

Light and life form interact to shape stomatal ratio among British angiosperms¹

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1 Abstract

- 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). Vari-
4 ation in stomatal ratio (the ratio of ab- and adaxial stomatal densities) is
5 probably adaptive, but the ecological conditions that favor amphistomy are
6 not well understood. In particular, high light and herbaceous growth form
7 have been hypothesized to favor amphistomy, but these hypotheses have not
8 been rigourously tested together.
- 9 • I leveraged a large dataset including stomatal ratio, Ellenberg light indica-
10 tor value, Raunkiaer lifeform, and phylogenetic relationships on 372 species of
11 British angiosperms. I used phylogenetic comparative methods to test how
12 light and/or growth form influence stomatal ratio.
- 13 • (return to this) key results: L-value, growth form, and interaction are important
- 14 • I show for the first time that light and growth form interact to shape variation
15 in stomatal ratio; amphistomy is advantageous in high light, but mostly for
16 herbs. These results improve our understanding of the adaptive significance of

17 stomatal ratio, use stomatal ratio as proxy for paleo vegetation, and as a target
18 for crop improvement.

19 **Keywords**

20 Adaptation, amphistomy, Ellenberg light indicator value, growth form, phylogenetic
21 comparative methods, stomata, stomatal ratio

22 **INTRODUCTION**

23 Natural selection shapes leaf anatomy in order to optimize its photosynthetic func-
24 tion in a given environment (Haberlandt, 1914; Givnish, 1987; Smith et al., 1997).
25 By understanding the adaptive significance of leaf anatomical variation we can learn
26 about natural history, find targets for crop improvement, and identify anatomical
27 proxies for paleoclimates preserved in the fossil record [CITE]. The size, density, and
28 distribution of stomata on a leaf vary widely and impact functions like the maximum
29 photosynthetic rate, water-use efficiency, photosynthetic nitrogen-use efficiency, and
30 susceptibility to foliar pathogens that infect through stomata [CITATIONS]. Hence,
31 stomata have been especially useful in understanding plastic and evolutionary re-

32 sponse to climate change and domestication (Royer, Ward, Woodward, Beerling,
33 Milla et al...).

34 While the density and size of stomata have been researched extensively [CITA-
35 TIONS], the adaptive significance of stomatal distribution is less well understood.
36 Stomata are most often found only on the lower leaf surface (hypostomy), but oc-
37 cur on both surfaces (amphistomy) in many species (Metcalf and Chalk, 1950;
38 Parkhurst, 1978; Mott et al., 1984). Theory and experiments demonstrate that
39 amphistomy increases photosynthetic rates under many conditions. By creating a
40 second parallel pathway for CO₂ diffusion within the mesophyll, amphistomy opti-
41 mally supplies CO₂ (Parkhurst, 1978; Gutschick, 1984; Jones, 1985). Amphistomy is
42 correlated with greater CO₂ diffusion (Beerling and Kelly, 1996) and higher photo-
43 synthetic rates (McKown et al., 2014). These observations are corroborated by ex-
44 periments demonstrating that amphistomy increases maximum photosynthetic rates
45 by up to 20% (Parkhurst and Mott, 1990). However, amphistomy can increase tran-
46 spiration (Jones, 1985; Foster and Smith, 1986), though empirical studies suggest
47 great water-use efficiency in amphistomatous species (Bucher et al., 2017). Hence,
48 amphistomy clearly should be favored when CO₂ limits photosynthetic rate, but the
49 open questions are under what ecological conditions does CO₂ supply most strongly
50 limit photosynthetic rate (Peat and Fitter, 1994) and when is photosynthetic rate

51 most important to fitness?

