

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

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Short title: Shedding light on stomatal evolution

Word count:

Summary: 190

Introduction: 1179

Materials and Methods: 1176

Results: 433

Discussion: 1504

Acknowledgement: 24

3 Figures and 1 Table, 1 Supplemental Figure

# Summary

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

## 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 CO<sub>2</sub> 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 CO<sub>2</sub> diffusion within the meso-  
44 phyll, amphistomy optimally supplies CO<sub>2</sub> (Parkhurst, 1978; Gutschick, 1984; Jones,  
45 1985). Amphistomy is correlated with greater CO<sub>2</sub> 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 CO<sub>2</sub> limits photo-  
54 synthetic rate. The open questions are under what ecological conditions does CO<sub>2</sub>  
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 CO<sub>2</sub> 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 CO<sub>2</sub>  
65 most strongly limits photosynthesis in high light and/or photosynthesis contributes  
66 most to fitness in herbaceous plants. Under high light, CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 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 C<sub>4</sub> (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  $\alpha$  parameter was extremely high. In the Ornstein-  
 199 Uhlenbeck model,  $\alpha$  is proportional to the inverse of the phylogenetic half-life (i.e.  
 200 phylogenetic signal). When there is no phylogenetic signal (i.e. high  $\alpha$ ), 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  $SR_{\text{even}}$ . Unlike the analysis above, there was  
208 significant phylogenetic signal in this comparison (see R code). I used  $SR_{\text{even}}$  rather  
209 than  $SR_{\text{propAd}}$  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 **Does ab- or adaxial stomatal density contribute more to stom-** 218 **atal ratio evolution?**

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

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

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

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

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

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

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

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

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

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

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

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

## Results

### Light tolerance varies among growth forms

Ellenberg light indicator values (L-value) differed significantly among growth forms. Among Raunkiaer life forms, therophytes (annuals), hemicryptophytes (perennial herbs with buds near the soil surface), and chamaephytes (subshrubs) had greater L-values than phanerophytes (woody plants) and geophytes (perennial herbs with storage organs) (Fig. 2; ANOVA -  $F_{4,367} = 18.3$ ,  $P = 1.1 \times 10^{-13}$ ). Likewise, herbaceous plants (annual, biennial, and perennials) had greater L-values than shrubs and trees (Fig. S3; ANOVA -  $F_{4,367} = 10.8$ ,  $P = 2.6 \times 10^{-8}$ ).

### Interactions between light and growth form determine stomatal ratio

Overall,  $SR_{\text{even}}$  increased with L-value, but there was a significant interaction ( $\Delta AIC > 2$ , Table 1) between Raunkiaer life form and L-value (Fig. 3). When classified based on plant habit, growth form interacted with L-valueless ( $\Delta AIC = 2.4$ ; Fig. S4). Raunkiaer life form explained variation in stomatal ratio better than habit (lower AIC; Table 1), therefore we focus hereafter on those analyses. Both life form and L-value significantly increased model fit, though L-value had a markedly larger effect on model AIC (Table 1). The significant interaction is caused by different slopes between life forms. Among life forms with the overall greatest L-value (therophytes, hemicryptophytes, and chamaephytes, see Fig. 2), there was a strong positive relationship between L-value and  $SR_{\text{even}}$ . Parametrically bootstrapped 95% confidence

intervals for the slope did not overlap zero (Fig. 3). The slope was weakly positive or not significantly different from zero in the most shade-adapted life forms (geophytes and phanerophytes), albeit the patterns were distinct in these groups. There were both hypostomatous ( $SR_{\text{even}} \approx 0$ ) and amphistomatous ( $SR_{\text{even}} \approx 1$ ) geophytes, but these were distributed across L-values. In contrast, phanerophytes were nearly always hypostomatous regardless of L-value.

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

Adaxial ('upper') stomatal density contributed most to the evolutionary variation in stomatal ratio. The contributions of adaxial density, abaxial density, and their covariance are 1.14, 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  $sd_{\text{ad}}$  and  $sd_{\text{ab}}$  increased with L-value ( $P = 6.1 \times 10^{-7}$  and  $2.9 \times 10^{-5}$ , respectively). However, the regression of L-value on  $sd_{\text{ad}}$  was  $2.1\times$  that of L-value on  $sd_{\text{ab}}$  (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

301 density.

