, light habitat, and growth form in British angiosperms (Salisbury, 1927; Fitter and Peat, 1994,

2017) using phylogenetic comparative methods. The British angiosperm flora is well suited for these questions because this flora has been comprehensively surveyed for many ecologically important traits, meaning it is probably the least biased survey of stomatal trait variation. Salisbury's observations on stomata and ecology in the British flora have heavily influenced plant ecophysiology, but many of his and subsequent authors' analyses have significant limitations because of inadequate statistical methods. For example, few analyses until recently account for phylogenetic nonindependence (Felsenstein, 1985), which can strongly influence inferences on stomatal traits and growth form (Kelly and Beerling, 1995, this study did not consider light). A species-level phylogeny of the entire British flora (Lim et al., 2014) now allows for the first time rigorous analysis of evolutionary relationships among stomatal ratio, light, and growth form.

Materials and Methods

Data and annotated source code to generate this manuscript are available on GitHub (https://github.com/cdmuir/britstom) and Dryad (Muir, 2017).

Data on stomatal ratio, light habitat, growth form, and phylogenetic relationships

I obtained data on ab- and adaxial stomatal density on 395 species from British Ecological Flora (Salisbury, 1927; Fitter and Peat, 1994, 2017). Following recent comparative analyses (e.g. Bartelheimer and Poschlod, 2016; Salguero-Gómez et al., 2016; Shipley et al.), I used Ellenberg light indicator values (Ellenberg, 1974) and

Raunkiær life form (Raunkiær, 1934) as measures of light habitat and growth form, respectively. Hence, I am assuming that the species' light habitat is closely related to the type of habitat (open versus closed) where that species is found. Both attributes have been recently updated by taxonomic experts of the British flora (PLANTATT, Hill et al. (2004)). Ellenberg light indicator values are hereafter abbreviated L-value. I used a dated molecular phylogeny of the British flora (Lim et al., 2014) available from TreeBASE (http://treebase.org/; accession number 15105). 14 species (3.5%) in the dataset were not present in the phylogeny. For 8 of these species, I used the position of a congeneric species as a proxy for the focal species (following Pennell et al., 2016). When multiple congeneric species were present, I consulted the phylogenetic literature to identify the most closely related proxy species (Scheen et al., 2004; Salmaki et al., 2013). For the remaining 6 missing species, I positioned them in the tree based on phylogenetic relationships to other genera or families present in the tree (Fior et al., 2006). Because many phylogenetic comparative methods do not allow polytomies, zero-length branches, and non-ultrametric trees, I made several small adjustments to the tree. I resolved polytomies randomly using the 'multi2di' function in **phytools** version 0.6-00 (Revell, 2012). I added 0.02 my to all zero-length branches, as this was approximately the length of the shortest nonzero branch length in the tree. After these changes, I slightly altered terminal branch lengths to make the tree precisely ultrametric.

I excluded data on hyrdrophytes (14 species) because many of these species are hyperstomatous (Fig. S1) due to the fact that leaves may rest on the water's surface, selecting for stomata to be present on the upper surface only. I also excluded C_4 (3 species) and CAM (2 species) plants. I limited this investigation to angiosperms because only 4 non-angiosperms had stomata data. The final dataset contained 372

species (Fig. 1). The R code accompanying this paper documents these decisions with citations to the relevant literature.

Following Muir (2015), I calculated stomatal ratio in two different ways depending on what was most appropriate for the question:

$$\mathrm{SR}_{\mathrm{propAd}} = \frac{\mathrm{SD}_{\mathrm{ad}}}{\mathrm{SD}_{\mathrm{total}}} \tag{1}$$

$$\mathrm{SR}_{\mathrm{even}} = \frac{\min\{\mathrm{SD}_{\mathrm{ab}}, \mathrm{SD}_{\mathrm{ad}}\}}{\max\{\mathrm{SD}_{\mathrm{ab}}, \mathrm{SD}_{\mathrm{ad}}\}} \tag{2}$$

$\mathrm{SD}_{\mathrm{ab}}$ and $\mathrm{SD}_{\mathrm{ad}}$ are the stomatal densities on abaxial or adaxial surface, respectively. $\mathrm{SD}_{\mathrm{total}} = \mathrm{SD}_{\mathrm{ab}} + \mathrm{SD}_{\mathrm{ad}}$. $\mathrm{SR}_{\mathrm{propAd}}$ is the proportion of stomata density on the adaxial surface, which is useful for discriminating among hypostomatous ($\mathrm{SR}_{\mathrm{propAd}} = 0$), amphistomatous ($0 < \mathrm{SR}_{\mathrm{propAd}} < 1$), and hyperstomatous species ($\mathrm{SR}_{\mathrm{propAd}} = 1$). $\mathrm{SR}_{\mathrm{even}}$ indicates how evenly stomatal densities are distributed across both leaf surfaces. This expression is useful because several hypotheses are based on the fact that a more even distribution should optimize leaf CO_2 diffusion.

Testing for an association between open habitat and growth form

I tested whether Raunkiær life form was associated with L-value among British angiosperms using ANOVA with Type-2 sum of squares. I did not use phylogenetic ANOVA for this test because there was no phylogenetic signal in the regression fit using **phylolm** version 2.5 (Ho and Ané, 2014). See the R code accompanying this

paper for further detail. I predicted that species with faster life histories, especially therophytes (annuals), would have greater L-values than species with slower life histories, especially phanerophytes, which are mostly long-lived trees.

Open habitat, growth form, and stomatal ratio

I compared phylogenetic linear models to test whether Raunkiær life form, L-value, or interactions between them predicted SR_{even}. Unlike the analysis above, there was significant phylogenetic signal in this comparison (see R code). I used SR_{even} rather than SR_{propAd} as the response variable because the hypothesis is that faster life history and/or high light favor more even stomatal densities on each surface. I fit models using **phylolm** and extracted Akaike Information Criteria (AIC). For these and subsequent analyses, I assumed an Ornstein-Uhlenbeck process model for the residuals with the root character state integrated over the stationary distribution. I used a 10^4 parametric bootstrap samples of the full model (including main effects and interactions) to calculate parameter confidence intervals (Boettiger et al., 2012).

