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

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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.

Meywords

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

24 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 27 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 CO₂ and water vapour (recently reviewed in Sack and Buckley, 2016), as well as susceptibility to foliar pathogens that infect 32 through stomata (McKown et al., 2014; Melotto et al., 2017). Hence, stomata have 33 been especially useful in understanding plastic and evolutionary response to climate change and domestication (Woodward, 1987; Beerling and Royer, 2011; Milla et al., 2013). 36

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 CO₂ diffusion within the mesophyll, amphistomy optimally supplies CO₂ (Parkhurst, 1978; Gutschick, 1984; Jones, 1985). Amphistomy is correlated with greater CO₂ diffusion (Beerling and Kelly, 45 1996) and higher photosynthetic rates (McKown et al., 2014). These observations 46 are corroborated by experiments demonstrating that amphistomy increases maxi-47 mum photosynthetic rates by up to 20% (Parkhurst and Mott, 1990). On the other 48 hand, amphistomy can increase transpiration (Jones, 1985; Foster and Smith, 1986; Buckley et al., 2015). While transition to amphistomy is thus thought to increase 50 transpiration, empirical studies suggest greater water-use efficiency in amphistoma-51 tous species (Bucher et al., 2017). Hence, amphistomy appears to benefit a plant's 52 carbon use relative to water loss and should be favored when CO₂ limits photo-53 synthetic rate. The open questions are under what ecological conditions does CO₂ supply most strongly limit photosynthetic rate (Peat and Fitter, 1994) and when is 55 photosynthetic rate most important to fitness?

The leading, nonmutually exclusive hypotheses are that 1) open habitats favour 57 amphistomy because CO₂ diffusion most strongly limits photosynthetic rate under 58 high light and 2) herbaceous growth form favours amphistomy because traits that 59 maximize photosynthetic rate are often under stronger selection in herbs. Salisbury (1927) first noted that amphistomy is most common in herbaceous plants from open 61 habitats (i.e., with high light) of the British flora. These observations have been 62 replicated in other studies (Mott et al., 1984; Peat and Fitter, 1994; Jordan et al., 63 2014; Muir, 2015) and may support physiological and ecological hypotheses that CO₂ most strongly limits photosynthesis in high light and/or photosynthesis contributes most to fitness in herbaceous plants. Under high light, CO₂ 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 CO₂ 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 76 from open habitats are amphistomatous. This pattern holds when data are averaged 77 by family to coarsely control for phylogenetic nonindependence (Peat and Fitter, 78 1994) or when using alternative classification schemes, such as Raunkiær life form 79 (Peat and Fitter, 1994). Across plants from ~ 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 82 patterns are consistent with other data indicating that many herbaceous plants are 83 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 CO₂ 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?

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- 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 co-105 ordinated syndrome of traits that promote higher photosynthetic rate, as both the 106 light and growth form hypotheses assume. If evolved increases in stomatal ratio are 107 mediated by shifting abaxial stomata to the adaxial surface, holding total stomatal 108 density constant, then the overall increase in CO₂ diffusion would be small. In con-109 trast, if amphistomy evolves by increasing adaxial stomatal density while holding abaxial density constant, then total stomatal density must increase as well. Evolu-111 tionary coordination of amphistomy and high stomatal density would reinforce one 112 another, increasing CO₂ supply to chloroplasts more than changes in either trait 113 would in isolation. 114
- 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 119 of stomatal trait variation. Salisbury's observations on stomata and ecology in the 120 British flora have heavily influenced plant ecophysiology, but many of his and subse-121 quent authors' analyses have significant limitations because of inadequate statistical 122 methods. For example, few analyses until recently account for phylogenetic nonin-123 dependence (Felsenstein, 1985), which can strongly influence inferences on stomatal 124 traits and growth form (Kelly and Beerling, 1995, this study did not consider light). 125 A species-level phylogeny of the entire British flora (Lim et al., 2014) now allows for 126 the first time rigorous analysis of evolutionary relationships among stomatal ratio, 127 light, and growth form.

129 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. Niinemets and Valladares, 2006; Bartelheimer and Poschlod, 2016; Shipley et al.), I used Ellenberg light indicator values (Ellenberg, 1974) as measures of light habitat. 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. Ellenberg light indicator values, hereafter abbreviated L-value, have been recently updated by taxonomic experts of the British flora (PLANTATT, Hill et al. (2004)).