## 302 Discussion

303 The ratio of stomatal densities on the abaxial ('lower') to that of the adaxial ('up-  
304 per') surface varies greatly across plant species, but the adaptive significance is not  
305 clear. Comparative studies correlating stomatal ratio to ecological factors can distin-  
306 guish among competing hypotheses and reveal critical experiments for future work.  
307 Previous comparative studies suggested that high light and herbaceous growth form  
308 favor amphistomy (Mott et al., 1984; Jordan et al., 2014; Muir, 2015; Bucher et al.,  
309 2017), particularly in the British flora (Salisbury, 1927; Peat and Fitter, 1994). How-  
310 ever, none of these studies have accounted for the fact that light and growth form  
311 are often confounded – open, high light habitats are often dominated by herbs – or  
312 the fact that species are not independent because of shared evolutionary history. By  
313 bringing together datasets on stomata, light tolerance, growth form, and phylogeny  
314 of British angiosperms, I tested new hypotheses and reevaluated previous results  
315 using modern phylogenetic comparative methods. As expected, species' light toler-  
316 ance (Ellenberg light indicator or L-value) is confounded with growth form (Fig. 2,  
317 Fig. S3). Nevertheless, both L-value and growth form affect stomatal ratio, but  
318 these factors also interact; the influence of L-value on stomatal ratio varies across  
319 forms. Finally, I show for the first time that adaxial stomatal density in particular  
320 accounts for most of the coordinated evolution between light tolerance and stomatal  
321 density. These novel findings provide further evidence that variation in stomatal ra-  
322 tio is adaptive and have important implications for interpreting changes in stomatal  
323 ratio through the paleo record (Jordan et al., 2014) and during domestication (Milla



et al., 2013).

## Adaptive significance of amphistomy

Previously, associations between light, growth form, and stomatal ratio have been interpreted in isolation as indicating that either high light and/or herbaceous growth form favors amphistomy. In British angiosperms, both factors are important, though light may be a stronger determinant than growth form based on information criterion comparisons (Table 1). Unlike previous studies, I found a significant interaction between light and growth form among British angiosperms, which suggests that amphistomy may only be strongly favored when CO<sub>2</sub> strongly limits photosynthesis (as in open habitat) *and* photosynthesis strongly limits fitness (as in herbs). This is consistent with life history theory predicting that the demography of open habitat herbs is strongly limited by plant growth (Franco and Silvertown, 1996). The ideal way to test this would be to measure selection on stomatal ratio in a species that varied quantitatively in both stomatal ratio and life history (e.g., containing both annual and perennial forms). I predict that amphistomy will be favored more strongly in the annual form grown under high light compared to an annual under low light or a perennial in high light, and much more strongly than a perennial grown in low light. Similar experiments could also be performed to test if and when light-mediated plasticity in stomatal ratio is adaptive (Gay and Hurd, 1975; Mott and Michaelson, 1991; Fontana et al., 2017).

The prevalence of amphistomatous species in high light habitats supports the hypothesis that amphistomy is an adaptation to maximize photosynthetic rates by increasing CO<sub>2</sub> diffusion (Jones, 1985). It is also evidence against the hypothesis

347 that the principle fitness cost of amphistomy is water loss (Darwin, 1886; Foster and  
 348 Smith, 1986) or dehydration of palisade mesophyll (Buckley et al., 2015), though  
 349 these factors are likely very important in determining differential regulation of stom-  
 350 ata on each surface. Since evaporative demand increases under high light, under  
 351 these hypotheses we would expect plants in high light to be hypostomatous. Because  
 352 stomatal conductances on each surface can be regulated independently in response  
 353 to the environment (Darwin, 1898; Pospíšilová and Solárová, 1984; Smith, 1981; Re-  
 354 ich, 1984; Mott and O’Leary, 1984), amphistomatous leaves likely cope with these  
 355 stresses by rapidly closing adaxial stomata when water supply cannot match evapo-  
 356 rative demands (Richardson et al., 2017). Instead, patterns in the British flora are  
 357 at least consistent with the idea that adaxial stomata increase susceptibility to foliar  
 358 pathogens (Gutschick, 1984; McKown et al., 2014). The cost of adaxial stomata  
 359 may be greater in the shade because greater leaf wetness and lower ultraviolet light  
 360 provide a more suitable microclimate for many foliar pathogens.