Does ab- or adaxial stomatal density contribute more to stomatal ratio evolution?

I used two related phylogenetic methods, variance decomposition and structural equation modeling (SEM), to assess the relative contribution of ab- versus adaxial stomatal density to light-mediated stomatal ratio evolution. First, the contribution of ab- versus adaxial stomatal density can be calculated using phylogenetic variance de-

composition methods as derived below. Because stomatal density is highly skewed, I log-transformed values for normality:

$$\mathrm{SR}_{\mathrm{even}} = \frac{\mathrm{SD}_{\mathrm{ad}}}{\mathrm{SD}_{\mathrm{ab}}} \tag{3}$$

$$\log(\mathrm{SR}_{\mathrm{even}}) = \log(\mathrm{SD}_{\mathrm{ad}}) - \log(\mathrm{SD}_{\mathrm{ad}}) \tag{4}$$

$$\mathrm{sr}_{\mathrm{even}} = \mathrm{sd}_{\mathrm{ad}} - \mathrm{sd}_{\mathrm{ad}} \tag{5}$$

Lowercase variables (sr, sd) indicate log-transformed values. Because some species had zero adaxial stomata, I added one to all values prior to log-transformation. To make the variance decomposition calculations tractable, I have defined $\mathrm{SR}_{\mathrm{even}}$ here as the ratio of ad- to abaxial stomatal density because in most cases adaxial stomatal density is lower than abaxial (see Eq. 2). This differs from analyses described above because in those I wanted to test what factors influenced the evenness of stomatal densities, regardless of which surface had higher density. With this modified form, the variance in $\mathrm{sr}_{\mathrm{even}}$ can readily be decomposed into contributions of $\mathrm{sd}_{\mathrm{ad}}$, $\mathrm{sd}_{\mathrm{ab}}$, and their covariance:

$$\mathrm{Var}(\mathrm{sr}_{\mathrm{even}}) = \mathrm{Var}(\mathrm{sd}_{\mathrm{ad}}) + \mathrm{Var}(\mathrm{sd}_{\mathrm{ad}}) - 2\mathrm{Cov}(\mathrm{sd}_{\mathrm{ad}}, \mathrm{sd}_{\mathrm{ab}}) \tag{6}$$

I did not use the raw covariance, but rather estimated the phylogenetic covariance matrix between L-value, $\mathrm{sd}_{\mathrm{ab}}$, and $\mathrm{sd}_{\mathrm{ad}}$ using a multivariate Ornstein-Uhlenbeck model fit in **Rphylopars** version 0.2.9 (Goolsby et al., 2016, 2017). From the co-

variance matrix, I estimated the contribution of abaxial density, adaxial density, and their covariance as:

$$\text{Contribution of } \text{sd}_{\text{ad}} = \frac{\text{Var}(\text{sd}_{\text{ad}})}{\text{Var}(\text{sr}_{\text{even}})} \tag{7}$$

$$\text{Contribution of } \text{sd}_{\text{ab}} = \frac{\text{Var}(\text{sd}_{\text{ab}})}{\text{Var}(\text{sr}_{\text{even}})} \tag{8}$$

$$\text{Contribution of } \text{Cov}(\text{sd}_{\text{ad}}, \text{sd}_{\text{ab}}) = \frac{\text{Cov}(\text{sd}_{\text{ad}}, \text{sd}_{\text{ab}})}{\text{Var}(\text{sr}_{\text{even}})} \tag{9}$$

respectively. Note that when ab- and adaxial densities positively covary, the contribution will be negative because this reduces the variance in stomatal ratio.

I also wanted to test whether light-mediated evolution of stomatal ratio acted mostly by 1) increasing adaxial stomatal density while maintaining abaxial density, or 2) keeping total stomatal density the same, but shifting a greater proportion to the adaxial surface. The first scenario predicts that the phylogenetic regression of L-value on sd_{ad} is stronger than that for sd_{ab}. The second scenario predicts that L-value acts similarly on both and that there is a negative covariance ($\text{Cov}(\text{sd}_{\text{ad}}, \text{sd}_{\text{ab}}) < 0$). I tested these competing predictions by fitting a very simple phylogenetic SEM (see Mason et al., 2016, for a similar approach). The model uses the phylogenetic covariance matrix, as described above, to simultaneously estimate regressions of L-value on sd_{ad} and sd_{ab} while allowing covariance between them (i.e. estimating $\text{Cov}(\text{sd}_{\text{ad}}, \text{sd}_{\text{ab}})$). To fit the SEM, I used the R package **lavaan** version 0.5-23.1097 (Rosseel, 2012). I tested whether parameter estimates were significantly different from zero using z-scores.

Results

Light tolerance varies among Raunkiær life forms

Ellenberg light indicator values (L-value) differed significantly among life forms (Fig. 2;ANOVA - $F_{4,367} = 18.3$, $P = 1.1 \times 10^{-13}$). Therophytes (annuals), hemicryptophytes (perennial herbs with buds near the soil surface), and chamaephytes (subshrubs) had greater L-values than phanerophytes (large woody plants) and geophytes (perennial herbs with storage organs) (Fig. 2).

Interactions between light and Raunkiær life form determine stomatal ratio

Overall, SR_{even} increased with L-value, but there was a significant interaction (ΔAIC > 2, Table 1) between Raunkiær life form and L-value (Fig. 3). Both life form and L-value significantly increased model fit, though L-value had a markedly larger effect on model AIC (Table 1). The significant interaction is caused by different slopes between life forms. Among life forms with the overall greatest L-value (therophytes, hemicryptophytes, and chamaephytes, see Fig. 2), there was a strong positive relationship between L-value and SR_{even}. Parametrically bootstrapped 95% confidence intervals for the slope did not overlap zero (Fig. 3). The slope was weakly positive or not significantly different from zero in the most shade-adapted life forms (geophytes and phanerophytes), albeit the patterns were distinct in these groups. There were both hypostomatous ($SR_{even} \approx 0$) and amphistomatous ($SR_{even} \approx 1$) geophytes, but these were distributed across L-values. In contrast, phanerophytes were nearly

always hypostomatous regardless of L-value.