There is no universally adopted scientific classification scheme for plant growth form, 142 therefore I statistically competed two widely used schemes. First, I used PLANTATT 143 data on perennation, woodiness, and height to classify species as herbaceous (annual, 144 biennial, or perennial) or woody (shrub or tree). Following Muir (2015), 'biennial' 145 includes true biennials as well as species that have a mix of perennation forms (e.g. a 146 species with both annual and perennial forms would be classified as a biennial here). 147 Woody species were classified as shrubs (plant height less than 4 m) or trees (plant 148 height greater than 4 m). Next, I compared this scheme to PLANTATT data on 149 Raunkiær life form (Raunkiær, 1934), which is another way to classify growth form in 150 comparative ecology (e.g. Salguero-Gómez et al., 2016). [NOTE: SUPPLEMENTAL 151 FIGURE OF OVERLAP BETWEEN THESE 152

I used a dated molecular phylogeny of the British flora (Lim et al., 2014) available 153 from TreeBASE (http://treebase.org/; accession number 15105). 14 species (3.5%) 154 in the dataset were not present in the phylogeny. For 8 of these species, I used the 155 position of a congeneric species as a proxy for the focal species (following Pennell 156 et al., 2016). When multiple congeneric species were present, I consulted the phy-157 logenetic literature to identify the most closely related proxy species (Scheen et al., 158 2004; Salmaki et al., 2013). For the remaining 6 missing species, I positioned them 159 in the tree based on phylogenetic relationships to other genera or families present in 160 the tree (Fior et al., 2006). Because many phylogenetic comparative methods do not 161 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. S2) 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 C₄ (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:

$$SR_{propAd} = \frac{SD_{ad}}{SD_{total}}$$
 (1)

$$SR_{even} = \frac{\min\{SD_{ab}, SD_{ad}\}}{\max\{SD_{ab}, SD_{ad}\}}$$
(2)

 SD_{ab} and SD_{ad} are the stomatal densities on abaxial or adaxial surface, respectively. $SD_{total} = SD_{ab} + SD_{ad}$. SR_{propAd} is the proportion of stomata density on the adaxial surface, which is useful for discriminating among hypostomatous ($SR_{propAd} = 0$), amphistomatous ($SR_{propAd} < 1$), and hyperstomatous species ($SR_{propAd} = 1$). SR_{even} indicates how evenly stomatal densities are distributed across both leaf sur-

faces. This expression is useful because several hypotheses are based on the fact that a more even distribution should optimize leaf CO₂ 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, 194 or interactions between them predicted SR_{even}. Unlike the analysis above, there 195 was significant phylogenetic signal in this comparison (see R code). I used SR_{even} 196 rather than SR_{propAd} as the response variable because the hypothesis is that faster 197 life history and/or high light favor more even stomatal densities on each surface. 198 I fit models using **phylolm** and extracted Akaike Information Criteria (AIC). For 199 these and subsequent analyses, I assumed an Ornstein-Uhlenbeck process model for 200 the residuals with the root character state integrated over the stationary distribu-201 tion. I used a 10⁴ 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 abversus adaxial stomatal density can be calculated using phylogenetic variance decomposition methods as derived below. Because stomatal density is highly skewed,
I log-transformed values for normality:

$$SR_{even} = \frac{SD_{ad}}{SD_{ab}}$$
 (3)

$$\log(SR_{even}) = \log(SD_{ad}) - \log(SD_{ad})$$
(4)

$$sr_{even} = sd_{ad} - sd_{ad}$$
 (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 SR_{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 sr_{even} can readily be decomposed into contributions of sd_{ad}, sd_{ab}, and their covariance:

$$Var(sr_{even}) = Var(sd_{ad}) + Var(sd_{ad}) - 2Cov(sd_{ad}, sd_{ab})$$
(6)

I did not use the raw covariance, but rather estimated the phylogenetic covariance matrix between L-value, sd_{ab}, and sd_{ad} using a multivariate Ornstein-Uhlenbeck model fit in **Rphylopars** version 0.2.9 (Goolsby et al., 2016, 2017). From the covariance matrix, I estimated the contribution of abaxial density, adaxial density, and their covariance as:

Contribution of
$$sd_{ad} = \frac{Var(sd_{ad})}{Var(sr_{even})}$$
 (7)

Contribution of
$$sd_{ab} = \frac{Var(sd_{ab})}{Var(sr_{even})}$$
 (8)