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

362 These observations from the British flora partially support the hypothesis that am-  
 363 phistomy can be used a proxy for open habitat in paleoenvironment reconstruction  
 364 (Carpenter, 1994; Jordan et al., 2014; Carpenter et al., 2015) but also point out pre-  
 365 viously unknown subtleties. These previous studies based their conclusions on data  
 366 from Proteaceae, in which there is little quantitative variation in stomatal ratio;  
 367 species are either completely hypostomatous ( $SR_{propAd} \approx 0$ ) or completely amphis-  
 368 tomatus ( $SR_{propAd} \approx 0.5$ ). Stomatal ratio in British angiosperms is also bimodal  
 369 (Peat and Fitter, 1994), but across many families there is also quantitative variation.  
 370 Importantly, this means that quantitative variation in stomatal ratio may provide a

371 more precise, quantitative indicator of vegetation type, rather than simply ‘open’ or  
372 ‘closed’. A quantitative relationship between L-value and stomatal ratio has already  
373 been shown for herbaceous perennials (Bucher et al., 2017), but we now know that  
374 it holds among annuals (therophytes), subshrubs (chamaephytes), and, to a lesser  
375 extent, geophytes as well (Fig. 3).

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

388 Finally, phylogenetic information should improve inferences about paleoclimates be-  
389 cause there is appreciable phylogenetic signal in stomatal ratio. The phylogenetic  
390 half-life of stomatal ratio evolution, after accounting for L-value and Raunkiær life  
391 form, is  $\log(2)/\alpha = 1.5$  my (see Table 1 for maximum likelihood estimates of  $\alpha$ , the  
392 return rate in the Ornstein-Uhlenbeck model of trait evolution). This lag time may  
393 indicate that evolving to the ‘optimum’ is constrained by the shape of the fitness  
394 landscape (Muir, 2015) or that other unmeasured factors which affect stomatal ra-  
395 tio have some phylogenetic signal. Regardless of the mechanism, this fact means

396 that researchers may be able to use data from closely related species to improve  
397 paleoenvironment reconstruction.

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

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

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

417 I refer to the second hypothesis as the ‘coordination’ hypothesis. In this scenario,  
418 ecological conditions such as high light select for both increased total stomatal density

419 and for amphistomy because these traits work well in coordination with one another.  
420 For example, if stomatal density were very high on a hypostomatous plant, then CO<sub>2</sub>  
421 would be more strongly limited by the mesophyll. Adding a second parallel pathway  
422 for diffusion by developing stomata on both surfaces would restore a more optimal  
423 balance between stomatal and mesophyll limitations. Conversely, there would be  
424 little benefit to amphistomy when total stomatal density is low because CO<sub>2</sub> diffusion  
425 is strongly limited by stomatal resistance, and therefore photosynthetic rate is not  
426 sensitive to changes in mesophyll diffusion mediated by stomatal ratio. A related  
427 prediction is that increased atmospheric CO<sub>2</sub> may select for reduced stomatal ratio  
428 and density primarily by decreasing adaxial stomatal density, but this has not been  
429 well tested (but see Woodward and Bazzaz, 1988).

## 430 **Conclusions**

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

## 437 **Acknowledgements**

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

## <sup>440</sup> **Author contribution statement**

<sup>441</sup> 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 $\times$ 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



