

# Light and life form interact to shape stomatal ratio among British angiosperms

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# 1 Summary

2       • In most plants, stomata are located only on the abaxial leaf surface (hypos-  
3       tomy), but many plants have stomata on both surfaces (amphistomy). High  
4       light and herbaceous growth form have been hypothesized to favor amphis-  
5       tomy, but these hypotheses have not been rigourously tested together using  
6       phylogenetic comparative methods.

7       • I leveraged a large dataset including stomatal ratio, Ellenberg light indica-  
8       tor value, Raunkiaer lifeform, and phylogenetic relationships for 372 species of  
9       British angiosperms. I used phylogenetic comparative methods to test how  
10      light and/or growth form influence stomatal ratio.

11      • High light and herbaceous growth form are correlated with amphistomy, as  
12      predicted, but they also interact; the effects of light are most pronounced in  
13      therophytes (annuals) and hemocryptophytic perennials, but less so in phanero-  
14      phytes (mostly trees). Interestingly, amphistomy and stomatal density evolve  
15      together in response to light, suggesting coordinated selection on this trait  
16      combination.

17      • I show for the first time that light and growth form interact to shape variation  
18      in stomatal ratio; amphistomy is advantageous in high light, but mostly for

19       herbs. These results improve our understanding of the adaptive significance of  
20       stomatal ratio as well as its use as functional trait for paleovegetation recon-  
21       struction and crop improvement.

## 22   **Keywords**

23   Adaptation, amphistomy, Ellenberg light indicator value, growth form, phylogenetic  
24   comparative methods, stomata, stomatal ratio

## 25   **Introduction**

26   Natural selection shapes leaf anatomy in order to optimize its photosynthetic function  
27   in a given environment (Haberlandt, 1914; Givnish, 1987; Smith et al., 1997). By  
28   understanding the adaptive significance of leaf anatomical variation we can learn  
29   about natural history, find targets for crop improvement, and identify anatomical  
30   proxies for paleoclimates preserved in the fossil record (e.g. Wolfe, 1971; Royer, 2001;  
31   McElwain and Steinthorsdottir, 2017). The size, density, and distribution of stomata  
32   on a leaf vary widely and impact the flux of CO<sub>2</sub> and water vapour (recently reviewed  
33   in Sack and Buckley, 2016), as well as susceptibility to foliar pathogens that infect

34 through stomata (McKown et al., 2014; Melotto et al., 2017). Hence, stomata have  
35 been especially useful in understanding plastic and evolutionary response to climate  
36 change and domestication (Woodward, 1987; Beerling and Royer, 2011; Milla et al.,  
37 2013).

38 While the density and size of stomata have been researched extensively (Sack and  
39 Buckley, 2016, and references therein), the adaptive significance of stomatal distri-  
40 bution is less well understood. Stomata are most often found only on the lower  
41 leaf surface (hypostomy) but occur on both surfaces (amphistomy) in many species  
42 (Metcalf and Chalk, 1950; Parkhurst, 1978; Mott et al., 1984). Theory and ex-  
43 periments demonstrate that amphistomy increases photosynthetic rates under many  
44 conditions. By creating a second parallel pathway for CO<sub>2</sub> diffusion within the meso-  
45 phyll, amphistomy optimally supplies CO<sub>2</sub> (Parkhurst, 1978; Gutschick, 1984; Jones,  
46 1985). Amphistomy is correlated with greater CO<sub>2</sub> diffusion (Beerling and Kelly,  
47 1996) and higher photosynthetic rates (McKown et al., 2014). These observations  
48 are corroborated by experiments demonstrating that amphistomy increases maxi-  
49 mum photosynthetic rates by up to 20% (Parkhurst and Mott, 1990). On the other  
50 hand, amphistomy can increase transpiration (Jones, 1985; Foster and Smith, 1986;  
51 Buckley et al., 2015). While transition to amphistomy is thus thought to increase  
52 transpiration, empirical studies suggest greater water-use efficiency in amphistoma-

53 tous species (Bucher et al., 2017). Hence, amphistomy appears to benefit a plant's  
54 carbon use relative to water loss and should be favored when CO<sub>2</sub> limits photo-  
55 synthetic rate. The open questions are under what ecological conditions does CO<sub>2</sub>  
56 supply most strongly limit photosynthetic rate (Peat and Fitter, 1994) and when is  
57 photosynthetic rate most important to fitness?

