

Light and growth 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, Raunkiær 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 effect of light is pronounced in therophytes
13 (annuals) and perennial herbs, but muted in phanerophytes (mostly trees).
14 Interestingly, amphistomy and stomatal density evolve together in response to
15 light, suggesting coordinated selection on this trait combination.
- 16 • I show for the first time that light and growth form interact to shape variation
17 in stomatal ratio; amphistomy is advantageous in high light, but mostly for
18 herbs. These results improve our understanding of the adaptive significance of
19 stomatal ratio as well as its use as functional trait for paleoecology and crop
20 improvement.

21 **Keywords**

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

24 **Introduction**

25 Natural selection shapes leaf anatomy in order to optimize its photosynthetic function
26 in a given environment (Haberlandt, 1914; Givnish, 1987; Smith et al., 1997). By
27 understanding the adaptive significance of leaf anatomical variation we can learn
28 about natural history, find targets for crop improvement, and identify anatomical
29 proxies for paleoclimates preserved in the fossil record (e.g. Wolfe, 1971; Royer, 2001;
30 McElwain and Steinthorsdottir, 2017). The size, density, and distribution of stomata
31 on a leaf vary widely and impact the flux of CO₂ and water vapour (recently reviewed
32 in Sack and Buckley, 2016), as well as susceptibility to foliar pathogens that infect
33 through stomata (McKown et al., 2014; Melotto et al., 2017). Hence, stomata have
34 been especially useful in understanding plastic and evolutionary response to climate
35 change and domestication (Woodward, 1987; Beerling and Royer, 2011; Milla et al.,
36 2013).

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

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

57 The leading, nonmutually exclusive hypotheses are that 1) open habitats favour
58 amphistomy because CO₂ diffusion most strongly limits photosynthetic rate under
59 high light and 2) herbaceous growth form favours amphistomy because traits that
60 maximize photosynthetic rate are often under stronger selection in herbs. Salisbury
61 (1927) first noted that amphistomy is most common in herbaceous plants from open
62 habitats (i.e., with high light) of the British flora. These observations have been
63 replicated in other studies (Mott et al., 1984; Peat and Fitter, 1994; Jordan et al.,
64 2014; Muir, 2015) and may support physiological and ecological hypotheses that CO₂
65 most strongly limits photosynthesis in high light and/or photosynthesis contributes
66 most to fitness in herbaceous plants. Under high light, CO₂ can strongly limit max-
67 imum photosynthetic rates, especially in thick leaves (Jones, 1985). Hence, having

68 stomata on both surfaces relieves this limitation by adding a second parallel pathway
69 for CO₂ diffusion. Parkhurst (1978) argued that greater leaf thickness *per se* selected
70 for amphistomy, but there is little evidence for correlations between leaf thickness
71 and stomatal ratio independent of light (Mott et al., 1984; Gibson, 1996; Muir, 2015).
72 Amphistomy is correlated with open habitat in warm desert plants of western North
73 America (Mott et al., 1984; Gibson, 1996), among the Proteaceae (Jordan et al.,
74 2014), and in continental European herbs (Bucher et al., 2017).

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

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

93 traits simply because they are more closely related to each other than they are to
94 high light species. Finally, open habitat and growth form may also interact with one
95 another. For example, amphistomy may only be favored when CO₂ strongly limits
96 photosynthetic rate (e.g. in high light) *and* photosynthetic rate strongly limits fitness
97 (e.g. in herbs).

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

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

105 The final question is important for addressing whether amphistomy is part of a co-
106 ordinated syndrome of traits that promote higher photosynthetic rate, as both the
107 light and growth form hypotheses assume. If evolved increases in stomatal ratio are
108 mediated by shifting abaxial stomata to the adaxial surface, holding total stomatal
109 density constant, then the overall increase in CO₂ diffusion would be small. In con-
110 trast, if amphistomy evolves by increasing adaxial stomatal density while holding
111 abaxial density constant, then *total* stomatal density must increase as well. Evolu-
112 tionary coordination of amphistomy and high stomatal density would reinforce one
113 another, increasing CO₂ supply to chloroplasts more than changes in either trait
114 would in isolation.