Contribution of
$$Cov(sd_{ad}, sd_{ab}) = \frac{Cov(sd_{ad}, sd_{ab})}{Var(sr_{even})}$$
 (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 sim-233 ilarly on both and that there is a negative covariance ($Cov(sd_{ad}, sd_{ab}) < 0$). I tested 234 these competing predictions by fitting a very simple phylogenetic SEM (see Mason 235 et al., 2016, for a similar approach). The model uses the phylogenetic covariance 236 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 $Cov(sd_{ad}, sd_{ab})$). 238 To fit the SEM, I used the R package lavaan version 0.5-23.1097 (Rosseel, 2012). 239 I tested whether parameter estimates were significantly different from zero using 240 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 ($\Delta AIC >$ 251 2, Table 1) between Raunkiær life form and L-value (Fig. 3). Both life form and L-252 value significantly increased model fit, though L-value had a markedly larger effect 253 on model AIC (Table 1). The significant interaction is caused by different slopes 254 between life forms. Among life forms with the overall greatest L-value (therophytes, 255 hemicryptophytes, and chamaephytes, see Fig. 2), there was a strong positive rela-256 tionship 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 258 or not significantly different from zero in the most shade-adapted life forms (geo-259 phytes and phanerophytes), albeit the patterns were distinct in these groups. There 260 were both hypostomatous (SR_{even} ≈ 0) and amphistomatous (SR_{even} ≈ 1) geophytes, 261 but these were distributed across L-values. In contrast, phanerophytes were nearly 262 always hypostomatous regardless of L-value. 263

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} , 273 respectively). However, the regression of L-value on sd_{ad} was 2× that of L-value on 274 sd_{ab} (0.24 versus 0.12). Because stomatal densities were natural log-transformed, this 275 implies an increase in L-value by one leads to a 1.27-fold change in adaxial stom-276 atal 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 278 $(P = 2.5 \times 10^{-10})$. These results together imply that total stomatal density increases 279 with L-value, but the response is mediated mostly by increases in adaxial stomatal 280 density. 281

Discussion

The ratio of stomatal densities on the abaxial ('lower') to that of the adaxial ('upper') 283 surface varies greatly across plant species, but the adaptive significance is not clear. 284 Comparative studies correlating stomatal ratio to ecological factors can distinguish 285 among competing hypotheses and reveal critical experiments for future work. Previ-286 ous comparative studies suggested that high light and herbaceous growth form favor 287 amphistomy (Mott et al., 1984; Jordan et al., 2014; Muir, 2015; Bucher et al., 2017), 288 particularly in the British flora (Salisbury, 1927; Peat and Fitter, 1994). However, 289 none of these studies have accounted for the fact that light and growth form are 290 often confounded – open, high light habitats are often dominated by herbs – or the 291 fact that species are not independent because of shared evolutionary history. Here, I 292 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 304 form favors amphistomy. In British angiosperms, both factors are important, though 305 statistical analyses suggest that light may be a stronger determinant than growth 306 form (Table 1). Unlike previous studies, I found a significant interaction between 307 light and growth form among British angiosperms, which suggests that amphistomy 308 may only be strongly favored when CO₂ strongly limits photosynthesis (as in open 309 habitat) and photosynthesis strongly limits fitness (as in herbs). This is consistent 310 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 312 test this would be to measure selection on stomatal ratio in a species that varied 313 quantitatively in both stomatal ratio and life history (e.g., containing both annual 314 and perennial forms). I predict that amphistomy will be favored more strongly in 315 the annual form grown under high light compared to an annual under low light 316 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 hy-321 pothesis that amphistomy is an adaptation to maximize photosynthetic rates by 322 increasing CO₂ diffusion (Jones, 1985). It is also evidence against the hypothesis 323 that the principle fitness cost of amphistomy is water loss (Darwin, 1886; Foster and 324 Smith, 1986) or dehydration of pallisade mesophyll (Buckley et al., 2015), though 325 these factors are likely very important in determining differential regulation of stom-326 ata on each surface. Since evaporative demand increases under high light, under 327 these hypotheses we would expect plants in high light to be hypostomatous. Because 328 stomatal conductances on each surface can be regulated independently in response 329 to the environment (Darwin, 1898; Pospíŝilová and Solárová, 1984; Smith, 1981; Re-330 ich, 1984; Mott and O'Leary, 1984), amphistomatous leaves likely cope with these 331 stresses by rapidly closing adaxial stomata when water supply cannot match evapo-332 rative demands (Richardson et al., 2017). Instead, patterns in the British flora are 333 at least consistent with the idea that adaxial stomata increase susceptibility to foliar 334 pathogens (Gutschick, 1984; McKown et al., 2014). The cost of adaxial stomata 335 may be greater in the shade because greater leaf wetness and lower ultraviolet light 336 provide a more suitable microclimate for many foliar pathogens. 337