58 The leading, nonmutually exclusive hypotheses are that 1) open habitats favour  
59 amphistomy because CO<sub>2</sub> diffusion most strongly limits photosynthetic rate under  
60 high light and 2) herbaceous growth form favours amphistomy because traits that  
61 maximize photosynthetic rate are often under stronger selection in herbs. Salisbury  
62 (1927) first noted that amphistomy is most common in herbaceous plants from open  
63 habitats (i.e., with high light) of the British flora. These observations have been  
64 replicated in other studies (Mott et al., 1984; Peat and Fitter, 1994; Jordan et al.,  
65 2014; Muir, 2015) and may support physiological and ecological hypotheses that CO<sub>2</sub>  
66 most strongly limits photosynthesis in high light and/or photosynthesis contributes  
67 most to fitness in herbaceous plants. Under high light, CO<sub>2</sub> can strongly limit max-  
68 imum photosynthetic rates, especially in thick leaves (Jones, 1985). Hence, having  
69 stomata on both surfaces relieves this limitation by adding a second parallel pathway  
70 for CO<sub>2</sub> diffusion. Parkhurst 1978 argued that greater leaf thickness *per se* selected  
71 for amphistomy, but there is little evidence for correlations between leaf thickness

72 and stomatal ratio independent of light (Mott et al., 1984; Gibson, 1996; Muir, 2015).

73 Amphistomy is correlated with open habitat in warm desert plants of western North  
74 America (Mott et al., 1984; Gibson, 1996), among the Proteaceae (Jordan et al.,  
75 2014), and in continental European herbs (Bucher et al., 2017).

76 Stomatal ratio is also associated with growth form. In the British flora, Salisbury  
77 (1927) found that trees and shrubs are nearly always hypostomatous, whereas herbs  
78 from open habitats are amphistomatous. This pattern holds when data are averaged  
79 by family to coarsely control for phylogenetic nonindependence (Peat and Fitter,  
80 1994) or when using alternative classification schemes, such as Raunkiaer life form  
81 (Peat and Fitter, 1994). Across plants from  $\sim 90$  families worldwide, growth form is  
82 the strongest predictor of stomatal ratio when multiple factors are estimated simulta-  
83 neously and controlling for phylogenetic nonindependence (Muir, 2015). These pat-  
84 terns are consistent with other data indicating that many herbaceous plants are un-  
85 der strong selection for high maximum photosynthetic rates. (Bazzaz, 1979; Körner  
86 et al., 1989; Wullschleger, 1993).

87 Although previous comparative studies have tested whether open habitat and growth  
88 form influence stomatal ratio, we do not know if these effects are independent of one  
89 another. Open habitat and growth form may not be independent because open habi-  
90 tats generally consist of more short-statured, herbaceous plants. Some authors have

91 attempted to disentangle light and growth form by contrasting herbs from open and  
92 understory habitats (Salisbury, 1927). However, this is problematic if phylogenetic  
93 relationships are not controlled for, because shade species may share traits simply  
94 because they are more closely related to each other than they are to high light  
95 species. Finally, open habitat and growth form may also interact with one another.  
96 For example, amphistomy may only be favored when CO<sub>2</sub> strongly limits photosyn-  
97 thetic rate (e.g. in high light) *and* photosynthetic rate strongly limits fitness (e.g. in  
98 herbs).

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

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

106 The final question is important for telling whether amphistomy is part of a coordi-  
107 nated syndrome of traits that promote higher photosynthetic rate, as both the light  
108 and growth form hypotheses assume. If evolved increases in stomatal ratio are medi-

109 ated by shifting abaxial stomata to the adaxial surface, holding total stomatal density  
110 constant, then the overall increase in CO<sub>2</sub> diffusion would be limited. In contrast,  
111 if amphistomy evolves by increasing adaxial stomatal density while holding abaxial  
112 density constant, then *total* stomatal density must increase as well. Evolutionary  
113 coordination of amphistomy and high stomatal density would reinforce one another,  
114 increasing CO<sub>2</sub> supply to chloroplasts more than changes in either trait would in  
115 isolation.