115 To address these questions, I reanalyzed existing data on stomatal ratio, light habi-
116 tat, 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. Niinemets and Valladares, 2006; Bartelheimer and Poschlod, 2016; Shipley et al.), I used Ellenberg light indicator values (Ellenberg, 1974) as mea-

138 sures of light habitat. Hence, I am assuming that the species' light habitat is closely
139 related to the type of habitat (open versus closed) where that species is found. Ellen-
140 berg light indicator values, hereafter abbreviated L-value, have been recently updated
141 by taxonomic experts of the British flora (PLANTATT, Hill et al. (2004)).

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

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

163 small adjustments to the tree. I resolved polytomies randomly using the ‘multi2di’
 164 function in **phytools** version 0.6-00 (Revell, 2012). I added 0.02 my to all zero-length
 165 branches, as this was approximately the length of the shortest nonzero branch length
 166 in the tree. After these changes, I slightly altered terminal branch lengths to make
 167 the tree precisely ultrametric.

168 I excluded data on hydropytes (14 species) because many of these species are
 169 hyperstomatous (Fig. S2) due to the fact that leaves may rest on the water’s surface,
 170 selecting for stomata to be present on the upper surface only. I also excluded C₄
 171 (3 species) and CAM (2 species) plants. I limited this investigation to angiosperms
 172 because only 4 non-angiosperms had stomata data. The final dataset contained 372
 173 species (Fig. 1). The R code accompanying this paper documents these decisions
 174 with citations to the relevant literature.

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

$$\text{SR}_{\text{propAd}} = \frac{\text{SD}_{\text{ad}}}{\text{SD}_{\text{total}}} \quad (1)$$

$$\text{SR}_{\text{even}} = \frac{\min\{\text{SD}_{\text{ab}}, \text{SD}_{\text{ad}}\}}{\max\{\text{SD}_{\text{ab}}, \text{SD}_{\text{ad}}\}} \quad (2)$$

177 SD_{ab} and SD_{ad} are the stomatal densities on abaxial or adaxial surface, respectively.
 178 $\text{SD}_{\text{total}} = \text{SD}_{\text{ab}} + \text{SD}_{\text{ad}}$. $\text{SR}_{\text{propAd}}$ is the proportion of stomata density on the adaxial
 179 surface, which is useful for discriminating among hypostomatous ($\text{SR}_{\text{propAd}} = 0$),
 180 amphistomatous ($0 < \text{SR}_{\text{propAd}} < 1$), and hyperstomatous species ($\text{SR}_{\text{propAd}} = 1$).
 181 SR_{even} indicates how evenly stomatal densities are distributed across both leaf sur-

182 faces. This expression is useful because several hypotheses are based on the fact that
183 a more even distribution should optimize leaf CO₂ diffusion.

184 **Testing for an association between open habitat and growth** 185 **form**

186 I tested whether Raunkiaer life form was associated with L-value among British an-
187 giosperms using ANOVA with Type-2 sum of squares. I did not use phylogenetic
188 ANOVA for this test because there was no phylogenetic signal in the regression fit
189 using **phylolm** version 2.5 (Ho and Ané, 2014). See the R code accompanying this
190 paper for further detail. I predicted that species with faster life histories, especially
191 therophytes (annuals), would have greater L-values than species with slower life his-
192 tories, especially phanerophytes, which are mostly long-lived trees.

193 **Open habitat, growth form, and stomatal ratio**

194 I compared phylogenetic linear models to test whether Raunkiaer life form, L-value,
195 or interactions between them predicted SR_{even}. Unlike the analysis above, there
196 was significant phylogenetic signal in this comparison (see R code). I used SR_{even}
197 rather than SR_{propAd} as the response variable because the hypothesis is that faster
198 life history and/or high light favor more even stomatal densities on each surface.
199 I fit models using **phylolm** and extracted Akaike Information Criteria (AIC). For
200 these and subsequent analyses, I assumed an Ornstein-Uhlenbeck process model for
201 the residuals with the root character state integrated over the stationary distribu-
202 tion. I used a 10⁴ parametric bootstrap samples of the full model (including main

203 effects and interactions) to calculate parameter confidence intervals (Boettiger et al.,
204 2012).

