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Paper

Does stomatal patterning in amphistomatous leaves minimize the CO₂ diffusion path length within leaves?

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Abstract

Photosynthesis is co-limited by multiple factors depending on the plant and its environment. These include biochemical rate limitations, internal and external water potentials, temperature, irradiance, and carbon dioxide (CO₂). Amphistomatous leaves have stomata on both abaxial and adaxial leaf surfaces. This feature is considered an adaptation to alleviate CO₂ diffusion limitations in productive environments as the diffusion path length from stomate to chloroplast is effectively halved in amphistomatous leaves. Plants may also reduce CO₂ limitations through other aspects of optimal stomatal anatomy: stomatal density, distribution, patterning, and size. A number of studies have demonstrated that stomata are overdispersed compared to a random distribution on a single leaf surface; however, despite their prevalence in nature and near ubiquity among crop species, much less is known about stomatal anatomy in amphistomatous leaves, especially the coordination between leaf surfaces. Here we use novel spatial statistics based on simulations and photosynthesis modeling to test hypotheses about how amphistomatous plants may optimize CO₂ diffusion in the model angiosperm *Arabidopsis thaliana* grown in different light environments. We find that 1) stomata are overdispersed, but not ideally dispersed, on both leaf surfaces across all light treatments; 2) the patterning of stomata on abaxial and adaxial leaf surfaces is independent; and 3) the theoretical improvements to photosynthesis from abaxial-adaxial stomatal coordination are miniscule ($\ll 1\%$) across the range of feasible parameter space. However, we also find that 4) stomatal size is correlated with the mesophyll volume that it supplies with CO₂, suggesting that plants may optimize CO₂ diffusion limitations through alternative pathways other than ideal, uniform stomatal spacing. We discuss the developmental, physical, and evolutionary constraints which may prohibit plants from reaching this theoretical adaptive peak of uniform stomatal spacing and inter-surface stomatal coordination. These findings contribute to our understanding of variation in the anatomy of amphistomatous leaves.

Key words: amphistomy; *Arabidopsis thaliana*; CO₂ diffusion; finite element method; optimality; photosynthesis; stomata

Introduction

Stomatal anatomy (e.g. size, density, distribution, and patterning) and movement regulate gas exchange during photosynthesis, namely CO₂ assimilation and water loss through transpiration. Since waxy cuticles are mostly impermeable to CO₂ and H₂O, stomata are the primary entry and exit points through which gas exchange occurs despite making up a small percentage of the leaf area (Lange et al., 1971). Stomata consist of two guard cells which open and close upon changes in turgor pressure or hormonal cues (McAdam and Brodribb, 2016). The stomatal pore leads to an internal space known as the substomatal cavity where gases contact the mesophyll. Once in the mesophyll, CO₂ diffuses throughout a network of intercellular air space (IAS) and into mesophyll cells where CO₂ assimilation (A) occurs within the chloroplasts (Lee and Gates, 1964). Stomatal conductance and transpiration are determined by numerous environmental and anatomical parameters such as vapor pressure deficit (VPD), irradiance, temperature, wind speed, leaf water potential, IAS geometry, mesophyll cell anatomy, and stomatal anatomy. The latter of these is the focus of this study, with discussion of other interacting variables.

