

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,

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

129 **Materials and Methods**

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

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

134 I obtained data on ab- and adaxial stomatal density on 395 species from British Eco-
135 logical Flora (Salisbury, 1927; Fitter and Peat, 1994, 2017). Following recent com-
136 parative analyses (e.g. Niinemets and Valladares, 2006; Bartelheimer and Poschlod,
137 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 based on plant habit and
144 Raunkiær life form. First, I used PLANTATT data on perennation, woodiness, and
145 height to classify species' growth form based on habit. I categorized herbaceous
146 species as annual, biennial, or perennial and woody species as shrub or tree. Fol-
147 lowing Muir (2015), 'biennial' includes true biennials as well as species that have
148 a mix of perennation forms (e.g. a species with both annual and perennial forms
149 would be classified as a biennial here). Woody species are shrubs (plant height less
150 than 4 m) or trees (plant height greater than 4 m). Next, I compared this scheme
151 to PLANTATT data on Raunkiær life form (Raunkiær, 1934), which is another way
152 to classify growth form in comparative ecology (e.g. Peat and Fitter, 1994; Salguero-
153 Gómez et al., 2016). I retained phanerophytes, geophytes, chamaephytes, hemicryp-
154 tophytes, and therophytes, but excluded data on hydrophytes (14 species) because
155 many of these species are hyperstomatous (Fig. S1) due to the fact that leaves may
156 rest on the water's surface, selecting for stomata to be present on the upper surface
157 only. The two main differences between these growth form classifications are that
158 1) most shrubs and trees are lumped together as phanerophytes and 2) many geo-
159 phytes and chamaephytes are lumped together with hemicryptophytes as perennials
160 (Fig. S2).

161 I used a dated molecular phylogeny of the British flora (Lim et al., 2014) available
162 from TreeBASE (<http://treebase.org/>; accession number 15105). 14 species (3.5%)

163 in the dataset were not present in the phylogeny. For 8 of these species, I used the
 164 position of a congeneric species as a proxy for the focal species (following Pennell
 165 et al., 2016). When multiple congeneric species were present, I consulted the phy-
 166 logenetic literature to identify the most closely related proxy species (Scheen et al.,
 167 2004; Salmaki et al., 2013). For the remaining 6 missing species, I positioned them
 168 in the tree based on phylogenetic relationships to other genera or families present in
 169 the tree (Fior et al., 2006). Because many phylogenetic comparative methods do not
 170 allow polytomies, zero-length branches, and non-ultrametric trees, I made several
 171 small adjustments to the tree. I resolved polytomies randomly using the ‘multi2di’
 172 function in **phytools** version 0.6-00 (Revell, 2012). I added 0.02 my to all zero-length
 173 branches, as this was approximately the length of the shortest nonzero branch length
 174 in the tree. After these changes, I slightly altered terminal branch lengths to make
 175 the tree precisely ultrametric.

176 I excluded C₄ (3 species) and CAM (2 species) plants. I limited this investigation to
 177 angiosperms because only 4 non-angiosperms had stomata data. The final dataset
 178 contained 372 species (Fig. 1). The R code accompanying this paper documents
 179 these decisions in greater detail and citations to the relevant literature.

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

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

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

182 SD_{ab} and SD_{ad} are the stomatal densities on abaxial or adaxial surface, respectively.
 183 $SD_{total} = SD_{ab} + SD_{ad}$. SR_{propAd} is the proportion of stomata density on the adaxial
 184 surface, which is useful for discriminating among hypostomatous ($SR_{propAd} = 0$),
 185 amphistomatous ($0 < SR_{propAd} < 1$), and hyperstomatous species ($SR_{propAd} = 1$).
 186 SR_{even} indicates how evenly stomatal densities are distributed across both leaf sur-
 187 faces. This expression is useful because several hypotheses are based on the fact that
 188 a more even distribution should optimize leaf CO_2 diffusion.

189 **Testing for an association between open habitat and growth** 190 **form**

191 I tested whether growth form, under either classification scheme, was associated
 192 with L-value among British angiosperms. I predicted that species with faster life
 193 histories, especially therophytes (annuals), would have greater L-values than species
 194 with slower life histories, especially phanerophytes (shrubs and trees). I first used
 195 a phylogenetic ANOVA assuming an Ornstein-Uhlenbeck process model fit using
 196 **phylolm** version 2.5 (Ho and Ané, 2014). However, this analysis indicated no phylo-
 197 genetic signal in the regression (See the R code accompanying this paper for further
 198 detail). Specifically, the estimated α parameter was extremely high. In the Ornstein-
 199 Uhlenbeck model, α is proportional to the inverse of the phylogenetic half-life (i.e.
 200 phylogenetic signal). When there is no phylogenetic signal (i.e. high α), regular and
 201 phylogenetic ANOVA converge on the same parameters estimates. Furthermore, sta-
 202 tistical tests assuming there is phylogenetic signal when in fact none exists performs
 203 worse than nonphylogenetic tests (Revell, 2010). Therefore, I used a regular ANOVA
 204 with Type-2 sum of squares.