52 The leading, nonmutually exclusive hypotheses are that 1) open habitats favour
53 amphistomy because CO₂ diffusion most strongly limits photosynthetic rate under
54 high light and 2) herbaceous growth form favours amphistomy because traits that
55 maximize photosynthetic rate are often under stronger selection in herbs. Salisbury
56 (1927) first noted that amphistomy is most common in herbaceous plants from open
57 habitat (i.e. high light) of the British flora. These observations have been repli-
58 cated in other studies (Mott et al., 1984; Peat and Fitter, 1994; Jordan et al., 2014;
59 Muir, 2015) and may support physiological and ecological hypotheses that CO₂ most
60 strongly limits photosynthesis in high light and/or photosynthesis contributes most
61 to fitness in herbaceous plants. Under high light, CO₂ can strongly limit maximum
62 photosynthetic rates, espcecially in thick leaves (Jones, 1985). Hence, having stom-
63 ata on both surfaces relieves this limitation by adding a second parallel pathway for
64 CO₂ diffusion. Parkhurst 1978 argued that greater leaf thickness *per se* selected for
65 amphistomy, but there is little evidence for correlations between leaf thickness and
66 stomatal ratio independent of light (Mott et al., 1984; Gibson, 1996; Muir, 2015).
67 Amphistomy is correlated with open habitat in warm desert plants of western North
68 America (Mott et al., 1984; Gibson, 1996), among the Proteaceae (Jordan et al.,
69 2014), and in continental European herbs (Bucher et al., 2017).

70 Stomatal ratio is also associated with growth form. In the British flora, Salisbury
 71 (1927) found that trees and shrubs are nearly always hypostomatous, whereas herbs
 72 from open habitat are amphistomatous. This pattern holds when data are averaged
 73 by family to coarsely control for phylogenetic nonindependence (Peat and Fitter,
 74 1994) or when using alternative classification schemes, such as Raunkiaer life form
 75 (Peat and Fitter, 1994). Across plants from 90 families worldwide, growth form is
 76 the strongest predictor of stomatal ratio when multiple factors are estimated simulta-
 77 neously and controlling for phylogenetic nonindependence (Muir, 2015). These pat-
 78 terns are consistent with other data indicating that many herbaceous plants are un-
 79 der strong selection for high maximum photosynthetic rates. (Bazzaz, 1979; Körner
 80 et al., 1989). NEED MORE RECENT CITATIONS ON THIS.

81 Although previous comparative studies have tested whether open habitat and growth
 82 form influence stomatal ratio, we do not know if these effects are independent of
 83 one another. Open habitat and growth form are probably not independent because
 84 open habitat by definition consists of more short-statured, herbaceous plants. Even
 85 attempts to disentangle light and growth form by contrasting herbs from open and
 86 understory habitats (Salisbury, 1927) are problematic because high light habitats
 87 select for faster life history strategies (Galloway and Etterson, 2007) [\leftarrow OTHER
 88 REFS IN THAT PAPER?]. Open habitat and growth form may also interact with

89 one another. For example, amphistomy may only be favored when CO₂ strongly limits
90 photosynthetic rate *and* photosynthetic rate strongly limits fitness.

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

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

98 The final question is important for telling whether amphistomy is part of a coordi-
99 nated syndrome of traits that promote higher photosynthetic rate, as both the light
100 and growth form hypotheses assume. If evolved increases in stomatal ratio are medi-
101 ated by shifting abaxial stomata to the adaxial surface, holding total stomatal density
102 constant, then the overall increase in CO₂ diffusion would be limited. In contrast,
103 if amphistomy evolves by increasing adaxial stomatal density while holding abaxial
104 density constant, then *total* stomatal density must increase as well. Evolutionary
105 coordination of amphistomy and high stomatal density would reinforce one another,
106 increasing CO₂ supply to chloroplasts more than changes in either trait would in

107 isolation.

108 To address these questions, I reanalyzed existing data on stomatal ratio, light habitat,
109 and growth form in British angiosperms (Fitter and Peat, 1994; BEF) using phylo-
110 genetic comparative methods. The British angiosperm flora is well suited for these
111 questions because this flora has been comprehensively surveyed for many ecologically
112 important traits, meaning it is probably the least biased survey of stomatal trait vari-
113 ation. Salisbury’s observations on stomata and ecology in the British flora have heav-
114 ily influenced plant ecophysiology, but many of his and subsequent authors’ analyses
115 have significant limitations because of inadequate statistical methods. For example,
116 few analyses until recently account for phylogenetic nonindependence (Felsenstein,
117 1985), which can strongly influence inferences on stomatal traits and growth form
118 (Kelly and Beerling, 1995). A species-level phylogeny of the entire British flora (Lim
119 et al., 2014) now allows me to rigorously analyze evolutionary relationships between
120 stomatal ratio, light, and growth form.