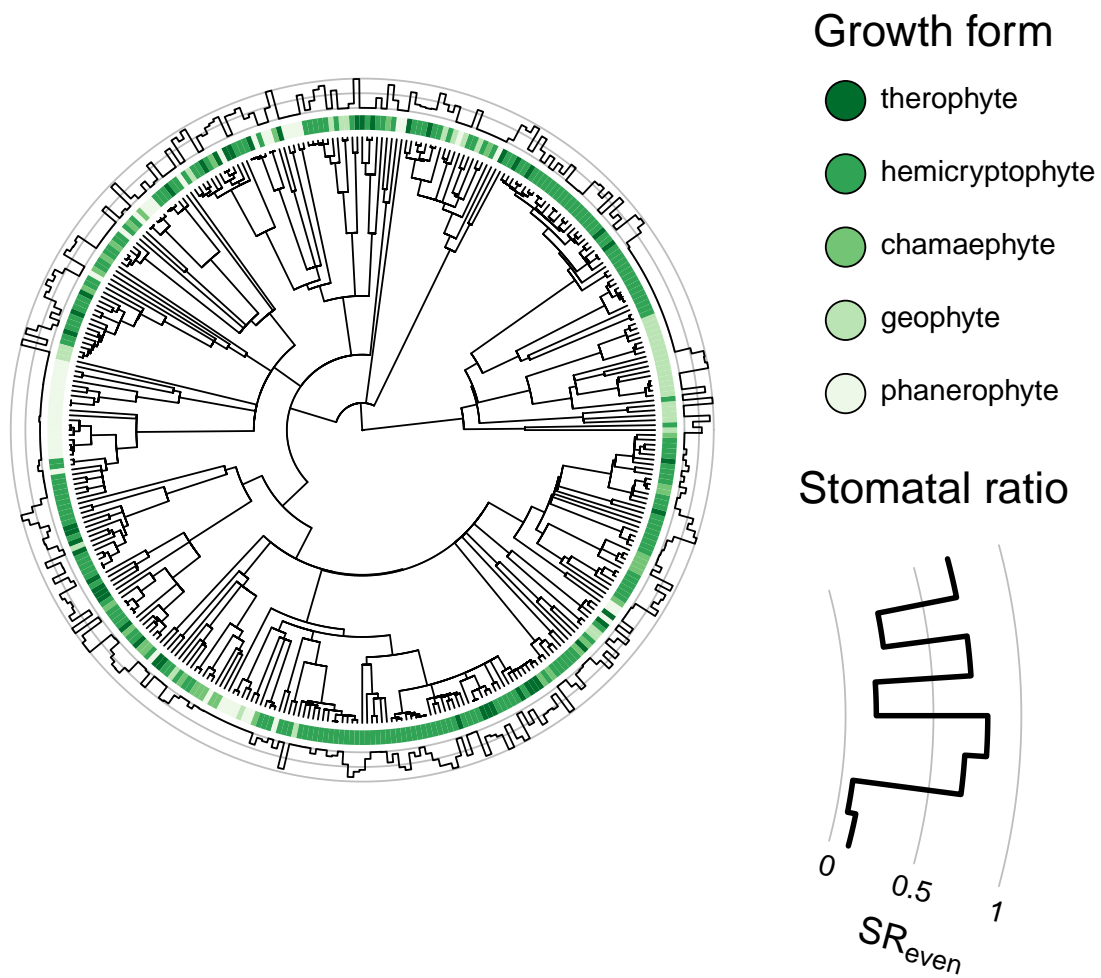


Figure 1: CAPTION

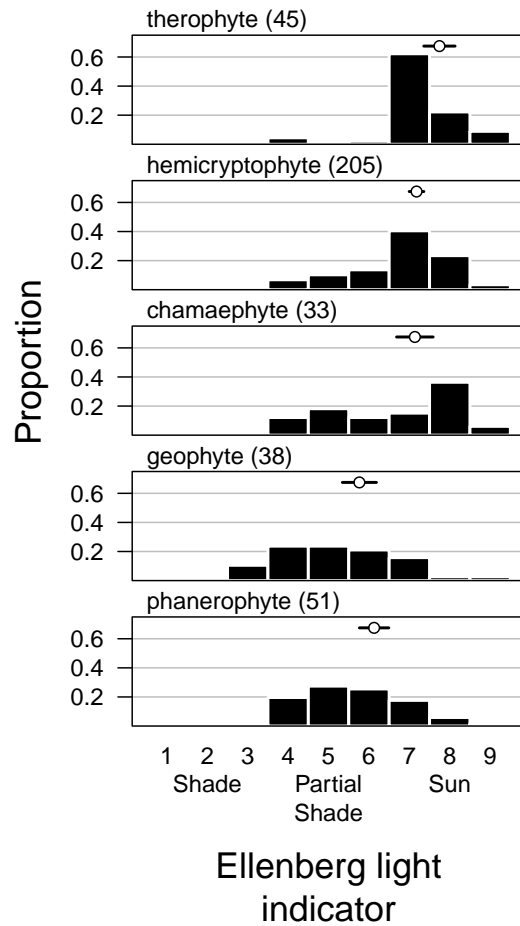


Figure 2: Lifeforms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different Raunkiær life forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform.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