205 Open habitat, growth form, and stomatal ratio

206 I compared phylogenetic linear models to test whether growth form, L-value, or
207 interactions between them predicted SR_{even} . Unlike the analysis above, there was
208 significant phylogenetic signal in this comparison (see R code). I used SR_{even} rather
209 than SR_{propAd} as the response variable because the hypothesis is that faster life his-
210 tory and/or high light favor more even stomatal densities on each surface. I fit
211 models using **phylolm** and calculated Akaike Information Criteria (AIC). For these
212 and subsequent analyses, I assumed an Ornstein-Uhlenbeck process model for the
213 residuals with the root character state integrated over the stationary distribution.
214 I used a 10^4 parametric bootstrap samples of the full model (including main ef-
215 fects and interactions) to calculate parameter confidence intervals (Boettiger et al.,
216 2012).

217 I also tested whether phylogenetic could explain the residual variation in stomatal
218 ratio after accounting for growth form and L-value. Specifically, I compared the
219 expected residual variation given the actual tree versus a hypothetical tree where trait
220 evolution has reached stationarity (i.e. a star phylogeny with infinite branch lengths).
221 If phylogeny explains much of the variation, then the simulated residual variance
222 from the actual tree should be greater than that of the stationary tree. I simulated
223 trait values from 10^4 parametric bootstrap samples of the model with the lowest
224 AIC (this was the model including Raunkiær lifeform, L-value, and their interaction;
225 see Results). I performed the first set of simulations using the actual phylogenetic
226 tree in **OUwie** version 1.50 (Beaulieu and O’Meara, 2016). Each simulation used
227 a different bootstrap parameter sample of α and σ^2 , where α is the return rate to
228 the mean and σ^2 is the diffusion rate. At stationarity, the variance of an Ornstein-
229 Uhlenbeck trait is equal to $\sigma^2/2\alpha$. Therefore, I simulated stationary data by assuming

230 a normal distribution with this variance estimated from the bootstrap samples. For
 231 comparability, I set the mean of simulations from both actual phylogeny and the
 232 stationary to zero. I compared the actual to stationary variance across simulated
 233 datasets using a paired *t*-test.

234 **Does ab- or adaxial stomatal density contribute more to stom-** 235 **atal ratio evolution?**

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

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

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

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

242 Lowercase variables (sr, sd) indicate log-transformed values. Because some species
 243 had zero adaxial stomata, I added one to all values prior to log-transformation. To
 244 make the variance decomposition calculations tractable, I have defined SR_{even} here

245 as the ratio of ad- to abaxial stomatal density because in most cases adaxial stomatal
 246 density is lower than abaxial (see Eq. 2). This differs from analyses described above
 247 because in those I wanted to test what factors influenced the evenness of stomatal
 248 densities, regardless of which surface had higher density. With this modified form,
 249 the variance in sr_{even} can readily be decomposed into contributions of sd_{ad} , sd_{ab} , and
 250 their covariance:

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

251 I did not use the raw covariance, but rather estimated the phylogenetic covariance
 252 matrix between L-value, sd_{ab} , and sd_{ad} using a multivariate Ornstein-Uhlenbeck
 253 model fit in **Rphylopars** version 0.2.9 (Goolsby et al., 2016, 2017). The phylogenetic
 254 covariance measures how strongly a set of traits evolve together over macroevolution-
 255 ary timescales. From the covariance matrix, I estimated the contribution of abaxial
 256 density, adaxial density, and their covariance as:

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

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

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

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

259 I also tested whether light-mediated evolution of stomatal ratio acted mostly by 1)
 260 increasing adaxial stomatal density while maintaining abaxial density, or 2) keeping
 261 total stomatal density the same, but shifting a greater proportion to the adaxial
 262 surface. The first scenario predicts that the phylogenetic regression of L-value on
 263 sd_{ad} is stronger than that for sd_{ab} . The second scenario predicts that L-value acts
 264 similarly on both and that there is a negative covariance ($Cov(sd_{ad}, sd_{ab}) < 0$). I
 265 tested these competing predictions by fitting a very simple phylogenetic SEM (see
 266 Mason et al., 2016, for a similar approach). In general, SEMs attempt to deter-
 267 mine whether variables are related causally or whether a relationship is mediated
 268 by another correlated variable. Phylogenetic SEMs use the phylogenetic covariance
 269 matrix, as described above, rather than the raw covariance. Here, I used a phyloge-
 270 netic SEM to simultaneously estimate regressions of L-value on sd_{ad} and sd_{ab} while
 271 allowing covariance between them (i.e. estimating $Cov(sd_{ad}, sd_{ab})$). To fit the SEM,
 272 I used the R package **lavaan** version 0.5-23.1097 (Rosseel, 2012). I tested whether
 273 parameter estimates were significantly different from zero using z -scores.