Many successful predictions about stomata and other C₃ leaf traits can be made by hypothesizing that natural selection should optimize CO₂ gain per unit of water loss for any given set of environmental parameters, including their variability (Cowan and Farquhar, 1977; Buckley et al., 2017; Sperry et al., 2017). Total stomatal area (size \times density) is optimized for operational conductance ($g_{s,op}$) rather than maximum conductance ($g_{s,max}$) such that stomatal apertures are most responsive to changes in the environment at their operational aperture (Franks et al., 2012; Liu et al., 2021). Stomatal aperture can compensate for suboptimal stomatal densities to an extent (Büßis et al., 2006), but stomatal density and size ultimately determine a leaf's theoretical $g_{s,max}$ (Sack and Buckley, 2016), which is proportional to $g_{s,op}$ under typical conditions (McElwain et al., 2016; Murray et al., 2020). Additionally, low stomatal densities lead to irregular and insufficient CO₂ supply and reduced photosynthetic efficiency in leaf areas far from stomata (Pieruschka et al., 2006; Morison et al., 2005), while high stomatal densities can reduce water use efficiency (WUE) (Büßis et al., 2006) and incur excessive metabolic costs (de Boer et al., 2016; Deans et al., 2020). Stomatal density positively co-varies with irradiance during leaf development and negatively co-varies with CO₂ concentration (Gay and Hurd, 1975; Schoch et al., 1980; Woodward, 1987; Royer, 2001), consistent with optimality predictions. In most species, stomata occur only on the abaxial (usually lower) leaf surface; but amphistomy, the occurrence of stomata on both abaxial and adaxial leaf surfaces, is also prevalent in high light environments with constant or intermittent access to sufficient water (Mott et al., 1982; Jordan et al., 2014; Muir, 2018; Drake et al., 2019; Muir, 2019). Amphistomy effectively halves the CO₂ diffusion path length and boundary layer resistance by doubling boundary layer conductance (Parkhurst, 1978; Mott and Michaelson, 1991; Harrison et al. (2020)). Ab- and adaxial leaf surfaces were found to function independent of one another in wheat, an important crop, with the adaxial surface demonstrating higher photosynthetic capacity (Wall et al., 2022). These results highlight the utmost importance of amphistomy for some plants.

Despite the success of optimality predictions, stomatal anatomy may be partially constrained by physical and developmental limits on phenotypic expression (Croxdale, 2000; Harrison et al., 2020; Muir et al., 2023). A number of physical and developmental processes constrain stomatal anatomy trait space. For example, almost all stomata follow the one cell spacing rule to maintain proper stomatal functioning as guard cell movement requires the rapid exchange of ions with neighboring epidermal cells (i.e. subsidiary cells) (Geisler et al., 2000; Dow et al., 2014). This would prevent stomata from being strongly clustered; however, some species (notably in *Begonia*) appear to benefit from the overlapping vapor shells caused by stomatal clustering in dry environments (Yi Gan et al., 2010; Lehmann and Or, 2015; Papanatsiou et al., 2017). Historically, stomatal patterning in eudicot angiosperms was thought to be random with an exclusionary distance surrounding each stomate (Sachs, 1974); however, the developmental controls of stomatal patterning are more complex. Croxdale (2000) reviews three developmental theories which attempt to explain stomatal patterning in angiosperms: inhibition, cell lineage, and cell cycle, ultimately arguing for a cell cycle based control of stomatal patterning. Pillitteri and Torii (2012) review the short and long distance signalling pathways associated with stomatal spacing and development, which include cell to cell communication and whole plant integration to ensure the proper spacing of stomata across a single leaf surface depending on environmental cues. Much less is known about the development of stomata on the adaxial leaf surface in amphistomatous plants. Stomatal size is additionally constrained by genome size with larger genomes leading to larger minimum guard cell size (Jordan et al., 2015; Roddy et al., 2020). Despite these limitations, ecophysiological theory still predicts optimal stomatal anatomy, the details of which are discussed below.

The patterning and spacing of stomata on the leaf affects photosynthesis in C₃ leaves by altering the CO₂ diffusion path length from stomata to sites of carboxylation in the mesophyll. Maximum photosynthetic rate (A_{max}) in C₃ plants is generally co-limited by biochemistry and diffusion, but modulated by light availability (Parkhurst and Mott, 1990; Manter, 2004; Carriqui et al., 2015). Low light decreases CO₂ demand by limiting electron transport rate, leading to relatively high internal CO₂ concentration (C_i) and low A_{max} (Kaiser et al., 2016). In contrast, well hydrated leaves with open stomata in high light, photosynthesis is often limited by CO₂ supply

as resistances from the boundary layer, stomatal pore, sub-stomatal cavity, and mesophyll can result in insufficient CO_2 supply at the chloroplast to maximize photosynthesis (Farquhar et al., 1980; Lehmeier et al., 2017). In this study, we focus primarily on how stomatal patterning affects diffusion.