205 **Does ab- or adaxial stomatal density contribute more to stom-** 206 **atal ratio evolution?**

207 I used two related phylogenetic methods, variance decomposition and structural equa-
208 tion modeling (SEM), to assess the relative contribution of ab- versus adaxial stom-
209 atal density to light-mediated stomatal ratio evolution. First, the contribution of ab-
210 versus adaxial stomatal density can be calculated using phylogenetic variance de-
211 composition methods as derived below. Because stomatal density is highly skewed,
212 I log-transformed values for normality:

$$\text{SR}_{\text{even}} = \frac{\text{SD}_{\text{ad}}}{\text{SD}_{\text{ab}}} \quad (3)$$

$$\log(\text{SR}_{\text{even}}) = \log(\text{SD}_{\text{ad}}) - \log(\text{SD}_{\text{ab}}) \quad (4)$$

$$\text{sr}_{\text{even}} = \text{sd}_{\text{ad}} - \text{sd}_{\text{ab}} \quad (5)$$

213 Lowercase variables (sr, sd) indicate log-transformed values. Because some species
214 had zero adaxial stomata, I added one to all values prior to log-transformation. To
215 make the variance decomposition calculations tractable, I have defined SR_{even} here
216 as the ratio of ad- to abaxial stomatal density because in most cases adaxial stomatal
217 density is lower than abaxial (see Eq. 2). This differs from analyses described above

218 because in those I wanted to test what factors influenced the evenness of stomatal
 219 densities, regardless of which surface had higher density. With this modified form,
 220 the variance in sr_{even} can readily be decomposed into contributions of sd_{ad} , sd_{ab} , and
 221 their covariance:

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

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

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

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

$$\text{Contribution of } \text{Cov}(sd_{\text{ad}}, sd_{\text{ab}}) = \frac{\text{Cov}(sd_{\text{ad}}, sd_{\text{ab}})}{\text{Var}(sr_{\text{even}})} \quad (9)$$

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

229 I also wanted to test whether light-mediated evolution of stomatal ratio acted mostly
 230 by 1) increasing adaxial stomatal density while maintaining abaxial density, or 2)

231 keeping total stomatal density the same, but shifting a greater proportion to the adax-
 232 ial surface. The first scenario predicts that the phylogenetic regression of L-value on
 233 sd_{ad} is stronger than that for sd_{ab} . The second scenario predicts that L-value acts sim-
 234 ilarly on both and that there is a negative covariance ($Cov(sd_{ad}, sd_{ab}) < 0$). I tested
 235 these competing predictions by fitting a very simple phylogenetic SEM (see Mason
 236 et al., 2016, for a similar approach). The model uses the phylogenetic covariance
 237 matrix, as described above, to simultaneously estimate regressions of L-value on sd_{ad}
 238 and sd_{ab} while allowing covariance between them (i.e. estimating $Cov(sd_{ad}, sd_{ab})$).
 239 To fit the SEM, I used the R package **lavaan** version 0.5-23.1097 (Rosseel, 2012).
 240 I tested whether parameter estimates were significantly different from zero using
 241 z -scores.

242 Results

243 Light tolerance varies among Raunkiaer life forms

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

249 Interactions between light and Raunkiær life form determine 250 stomatal ratio

251 Overall, SR_{even} increased with L-value, but there was a significant interaction ($\Delta AIC >$
252 2, Table 1) between Raunkiær life form and L-value (Fig. 3). Both life form and L-
253 value significantly increased model fit, though L-value had a markedly larger effect
254 on model AIC (Table 1). The significant interaction is caused by different slopes
255 between life forms. Among life forms with the overall greatest L-value (therophytes,
256 hemicryptophytes, and chamaephytes, see Fig. 2), there was a strong positive rela-
257 tionship between L-value and SR_{even} . Parametrically bootstrapped 95% confidence
258 intervals for the slope did not overlap zero (Fig. 3). The slope was weakly positive
259 or not significantly different from zero in the most shade-adapted life forms (geo-
260 phytes and phanerophytes), albeit the patterns were distinct in these groups. There
261 were both hypostomatous ($SR_{\text{even}} \approx 0$) and amphistomatous ($SR_{\text{even}} \approx 1$) geophytes,
262 but these were distributed across L-values. In contrast, phanerophytes were nearly
263 always hypostomatous regardless of L-value.