121 METHODS

122 Data on stomatal ratio, light habitat, growth form, and phy- 123 logenetic relationships

124 I obtained data on ab- and adaxial stomatal density on 370 species from British
125 Ecological Flora (Fitter and Peat, 1994; BEF). I used Ellenberg light indicator values
126 (Ellenberg, 1974) and Raunkiær life form (Raunkiær, 1934) as measures of light
127 habitat and growth form, respectively. Hence, I am assuming that the species' light
128 habitat is closely related to the type of habitat (open versus closed) where that species
129 is found. Both attributes have been recently updated by taxonomic experts of the
130 British flora (PLANTATT, Hill et al. (2004)). Ellenberg light indicator values are
131 hereafter abbreviated L-value. I used a dated molecular phylogeny of the British flora
132 (Lim et al., 2014) available from TreeBASE (<http://treebase.org/>; accession number
133 15105). Seventeen species (4.6%) in the dataset were not present in the phylogeny.
134 For nine species, I used the position a congeneric species as a proxy for the focal
135 species. When multiple congeneric species were present, I consulted the phylogenetic
136 literature to identify the most closely related proxy species. For the remaining eight
137 missing species, I positioned them in the tree based on phylogenetic relationships to
138 other genera or families present in the tree. Because many phylogenetic comparative

139 methods do not allow polytomies, zero-length branches, and non-ultrametric trees, I
140 made several small adjustments to the tree. I resolved polytomies randomly using the
141 ‘multi2di’ function in **phytools** version 0.5-64 (Revell, 2012). I added 0.02 my to all
142 zero-length branches, as this was approximately the length of the shortest nonzero
143 branch length in the tree. After these changes, I slightly altered terminal branch
144 lengths to make the tree precisely ultrametric.

145 I excluded data from hyrdrophytes (14 species) because many of these species are
146 hyperstomatous (Fig. S1) due to the fact that leaves may rest on the water’s surface,
147 selecting for stomata to be present on the upper surface only. I also excluded C₄
148 (3 species) and CAM (2 species) plants.. I limited this investigation to angiosperms
149 because only 4 non-angiosperms had stomata data. The final dataset contained
150 372 species. The R code accompanying this paper documents these decisions with
151 citations to the relevant literature (Muir, 2017).

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

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

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

154 SD_{ab} and SD_{ad} are the stomatal densities on abaxial or adaxial surface, respectively.
 155 $SD_{\text{total}} = SD_{\text{ab}} + SD_{\text{ad}}$. SR_{propAd} is the proportion of stomata density on the adaxial
 156 surface, which is useful for discriminating among hypostomatous ($SR_{\text{propAd}} = 0$),
 157 amphistomatous ($0 < SR_{\text{propAd}} < 1$), and hyperstomatous species ($SR_{\text{propAd}} = 1$).
 158 SR_{even} indicates how evenly stomatal densities are distributed across both leaf sur-
 159 faces. This expression is useful because several hypotheses are based on the fact that
 160 a more even distribution should optimize leaf CO_2 diffusion.

161 **Testing for an association between open habitat and growth** 162 **form**

163 I tested whether Raunkiaer life form was associated L-value values among British
 164 angiosperms using ANOVA with Type-2 sum of squares. I did not use phylogenetic
 165 ANOVA for this test because there was no phylogenetic signal in the regression fit
 166 using **phylolm** version 2.5 (Ho and Ané, 2014). See the R code accompanying this
 167 paper for further detail (Muir, 2017). I predicted that species with faster life histories,
 168 especially therophytes (annuals), would have greater L-value than species with slower

169 life histories, especially phanerophytes, which are mostly long-lived trees.

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

171 I compared phylogenetic linear models to test whether Raunkiær life form, L-value,
172 or interactions between them predicted SR_{even} . I used SR_{even} rather than SR_{propAd}
173 as the response variable because the hypothesis is that faster life history and/or high
174 light favor more even stomatal densities on each surface. I fit models using **phylolm**
175 and extracted Akaike Information Criteria (AIC). For these and subsequent analy-
176 ses, I assumed an Ornstein-Uhlenbeck process model for the residuals with the root
177 character state integrated over the stationary distribution. I used a 10,000 para-
178 metric bootstrap samples of the full model (including main effects and interactions)
179 to calculate parameter confidence intervals (Boettiger et al., 2012). Likewise, to
180 determine whether the interaction between Raunkiær life form and L-value was sta-
181 tistically significant, I used a parametric bootstrap to generate the null distribution
182 of ΔAIC values (ΔAIC is the difference in AIC between competing models). Specif-
183 ically, I sampled 1000 random datasets from the estimated model with main effects
184 of Raunkiær life form and L-value but no interaction. I fit these simulated datasets
185 to models with and without interactions and calculated ΔAIC . The statistical signif-
186 icance of the observed ΔAIC is the proportion of simulated ΔAIC greater than the

187 observed.