Adaxial stomatal density contributes most of the variation in stomatal ratio

Adaxial ('upper') stomatal density contributed most to the evolutionary variation in stomatal ratio. The contributions of adaxial density, abaxial density, and their covariance are 1.12, 0.38, and -0.5, respectively. This implies that evolutionary variation in adaxial stomatal density is greater than that for stomatal ratio due to positive covariance between ab- and adaxial stomatal density.

Similarly, the phylogenetic SEM showed that changes in stomatal ratio associated with L-value can be attributed mostly to evolution of adaxial stomatal density (Fig. 4). Both sd_{ad} and sd_{ab} increased with L-value ($P = 1.2 \times 10^{-8}$ and 8.9×10^{-7}, respectively). However, the regression of L-value on sd_{ad} was 2× that of L-value on sd_{ab} (0.24 versus 0.12). Because stomatal densities were natural log-transformed, this implies an increase in L-value by one leads to a 1.27-fold change in adaxial stomatal density versus a 1.13-fold change in abaxial stomatal density. The SEM also showed a significant positive covariance between stomatal densities on each surface ($P = 2.5 \times 10^{-10}$). These results together imply that total stomatal density increases with L-value, but the response is mediated mostly by increases in adaxial stomatal density.

Discussion

The ratio of stomatal densities on the abaxial ('lower') to that of the adaxial ('upper') surface varies greatly across plant species, but the adaptive significance is not clear. Comparative studies correlating stomatal ratio to ecological factors can distinguish among competing hypotheses and reveal critical experiments for future work. Previous comparative studies suggested that high light and herbaceous growth form favor amphistomy (Mott et al., 1984; Jordan et al., 2014; Muir, 2015; Bucher et al., 2017), particularly in the British flora (Salisbury, 1927; Peat and Fitter, 1994). However, none of these studies have accounted for the fact that light and growth form are often confounded – open, high light habitats are often dominated by herbs – or the fact that species are not independent because of shared evolutionary history. Here, I reanalyzed data on stomata, light tolerance, and growth form in British angiosperms using phylogenetic comparative methods. As expected, species' light tolerance (Ellenberg light indicator or L-value) is confounded with growth form (Raunkiær life form; Fig. 2). Nevertheless, both L-value and Raunkiær life form affect stomatal ratio, but these factors also interact; the influence of L-value on stomatal ratio varies across forms. These novel findings provide further evidence that variation in stomatal ratio is adaptive and have important implications for interpreting changes in stomatal ratio through the paleo record (Jordan et al., 2014) and during domestication (Milla et al., 2013).

Adaptive significance of amphistomy

Previously, associations between light, growth form, and stomatal ratio have been interpreted in isolation as indicating that either high light and/or herbaceous growth

form favors amphistomy. In British angiosperms, both factors are important, though statistical analyses suggest that light may be a stronger determinant than growth form (Table 1). Unlike previous studies, I found a significant interaction between light and growth form among British angiosperms, which suggests that amphistomy may only be strongly favored when CO_2 strongly limits photosynthesis (as in open habitat) *and* photosynthesis strongly limits fitness (as in herbs). This is consistent with life history theory predicting that the demography of open habitat herbs is strongly limited by plant growth (Franco and Silvertown, 1996). The ideal way to test this would be to measure selection on stomatal ratio in a species that varied quantitatively in both stomatal ratio and life history (e.g., containing both annual and perennial forms). I predict that amphistomy will be favored more strongly in the annual form grown under high light compared to an annual under low light or a perennial in high light, and much more strongly than a perennial grown in low light. Similar experiments could also be performed to test if and when light-mediated plasticity in stomatal ratio is adaptive (Gay and Hurd, 1975; Mott and Michaelson, 1991; Fontana et al., 2017).

The prevalence of amphistomatous species in high light habitats supports the hypothesis that amphistomy is an adaptation to maximize photosynthetic rates by increasing CO_2 diffusion (Jones, 1985). It is also evidence against the hypothesis that the principle fitness cost of amphistomy is water loss (Darwin, 1886; Foster and Smith, 1986) or dehydration of pallisade mesophyll (Buckley et al., 2015), though these factors are likely very important in determining differential regulation of stomata on each surface. Since evaporative demand increases under high light, under these hypotheses we would expect plants in high light to be hypostomatous. Because stomatal conductances on each surface can be regulated independently in response

to the environment (Darwin, 1898; Pospíšilová and Solárová, 1984; Smith, 1981; Reich, 1984; Mott and O'Leary, 1984), amphistomatous leaves likely cope with these stresses by rapidly closing adaxial stomata when water supply cannot match evaporative demands (Richardson et al., 2017). Instead, patterns in the British flora are at least consistent with the idea that adaxial stomata increase susceptibility to foliar pathogens (Gutschick, 1984; McKown et al., 2014). The cost of adaxial stomata may be greater in the shade because greater leaf wetness and lower ultraviolet light provide a more suitable microclimate for many foliar pathogens.

Amphistomy as a proxy for open habitat

These observations from the British flora partially support the hypothesis that amphistomy can be used a proxy for open habitat in paleoenvironment reconstruction (Carpenter, 1994; Jordan et al., 2014; Carpenter et al., 2015) but also point out previously unknown subtleties. These previous studies based their conclusions on data from Proteaceae, in which there is little quantitative variation in stomatal ratio; species are either completely hypostomatous ($SR_{propAd} \approx 0$) or completely amphistomatous ($SR_{propAd} \approx 0.5$). Stomatal ratio in British angiosperms is also bimodal (Peat and Fitter, 1994), but across many families there is also quantitative variation. Importantly, this means that quantitative variation in stomatal ratio may provide a more precise, quantitative indicator of vegetation type, rather than simply 'open' or 'closed'. A quantitative relationship between L-value and stomatal ratio has already been shown for herbaceous perennials (Bucher et al., 2017), but we now know that it holds among annuals (therophytes), subshrubs (chamaephytes), and, to a lesser extent, geophytes as well (Fig. 3).