264 Adaxial stomatal density contributes most of the variation in 265 stomatal ratio

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

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

282 Discussion

283 The ratio of stomatal densities on the abaxial ('lower') to that of the adaxial ('upper')
 284 surface varies greatly across plant species, but the adaptive significance is not clear.
 285 Comparative studies correlating stomatal ratio to ecological factors can distinguish
 286 among competing hypotheses and reveal critical experiments for future work. Previ-
 287 ous comparative studies suggested that high light and herbaceous growth form favor
 288 amphistomy (Mott et al., 1984; Jordan et al., 2014; Muir, 2015; Bucher et al., 2017),
 289 particularly in the British flora (Salisbury, 1927; Peat and Fitter, 1994). However,
 290 none of these studies have accounted for the fact that light and growth form are
 291 often confounded – open, high light habitats are often dominated by herbs – or the
 292 fact that species are not independent because of shared evolutionary history. Here, I
 293 reanalyzed data on stomata, light tolerance, and growth form in British angiosperms

294 using phylogenetic comparative methods. As expected, species' light tolerance (El-
295 lenberg light indicator or L-value) is confounded with growth form (Raunkiaer life
296 form; Fig. 2). Nevertheless, both L-value and Raunkiaer life form affect stomatal
297 ratio, but these factors also interact; the influence of L-value on stomatal ratio varies
298 across forms. These novel findings provide further evidence that variation in stomatal
299 ratio is adaptive and have important implications for interpreting changes in stom-
300 atal ratio through the paleo record (Jordan et al., 2014) and during domestication
301 (Milla et al., 2013).

302 **Adaptive significance of amphistomy**

303 Previously, associations between light, growth form, and stomatal ratio have been
304 interpreted in isolation as indicating that either high light and/or herbaceous growth
305 form favors amphistomy. In British angiosperms, both factors are important, though
306 statistical analyses suggest that light may be a stronger determinant than growth
307 form (Table 1). Unlike previous studies, I found a significant interaction between
308 light and growth form among British angiosperms, which suggests that amphistomy
309 may only be strongly favored when CO₂ strongly limits photosynthesis (as in open
310 habitat) *and* photosynthesis strongly limits fitness (as in herbs). This is consistent
311 with life history theory predicting that the demography of open habitat herbs is
312 strongly limited by plant growth (Franco and Silvertown, 1996). The ideal way to
313 test this would be to measure selection on stomatal ratio in a species that varied
314 quantitatively in both stomatal ratio and life history (e.g., containing both annual
315 and perennial forms). I predict that amphistomy will be favored more strongly in
316 the annual form grown under high light compared to an annual under low light
317 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 CO₂ 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 palisade 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 as a proxy for open habitat in paleoenvironment reconstruction

341 (Carpenter, 1994; Jordan et al., 2014; Carpenter et al., 2015) but also point out pre-
 342 viously unknown subtleties. These previous studies based their conclusions on data
 343 from Proteaceae, in which there is little quantitative variation in stomatal ratio;
 344 species are either completely hypostomatous ($SR_{propAd} \approx 0$) or completely amphis-
 345 tomatus ($SR_{propAd} \approx 0.5$). Stomatal ratio in British angiosperms is also bimodal
 346 (Peat and Fitter, 1994), but across many families there is also quantitative variation.
 347 Importantly, this means that quantitative variation in stomatal ratio may provide a
 348 more precise, quantitative indicator of vegetation type, rather than simply ‘open’ or
 349 ‘closed’. A quantitative relationship between L-value and stomatal ratio has already
 350 been shown for herbaceous perennials (Bucher et al., 2017), but we now know that
 351 it holds among annuals (therophytes), subshrubs (chamaephytes), and, to a lesser
 352 extent, geophytes as well (Fig. 3).

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

365 Finally, phylogenetic information should improve inferences about paleoclimates be-

366 cause there is appreciable phylogenetic signal in stomatal ratio. The phylogenetic
367 half-life of stomatal ratio evolution, after accounting for L-value and Raunkiaer life
368 form, is $\log(2)/\alpha = 1.5$ my (see Table 1 for maximum likelihood estimates of α , the
369 return rate in the Ornstein-Uhlenbeck model of trait evolution). This lag time may
370 indicate that evolving to the ‘optimum’ is constrained by the shape of the fitness
371 landscape (Muir, 2015) or that other unmeasured factors which affect stomatal ra-
372 tio have some phylogenetic signal. Regardless of the mechanism, this fact means
373 that researchers may be able to use data from closely related species to improve
374 paleoenvironment reconstruction.