Assuming uniform mesophyll diffusion resistance in all directions (homogenous porous medium), an ideal stomatal anatomy can be predicted. To maximize CO_2 supply from the stomatal pore to chloroplasts, stomata should be uniformly distributed in an equilateral triangular grid on the leaf surface so as to minimize stomatal number and CO_2 diffusion path length (Parkhurst, 1994). An equilateral triangular grid is ideal because it maximizes the average distance between stomata, for a given stomatal density, and thereby minimizes the average distance between any point in the mesophyll to its nearest stomate. Assuming a homogenous mesophyll, this is the most efficient pattern to supply CO_2 to a leaf volume.

Such an assumption, though an oversimplification, is a powerful tool for photosynthesis modelling, and may provide insight into how real leaves diverge from this. In real leaves, as the diffusion rate of CO_2 through liquid is approximately $10^4\times$ slower than CO_2 diffusion through air, mesophyll resistance is generally thought to be primarily limited by liquid diffusion (Aalto and Juurola, 2002; Evans et al., 2009), but diffusion through the IAS has also been shown to be a rate limiting process because the tortuous, disjunct nature of the IAS can greatly increase diffusion path lengths (Harwood et al., 2021). Additionally, tortuosity is higher in horizontal directions (parallel to leaf surface) than vertical directions (perpendicular to leaf surface) because of the cylindrical shape and vertical arrangement of palisade mesophyll cells (Earles et al., 2018; Harwood et al., 2021). However, the ratio of lateral to vertical diffusion rate is still largely unknown and may be a highly variable trait in leaves (Morison et al., 2005; Pieruschka, 2005; Pieruschka et al., 2006; Morison and Lawson, 2007). Depending on the thickness of the leaf, porosity of the leaf mesophyll, tortuosity of the IAS, and lateral to vertical diffusion rate ratio, minimizing diffusion path length for CO_2 via optimally distributed stomata may yield significant increases in CO_2 supply for photosynthesis and higher A_{max} . Or plants may simply coordinate the development of stomata and mesophyll IAS to reach another optimal solution which does not rely on uniformly distributed stomata (Baillie and Fleming, 2020).

We hypothesized that, in the absence of any constraint and assuming homogenous mesophyll diffusion resistance, natural selection will favor stomatal patterning and distribution to minimize the diffusion path length. In amphistomatous leaves, this would be accomplished by 1) a uniform, equilateral triangular distribution of stomata on both abaxial and adaxial leaf surfaces and 2) coordinated stomatal spacing on each surface that offsets the position of stomata (Fig. 1). Coordination between leaf surfaces is defined in this study as the occurrence of stomata in areas farthest from stomata on the opposite leaf surface. Additionally, because CO_2 is more limiting for photosynthesis under high light, we hypothesize that in high light 3) there should be more stomata, and 4) stomata should be more overdispersed (closer to equilateral triangular grid) compared to a random distribution than in low light. Finally, since, in measures of whole leaves, stomatal area rather than stomatal density is optimized for operational conductance, we hypothesize that 5) stomatal length (and hence its area) will be positively correlated with the area of the leaf surface to which it is spatially closest as defined by Voronoi tessellation techniques. We refer to this as the ‘stomatal zone’, the leaf area surrounding a focal stomate closest to that stomate and therefore the zone it supplies with CO_2). This way, each stomate may be optimally sized relative to the mesophyll volume it supplies. Hypothesis 3 is already well supported in many species (Poorter et al., 2019), but it is useful here to confirm that light treatments induced plasticity in the expected direction.