274 Results

275 Light tolerance varies among growth forms

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

281 ceous plants (annual, biennial, and perennials) had greater L-values than shrubs and
282 trees (Fig. S3; ANOVA - $F_{4,367} = 10.8$, $P = 2.6 \times 10^{-8}$)

283 **Interactions between light and growth form determine stom-** 284 **atal ratio**

285 Overall, SR_{even} increased with L-value, but there was a significant interaction ($\Delta AIC >$
286 2, Table 1) between Raunkiaer life form and L-value (Fig. 3). When classified based
287 on plant habit, growth form interacted with L-valueless ($\Delta AIC = 2.4$; Fig. S4).
288 Raunkiaer life form explained variation in stomatal ratio better than habit (lower
289 AIC; Table 1), therefore we focus hereafter on those analyses. Both life form and
290 L-value significantly increased model fit, though L-value had a markedly larger effect
291 on model AIC (Table 1). The significant interaction is caused by different slopes
292 between life forms. Among life forms with the overall greatest L-value (therophytes,
293 hemicryptophytes, and chamaephytes, see Fig. 2), there was a strong positive rela-
294 tionship between L-value and SR_{even} . Parametrically bootstrapped 95% confidence
295 intervals for the slope did not overlap zero (Fig. 3). The slope was weakly positive
296 or not significantly different from zero in the most shade-adapted life forms (geo-
297 phytes and phanerophytes), albeit the patterns were distinct in these groups. There
298 were both hypostomatous ($SR_{\text{even}} \approx 0$) and amphistomatous ($SR_{\text{even}} \approx 1$) geophytes,
299 but these were distributed across L-values. In contrast, phanerophytes were nearly
300 always hypostomatous regardless of L-value.

301 Although there was significant phylogenetic signal in the residual variation of stom-
302 atal ratio (see R code), the total variation among these species was consistent with a
303 trait at stationarity. Specifically, the simulated residual trait variation, after account-

ing for Raunkiaer life form and L-value, from the actual tree was not significantly greater than that simulated from a tree where traits had reached stationarity (paired t -test, $P = 0.331$). Hence, phylogenetic nonindependence is an important statistical consideration, but phylogeny does not explain stomatal trait variation among British angiosperms.

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 1.14, 0.38, and -0.53, 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 ($P = 6.1 \times 10^{-7}$ and 2.9×10^{-5} , respectively). However, the regression of L-value on sd_{ad} was $2.1\times$ that of L-value on sd_{ab} (0.21 versus 0.1). Because stomatal densities were natural log-transformed, this implies an increase in L-value by one leads to a 1.23-fold change in adaxial stomatal density versus a 1.1-fold change in abaxial stomatal density. The SEM also showed a significant positive covariance between stomatal densities on each surface ($P = 1.7 \times 10^{-11}$). These results together imply that total stomatal density increases with L-value, but the response is mediated mostly by increases in adaxial stomatal density.

Discussion

The ratio of stomatal densities on the abaxial ('lower') to that of the adaxial ('upper') surface varies greatly across plant species, but the adaptive significance is not clear. Comparative studies correlating stomatal ratio to ecological factors can distinguish among competing hypotheses and reveal critical experiments for future work. Previous comparative studies suggested that high light and herbaceous growth form favor amphistomy (Mott et al., 1984; Jordan et al., 2014; Muir, 2015; Bucher et al., 2017), particularly in the British flora (Salisbury, 1927; Peat and Fitter, 1994). However, none of these studies have accounted for the fact that light and growth form are often confounded – open, high light habitats are often dominated by herbs – or the fact that species are not independent because of shared evolutionary history. By bringing together datasets on stomata, light tolerance, growth form, and phylogeny of British angiosperms, I tested new hypotheses and reevaluated previous results using modern phylogenetic comparative methods. As expected, species' light tolerance (Ellenberg light indicator or L-value) is confounded with growth form (Fig. 2, Fig. S3). Nevertheless, both L-value and growth form affect stomatal ratio, but these factors also interact; the influence of L-value on stomatal ratio varies across forms. Finally, I show for the first time that adaxial stomatal density in particular accounts for most of the coordinated evolution between light tolerance and stomatal density. 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).

350 Adaptive significance of amphistomy

351 Among British angiosperms, phylogenetic comparative analyses suggest that selec-
352 tion favors amphistomy in high light habitats among fast-growing plants, but not
353 shrubs and trees. This is a significant advance over previous studies that considered
354 each factor in isolation and/or did not use modern approaches to control for phy-
355 logenetic nonindependence. For example, pioneering studies by Salisbury (1927)
356 first suggested that amphistomy is associated with herbs in open habitats, albeit
357 without formal statistical tools to disentangle these effects. Later work by Peat and
358 Fitter (1994) demonstrated these trends again using family-level comparisons, a ba-
359 sic method to account for phylogenetic nonindependence (see also Mott et al., 1984;
360 Beerling and Kelly, 1996). However, this approach is still problematic because traits
361 like growth form can be highly phylogenetically conserved. For example, orders like
362 Fagales contain multiple families dominated by hypostomatous trees, hence it is pre-
363 mature to conclude that this correlation is biologically meaningful without properly
364 accounting for phylogenetic nonindependence. By combining trait, ecological, and phy-
365 logenetic datasets on British angiosperms, we now know that not only do both light
366 and growth form influence stomatal ratio, but in fact their effects may reinforce one
367 another. Based on information criteria, light may be a more important factor than
368 growth form or their interaction (Table 1), consistent with previous studies indicat-
369 ing a dominant role of light (Mott et al., 1984; Jordan et al., 2014; Bucher et al.,
370 2017).