116 To address these questions, I reanalyzed existing data on stomatal ratio, light habi-  
117 tat, and growth form in British angiosperms (Salisbury, 1927; Fitter and Peat, 1994,  
118 2017) using phylogenetic comparative methods. The British angiosperm flora is well  
119 suited for these questions because this flora has been comprehensively surveyed for  
120 many ecologically important traits, meaning it is probably the least biased survey  
121 of stomatal trait variation. Salisbury’s observations on stomata and ecology in the  
122 British flora have heavily influenced plant ecophysiology, but many of his and subse-  
123 quent authors’ analyses have significant limitations because of inadequate statistical  
124 methods. For example, few analyses until recently account for phylogenetic nonin-  
125 dependence (Felsenstein, 1985), which can strongly influence inferences on stomatal  
126 traits and growth form (Kelly and Beerling, 1995, this study did not consider light).  
127 A species-level phylogeny of the entire British flora (Lim et al., 2014) now allows for



128 the first time rigorous analysis of evolutionary relationships among stomatal ratio,  
129 light, and growth form.

## 130 **Materials and Methods**

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

### 133 **Data on stomatal ratio, light habitat, growth form, and phy-** 134 **logenetic relationships**

135 I obtained data on ab- and adaxial stomatal density on 395 species from British  
136 Ecological Flora (Salisbury, 1927; Fitter and Peat, 1994, 2017). Following recent  
137 comparative analyses (e.g. Bartelheimer and Poschlod, 2016; Salguero-Gómez et al.,  
138 2016), I used Ellenberg light indicator values (Ellenberg, 1974) and Raunkiær life  
139 form (Raunkiær, 1934) as measures of light habitat and growth form, respectively.  
140 Hence, I am assuming that the species' light habitat is closely related to the type of  
141 habitat (open versus closed) where that species is found. Both attributes have been  
142 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 a congeneric species as a proxy for the focal species. 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 0.5-64 (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 hydrophytes (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<sub>4</sub> (3 species) and CAM (2 species) plants. I limited this investigation to angiosperms

162 because only 4 non-angiosperms had stomata data. The final dataset contained  
 163 372 species. The R code accompanying this paper documents these decisions with  
 164 citations to the relevant literature.

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

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

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

167  $SD_{ab}$  and  $SD_{ad}$  are the stomatal densities on abaxial or adaxial surface, respectively.  
 168  $SD_{total} = SD_{ab} + SD_{ad}$ .  $SR_{propAd}$  is the proportion of stomata density on the adaxial  
 169 surface, which is useful for discriminating among hypostomatous ( $SR_{propAd} = 0$ ),  
 170 amphistomatous ( $0 < SR_{propAd} < 1$ ), and hyperstomatous species ( $SR_{propAd} = 1$ ).  
 171  $SR_{even}$  indicates how evenly stomatal densities are distributed across both leaf sur-  
 172 faces. This expression is useful because several hypotheses are based on the fact that  
 173 a more even distribution should optimize leaf  $CO_2$  diffusion.

## 174 **Testing for an association between open habitat and growth** 175 **form**

176 I tested whether Raunkiaer life form was associated L-value among British angiosperms  
177 using ANOVA with Type-2 sum of squares. I did not use phylogenetic ANOVA for  
178 this test because there was no phylogenetic signal in the regression fit using **phylolm**  
179 version 2.5 (Ho and Ané, 2014). See the R code accompanying this paper for further  
180 detail. I predicted that species with faster life histories, especially therophytes (an-  
181 nuals), would have greater L-values than species with slower life histories, especially  
182 phanerophytes, which are mostly long-lived trees.

## 183 **Open habitat, growth form, and stomatal ratio**

184 I compared phylogenetic linear models to test whether Raunkiaer life form, L-value,  
185 or interactions between them predicted  $SR_{\text{even}}$ . I used  $SR_{\text{even}}$  rather than  $SR_{\text{propAd}}$   
186 as the response variable because the hypothesis is that faster life history and/or high  
187 light favor more even stomatal densities on each surface. I fit models using **phylolm**  
188 and extracted Akaike Information Criteria (AIC). For these and subsequent analy-  
189 ses, I assumed an Ornstein-Uhlenbeck process model for the residuals with the root  
190 character state integrated over the stationary distribution. I used a 10,000 para-

metric bootstrap samples of the full model (including main effects and interactions) to calculate parameter confidence intervals (Boettiger et al., 2012). Likewise, to determine whether the interaction between Raunkiær life form and L-value was statistically significant, I used a parametric bootstrap to generate the null distribution of  $\Delta\text{AIC}$  values ( $\Delta\text{AIC}$  is the difference in AIC between competing models). Specifically, I sampled 1000 random datasets from the estimated model with main effects of Raunkiær life form and L-value but no interaction. I fit these simulated datasets to models with and without interactions and calculated  $\Delta\text{AIC}$ . The statistical significance of the observed  $\Delta\text{AIC}$  is the proportion of simulated  $\Delta\text{AIC}$  greater than the observed.