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

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

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

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

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

195 Lowercase variables (sr, sd) indicate log-transformed values. Because some species

196 had zero adaxial stomata, I added one to all values prior to log-transformation. For
 197 simplicity, I have defined SR_{even} here as the ratio of ad- to abaxial stomatal density
 198 because in most cases adaxial stomatal density is lower than abaxial (see Eq. 2).
 199 The variance in sr_{even} can be decomposed into contributions of sd_{ad} , sd_{ab} , and their
 200 covariance:

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

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

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

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

207 I was interested in whether light-mediated evolution of stomatal ratio acted mostly
 208 by increasing adaxial stomatal density while maintaining abaxial density, or keeping

total stomatal density the same, but shifting a greater proportion to the adaxial surface. The first scenario predicts that the phylogenetic regression of L-value on sd_{ad} is stronger than that for sd_{ab} . The second scenario predicts that L-value acts similarly on both and that there is a negative covariance $Cov(sd_{ad}, sd_{ab}) < 0$. I tested these competing predictions by fitting a simple phylogenetic structural equation model (SEM). The model uses the phylogenetic covariance matrix to simultaneously estimate regressions of L-value on sd_{ad} and sd_{ab} while allowing covariance between them (i.e. estimating $Cov(sd_{ad}, sd_{ab})$). To fit the SEM, I used the R package **lavaan** version 0.5-23.1097 (Rosseel, 2012). I tested whether parameter estimates were significantly different than zero using z -scores.

RESULTS

Light tolerance varies among Raunkiær life forms

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

226 Interactions between light and Raunkiær life form determine 227 stomatal ratio

228 Overall, SR_{even} increased with L-value, but there was a significant interaction between
229 Raunkiær life form and L-value (Fig. 2). Both life form and L-value significantly
230 increased model fit, though L-value had a markedly larger effect on model AIC
231 (Table 1). The significant interaction is caused by different slopes between life forms.
232 Among life forms with the overall greatest L-value (therophytes, hemicryptophytes,
233 and chamaephytes, see Fig. 1), there was a strong positive relationship between
234 L-value and SR_{even} . Parametrically bootstrapped 95% confidence intervals did not
235 overlap zero (Fig. 2). The slope was weakly positive or not significantly different from
236 zero in the most shade-adapted life forms (geophytes and phanerophytes), albeit the
237 patterns were distinct in these groups. There were both hypostomatous ($SR_{\text{even}} \approx 0$)
238 and amphistomatous ($SR_{\text{even}} \approx 1$) geophytes, but these were distributed across L-
239 values. In contrast, phanerophytes were nearly always hypostomatous regardless of
240 L-value. Allowing slopes to vary across life form significantly increased model fit (lower
241 AIC, Table 1).

242 **Adaxial stomatal density contributes most of the variation in** 243 **stomatal ratio**

244 Adaxial (‘upper’) stomatal density contributed most to the evolutionary variation
245 in stomatal ratio. The contributions of adaxial density, abaxial density, and their
246 covariance are 1.14, 0.38, and -0.53, respectively. Recall that values can be greater
247 than one for adaxial stomatal density and negative for the covariance when the latter
248 value is positive. This implies that evolutionary variation in adaxial stomatal density
249 is greater than that for stomatal ratio due to positive covariance between ab- and
250 adaxial stomatal density.