371 The interaction between light and growth form among British angiosperms suggests
372 that amphistomy may only be strongly favored when CO₂ strongly limits photosyn-
373 thesis (as in open habitat) *and* photosynthesis strongly limits fitness (as in herbs).
374 This is consistent with life history theory predicting that the demography of open

375 habitat herbs is strongly limited by plant growth (Franco and Silvertown, 1996).
376 Along these lines, Raunkiaer lifeform may explain stomatal ratio better than plant
377 habit (Table 1) because it is a better proxy for life history characteristics. For ex-
378 ample, on an axis of ‘fast’ to ‘slow’ life history, geophytes more closely resemble
379 phanerophytes than do chamaephytes or hemicryptophytes (Salguero-Gómez et al.,
380 2016). Similarly, the relationship between light and stomatal ratio for geophytes was
381 intermediate between that for phanerophytes and chamaephytes/hemicryptophytes
382 (Fig. S3). These comparisons indirectly suggest that both high light and fast life
383 history are necessary to induce strong selection for amphistomy. The ideal way to
384 test this would be to measure selection on stomatal ratio in a species that varied
385 quantitatively in both stomatal ratio and life history (e.g., containing both thero-
386 phyte/annual and perennial forms). I predict that amphistomy will be favored more
387 strongly in the annual form grown under high light compared to an annual under low
388 light or a perennial in high light, and much more strongly than a perennial grown
389 in low light. Similar experiments could also be performed to test if and when light-
390 mediated plasticity in stomatal ratio is adaptive (Gay and Hurd, 1975; Mott and
391 Michaelson, 1991; Fontana et al., 2017).

392 The prevalence of amphistomatous species in high light habitats supports the hy-
393 pothesis that amphistomy is an adaptation to maximize photosynthetic rates by
394 increasing CO₂ diffusion (Jones, 1985). It is also evidence against the hypothesis
395 that the principle fitness cost of amphistomy is water loss (Darwin, 1886; Foster and
396 Smith, 1986) or dehydration of palisade mesophyll (Buckley et al., 2015), though
397 these factors are likely very important in determining differential regulation of stom-
398 ata on each surface. Since evaporative demand increases under high light, under
399 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 evapo-
 rative 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 am-
 phistomy 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 pre-
 viously unknown subtleties. These previous studies based their conclusions on data
 from Proteaceae, in which there is little quantitative variation in stomatal ratio;
 species are either completely hypostomatous ($SR_{propAd} \approx 0$) or completely amphis-
 tomatus ($SR_{propAd} \approx 0.5$). Stomatal ratio in British angiosperms is also bimodal
 (Peat and Fitter, 1994), but across many families there is also quantitative variation.
 Importantly, this means that quantitative variation in stomatal ratio may provide a
 more precise, quantitative indicator of vegetation type, rather than simply ‘open’ or
 ‘closed’. A quantitative relationship between L-value and stomatal ratio has already
 been shown for herbaceous perennials (Bucher et al., 2017), but we now know that
 it holds among annuals (therophytes), subshrubs (chamaephytes), and, to a lesser
 extent, geophytes as well (Fig. 3).

424 The weak or nonsignificant relationship between L-value and stomatal ratio in geo-
425 phytes and phanerophytes suggests that in some cases amphistomy may not reliably
426 indicate open habitat without further information. For example, perhaps amphis-
427 tomatous geophytes from partially shaded habitats are spring ephemerals, so they
428 experience high light during their growth phase, but this has not been tested. Like-
429 wise, phanerophytes (mostly tall trees) are almost always hypostomatous (see also
430 Muir, 2015). Most British phanerophytes are tall, hypostomatous trees, but the ex-
431 ceptions are telling. For example, the most amphistomatous phanerophyte in this
432 dataset is *Brassica oleracea*, a short-statured biennial that has more in common
433 physiologically with hemicryptophytes than other phanerophytes. The other am-
434 phistomatous phanerophytes in this data set (*Populus nigra* and *Lavatera arborea*)
435 are fast-growing pioneer species.