To test these hypotheses, we grew the model plant *Arabidopsis thaliana* in high, medium, and low light and measured stomatal density, size, and patterning on both leaf surfaces, and spatial coordination between them. We use Voronoi tessellation techniques to calculate stomatal zones. We also used a 2-D porous medium approximation of CO_2 diffusion and photosynthesis to predict the photosynthetic advantage of optimal versus suboptimal coordination in stomatal coordination between surfaces. Specifically, we predicted that traits which affect diffusion path length (leaf thickness, stomatal density, leaf porosity), diffusion rate (determined by temperature, pressure), and CO_2 demand (Rubisco concentration, light) would modulate the advantage of optimal stomatal arrangement following the relationships outlined in Table 1. Here, we integrate over reasonable parameter space to determine the ecophysiological context most likely to favor stomatal coordination in amphistomatous leaves.

Materials and methods

Data Preparation

Plant material, growth conditions, and three-dimensional confocal imaging are described in Dow et al. (2017). Briefly, Columbia (Col-0) ecotype of *Arabidopsis thaliana* (L.) Heynh. plants were grown in three different light environments: low light ($\text{PAR} = 50 \mu\text{mol m}^{-2} \text{s}^{-1}$), medium light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), and high light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$). PAR stands for photosynthetically active radiation. *A. thaliana*

responds strongly to light levels over this range (Bailey et al., 2001), though natural populations in open canopies can experience $\text{PAR} > 800 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Callahan and Pigliucci, 2002). Seeds were surface-sterilized and stratified at 4 °C for 3–5 d in 0.15% agarose solution and then sown directly into Pro-Mix HP soil (Premier Horticulture; Quakerstown, PA, USA) and supplemented with Scott’s Osmocote Classic 14-14-14 fertilizer (Scotts-Sierra, Marysville, OH, USA). At 10–14 d, seedlings were thinned so only one seedling per container remained. Plants were grown to maturity in growth chambers where the conditions were as follows: 16 : 8 h, 22 : 20°C, day : night cycle. Imaging of the epidermis and internal leaf structures was performed using a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) with the protocol developed by Wuyts et al. (2010) with additional modification described in Dow et al. (2017). We captured 132 images in total, making 66 abaxial-adaxial image pairs. Images were square with an area of 0.386 mm². We measured stomatal position and length using ImageJ (Schneider et al., 2012). A number of synthetic leaf surface data sets were also simulated (details below) to generate null distributions against which to test our hypotheses and to avoid any methodological influence on our results (e.g. boundary effects when calculating stomatal patterning). All synthetic leaf surfaces were simulated based on the size of the real leaf images and stomatal densities matched those of real leaf images.

Single surface analyses

We compared observed stomatal patterning to an ideal pattern (uniform equilateral triangular grid) and a null model (random uniform distribution). The terminology is unfortunately confusing because the word ‘uniform’ is used in different ways. A uniform equilateral triangular grid means that the distance between stomata is uniform; a random uniform distribution means that a stomate has an equal probability (i.e. uniform) of occurring anywhere on the leaf surface. To limit confusion, we refer to the ideal pattern (equilateral triangle grid) as uniform and the null pattern (random uniform) as random. When observed stomatal patterns are more dispersed than expected under random patterning, we refer to this as overdispersed. Note, however, that overdispersed compared to random is still less dispersed than ideal, because the ideal pattern is maximally dispersed.