251 Similarly, the phylogenetic SEM showed that changes in stomatal ratio associated
252 with L-value can be attributed mostly to evolution of adaxial stomatal density
253 (Fig. 3). Both sd_{ad} and sd_{ab} increased with L-value ($P = 6.1 \times 10^{-7}$ and 2.9×10^{-5} ,
254 respectively). However, the regression of L-value on sd_{ad} was $2.1\times$ that of L-value on
255 sd_{ab} (0.21 versus 0.1). Because stomatal densities were natural log-transformed, this
256 implies an increase in L-value by one leads to a 1.23-fold change in adaxial stomatal
257 density versus a 1.1-fold change in abaxial stomatal density. The SEM also showed
258 a significant positive covariance between stomatal densities on each surface ($P = 1.7$
259 $\times 10^{-11}$). These results together imply that total stomatal density increases with

260 L-value, but the response is mediated mostly by adaxial stomatal density.

261 **DISCUSSION**

262 The ratio of stomatal densities on the abaxial ('lower') to that of the adaxial ('upper')
263 surface varies greatly across plant species, but the adaptive significance is not clear.
264 Comparative studies correlating stomatal ratio to ecological factors can distinguish
265 among competing hypotheses and reveal critical experiments for future work. Previ-
266 ous comparative studies suggested that high light and herbaceous growth form favor
267 amphistomy (Mott et al., 1984; Jordan et al., 2014; Muir, 2015; Bucher et al., 2017),
268 particularly in the British flora (Salisbury, 1927; Peat and Fitter, 1994). However,
269 none of these studies have accounted for the fact that light and growth form are often
270 confounded – open, high light habitats are necessarily dominated by herbs – or the
271 fact that species are not independent because of shared evolutionary history. Here, I
272 reanalyzed data on stomata, light tolerance, and growth form in British angiosperms
273 using phylogenetic comparative methods. As expected, species' light tolerance (El-
274 lenberg light indicator or L-value) is confounded with growth form (Raunkiaer life
275 form; Fig. 1). Nevertheless, both L-value and Raunkiaer life form affect stomatal
276 ratio, but these factors also interact; the influence of L-value on stomatal ratio varies

277 across forms. These novel findings provide further evidence that variation in stomatal
278 ratio is adaptive and have important implications for interpreting changes in stom-
279 atal ratio through the paleo record (Jordan et al., 2014) and during domestication
280 (Milla et al., 2013).

281 **Adaptive significance of amphistomy**

282 Previously, associations between light, growth form, and stomatal ratio have been
283 interpreted in isolation as indicating that either high light and/or herbaceous growth
284 form favors amphistomy. In British angiosperms, both factors are important, though
285 statistical analyses suggest that light may be a stronger determinant than growth
286 form (Table 1). Unlike previous studies, I found a significant interaction between
287 light and growth form among British angiosperms, which suggests that amphistomy
288 may only be strongly favored when CO₂ strongly limits photosyntheses *and* pho-
289 tosynthesis strongly limits fitness. The ideal way to test this would be to measure
290 selection on stomatal ratio in a species that varied quantitatively in both stomatal
291 ratio and life history (e.g. containing both annual and perennial forms). I predict
292 that amphistomy will be favored much more strongly in the annual form grown under
293 high light compared to an annual under low light or a perennial in high light. Similar
294 experiments could also be performed to test if and when light-mediated plasticity in

295 stomatal ratio is adaptive (Gay and Hurd, 1975; Mott and Michaelson, 1991).

296 The prevalence of amphistomatous species in high light habitats supports the hy-
297 pothesis that amphistomy is an adaptation to maximize photosynthetic rates by
298 increasing CO₂ diffusion (Jones, 1985). It is also evidence against the hypothesis
299 that the principle fitness cost of amphistomy is water loss (Darwin, 1886; Foster
300 and Smith, 1986) or dehydration of palisade mesophyll (Buckley et al., 2015). Since
301 evaporative demand increases under high insolation, under these hypotheses we would
302 expect plants in high light to be hypostomatous. Because stomatal conductances on
303 each surface can be regulated independently in response to the environment (Darwin,
304 1898; Pospíšilová and Solárová, 1984; Smith, 1981; Reich, 1984; Mott and O’Leary,
305 1984), amphistomatous leaves likely cope with these stresses by rapidly closing adax-
306 ial stomata when water supply cannot match evaporative demands. Instead, pat-
307 terns in the British flora are at least consistent with the idea that adaxial stomata
308 increase susceptibility to foliar pathogens (Gutschick, 1984; McKown et al., 2014).

309 The cost of adaxial stomata may be greater in the shade because greater leaf wet-
310 ness and lower ultraviolet light provide a more suitable microclimate for many foliar
311 pathogens.