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

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

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

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

465 I refer to the second hypothesis as the ‘coordination’ hypothesis. In this scenario,
466 ecological conditions such as high light select for both increased total stomatal density
467 and for amphistomy because these traits work well in coordination with one another.
468 For example, if stomatal density were very high on a hypostomatous plant, then CO₂
469 would be more strongly limited by the mesophyll. Adding a second parallel pathway

470 for diffusion by developing stomata on both surfaces would restore a more optimal
471 balance between stomatal and mesophyll limitations. Conversely, there would be
472 little benefit to amphistomy when total stomatal density is low because CO₂ diffusion
473 is strongly limited by stomatal resistance, and therefore photosynthetic rate is not
474 sensitive to changes in mesophyll diffusion mediated by stomatal ratio. A related
475 prediction is that increased atmospheric CO₂ may select for reduced stomatal ratio
476 and density primarily by decreasing adaxial stomatal density, but this has not been
477 well tested (but see Woodward and Bazzaz, 1988).

478 **Conclusions**

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

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488 Author contribution statement

489 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 $\text{AIC} = 2k - 2\log(\mathcal{L})$. 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 (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: $\text{SR}_{\text{even}} \sim$	α	σ^2	r^2	k	$\log(\mathcal{L})$	AIC	ΔAIC
L-value \times Raunkiaer lifeform	0.46	0.068	0.34	12	-33.3	90.6	0
L-value \times growth form	0.46	0.07	0.32	12	-38.2	100.3	9.8
L-value + Raunkiaer lifeform	0.47	0.072	0.32	8	-40.3	96.5	6
L-value + growth form	0.51	0.08	0.31	8	-43.3	102.7	12.1
Raunkiaer lifeform	0.34	0.067	0.15	7	-79.2	172.4	81.8
growth form	0.35	0.069	0.13	7	-82.5	178.9	88.4
L-value	0.64	0.108	0.26	4	-59.3	126.6	36.1
null	0.29	0.067	0	3	-107.5	221	130.5