375 **Why does adaxial stomatal density control stomatal ratio?**

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

386 There are at least two hypotheses that could explain why adaxial stomatal density
387 is the most responsive. The first I refer to as the ‘real estate’ hypothesis. In hy-
388 postomatous plants, the lower surface is already crowded with stomata, and hence

389 plants must increase the real estate available for stomata by developing them on the
390 upper surface whenever there is selection for greater stomatal density. When stomata
391 are packed too densely on one surface, stomatal interference limits their function-
392 ing and hence may create a strong selective pressure for amphistomy (Parlange and
393 Waggoner, 1970; Dow et al., 2014).

394 I refer to the second hypothesis as the ‘coordination’ hypothesis. In this scenario,
395 ecological conditions such as high light select for both increased total stomatal density
396 and for amphistomy because these traits work well in coordination with one another.
397 For example, if stomatal density were very high on a hypostomatous plant, then CO₂
398 would be more strongly limited by the mesophyll. Adding a second parallel pathway
399 for diffusion by developing stomata on both surfaces would restore a more optimal
400 balance between stomatal and mesophyll limitations. Conversely, there would be
401 little benefit to amphistomy when total stomatal density is low because CO₂ diffusion
402 is strongly limited by stomatal resistance, and therefore photosynthetic rate is not
403 sensitive to changes in mesophyll diffusion mediated by stomatal ratio. A related
404 prediction is that increased atmospheric CO₂ may select for reduced stomatal ratio
405 and density primarily by decreasing adaxial stomatal density, but this has not been
406 well tested (but see Woodward and Bazzaz, 1988).

407 **Conclusions**

408 By revisiting this classic ecological dataset with modern phylogenetic comparative
409 methods, I have shown that amphistomy is strongly associated with both light and
410 growth form, but the interaction between these factors is also important. Fur-
411 thermore, amphistomy and high stomatal density are closely connected in species

412 from high light environments, suggesting selection for coordination between these
413 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 between species' Ellenberg light indicator value (L-value) and Raunkiaer 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: $SR_{\text{even}} \sim$	α	σ^2	r^2	k	$\log(\mathcal{L})$	AIC	ΔAIC
L-value \times Raunkiaer lifeform	0.46	0.068	0.34	12	-33.2	90.4	0
L-value \times growth form	0.46	0.07	0.32	12	-38.2	100.4	9.9
L-value + Raunkiaer lifeform	0.46	0.071	0.32	8	-40.2	96.4	6
L-value + growth form	0.51	0.08	0.31	8	-43.4	102.7	12.3
Raunkiaer lifeform	0.34	0.067	0.15	7	-79.2	172.4	82
growth form	0.35	0.069	0.13	7	-82.5	179.1	88.6
L-value	0.64	0.107	0.26	4	-59.3	126.6	36.2
null	0.29	0.067	0	3	-107.6	221.1	130.7