We tested whether stomata overdispersed by comparing each observed, real leaf stomatal pattern to an array of synthetic data simulated from a random distribution. For each observed leaf surface image with n stomata we generated 10³ synthetic surfaces with n stomata uniformly randomly distributed on the surface. For each sample image, we compared the observed Nearest Neighbor Index (NNI) to the null distribution of NNI values calculated from the synthetic data set. NNI is the ratio of observed mean distance (\overline{D}_O) to the expected mean distance (\overline{D}_E) where \overline{D}_E is:

$$\overline{D}_E = \frac{0.5}{\sqrt{A_{\text{leaf}}/n_{\text{stomata}}}}. \quad (1)$$

A_{leaf} is leaf area visible in the sampled field and n_{stomata} the number of stomata. \overline{D}_E is the theoretical average distance to the nearest neighbor of each stomate if stomata were uniformly randomly distributed (Clark and Evans, 1954). \overline{D}_O calculated for each synthetic data set is:

$$\overline{D}_O = \frac{\sum_{i=1}^{n_{\text{stomata}}} d_i}{n_{\text{stomata}}}, \quad (2)$$

where d_i is the distance between stomate_{*i*} and its nearest neighbor. We calculated NNI using the *R* package **spatialEco** version 2.0.2 (Evans and Murphy, 2023). The observed stomatal distribution is overdispersed relative to a random distribution if the observed *NNI* is greater than 95% of the synthetic *NNI* values (one-tailed test). We adjusted *P*-values to account for multiple comparisons using the Benjamini-Hochberg (Benjamini and Hochberg, 1995) false discovery rate procedure implemented in the *R* package **multtest** version 2.56.0 (Pollard et al., 2005).

For each sample image, we also simulated 10³ synthetic leaf surfaces with n stomata ideally, uniformly dispersed in an equilateral triangular grid. To account for uncertainty in the stomatal density of each sample image with n stomata, we integrated over plausible stomatal densities and then conditioned on synthetic leaf surfaces with exactly n stomata. The simulated stomatal count was drawn from a Poisson distribution with the mean parameter λ drawn from a Gamma distribution with shape n and scale 1 ($\lambda \sim \Gamma(n, 1)$). $\Gamma(n, 1)$ is the posterior distribution of λ with a flat prior distribution. This integration was necessary to remove any artifacts of uncertainty in the true stomatal density of the sample leaves.

We developed a dispersion index *DI* to quantify how close observed stomatal patterning is to random versus ideally patterned in an equilateral triangular grid. *DI* varies from zero to one, where zero is random and one is ideally patterned:

$$DI = \frac{NNI - \text{median}(NNI_{\text{random}})}{\text{median}(NNI_{\text{ideal}}) - \text{median}(NNI_{\text{random}})} \quad (3)$$

NNI is calculated for each sample image as described above; $\text{median}(NNI_{\text{random}})$ and $\text{median}(NNI_{\text{uniform}})$ are calculated from the synthetic data specific to each sample image as described above. We tested whether light treatment affects DI and stomatal density (D_S) using analysis of variance (ANOVA).

Finally, we examined the relationship between stomatal zone area and stomatal length using a Bayesian linear mixed-effects model fit with the *R* package **brms** version 2.20.4 (Bürkner, 2017, 2018) and *Stan* version 2.33.1 (Stan Development Team, 2023). Stomatal zone area was calculated using Voronoi tessellation (e.g. Fig. 2). The stomatal zone area, S_{area} , is the region of the leaf surface whose distance to stomate, S , is less than the distance to any other stomate, S . Stomatal length was measured in ImageJ (Schneider et al., 2012). We modeled fixed effects of surface, light treatment, stomatal length, and their 2- and 3-way interactions on $\sqrt{S_{\text{area}}}$. We included random intercepts, random effects of surface, random slopes, and random surface-by-slope interactions within both plant and individual to account for nonindependence of stomata within the same plant or individual. We also modeled residual variance as a function of light treatment. We sampled the posterior distribution from 4 chains with 1000 iterations each after 1000 warmup iterations. We calculated convergence diagnostics (\hat{R}) and effective sample sizes following Vehtari et al. (2021). We estimated the marginal slope and 95% highest posterior density (HPD) intervals between stomatal length and $\sqrt{S_{\text{area}}}$ using the *emtrends* function in the *R* package **emmeans** version 1.10.0 (Lenth, 2023).