The weak or nonsignificant relationship between L-value and stomatal ratio in geophytes and phanerophytes suggests that in some cases amphistomy may not reliably indicate open habitat without further information. For example, perhaps amphistomatous geophytes from partially shaded habitats are spring ephemerals, so they experience high light during their growth phase, but this has not been tested. Likewise, phanerophytes (most tall trees) are almost always hypostomatous (see also Muir (2015)). Most British phanerophytes are tall, hypostomatous trees, but the exceptions are telling. For example, the most amphistomatous phanerophyte in this dataset is *Brassica oleracea*, a short-statured biennial that has more in common physiologically with hemicryptophytes than other phanerophytes. The other amphistomatous phanerophytes in this data set (*Populus nigra* and *Lavatera arborea*) are fast-growing pioneer species.

Finally, phylogenetic information should improve inferences about paleoclimates because there is appreciable phylogenetic signal in stomatal ratio. The phylogenetic half-life of stomatal ratio evolution, after accounting for L-value and Raunkiær life form, is $\log(2)/\alpha = 1.5$ my (see Table 1 for maximum likelihood estimates of α, the return rate in the Ornstein-Uhlenbeck model of trait evolution). This lag time may indicate that evolving to the 'optimum' is constrained by the shape of the fitness landscape (Muir, 2015) or that other unmeasured factors which affect stomatal ratio have some phylogenetic signal. Regardless of the mechanism, this fact means that researchers may be able to use data from closely related species to improve paleoenvironment reconstruction.

Why does adaxial stomatal density control stomatal ratio?

Variation in stomatal ratio is determined primarily by evolution of adaxial stomatal density and is coordinated with increases in total leaf stomatal density summed across both surfaces. Note here that I am referring only to evolutionary variation in stomatal ratio among species; different processes may mediate within species variation or plastic responses. Phylogenetic analyses show that changes in stomatal ratio and total stomatal density, especially in response to L-value, are predominantly mediated by changes in adaxial stomatal density. This highly nonrandom pattern among British angiosperms mirrors evolutionary changes wrought by domestication (Milla et al., 2013); crop species tend to have higher adaxial stomatal density than their wild relatives.

There are at least two hypotheses that could explain why adaxial stomatal density is the most responsive. The first I refer to as the 'real estate' hypothesis. In hypostomatous plants, the lower surface is already crowded with stomata, and hence plants must increase the real estate available for stomata by developing them on the upper surface whenever there is selection for greater stomatal density. When stomata are packed too densely on one surface, stomatal interference limits their functioning and hence may create a strong selective pressure for amphistomy (Parlange and Waggoner, 1970; Dow et al., 2014).

I refer to the second hypothesis as the 'coordination' hypothesis. In this scenario, ecological conditions such as high light select for both increased total stomatal density and for amphistomy because these traits work well in coordination with one another. For example, if stomatal density were very high on a hypostomatous plant, then CO_2 would be more strongly limited by the mesophyll. Adding a second parallel pathway

for diffusion by developing stomata on both surfaces would restore a more optimal balance between stomatal and mesophyll limitations. Conversely, there would be little benefit to amphistomy when total stomatal density is low because CO_2 diffusion is strongly limited by stomatal resistance, and therefore photosynthetic rate is not sensitive to changes in mesophyll diffusion mediated by stomatal ratio. A related prediction is that increased atmospheric CO_2 may select for reduced stomatal ratio and density primarily by decreasing adaxial stomatal density, but this has not been well tested (but see Woodward and Bazzaz, 1988).

Conclusions

By revisiting this classic ecological dataset with modern phylogenetic comparative methods, I have shown that amphistomy is strongly associated with both light and growth form, but the interaction between these factors is also important. Furthermore, amphistomy and high stomatal density are closely connected in species from high light environments, suggesting selection for coordination between these traits.

Acknowledgements

I thank Sally Otto, Matt Pennell, and Rob Salguero-Gómez for feedback on this manuscript. I was supported by an NSERC CREATE grant.

Author contribution statement

CDM designed the study, collected data, analyzed the data, and wrote the manuscript.

References

Bartelheimer, M. and P. Poschlod, 2016. Functional characterizations of Ellenberg indicator values–a review on ecophysiological determinants. Functional Ecology 30:506–516.

Bazzaz, F., 1979. The physiological ecology of plant succession. Annual Review of Ecology and Systematics 10:351–71.

Beerling, D. J. and C. K. Kelly, 1996. Evolutionary comparative analyses of the relationship between leaf structure and function. New Phytologist 134:35–51.

Beerling, D. J. and D. L. Royer, 2011. Convergent Cenozoic CO_2 history. Nature Geoscience 4:418–420.

Boettiger, C., G. Coop, and P. Ralph, 2012. Is your phylogeny informative? Measuring the power of comparative methods. Evolution 66:2240–2251.

Bucher, S. F., K. Auerswald, C. Grün-Wenzel, S. I. Higgins, J. G. Jorge, and C. Römermann, 2017. Stomatal traits relate to habitat preferences of herbaceous species in a temperate climate. Flora 229:107–115.

Buckley, T. N., G. P. John, C. Scoffoni, and L. Sack, 2015. How does leaf anatomy influence water transport outside the xylem? Plant Physiology 168:1616–1635.

Carpenter, R. J., 1994. Cuticular morphology and aspects of the ecology and fossil history of North Queensland rainforest Proteaceae. Botanical Journal of the Linnean Society 116:249.

Carpenter, R. J., M. K. Macphail, G. J. Jordan, and R. S. Hill, 2015. Fossil evidence

for open, Proteaceae-dominated heathlands and fire in the Late Cretaceous of Australia. American Journal of Botany 102:2092–2107.

Darwin, F., 1886. On the relation between the "bloom" on leaves and the distribution of the stomata. Botanical Journal of the Linnean Society 22:99–116.

———, 1898. Observations on stomata. Philosophical Transactions of the Royal Society B: Biological Sciences 190:531–621.