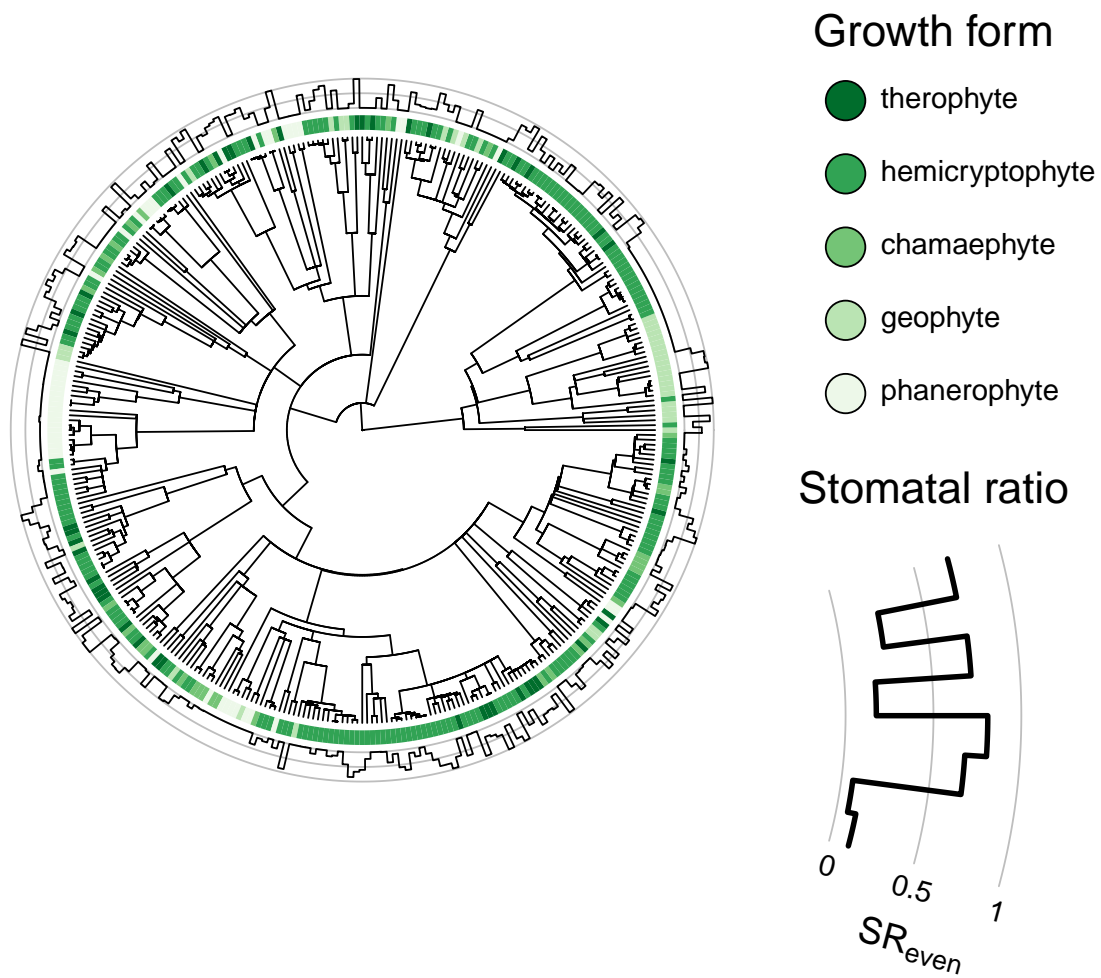


Figure 1: CAPTION

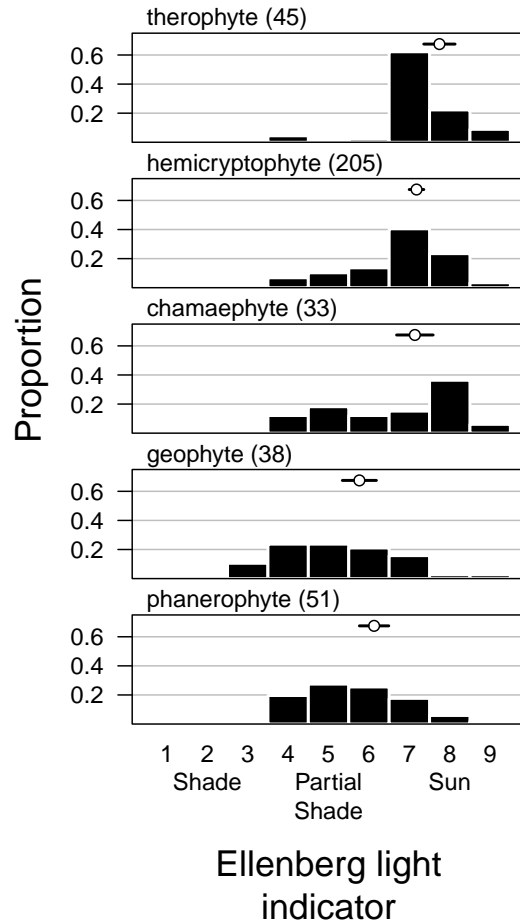


Figure 2: Lifeforms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different Raunkiær life forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform.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