Paired Abaxial and Adaxial Surface Analysis

To test whether the position of ab- and adaxial stomata are coordinated we compared the observed distribution to a null distribution where the positions on each surface are random. For each pair of surfaces (observed or synthetic) we calculated the distance squared between each pixel of the surface to the nearest stomatal centroid with the *R* package **raster** version 3.6.26 (Hijmans, 2023). We refer to this as the ‘nearest stomatal distance’ or NSD. Then we calculated the pixel-wise Pearson correlation coefficient. If stomatal positions on each surface are coordinated to minimize the distance between mesophyll and the nearest stomate, then we expect a negative correlation. A pixel that is far from a stomate on one surface should be near a stomate on the other surface (Fig. 1). We generated a null distribution of the correlation coefficient by simulating 10^3 synthetic data sets for each observed pair. For each synthetic data set, we simulated stomatal position using a random uniform distribution, as described above, matching the number of stomata on abaxial and adaxial leaf surfaces to the observed data. Stomatal positions on each surface are coordinated if the correlation coefficient of the NSD between observed ab- and adaxial surfaces is greater than 95% of the synthetic correlation values (one-tailed test).

Modeling Photosynthesis

We modeled photosynthesis CO_2 assimilation rate using a spatially-explicit two-dimensional reaction diffusion model using a porous medium approximation (Parkhurst, 1994) using the finite element method (FEM) following Earles et al. (2017). Consider a two-dimensional leaf where stomata occur on each surface in a regular sequence with interstomatal distance U . The main outcome we assessed is the advantage of offsetting the position of stomata on each surface compared to having stomata on the same x position on each surface. With these assumptions, by symmetry, we only need to model two stomata, one abaxial and one adaxial, from $x = 0$ to $x = U/2$ and from the adaxial surface at $y = 0$ to the abaxial surface at $y = L$, the leaf thickness. We arbitrarily set the adaxial stomate at $x = 0$ and toggled the abaxial stomata position between $x = U/2$ (offset) or $x = 0$ (below adaxial stomate). The ‘coordination advantage’ of offset stomatal position on each surface is the photosynthetic rate of the leaf with offset stomata compared to that with stomata aligned in the same x position:

$$\text{coordination advantage} = \frac{A_{\text{offset}}}{A_{\text{aligned}}} \quad (4)$$

We modeled the coordination advantage over a range of leaf thicknesses, stomatal densities, photosynthetic capacities, and light environments to understand when offsetting stomatal position on each surface might deliver a significant photosynthetic advantage (Table 1). The complete model description is available in the Supporting Information.

Results

Stomatal density of *Arabidopsis thaliana* varies among light treatments (ANOVA, $F_{2,126} = 682$, $P = 2.58 \times 10^{-68}$) because the density is much greater in the high light treatment (Fig. 3). Density is consistently greater on abaxial leaf surfaces across all light treatments (ANOVA, $F_{1,126} = 44.2$, $P = 8.21 \times 10^{-10}$; Fig. 3). There is no evidence for an interaction between light treatment and surface (ANOVA, $F_{2,126} = 2.75 \times 10^{-2}$, $P = 0.973$). Leaves are amphistomatous with a mean stomatal density ratio of 0.44.

Stomatal patterning is non-random, but far from uniform

Many leaf surfaces (34 of 132, 25.8%) are significantly overdispersed compared to a random uniform distribution, but none were close to an ideal, uniform equilateral triangular pattern (dispersion index = 1; Fig. 4). Before controlling for multiple comparisons, 40.9% are significantly overdispersed. The dispersion index differs significantly among light treatments (ANOVA, $F_{2,126} = 7.87$, $P = 6.02 \times 10^{-4}$) because the medium light treatment is significantly less than the low treatment (Fig. 4). Dispersion index is consistently greater on adaxial leaf surfaces across all light treatments (ANOVA, $F_{1,126} = 29.2$, $P = 3.19 \times 10^{-7}$; Fig. 4). There is no evidence for an interaction between light treatment and surface (ANOVA, $F_{2,126} = 0.594$, $P = 0.554$).