312 **Amphistomy as a proxy for open habitat**

313 These observations from the British flora strongly support the hypothesis that am-
314 phistomy can be used a proxy for open habitat in paleoenvironment reconstruction
315 (Carpenter, 1994; Jordan et al., 2014; Carpenter et al., 2015), but also point out pre-
316 viously unknown subtleties. These previous studies based their conclusions on data
317 from Proteaceae, in which there is little quantitative variation in stomatal ratio;
318 species are either completely hypostomatous ($SR_{propAd} \approx 0$) or completely amphis-
319 tomatus ($SR_{propAd} \approx 0.5$). Stomatal ratio in British angiosperms is also bimodal
320 (Peat and Fitter, 1994), but across many families there is also quantitative variation.
321 Importantly, this means that quantitative variation in stomatal ratio may provide a
322 more precise, quantitative indicator of vegetation type, rather than simply ‘open’ or
323 ‘closed’. A quantitative relationship between L-value and stomatal ratio has already
324 been shown for herbaceous perennials (Bucher et al., 2017), but we now know that
325 it holds among annuals (therophytes), subshrubs (chamaephytes), and, to a lesser
326 extent, geophytes as well (Fig. 2).

327 The nonsignificant relationship between L-value and stomatal ratio in geophytes and
328 phanerophytes suggests that in some cases amphistomy may not reliably indicate
329 open habitat without further information. For example, perhaps amphistomatous
330 geophytes from partially shaded habitats are spring ephemerals, so they experience

331 high light during their growth phase, but this has not been tested. Likewise, phanero-
332 phytes (most tall trees) are almost always hypostomatous (see also Muir (2015)).
333 Most British phanerophytes are tall, hypostomatous trees, but the exceptions are
334 telling. For example, the most amphistomatous phanerophyte in this dataset is
335 *Brassica oleracea*, a short-statured biennial that has more in common physiologi-
336 cally with hemicryptophytes than other phanerophytes. The other amphistomatous
337 phanerophytes in this data set (*Populus nigra* and *Lavatera arborea*) are fast-growing
338 pioneer species.

339 Finally, phylogenetic information should improve inferences about paleoclimates be-
340 cause there is appreciable phylogenetic signal in stomatal ratio. The phylogenetic
341 half-life of stomatal ratio evolution, after accounting for L-value and Raunkiaer life
342 form, is $\log(2)/\alpha = 1.5$ my (see Table 1 for maximum likelihood estimates of α).
343 This lag time may indicate that evolving to the ‘optimum’ is constrained by the
344 shape of the fitness landscape (Muir, 2015) or that other unmeasured factors which
345 affect stomatal ratio have some phylogenetic signal. Regardless of the mechanism,
346 this fact means that researchers may be able to use data from closely related species
347 to improve paleoenvironment reconstruction.

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

349 Variation in stomatal ratio is determined primarily by evolution of adaxial stomatal
350 density and is coordinated with increases in total leaf stomatal density summed across
351 both surfaces. Phylogenetic analyses show that changes in stomatal ratio and total
352 stomatal density, especially in response to L-value, are predominantly mediated by
353 changes in adaxial stomatal density. This highly nonrandom pattern among British
354 angiosperms mirrors evolutionary changes wrought by domestication (Milla et al.,
355 2013); crops species tend to have higher adaxial stomatal density than their wild
356 relatives. Note here that I am referring only to evolutionary variation in stomatal
357 ratio among species; different processes may mediate within species variation or
358 plastic responses.

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

367 I refer to the second hypothesis as the ‘coordination’ hypothesis. In this scenario,
368 ecological conditions such as high light select for both increased total stomatal density
369 and for amphistomy because these traits work well in coordination with one another.
370 For example, if stomatal density were very high on a hypostomatous plant, then CO₂
371 would be more strongly limited by the mesophyll. Adding a second parallel pathway
372 for diffusion by developing stomata on both surfaces would restore a more optimal
373 balance between stomatal and mesophyll limitations. Conversely, there would be
374 little benefit to amphistomy when total stomatal density is low because CO₂ diffusion
375 is strongly limited by stomatal resistance, and therefore photosynthetic rate is not
376 sensitive to changes in mesophyll diffusion mediated by stomatal ratio.