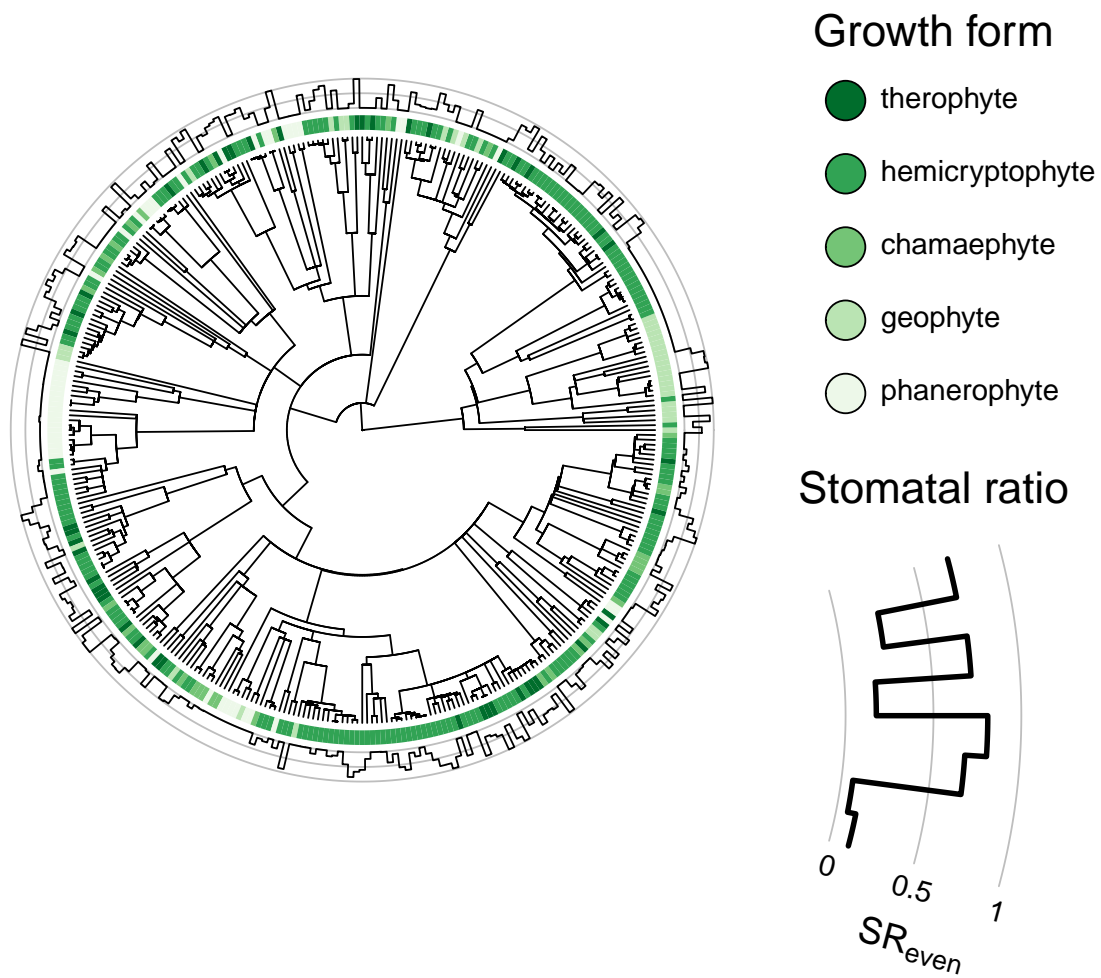


Figure 1: CAPTION

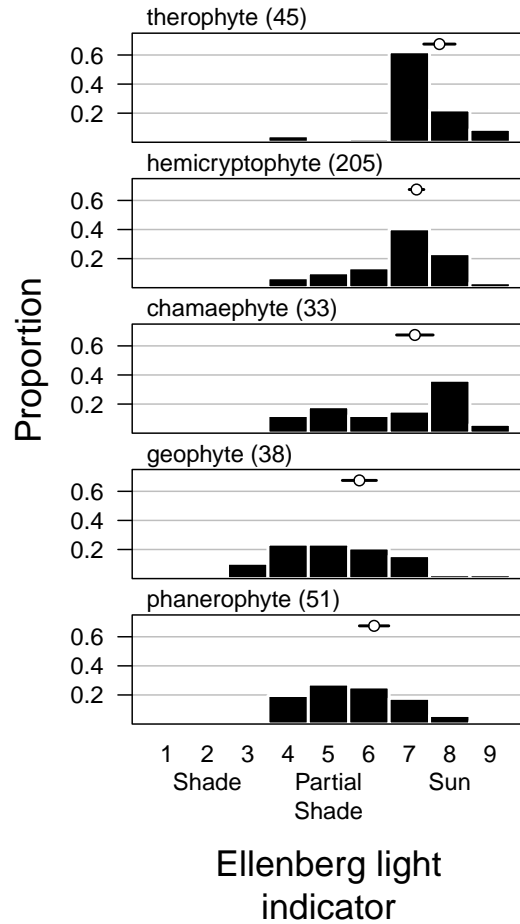


Figure 2: Lifeforms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different Raunkiær life forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform.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

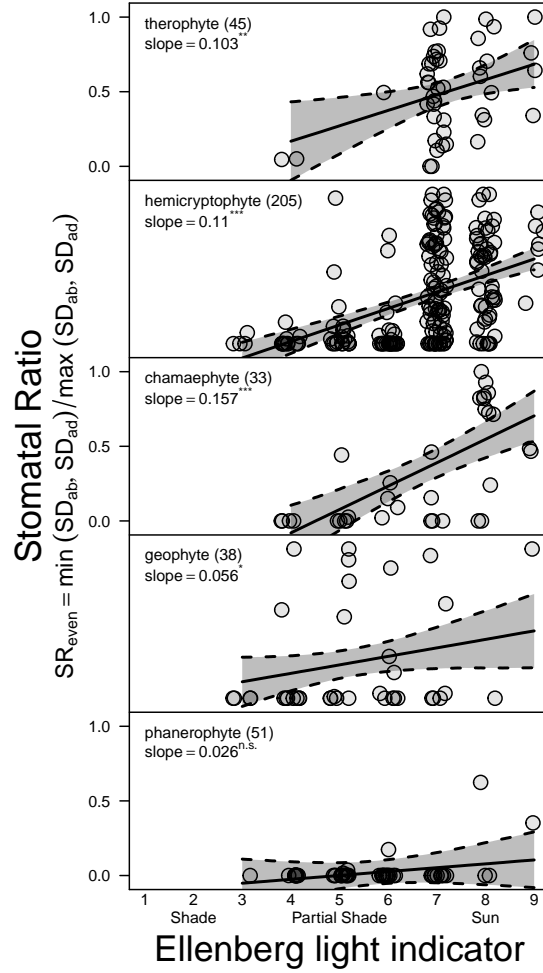


Figure 3: The effect of light on stomatal ratio depends on Raunkiaer life form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio (SR_{even}) in therophytes, hemicryptophytes, and chamaephytes but not geophytes and phanerophytes. The maximum likelihood slope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

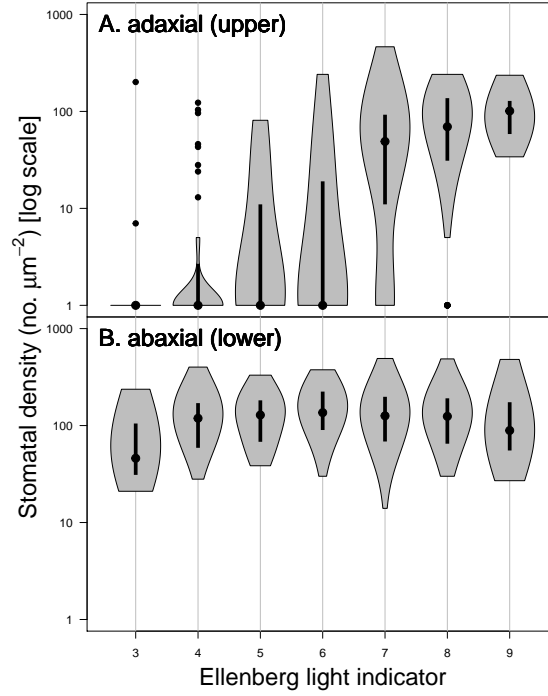


Figure 4: Light-mediated evolution of stomatal ratio is mostly driven by increased adaxial (‘upper’) stomatal density (Panel A), whereas abaxial (‘lower’) stomatal density (Panel B) is similar across Ellenberg light indicator values (L-value x -axis). The violin plot shows stomatal density (y -axis,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