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

I used two complementary phylogenetic methods to assess the relative contribution of ab- versus adaxial stomatal density to light-mediated stomatal ratio evolution. The contribution of each can be formalized using standard variance decomposition methods as derived below. Because stomatal density is highly skewed, I log-transformed values for normality:

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

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

$$sr_{\text{even}} = sd_{\text{ad}} - sd_{\text{ab}} \quad (5)$$

208 Lowercase variables (sr, sd) indicate log-transformed values. Because some species  
 209 had zero adaxial stomata, I added one to all values prior to log-transformation.  
 210 To make the variance decomposition calculations tractable, I have defined  $SR_{\text{even}}$   
 211 here as the ratio of ad- to abaxial stomatal density because in most cases adaxial  
 212 stomatal density is lower than abaxial (see Eq. 2). This was not done in previous  
 213 analyses because I wanted to test what factors influenced the evenness of stomatal  
 214 densities, regardless of which surface had higher density. With this modified form,  
 215 the variance in  $sr_{\text{even}}$  can be decomposed into contributions of  $sd_{\text{ad}}$ ,  $sd_{\text{ab}}$ , and their  
 216 covariance:

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

217 I estimated the phylogenetic covariance matrix between L-value,  $sd_{ab}$ , and  $sd_{ad}$  using  
 218 a multivariate Ornstein-Uhlenbeck model fit in **Rphylopars** version 0.2.9 (Goolsby  
 219 et al., 2016, 2017). From the covariance matrix, I estimated the contribution of  
 220 abaxial density, adaxial density, and their covariance as:

$$\frac{\text{Var}(sd_{ad})}{\text{Var}(sr_{even})}, \frac{\text{Var}(sd_{ab})}{\text{Var}(sr_{even})}, \text{ and } \frac{\text{Cov}(sd_{ad}, sd_{ab})}{\text{Var}(sr_{even})}, \quad (7)$$

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

223 I was interested in whether light-mediated evolution of stomatal ratio acted mostly  
 224 by increasing adaxial stomatal density while maintaining abaxial density, or keeping  
 225 total stomatal density the same, but shifting a greater proportion to the adaxial sur-  
 226 face. The first scenario predicts that the phylogenetic regression of L-value on  $sd_{ad}$  is  
 227 stronger than that for  $sd_{ab}$ . The second scenario predicts that L-value acts similarly  
 228 on both and that there is a negative covariance  $\text{Cov}(sd_{ad}, sd_{ab}) < 0$ . I tested these  
 229 competing predictions by fitting a simple phylogenetic structural equation model  
 230 (SEM). The model uses the phylogenetic covariance matrix to simultaneously esti-  
 231 mate regressions of L-value on  $sd_{ad}$  and  $sd_{ab}$  while allowing covariance between them  
 232 (i.e. estimating  $\text{Cov}(sd_{ad}, sd_{ab})$ ). To fit the SEM, I used the R package **lavaan** version

233 0.5-23.1097 (Rosseel, 2012). I tested whether parameter estimates were significantly  
234 different from zero using  $z$ -scores.

## 235 **Results**

### 236 **Light tolerance varies among Raunkiær life forms**

237 Ellenberg light indicator values (L-value) differed significantly among life forms (Fig. 1;ANOVA  
238 -  $F_{4,367} = 18.3$ ,  $P = 1.1 \times 10^{-13}$ ). Therophytes (annuals), hemicryptophytes (peren-  
239 nial herbs with buds near the soil surface), and chamaephytes (subshrubs) had greater  
240 L-values than phanerophytes (large woody plants) and geophytes (perennial herbs  
241 with storage organs) (Fig. 1).