377 **Conclusions - finish when analysis is complete**

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Table 1: Interaction between species' Ellenberg light indicator value (L-value) and Raunkiaer lifeform shape 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 lifeform	0.46	0.068	0.34	12	-33.3	90.6	0
L-value + lifeform	0.47	0.072	0.32	8	-40.3	96.5	6
L-value	0.64	0.108	0.26	4	-59.3	126.6	36.1
lifeform	0.34	0.067	0.15	7	-79.2	172.4	81.8
1	0.29	0.067	0	3	-107.5	221	130.5

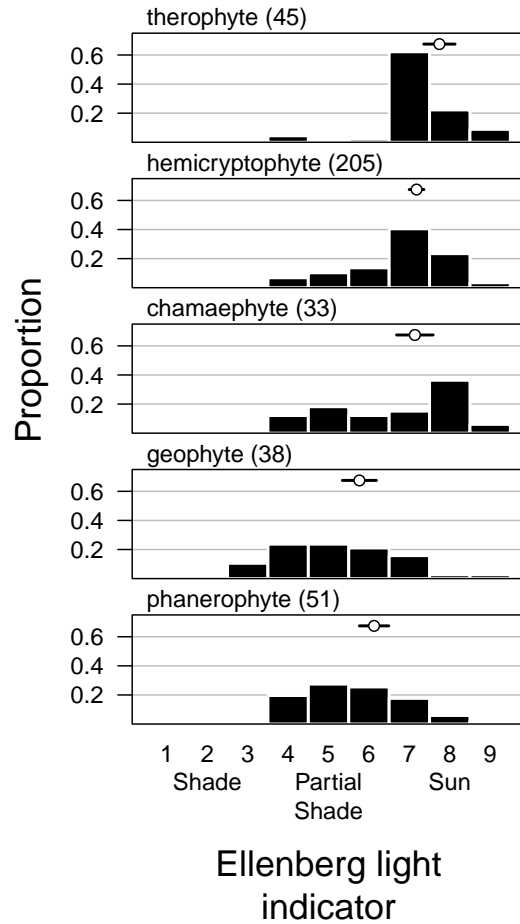


Figure 1: Lifeforms have different tolerances for sun and shade among British angiosperms. Each panel is the distribution of Ellenberg light indicator values on an integer scale of 1-9 for different Raunkiær life forms. Height of the bars indicate the raw proportion of species in each bin for that lifeform. The sample size for each lifeform is listed next in parentheses. The mean (open circle) and 95% confidence intervals (black line) around the mean Ellenberg light indicator value for each lifeform based on phylogenetic regression are above the histogram.

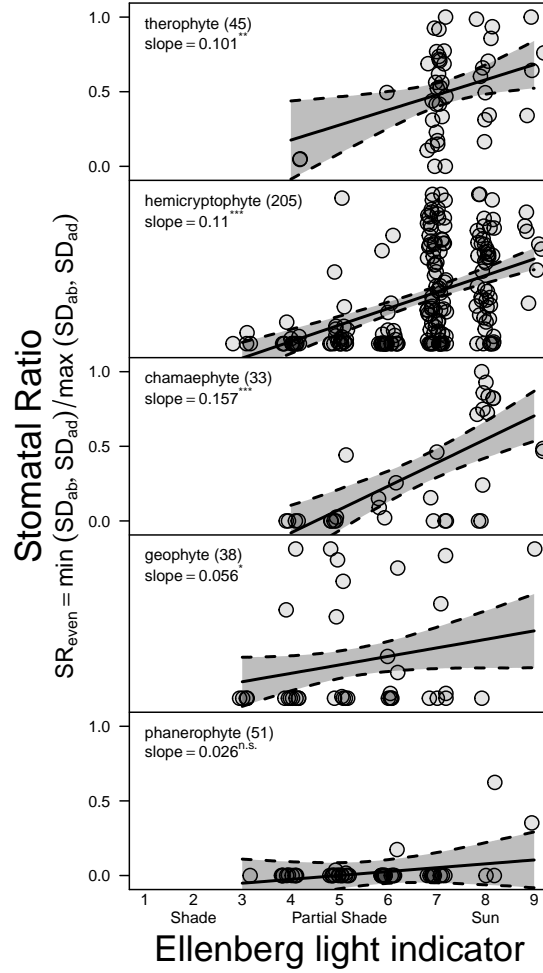


Figure 2: The effect of light on stomatal ratio depends on Raunkiaer life form. Greater Ellenberg light indicator values (L-value) are associated with greater stomatal ratio (SR_{even}) in therophytes, hemicryptophytes, and chamaephytes but not geophytes and phanerophytes. The maximum likelihood slope from phylogenetic regression is given with statistical significance based on 1000 parametric bootstrap samples. Numbers in parentheses next to Raunkiaer life form are the sample sizes in the final dataset. Estimated slopes (solid line) and 95% bootstrapped confidence intervals (gray polygon between dashed lines) are plotted against raw data. Points have been jittered for visual clarity.