Dow, G. J., J. A. Berry, and D. C. Bergmann, 2014. The physiological importance of developmental mechanisms that enforce proper stomatal spacing in *Arabidopsis thaliana*. New Phytologist 201:1205–1217.

Ellenberg, H., 1974. Indicator values of vascular plants in central Europe, *Scripta Geobotanica*, vol. 9. Springer-Verlag, Göttingen, Germany.

Felsenstein, J., 1985. Phylogenies and the comparative method. The American Naturalist 1:1–15.

Fior, S., P. O. Karis, G. Casazza, L. Minuto, and F. Sala, 2006. Molecular phylogeny of the Caryophyllaceae (Caryophyllales) inferred from chloroplast matk and nuclear rDNA ITS sequences. American Journal of Botany 93:399–411.

Fitter, A. and H. Peat, 1994. The ecological flora database. Journal of Ecology 82:415–425.

———, 2017. Ecological flora of the British isles. URL `http://www.ecoflora.co.uk`.

Fontana, M., M. Labrecque, A. Collin, and N. Bélanger, 2017. Stomatal distribution patterns change according to leaf development and leaf water status in *Salix miyabeana*. Plant Growth Regulation 81:63–70.

453 Foster, J. and W. Smith, 1986. Influence of stomatal distribution on transpiration
454 in low-wind environments. Plant, Cell & Environment 9:751–759.

455 Franco, M. and J. Silvertown, 1996. Life history variation in plants: an exploration
456 of the fast-slow continuum hypothesis. Philosophical Transactions: Biological Sci-
457 ences 351:1341–1348.

458 Gay, A. and R. Hurd, 1975. The influence of light on stomatal density in the tomato.
459 New Phytologist 75:37–46.

460 Gibson, A. C., 1996. Structure-Function Relations of Warm Desert Plants. Springer-
461 Verlag, Berlin.

462 Givnish, T. J., 1987. Comparative studies of leaf form: assessing the relative roles
463 of selective pressures and phylogenetic constraints. New Phytologist 106:131–160.

464 Goolsby, E. W., J. Bruggeman, and C. Ané, 2016. Rphylopars: Phyloge-
465 netic Comparative Tools for Missing Data and Within-Species Variation. URL
466 `https://CRAN.R-project.org/package=Rphylopars`. R package version 0.2.9.

467 ———, 2017. Rphylopars: fast multivariate phylogenetic comparative methods for
468 missing data and within-species variation. Methods in Ecology and Evolution
469 8:22–27.

470 Gutschick, V. P., 1984. Photosynthesis model for C_3 leaves incorporating CO_2 trans-
471 port, propagation of radiation, and biochemistry 2. ecological and agricultural
472 utility. Photosynthetica 18:569–595.

473 Haberlandt, G., 1914. Physiological Plant Anatomy. Macmillan and Co., London.

474 Hill, M., C. Preston, and D. Roy, 2004. PLANTATT - Attributes of British and Irish

475 Plants: Status, Size, Life History, Geography and Habitats. Centre for Ecology &
476 Hydrology, Huntingdon, Cambridgeshire.

477 Ho, L. S. T. and C. Ané, 2014. Intrinsic inference difficulties for trait evolution with
478 Ornstein-Uhlenbeck models. Methods in Ecology and Evolution 5:1133–1146.

479 Jones, H. G., 1985. Adaptive significance of leaf development and structural responses
480 to environment. Pp. 155–173, *in* N. R. Baker, W. Davies, and C. K. Ong, eds.
481 Control of Leaf Growth, *Society for Experimental Biology Seminar Series*, vol. 27.
482 Cambridge University Press, Cambridge.

483 Jordan, G. J., R. J. Carpenter, and T. J. Brodribb, 2014. Using fossil leaves as
484 evidence for open vegetation. Palaeogeography, Palaeoclimatology, Palaeoecology
485 395:168–175.

486 Kelly, C. and D. Beerling, 1995. Plant life form, stomatal density and taxonomic
487 relatedness: a reanalysis of Salisbury (1927). Functional Ecology 9:422–431.

488 Körner, C., M. Neumayer, S. P. Menendez-Riedl, and A. Smeets-Scheel, 1989. Func-
489 tional morphology of mountain plants. Flora 182:353–383.

490 Lim, J., M. J. Crawley, N. De Vere, T. Rich, and V. Savolainen, 2014. A phylogenetic
491 analysis of the British flora sheds light on the evolutionary and ecological factors
492 driving plant invasions. Ecology and Evolution 4:4258–4269.

493 Mason, C. M., E. W. Goolsby, D. P. Humphreys, and L. A. Donovan, 2016. Phy-
494 logenetic structural equation modelling reveals no need for an 'origin? of the leaf
495 economics spectrum. Ecology letters 19:54–61.

496 McElwain, J. C. and M. Steinthorsdottir, 2017. Paleoecology, ploidy, paleoatmo-

497 spheric composition, and developmental biology: a review of the multiple uses of
498 fossil stomata. Plant Physiology 174:650–664.

499 McKown, A. D., R. D. Guy, L. Quamme, J. Klápště, J. La Mantia, C. Constabel,
500 Y. A. El-Kassaby, R. C. Hamelin, M. Zifkin, and M. Azam, 2014. Association
501 genetics, geography and ecophysiology link stomatal patterning in *Populus tri-*
502 *chocarpa* with carbon gain and disease resistance trade-offs. Molecular Ecology
503 23:5771–5790.

504 Melotto, M., L. Zhang, P. R. Oblessuc, and S. Y. He, 2017. Stomatal defense a
505 decade later. Plant Physiology 174:561–571.

506 Metcalfe, C. R. and L. Chalk, 1950. Anatomy of the dicotyledons, Vols. 1 & 2. First
507 ed. Oxford University Press, Oxford.

508 Milla, R., N. de Diego-Vico, and N. Martín-Robles, 2013. Shifts in stomatal traits
509 following the domestication of plant species. Journal of Experimental Botany
510 64:3137–3146.

511 Mott, K. A., A. C. Gibson, and J. W. O'Leary, 1984. The adaptive significance of
512 amphistomatic leaves. Plant, Cell & Environment 5:455–460.