### 242 **Interactions between light and Raunkiær life form determine** 243 **stomatal ratio**

244 Overall,  $SR_{\text{even}}$  increased with L-value, but there was a significant interaction ( $\Delta AIC >$   
245 2, Table 1) between Raunkiær life form and L-value (Fig. 2). Both life form and L-  
246 value significantly increased model fit, though L-value had a markedly larger effect  
247 on model AIC (Table 1). The significant interaction is caused by different slopes



248 between life forms. Among life forms with the overall greatest L-value (therophytes,  
249 hemicryptophytes, and chamaephytes, see Fig. 1), there was a strong positive rela-  
250 tionship between L-value and  $SR_{\text{even}}$ . Parametrically bootstrapped 95% confidence  
251 intervals for the slope did not overlap zero (Fig. 2). The slope was weakly positive or  
252 not significantly different from zero in the most shade-adapted life forms (geophytes  
253 and phanerophytes), albeit the patterns were distinct in these groups. There were  
254 both hypostomatous ( $SR_{\text{even}} \approx 0$ ) and amphistomatous ( $SR_{\text{even}} \approx 1$ ) geophytes, but  
255 these were distributed across L-values. In contrast, phanerophytes were nearly always  
256 hypostomatous regardless of L-value.

## 257 **Adaxial stomatal density contributes most of the variation in** 258 **stomatal ratio**

259 Adaxial ('upper') stomatal density contributed most to the evolutionary variation  
260 in stomatal ratio. The contributions of adaxial density, abaxial density, and their  
261 covariance are 1.14, 0.38, and -0.53, respectively. This implies that evolutionary  
262 variation in adaxial stomatal density is greater than that for stomatal ratio due to  
263 positive covariance between ab- and adaxial stomatal density.

264 Similarly, the phylogenetic SEM showed that changes in stomatal ratio associated

265 with L-value can be attributed mostly to evolution of adaxial stomatal density  
 266 (Fig. 3). Both  $sd_{ad}$  and  $sd_{ab}$  increased with L-value ( $P = 6.1 \times 10^{-7}$  and  $2.9 \times 10^{-5}$ ,  
 267 respectively). However, the regression of L-value on  $sd_{ad}$  was  $2.1 \times$  that of L-value on  
 268  $sd_{ab}$  (0.21 versus 0.1). Because stomatal densities were natural log-transformed, this  
 269 implies an increase in L-value by one leads to a 1.23-fold change in adaxial stom-  
 270 atal density versus a 1.1-fold change in abaxial stomatal density. The SEM also  
 271 showed a significant positive covariance between stomatal densities on each surface  
 272 ( $P = 1.7 \times 10^{-11}$ ). These results together imply that total stomatal density increases  
 273 with L-value, but the response is mediated mostly by increases in adaxial stomatal  
 274 density.

## 275 Discussion

276 The ratio of stomatal densities on the abaxial ('lower') to that of the adaxial ('upper')  
 277 surface varies greatly across plant species, but the adaptive significance is not clear.  
 278 Comparative studies correlating stomatal ratio to ecological factors can distinguish  
 279 among competing hypotheses and reveal critical experiments for future work. Previ-  
 280 ous comparative studies suggested that high light and herbaceous growth form favor  
 281 amphistomy (Mott et al., 1984; Jordan et al., 2014; Muir, 2015; Bucher et al., 2017),

282 particularly in the British flora (Salisbury, 1927; Peat and Fitter, 1994). However,  
283 none of these studies have accounted for the fact that light and growth form are  
284 often confounded – open, high light habitats are often dominated by herbs – or the  
285 fact that species are not independent because of shared evolutionary history. Here, I  
286 reanalyzed data on stomata, light tolerance, and growth form in British angiosperms  
287 using phylogenetic comparative methods. As expected, species’ light tolerance (El-  
288 lenberg light indicator or L-value) is confounded with growth form (Raunkiær life  
289 form; Fig. 1). Nevertheless, both L-value and Raunkiær life form affect stomatal  
290 ratio, but these factors also interact; the influence of L-value on stomatal ratio varies  
291 across forms. These novel findings provide further evidence that variation in stomatal  
292 ratio is adaptive and have important implications for interpreting changes in stom-  
293 atal ratio through the paleo record (Jordan et al., 2014) and during domestication  
294 (Milla et al., 2013).