No evidence for coordinated stomatal position between surfaces

There is no evidence of spatial coordination between abaxial and adaxial leaf surfaces. The pixel-wise correlation between nearest stomatal distance (NSD) squared on paired abaxial and adaxial leaf surfaces is not significantly less than zero in any of the 66 leaves (Fig. 5). Before controlling for multiple comparisons, 3% are significantly *positively* correlated. The NSD correlation is not different among light treatments (ANOVA, $F_{2,63} = 2.28$, $P = 0.111$; Fig. 5).

Larger stomata supply larger mesophyll volumes

All parameters in the Bayesian linear mixed-effects model converged ($\hat{R} < 1.01$) and effective sample sizes exceeded 10^3 . Across all light treatments and leaf surfaces, stomatal length and stomatal area are weakly positively correlated (Fig. 6). The slope was significantly greater than zero for all abaxial surfaces, but not for the adaxial surface in low and medium light treatments. The estimated marginal slopes and 95% HPD intervals for each combination of light and surface is: low light, abaxial surface: 1.928 [0.779–3.133]; low light, adaxial surface: 1.745 [−0.041–3.373]; medium light, abaxial surface: 1.085 [0.328–1.957]; medium light, adaxial surface: 0.656 [−0.399–1.691]; high light, abaxial surface: 0.597 [0.316–0.911]; high light, adaxial surface: 1.269 [0.831–1.721].

Little benefit of coordinated stomatal arrangement

We used the finite element method (FEM) to model CO_2 diffusion within the leaf and photosynthesis as a 2-D porous medium. Across all realistic parts of parameter space, the coordination advantage is much less than 0.01 (Fig. 7). For reference, a log-response of ratio is 0.01 is approximately 1%. The only exception was for thin leaves ($T_{\text{leaf}} = 100 \mu\text{m}$) with few stomata ($U = 338 \mu\text{m}$, which corresponds to a stomatal density of $\approx 10 \text{ mm}^{-2}$), where lateral diffusion is major constraint on CO_2 supply. However, such thin leaves with so few stomata are uncommon among C_3 plants (some CAM plants have low stomatal density (Males and Griffiths, 2017)). In other areas of parameter space, lateral diffusion limitations were small relative to those along the ab-adaxial axis (see Fig. S1 for a representative model solution).

Discussion

Stomata cost resources to maintain (Deans et al., 2020) and expose leaves to risks such as hydraulic failure (Wang et al., 2020) or infection by plant pathogens (Melotto et al., 2017). Therefore leaves should develop enough stomata to adequately supply CO_2 to chloroplasts, but not overinvest. A widespread hypothesis in plant ecophysiology is that natural selection optimizes traits like stomatal size, density, and distribution to maximize carbon gain relative to any costs in a given environmental context. In principle, spacing stomata to minimize the average distance between stomatal pores and chloroplasts within the mesophyll should increase carbon gain, all else being equal. However, reducing this distance to its absolute minimum may be constrained by developmental processes or the photosynthetic benefit may be too small to be ‘seen’ by natural selection (i.e. the selection coefficient is less than drift barrier *sensu* Sung et al. (2012)). We also consider that our definition of optimal may be incorrect because it is based on overly simplistic assumptions about leaf mesophyll structure.