513 Mott, K. A. and O. Michaelson, 1991. Amphistomy as an adaptation to high light
514 intensity in *Ambrosia cordifolia* (Compositae). American Journal of Botany 78:76–
515 79.

516 Mott, K. A. and J. W. O'Leary, 1984. Stomatal behavior and CO_2 exchange char-
517 acteristics in amphistomatous leaves. Plant Physiology 74:47–51.

518 Muir, C. D., 2015. Making pore choices: repeated regime shifts in stomatal ratio.
519 Proc. R. Soc. B 282:20151498.

———, 2017. Data from: Light and life form interact to shape stomatal ratio among British angiosperms. URL http://dx.doi.org/10.5061/dryad.?????

Parkhurst, D. F., 1978. The adaptive significance of stomatal occurrence on one or both surfaces of leaves. The Journal of Ecology 66:367–383.

Parkhurst, D. F. and K. A. Mott, 1990. Intercellular diffusion limits to CO_2 uptake in leaves studied in air and helox. Plant Physiology 94:1024–1032.

Parlange, J.-Y. and P. E. Waggoner, 1970. Stomatal dimensions and resistance to diffusion. Plant Physiology 46:337–342.

Peat, H. and A. Fitter, 1994. A comparative study of the distribution and density of stomata in the British flora. Biological Journal of the Linnean Society 52:377–393.

Pennell, M. W., R. G. FitzJohn, and W. K. Cornwell, 2016. A simple approach for maximizing the overlap of phylogenetic and comparative data. Methods in Ecology and Evolution 7:751–758.

Pospíŝilová, J. and J. Solárová, 1984. Environmental and biological control of diffusive conductances of adaxial and abaxial leaf epidermes. Photosynthetica 18:445–453.

Raunkiær, C. C., 1934. The Life Forms of Plants and Statistical Plant Geography. Clarendon Press, Oxford.

Reich, P., 1984. Relationships between leaf age, irradiance, leaf conductance, CO_2 exchange, and water-use efficiency in hybrid poplar. Photosynthetica 18:445–453.

Revell, L. J., 2012. phytools: An R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution 3:217–223.

Richardson, F., T. J. Brodribb, and G. J. Jordan, 2017. Amphistomatic leaf surfaces independently regulate gas exchange in response to variations in evaporative demand. Tree Physiology Pp. 1–10.

Rosseel, Y., 2012. lavaan: An R package for structural equation modeling. Journal of Statistical Software 48:1–36.

Royer, D. L., 2001. Stomatal density and stomatal index as indicators of paleoatmospheric CO_2 concentration. Review of Palaeobotany and Palynology 114:1–28.

Sack, L. and T. N. Buckley, 2016. The developmental basis of stomatal density and flux. Plant Physiology 171:2358–2363.

Salguero-Gómez, R., O. R. Jones, E. Jongejans, S. P. Blomberg, D. J. Hodgson, C. Mbeau-Ache, P. A. Zuidema, H. de Kroon, and Y. M. Buckley, 2016. Fast–slow continuum and reproductive strategies structure plant life-history variation worldwide. Proceedings of the National Academy of Sciences of the United States of America 113:230–235.

Salisbury, E., 1927. On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. Philosophical Transactions of the Royal Society of London. Series B 216:1–65.

Salmaki, Y., S. Zarre, O. Ryding, C. Lindqvist, C. Bräuchler, G. Heubl, J. Barber, and M. Bendiksby, 2013. Molecular phylogeny of tribe Stachydeae (Lamiaceae subfamily Lamioideae). Molecular Phylogenetics and Evolution 69:535–551.

Scheen, A.-C., C. Brochmann, A. K. Brysting, R. Elven, A. Morris, D. E. Soltis, P. S. Soltis, and V. A. Albert, 2004. Northern hemisphere biogeography of *Cerastium*

(Caryophyllaceae): insights from phylogenetic analysis of noncoding plastid nucleotide sequences. American Journal of Botany 91:943–952.

Shipley, B., M. Belluau, I. Kühn, N. A. Soudzilovskaia, M. Bahn, J. Penuelas, J. Kattge, L. Sack, J. Cavender-Bares, W. A. Ozinga, B. Blonder, P. M. van Bodegom, P. Manning, T. Hickler, E. Sosinski, V. D. P. Pillar, and V. Onipchenko, ???? Predicting habitat affinities of plant species using commonly measured functional traits. Journal of Vegetation Science Pp. n/a–n/a. URL http://dx.doi.org/10.1111/jvs.12554.

Smith, W., 1981. Temperature and water relation patterns in subalpine understory plants. Oecologia 48:353–359.

Smith, W. K., T. C. Vogelmann, E. H. DeLucia, D. T. Bell, and K. A. Shepherd, 1997. Leaf form and photosynthesis. BioScience 11:785–793.

Wolfe, J. A., 1971. Tertiary climatic fluctuations and methods of analysis of Tertiary floras. Palaeogeography, Palaeoclimatology, Palaeoecology 9:27–57.

Woodward, F., 1987. Stomatal numbers are sensitive to increases in CO_2 from preindustrial levels. Nature 327:617–618.

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

Wullschleger, S. D., 1993. Biochemical limitations to carbon assimilation in C_3 plants? A retrospective analysis of the A/Ci curves from 109 species. Journal of Experimental Botany 44:907–920.

Table 1: Interaction beween species' Ellenberg light indicator value (L-value) and Raunkiær lifeform predict stomatal ratio (SR_{even}). I compared phylogenetic linear models using the Akaike Information Criterion (AIC), where AIC = $2k - 2\log(\mathcal{L})$. k is the number of model parameters and $\mathcal{L}$ is the model likelihood. Given a set of candidate models, the difference in AIC between a model and the lowest AIC (ΔAIC) indicates the relative fit of competing models. The correlation coefficient r^2 is another indicator of model fit. α and σ^2 are the return rate and diffusion parameters of the Ornstein-Uhlenbeck model of trait evolution.