## 295 **Adaptive significance of amphistomy**

296 Previously, associations between light, growth form, and stomatal ratio have been  
297 interpreted in isolation as indicating that either high light and/or herbaceous growth  
298 form favors amphistomy. In British angiosperms, both factors are important, though  
299 statistical analyses suggest that light may be a stronger determinant than growth

300 form (Table 1). Unlike previous studies, I found a significant interaction between  
301 light and growth form among British angiosperms, which suggests that amphistomy  
302 may only be strongly favored when CO<sub>2</sub> strongly limits photosyntheses (as in open  
303 habitat) *and* photosynthesis strongly limits fitness (as in herbs). This is consistent  
304 with life history theory predicting that the demography of open habitat herbs is  
305 strongly limited by plant growth (?). The ideal way to test this would be to measure  
306 selection on stomatal ratio in a species that varied quantitatively in both stomatal  
307 ratio and life history (e.g., containing both annual and perennial forms). I predict  
308 that amphistomy will be favored more strongly in the annual form grown under high  
309 light compared to an annual under low light or a perennial in high light, and much  
310 more strongly than a perennial grown in low light. Similar experiments could also be  
311 performed to test if and when light-mediated plasticity in stomatal ratio is adaptive  
312 (Gay and Hurd, 1975; Mott and Michaelson, 1991).

313 The prevalence of amphistomatous species in high light habitats supports the hy-  
314 pothesis that amphistomy is an adaptation to maximize photosynthetic rates by  
315 increasing CO<sub>2</sub> diffusion (Jones, 1985). It is also evidence against the hypothesis  
316 that the principle fitness cost of amphistomy is water loss (Darwin, 1886; Foster and  
317 Smith, 1986) or dehydration of palisade mesophyll (Buckley et al., 2015), though  
318 these factors are likely very important in determining differential regulation of stom-

319 ata on each surface. Since evaporative demand increases under high light, under  
320 these hypotheses we would expect plants in high light to be hypostomatous. Because  
321 stomatal conductances on each surface can be regulated independently in response  
322 to the environment (Darwin, 1898; Pospíšilová and Solárová, 1984; Smith, 1981; Re-  
323 ich, 1984; Mott and O’Leary, 1984), amphistomatous leaves likely cope with these  
324 stresses by rapidly closing adaxial stomata when water supply cannot match evapo-  
325 rative demands (Richardson et al., 2017). Instead, patterns in the British flora are  
326 at least consistent with the idea that adaxial stomata increase susceptibility to foliar  
327 pathogens (Gutschick, 1984; McKown et al., 2014). The cost of adaxial stomata  
328 may be greater in the shade because greater leaf wetness and lower ultraviolet light  
329 provide a more suitable microclimate for many foliar pathogens.

## 330 **Amphistomy as a proxy for open habitat**

331 These observations from the British flora partially support the hypothesis that am-  
332 phistomy can be used a proxy for open habitat in paleoenvironment reconstruction  
333 (Carpenter, 1994; Jordan et al., 2014; Carpenter et al., 2015) but also point out pre-  
334 viously unknown subtleties. These previous studies based their conclusions on data  
335 from Proteaceae, in which there is little quantitative variation in stomatal ratio;  
336 species are either completely hypostomatous ( $SR_{propAd} \approx 0$ ) or completely amphis-

337 tomatous ( $SR_{propAd} \approx 0.5$ ). Stomatal ratio in British angiosperms is also bimodal  
338 (Peat and Fitter, 1994), but across many families there is also quantitative variation.  
339 Importantly, this means that quantitative variation in stomatal ratio may provide a  
340 more precise, quantitative indicator of vegetation type, rather than simply ‘open’ or  
341 ‘closed’. A quantitative relationship between L-value and stomatal ratio has already  
342 been shown for herbaceous perennials (Bucher et al., 2017), but we now know that  
343 it holds among annuals (therophytes), subshrubs (chamaephytes), and, to a lesser  
344 extent, geophytes as well (Fig. 2).

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

356 pioneer species.

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

## 367 **Why does adaxial stomatal density control stomatal ratio?**

368 Variation in stomatal ratio is determined primarily by evolution of adaxial stom-  
369 atal density and is coordinated with increases in total leaf stomatal density summed  
370 across both surfaces. Note here that I am referring only to evolutionary variation in  
371 stomatal ratio among species; different processes may mediate within species vari-  
372 ation 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<sub>2</sub> 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



392 balance between stomatal and mesophyll limitations. Conversely, there would be  
393 little benefit to amphistomy when total stomatal density is low because CO<sub>2</sub> diffusion  
394 is strongly limited by stomatal resistance, and therefore photosynthetic rate is not  
395 sensitive to changes in mesophyll diffusion mediated by stomatal ratio. A related  
396 prediction is that increased atmospheric CO<sub>2</sub> may select for reduced stomatal ratio  
397 and density primarily by decreasing adaxial stomatal density, but this has not been  
398 well tested (but see Woodward and Bazzaz, 1988).