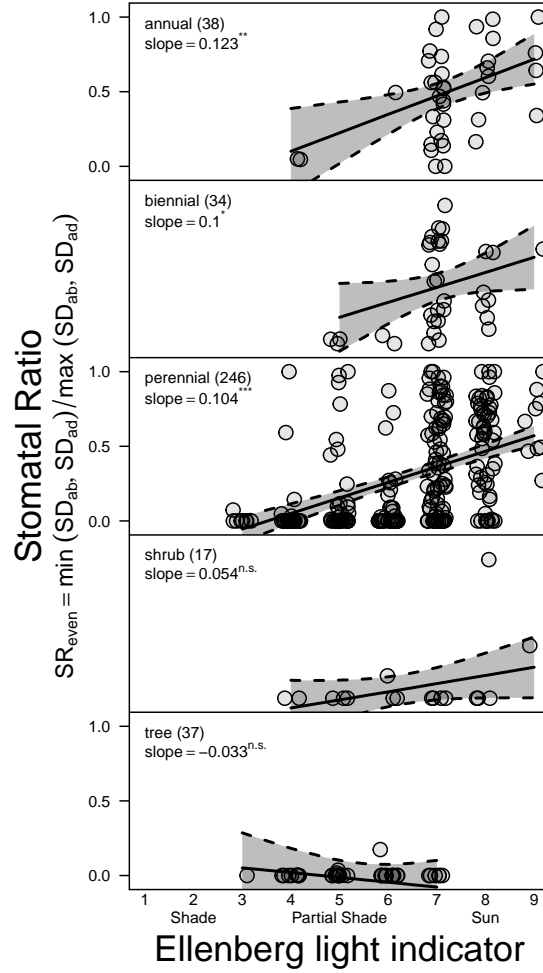


Figure 3: The effect of light on stomatal ratio depends on Raunkiaer life form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio (SR_{even}) in therophytes, hemicryptophytes, and chamaephytes but not geophytes and phanerophytes. The maximum likelihood slope from phylogenetic regression is given with statistical significance based on 10^4 parametric bootstrap samples. Numbers in parentheses next to 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.

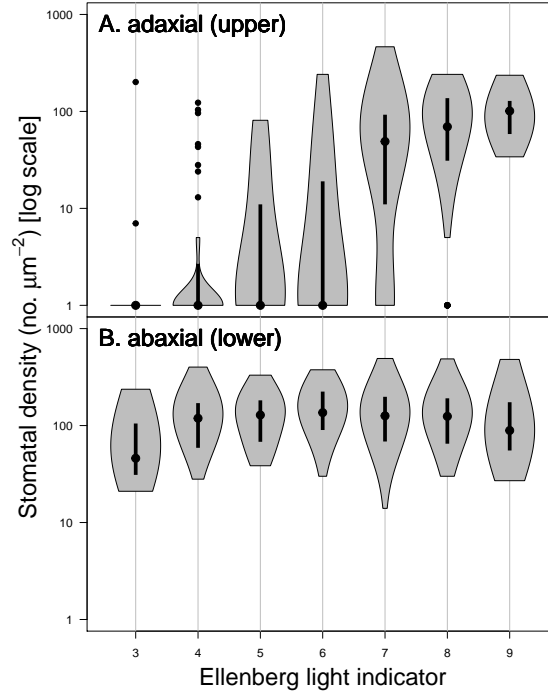


Figure 4: Light-mediated evolution of stomatal ratio is mostly driven by increased adaxial (‘upper’) stomatal density (Panel A), whereas abaxial (‘lower’) stomatal density (Panel B) is similar across Ellenberg light indicator values (L-value x -axis). The violin plot shows stomatal density (y -axis,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