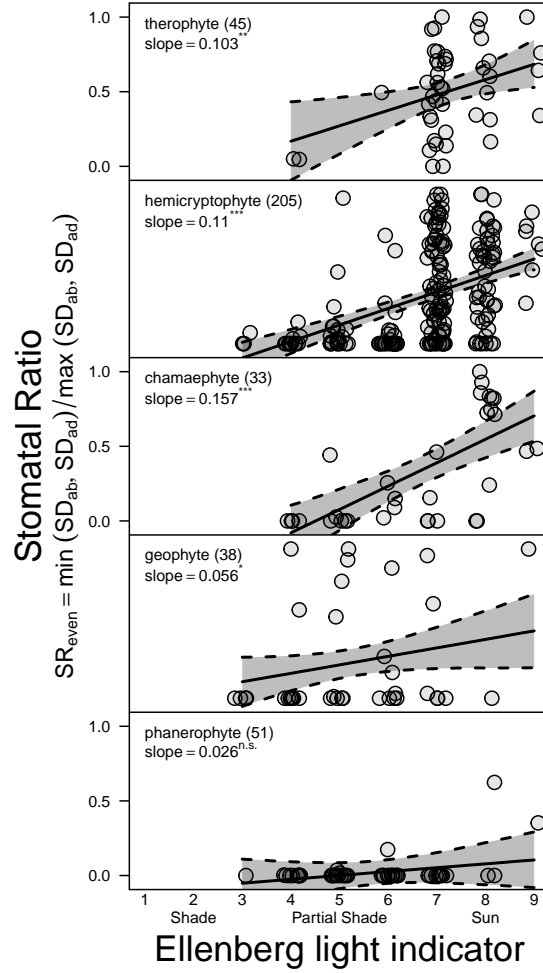


Figure 3: The effect of light on stomatal ratio depends on Raunkiaer life form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio ( $SR_{\text{even}}$ ) in therophytes, hemicryptophytes, and chamaephytes but not geophytes and phanerophytes. The maximum likelihood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

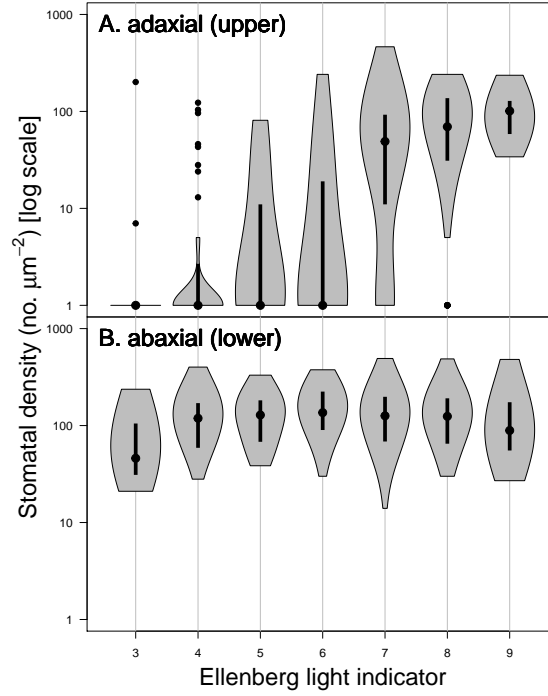


Figure 4: Light-mediated evolution of stomatal ratio is mostly driven by increased adaxial (‘upper’) stomatal density (Panel A), whereas abaxial (‘lower’) stomatal density (Panel B) is similar across Ellenberg light indicator values (L-value  $x$ -axis). The violin plot shows stomatal density ( $y$ -axis,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