## 399 **Conclusions**

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

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## 409 **Author contribution statement**

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

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Table 1: Interaction between species' Ellenberg light indicator value (L-value) and Raunkiaer lifeform predict stomatal ratio ( $\text{SR}_{\text{even}}$ ). I compared phylogenetic linear models using the Akaike Information Criterion (AIC), where  $\text{AIC} = 2k - 2\log(\mathcal{L})$ .  $k$  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 ( $\Delta\text{AIC}$ ) indicates the relative fit of competing models. The correlation coefficient  $r^2$  is another indicator of model fit.  $\alpha$  and  $\sigma^2$  are the return rate and diffusion parameters of the Ornstein-Uhlenbeck model of trait evolution.

Model: $\text{SR}_{\text{even}} \sim$	$\alpha$	$\sigma^2$	$r^2$	$k$	$\log(\mathcal{L})$	AIC	$\Delta\text{AIC}$
L-value $\times$ lifeform	0.46	0.068	0.34	12	-33.3	90.6	0
L-value + lifeform	0.47	0.072	0.32	8	-40.3	96.5	6
L-value	0.64	0.108	0.26	4	-59.3	126.6	36.1
lifeform	0.34	0.067	0.15	7	-79.2	172.4	81.8
null	0.29	0.067	0	3	-107.5	221	130.5

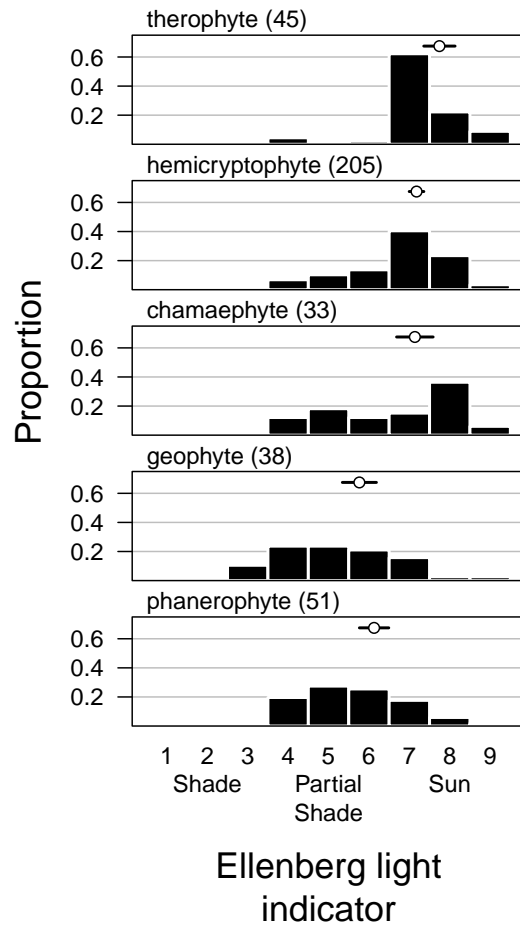


Figure 1: Lifeforms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different Raunkiær life forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform.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