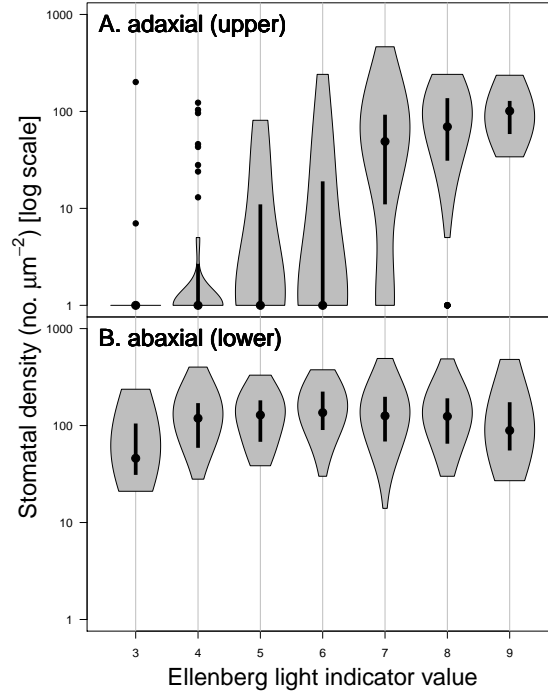


Figure 3: Light-mediated evolution of stomatal ratio is mostly driven by increased adaxial (‘upper’) stomatal density (Panel A), whereas abaxial (‘lower’) stomatal density (Panel B) is similar across Ellenberg light indicator values (L-value x -axis). The violin plot shows stomatal density (y -axis, log-scale) as a function of L-value. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range of the data; the point indicates the median; the thick lines indicate the 0.25 and 0.75 quantiles. Points outside the polygons are statistical outliers.

498 **Supporting Information**

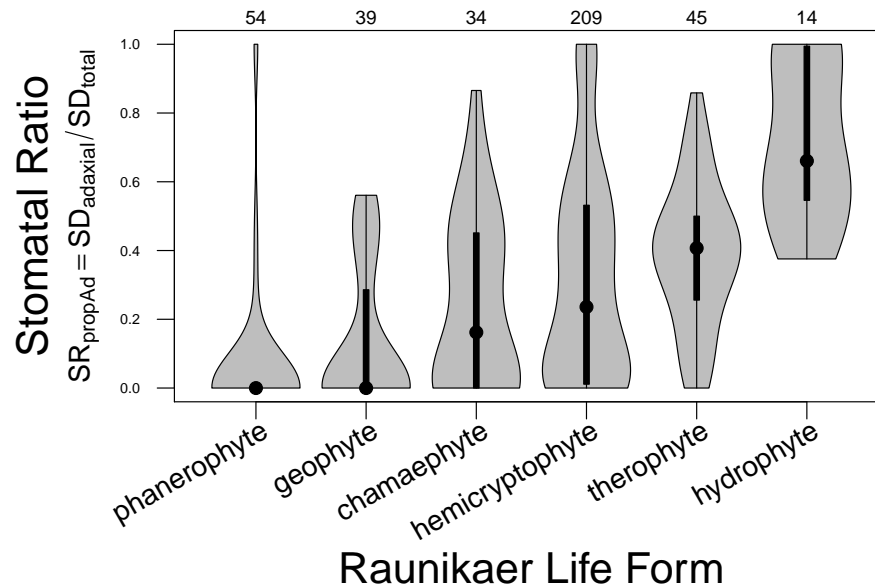


Figure S1: Most hydrophytes are hyperstomatous, having most stomata on the adaxial ('upper') surface (high SD_{propAd}). The violin plot shows stomatal ratio as a function of Raunkiaer lifeform. The width of the grey polygons indicates the density of data. Length of grey polygon indicate the range of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