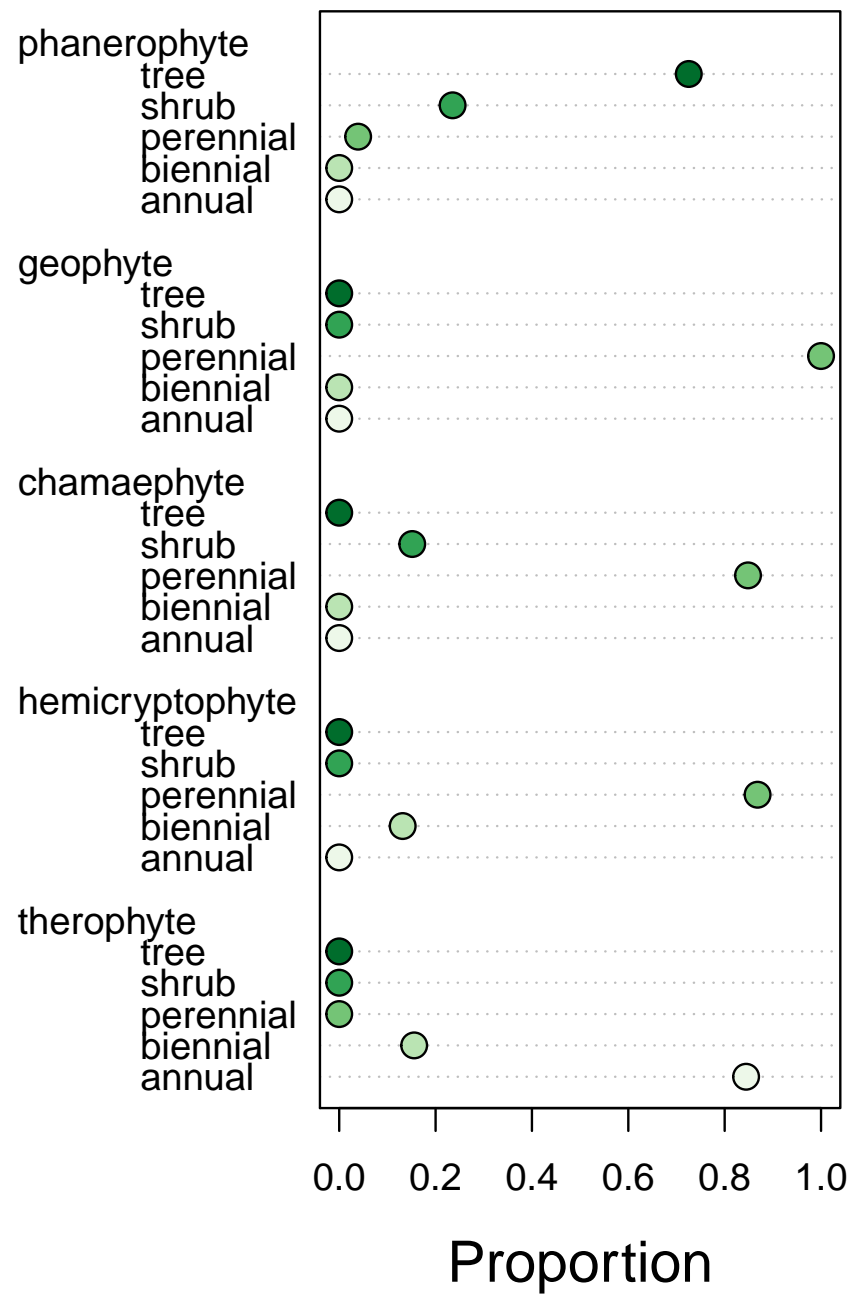


Figure S1: CAPTION

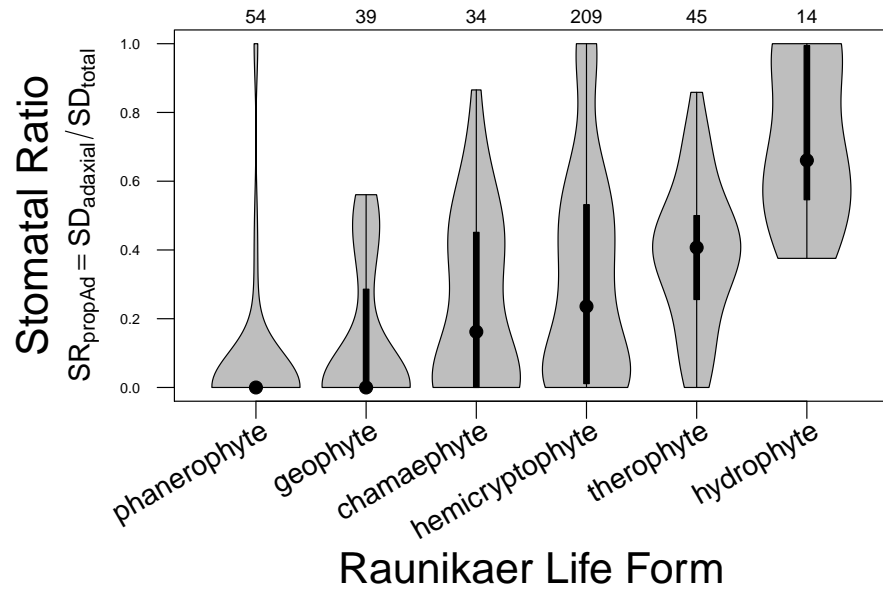


Figure S2: Most hydrophytes are hyperstomatous, having most stomata on the adaxial (‘upper’) surface (high SD_{propAd}). The violin plot shows stomatal ratio as a function of Raunkiaer lifeform. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range of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