## 623 Supporting Information

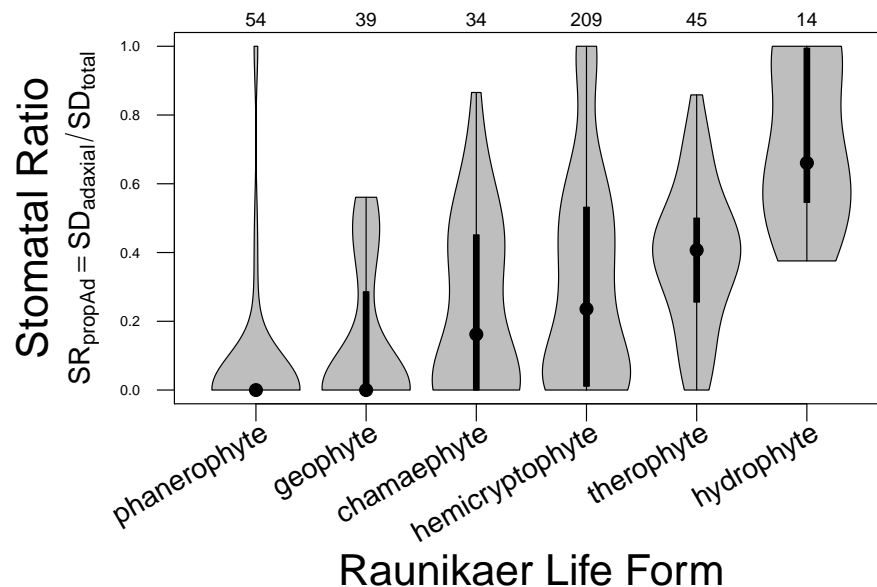


Figure S1: Most hydrophytes are hyperstomatous, having most stomata on the adaxial ('upper') surface (high  $SD_{propAd}$ ). The violin plot shows stomatal ratio as a function of Raunkiaer lifeform. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range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