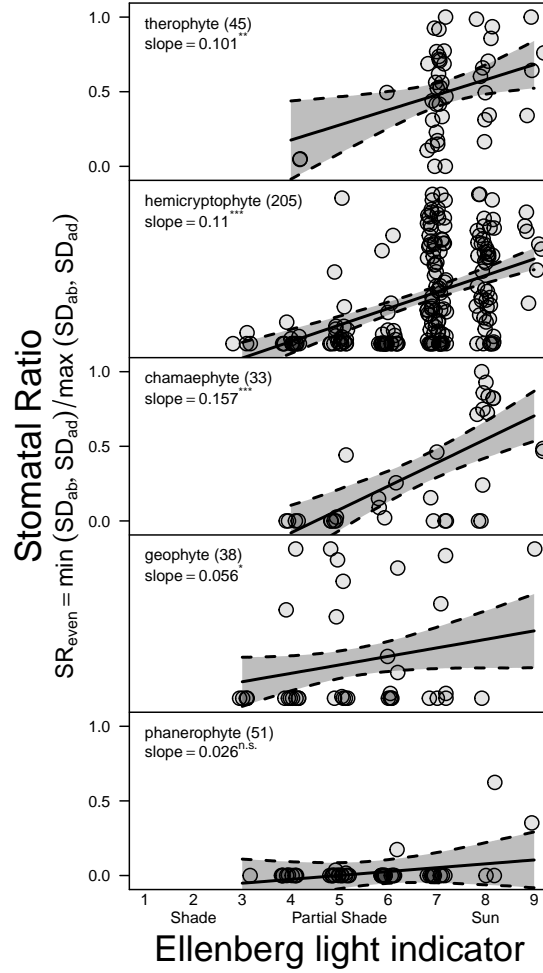


Figure 2: The effect of light on stomatal ratio depends on Raunkiaer life form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio ( $SR_{\text{even}}$ ) in therophytes, hemicryptophytes, and chamaephytes but not geophytes and phanerophytes. The maximum likelihood slope from phylogenetic regression is given with statistical significance based on 1000 parametric bootstrap samples. Numbers in parentheses next to Raunkiaer 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.

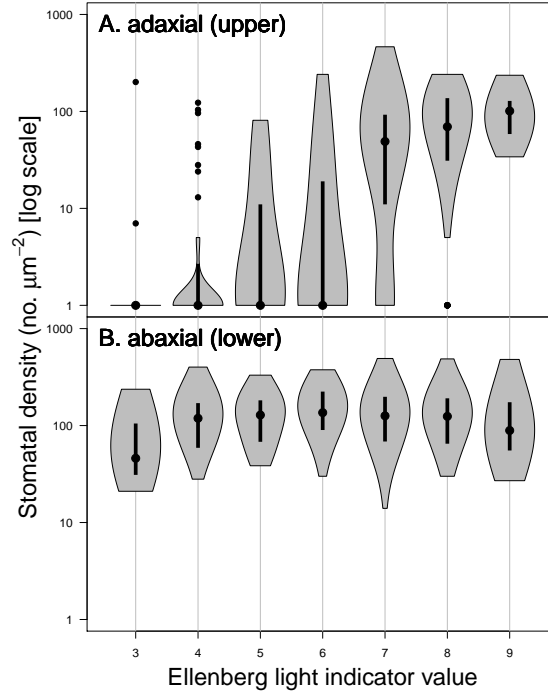


Figure 3: Light-mediated evolution of stomatal ratio is mostly driven by increased adaxial (‘upper’) stomatal density (Panel A), whereas abaxial (‘lower’) stomatal density (Panel B) is similar across Ellenberg light indicator values (L-value  $x$ -axis). The violin plot shows stomatal density ( $y$ -axis, 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.



## 570 Supporting Information

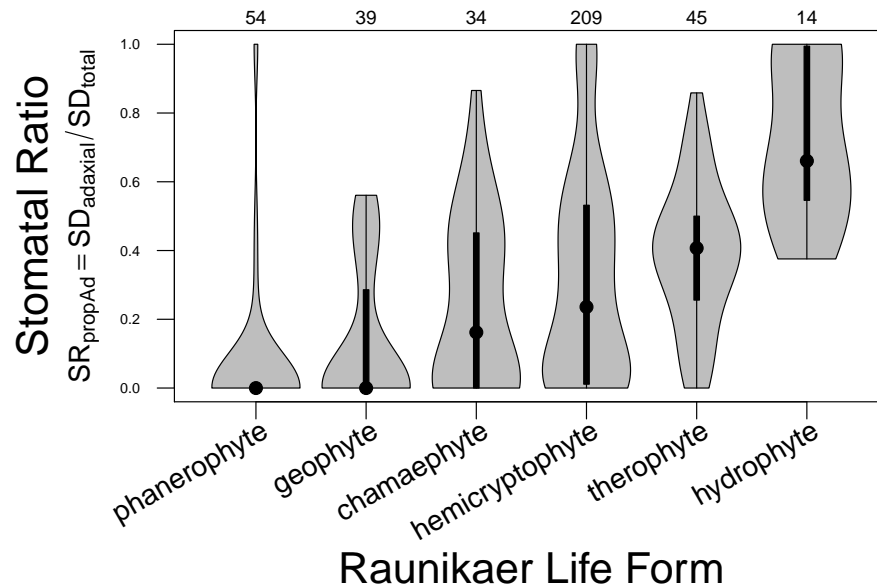


Figure S1: Most hydrophytes are hyperstomatous, having most stomata on the adaxial ('upper') surface (high  $SD_{propAd}$ ). The violin plot shows stomatal ratio as a function of Raunkiaer lifeform. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range 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.