Model: $SR_{even} \sim$	α	σ^2	r^2	k	$\log(\mathcal{L})$	AIC	ΔAIC
L-value × lifeform	0.46	0.068	0.34	12	-33.2	90.4	0
L-value + lifeform	0.46	0.071	0.32	8	-40.2	96.4	6
L-value	0.64	0.107	0.26	4	-59.3	126.6	36.2
lifeform	0.34	0.067	0.15	7	-79.2	172.4	82
null	0.29	0.067	0	3	-107.6	221.1	130.7

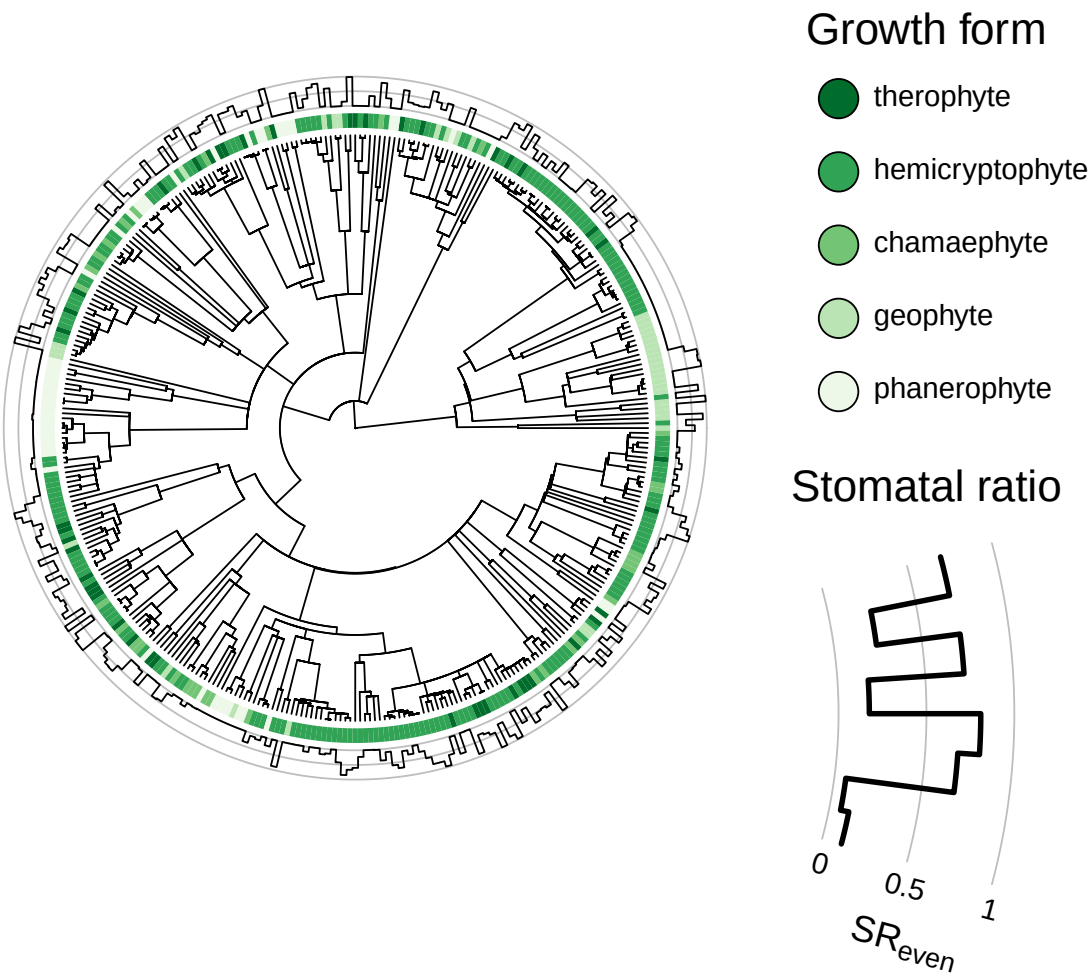

Figure 1: CAPTION

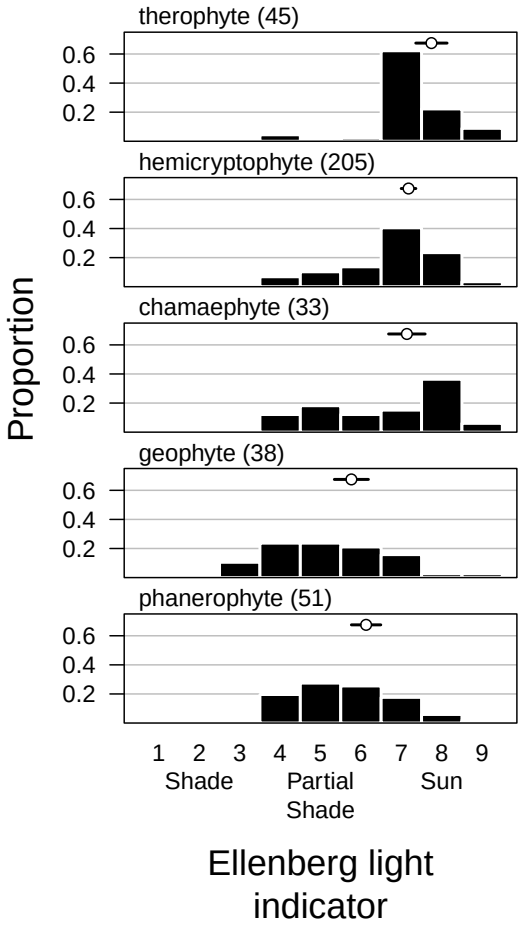

Figure 2: Lifeforms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different Raunkiær life forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform. The sample size for each lifeform is listed next in parentheses. The mean (open circle) and 95% confidence intervals (black line) around the mean Ellenberg light indicator value for each lifeform based on phylogenetic regression are above the histogram.