674 Supporting Information

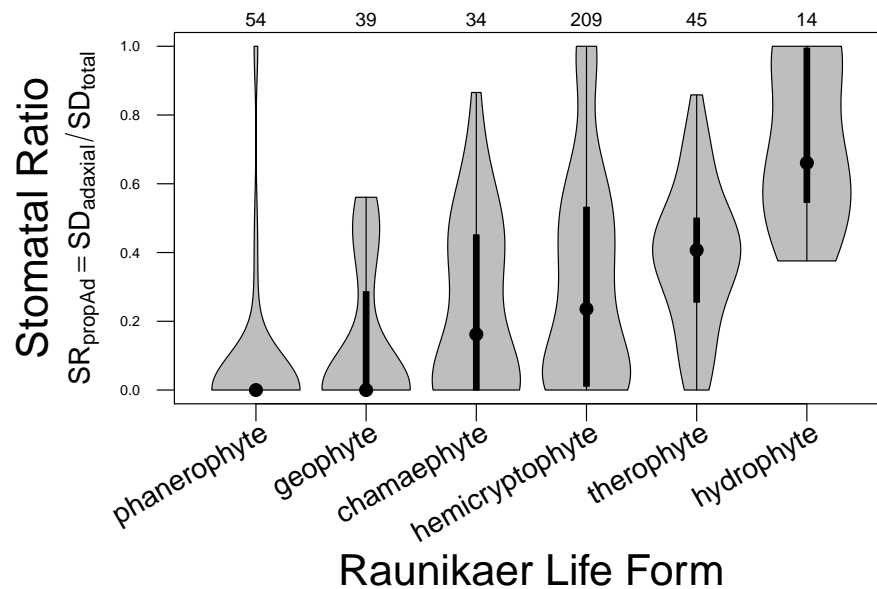


Figure S1: Most hydrophytes are hyperstomatous, having most stomata on the adaxial ('upper') surface (high SD_{propAd}). The violin plot shows stomatal ratio as a function of Raunkiaer lifeform. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range of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