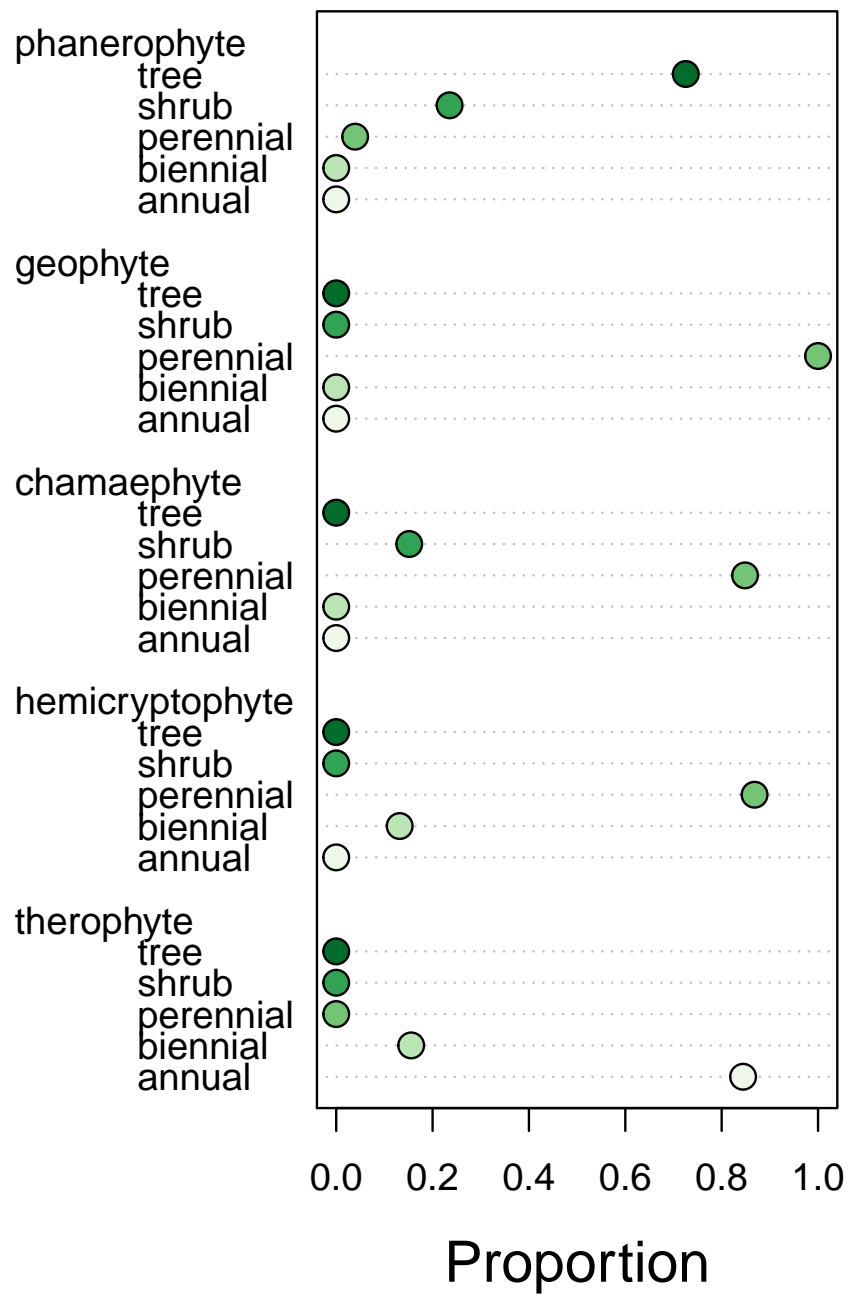


Figure S2: CAPTION

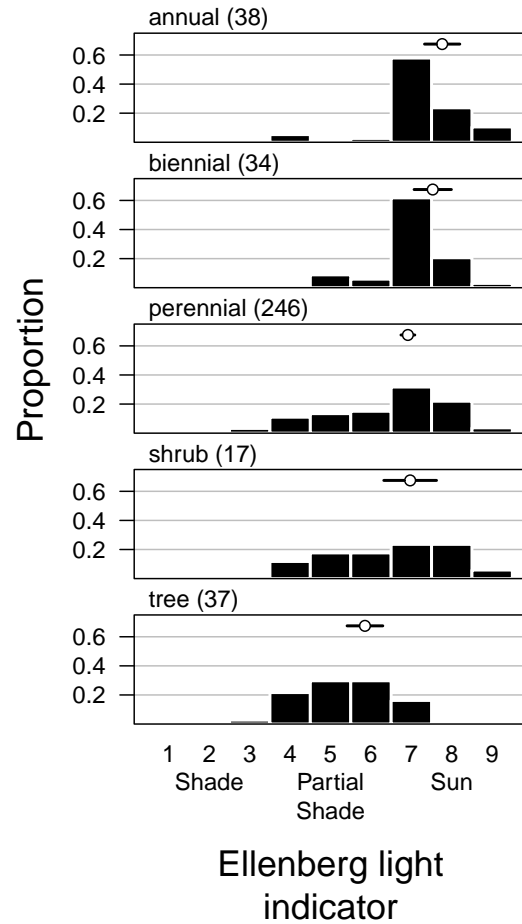


Figure S3: Growth forms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different growth forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform.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

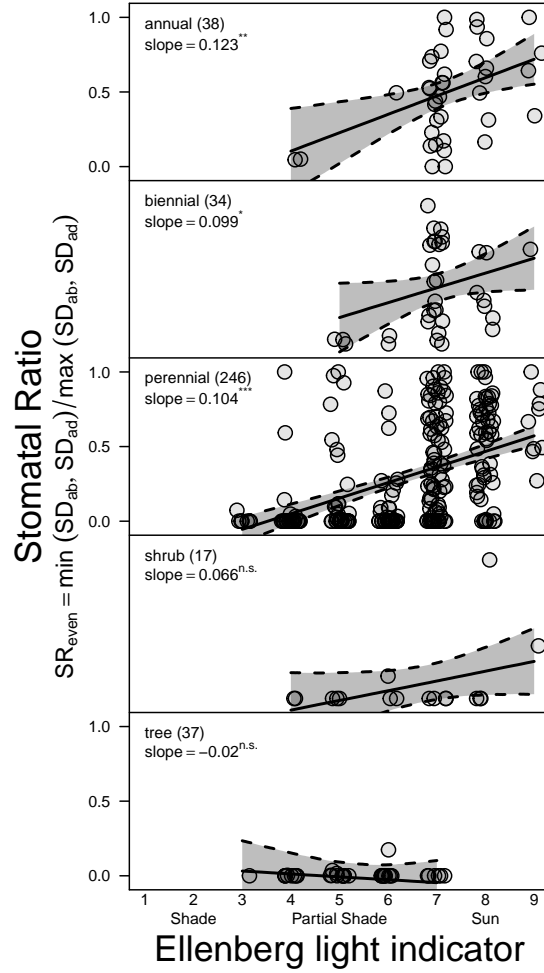


Figure S4: The effect of light on stomatal ratio depends on growth form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio ( $SR_{\text{even}}$ ) in annual, biennial, and perennial herbs, but not shrubs or trees. The maximum likelihood slope from phylogenetic 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.