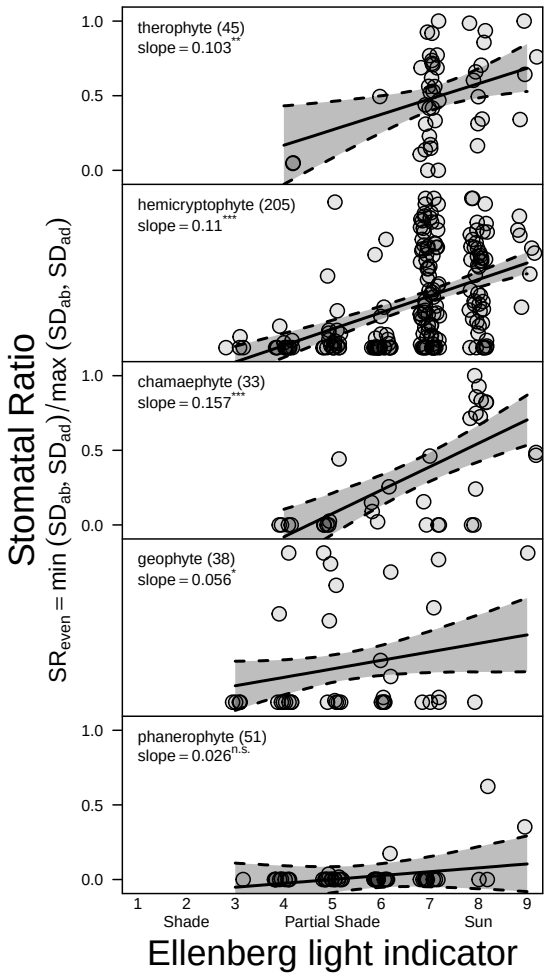

Figure 3: The effect of light on stomatal ratio depends on Raunkiær life form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio (SR_{even}) in therophytes, hemicryptophytes, and chamaephytes but not geophytes and phanerophytes. The maximum likelihood slope from phylogenetic regression is given with statistical significance based on 10^4 parametric bootstrap samples. Numbers in parentheses next to Raunkiær life form are the sample sizes in the final dataset. Estimated slopes (solid line) and 95% bootstrapped confidence intervals (gray polygon between dashed lines) are plotted against raw data. Points have been jittered for visual clarity.

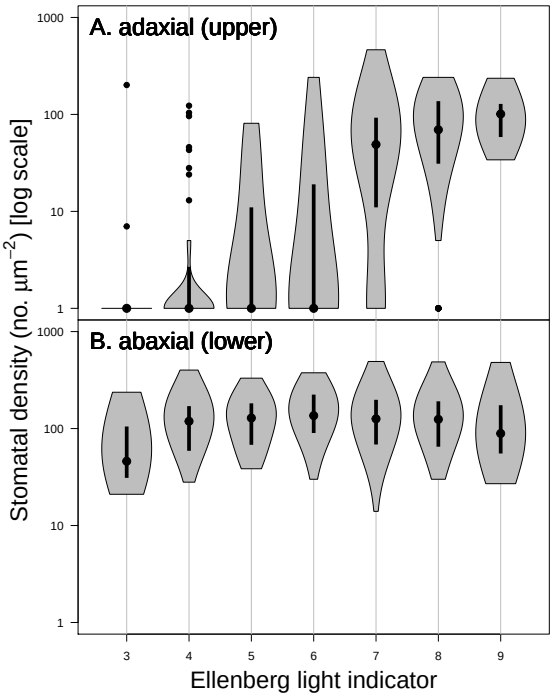

Figure 4: Light-mediated evolution of stomatal ratio is mostly driven by increased adaxial ('upper') stomatal density (Panel A), whereas abaxial ('lower') stomatal density (Panel B) is similar across Ellenberg light indicator values (L-value x-axis). The violin plot shows stomatal density (y-axis, log-scale) as a function of L-value. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range of the data; the dot indicates the median; the thick lines indicate the 0.25 and 0.75 quantiles. Points outside the polygons are statistical outliers.

Supporting Information

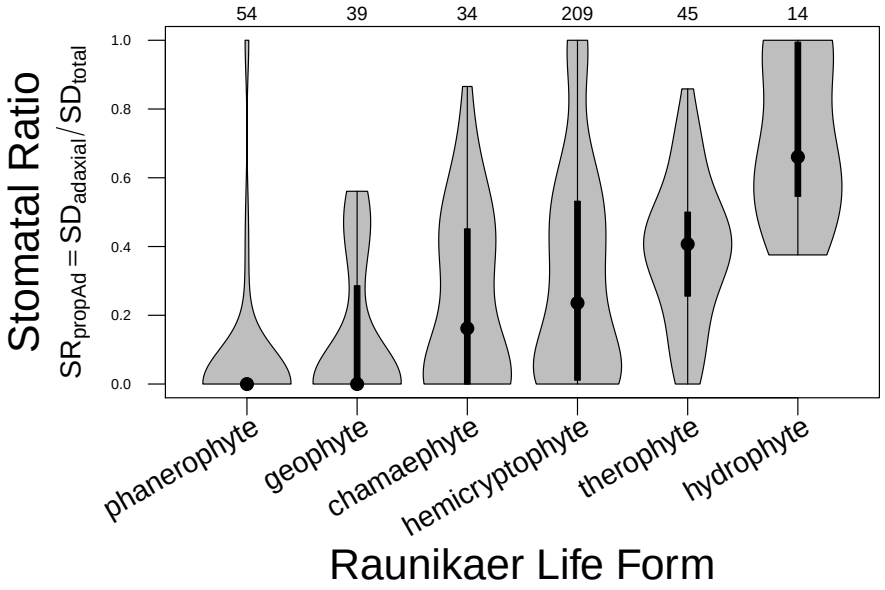

Figure S1: Most hydrophytes are hyperstomatous, having most stomata on the adaxial ('upper') surface (high SD_{propAd}). The violin plot shows stomatal ratio as a function of Raunkiær lifeform. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range of the data; the point indicates the median; the thick lines indicate the 0.25 and 0.75 quantiles. Sample sizes per lifeform in the dataset are given above the upper plot margin. SD_{ad} and SD_{total} stand for the stomatal density on the adaxial surface and the total leaf surface (adaxial plus abaxial), respectively.