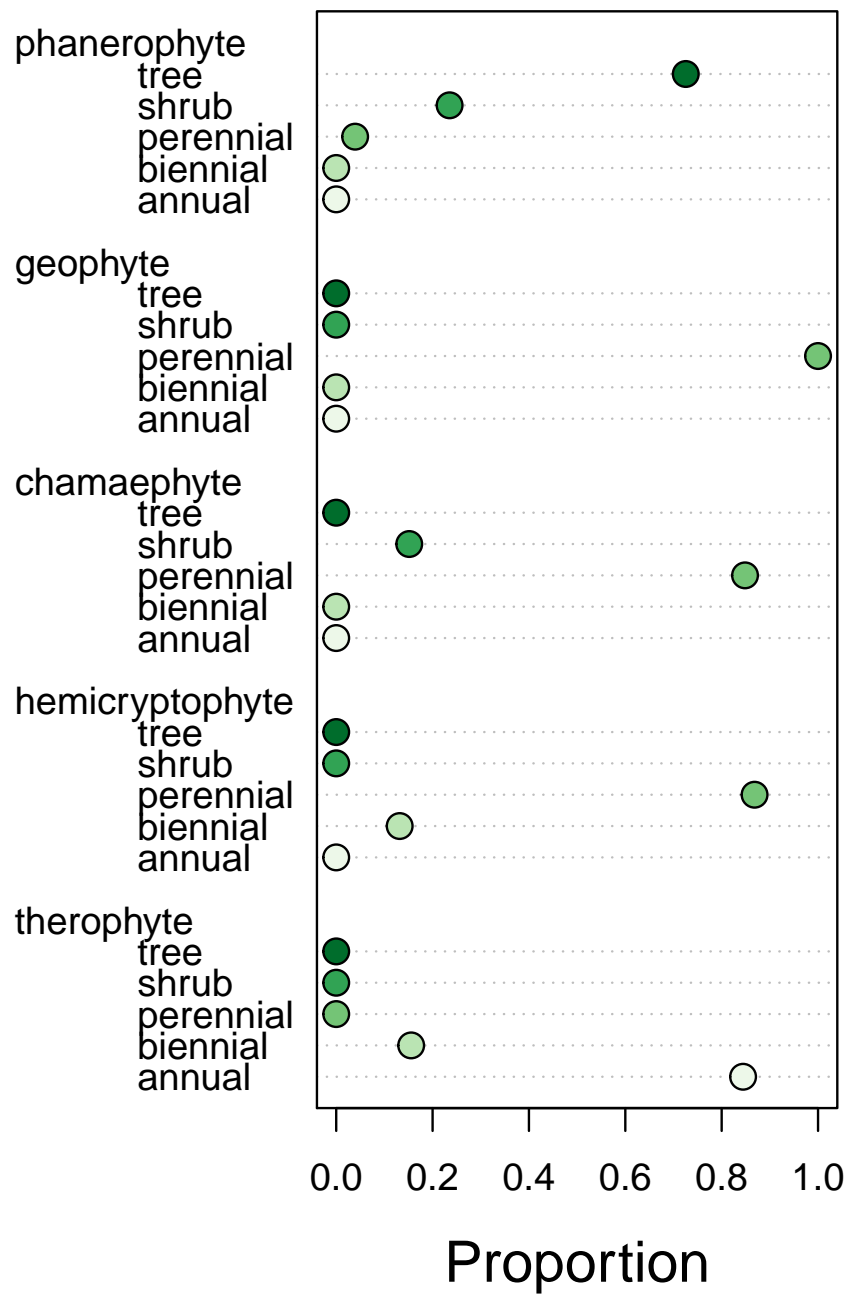


Figure S2: CAPTION

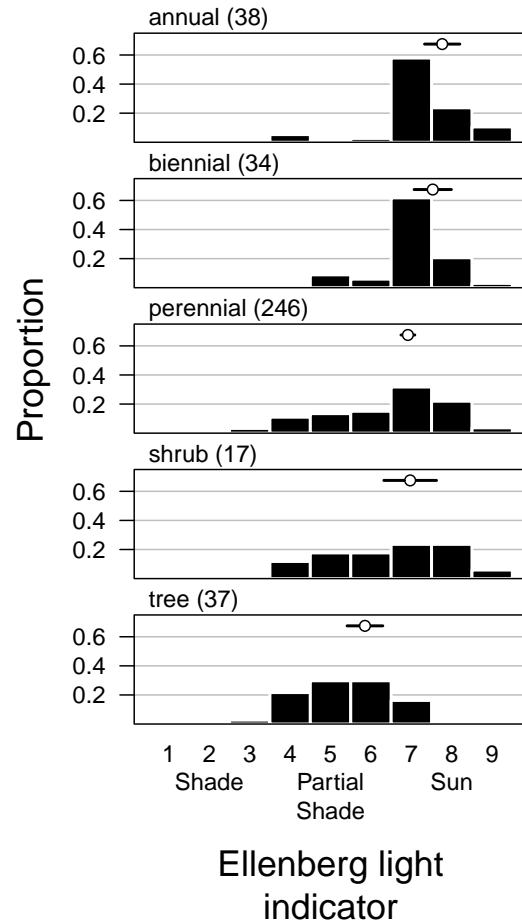


Figure S3: Growth forms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different growth forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform. 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.

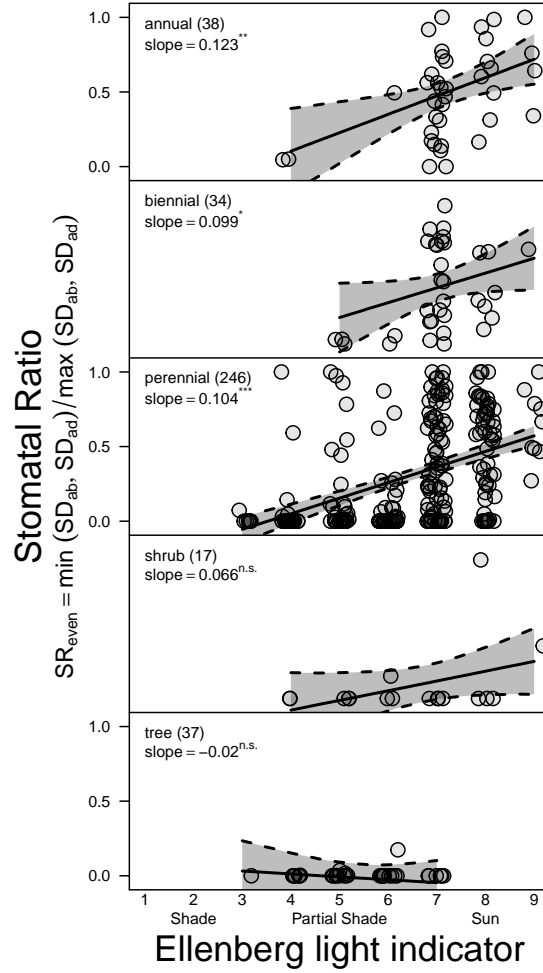


Figure S4: The effect of light on stomatal ratio depends on growth form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio (SR_{even}) in annual, biennial, and perennial herbs, but not shrubs or trees. The maximum likelihood slope from phylogenetic regression is given with statistical significance based on 10^4 parametric bootstrap samples. Numbers in parentheses next to growth 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.