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; 343 species are either completely hypostomatous ($SR_{propAd} \approx 0$) or completely amphis-344 tomatous ($SR_{propAd} \approx 0.5$). Stomatal ratio in British angiosperms is also bimodal 345 (Peat and Fitter, 1994), but across many families there is also quantitative variation. 346 Importantly, this means that quantitative variation in stomatal ratio may provide a 347 more precise, quantitative indicator of vegetation type, rather than simply 'open' or 348 'closed'. A quantitative relationship between L-value and stomatal ratio has already 349 been shown for herbaceous perennials (Bucher et al., 2017), but we now know that 350 it holds among annuals (therophytes), subshrubs (chamaephytes), and, to a lesser 351 extent, geophytes as well (Fig. 3). 352

The weak or nonsignificant relationship between L-value and stomatal ratio in geo-353 phytes and phanerophytes suggests that in some cases amphistomy may not reliably 354 indicate open habitat without further information. For example, perhaps amphis-355 tomatous geophytes from partially shaded habitats are spring ephemerals, so they 356 experience high light during their growth phase, but this has not been tested. Like-357 wise, phanerophytes (most tall trees) are almost always hypostomatous (see also 358 Muir (2015)). Most British phanerophytes are tall, hypostomatous trees, but the 359 exceptions are telling. For example, the most amphistomatous phanerophyte in this 360 dataset is Brassica oleracea, a short-statured biennial that has more in common 361 physiologically with hemicryptophytes than other phanerophytes. The other am-362 phistomatous phanerophytes in this data set (*Populus nigra* and *Lavatera arborea*) 363 are fast-growing pioneer species. 364

Finally, phylogenetic information should improve inferences about paleoclimates be-

cause there is appreciable phylogenetic signal in stomatal ratio. The phylogenetic 366 half-life of stomatal ratio evolution, after accounting for L-value and Raunkiær life 367 form, is $\log(2)/\alpha = 1.5$ my (see Table 1 for maximum likelihood estimates of α , the 368 return rate in the Ornstein-Uhlenbeck model of trait evolution). This lag time may 369 indicate that evolving to the 'optimum' is constrained by the shape of the fitness 370 landscape (Muir, 2015) or that other unmeasured factors which affect stomatal ra-371 tio have some phylogenetic signal. Regardless of the mechanism, this fact means 372 that researchers may be able to use data from closely related species to improve 373 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 377 across both surfaces. Note here that I am referring only to evolutionary variation in 378 stomatal ratio among species; different processes may mediate within species vari-379 ation or plastic responses. Phylogenetic analyses show that changes in stomatal 380 ratio and total stomatal density, especially in response to L-value, are predominantly 381 mediated by changes in adaxial stomatal density. This highly nonrandom pattern 382 among British angiosperms mirrors evolutionary changes wrought by domestication 383 (Milla et al., 2013); crop species tend to have higher adaxial stomatal density than 384 their wild relatives. 385

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 hypotheses postomatous 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, 394 ecological conditions such as high light select for both increased total stomatal density 395 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 CO₂ 397 would be more strongly limited by the mesophyll. Adding a second parallel pathway 398 for diffusion by developing stomata on both surfaces would restore a more optimal 390 balance between stomatal and mesophyll limitations. Conversely, there would be 400 little benefit to amphistomy when total stomatal density is low because CO₂ diffusion 401 is strongly limited by stomatal resistance, and therefore photosynthetic rate is not 402 sensitive to changes in mesophyll diffusion mediated by stomatal ratio. A related 403 prediction is that increased atmospheric CO_2 may select for reduced stomatal ratio 404 and density primarily by decreasing adaxial stomatal density, but this has not been 405 well tested (but see Woodward and Bazzaz, 1988).

407 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.

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417 Author contribution statement

⁴¹⁸ CDM designed the study, collected data, analyzed the data, and wrote the manuscript.

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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 (Δ AIC) 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
$\overline{\text{L-value} \times \text{lifeform}}$	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

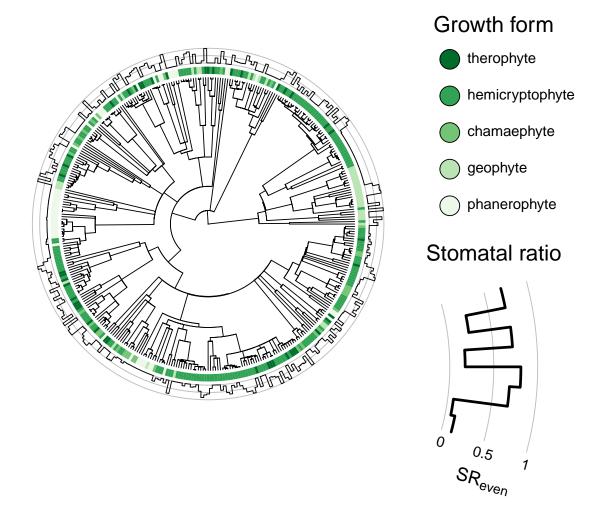


Figure 1: CAPTION

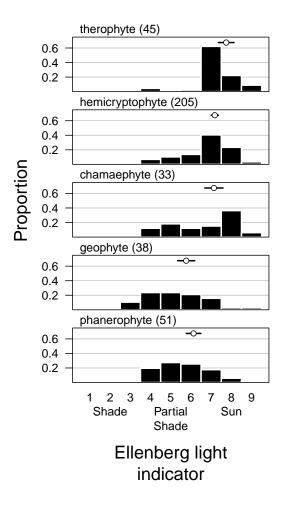


Figure 2: Lifeforms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different Raunkiær life forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform. 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.

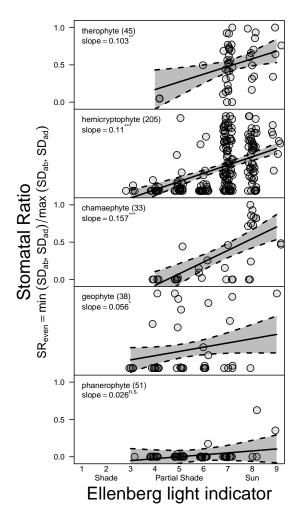


Figure 3: The effect of light on stomatal ratio depends on Raunkiær life form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio (SR_{even}) in therophytes, hemicryptophytes, and chamaephytes but not geophytes and phanerophytes. The maximum likelihood slope from phylogenetic regression is given with statistical significance based on 10⁴ 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.

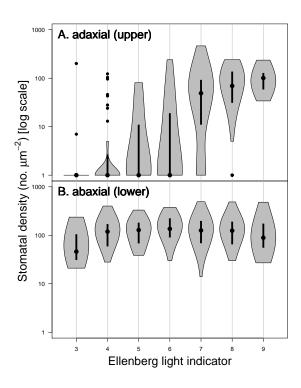


Figure 4: Light-mediated evolution of stomatal ratio is mostly driven by increased adaxial ('upper') stomatal density (Panel A), whereas abaxial ('lower') stomatal density (Panel B) is similar across Ellenberg light indicator values (L-value x-axis). The violin plot shows stomatal density (y-axis, log-scale) 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.

598 Supporting Information

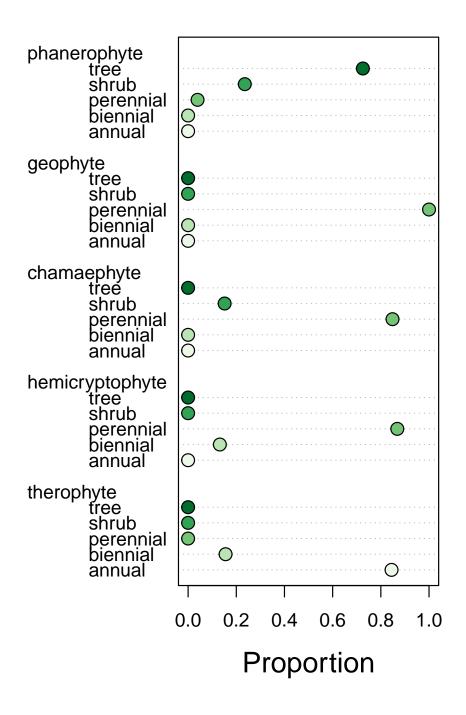


Figure S1: CAPTION

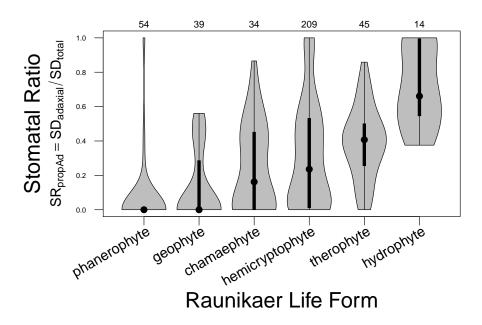


Figure S2: Most hydrophytes are hyperstomatous, having most stomata on the adaxial ('upper') surface (high SD_{propAd}). The violin plot shows stomatal ratio as a function of Raunkiær lifeform. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range of 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.