We tested five related hypotheses about stomatal spacing in amphistomatous leaves using the model angiosperm *Arabidopsis thaliana* grown under different light intensities. First, we predicted that stomata on each surface are overdispersed relative to a random distribution, which should increase CO₂ supply. Stomata on each surface are overdispersed (Fig. 4), but are not ideally, uniformly patterned in an equilateral triangular grid as would be optimal to minimize CO₂ diffusion path length and equalize the area supplied by each stomate (Fig. 2). Second, we predicted that an optimal amphistomatous leaf has offset stomata such that stomata are more likely to appear on one leaf surface if there is not a stomata directly opposite it on the other surface as shown in Fig. 1. However, there is no evidence for coordination and the positions on each surface appear independent, regardless of light treatment (Fig. 5). Third, we predicted that plants respond plastically to higher light intensity by increasing stomatal density. *Arabidopsis* plants grown under high light had higher stomatal density than the same genotype grown under low and medium light intensity (Fig. 3). However, we found no support for our fourth prediction that stomatal patterning would be overdispersed at high light intensity (Fig. 4). Finally, we predicted that within leaf variation in stomatal size would correlate with stomatal spacing, as larger stomata can supply larger volumes of adjacent mesophyll. In all three light treatments, stomatal size positively co-varied with the stomatal zone, i.e. adjacent region of mesophyll that would be supplied by that stomate (Fig. 6).

Stomatal spacing on *A. thaliana* leaves partially supports our overall hypothesis that natural selection minimizes the average distance between stomata and chloroplasts, for a given overall stomatal density. There are three non-mutually exclusive hypotheses for why several of our predictions were wrong. First, our predictions must be wrong because they are based on overly simplistic assumption of a homogenous porous medium within the mesophyll. Real leaf mesophylls are spatially heterogenous and chloroplasts are distributed as discrete nodes. The intercellular air space conductance is determined by its porosity and tortuosity, both of which are heterogenous within the leaf. The palisade is typically less porous than the spongy mesophyll (e.g. Théroux-Rancourt et al. (2017)), which should impact the optimal patterning on stomata on ab- versus adaxial surfaces. Tortuosity is also systematically greater in the palisade in the lateral direction parallel to the leaf plane (Harwood et al., 2021). We might predict a greater coordination advantage of offset stomata by accounting for greater lateral tortuosity, but it is likely that benefit is still very small under realistic parameter space. Quantifying the patterns of heterogeneity in porosity, tortuosity, and other factors (Earles et al., 2018) using 3D imaging (e.g. Borsuk et al. (2022)) will be needed to generate more realistic hypotheses about optimal stomatal spacing.

Second, spatio-temporal variation of internal conditions within leaves and between stomatal responses may make uniform, coordinated stomatal surfaces less beneficial (Weyers and Lawson, 1997; Lawson et al., 1998; Lawson and Weyers, 1999). This is because our model assumes a uniform leaf, the internal conditions of which are periodic and solved empirically and therefore stable. Any horizontal concentration gradients due to environmental heterogeneity and variable induction times for interacting leaf processes may reduce the benefit of uniform stomatal patterning. Third, natural selection may be constrained by developmental processes that prevent phenotypes from reaching their adaptive optima. Stomatal development must be plastic to environmental cues interpreted through long distance and cell-to-cell signalling pathways (Pillitteri and Torii, 2012). This plasticity may come with the cost of being unable to orchestrate the development of an absolutely uniform stomatal grid. Fourth, the benefit of some traits may be of too little consequence to result in fitness differences large enough to respond to selection. We consider the plausibility of these alternative hypotheses below and present ideas for future work to test them.

We assume an idealized leaf epidermal and mesophyll structure that is homogenous and unconstrained by other tradeoffs. Real leaves not only provide pathways for CO₂ diffusion, but must supply water, intercept light, and deter herbivores and pathogens. All of these competing processes also happen on different time scales, and can be observed as heterogeneity in stomatal density, aperture, and internal leaf conditions across the leaf at any given moment (Lawson et al., 1998; Lawson and Weyers, 1999). These competing interests result in heterogeneous epidermal and mesophyll structure that could alter predictions about optimal stomatal spacing. In order to maintain consistent leaf water potential across the lamina, stomatal density must be coordinated with vein density (Fiorin et al., 2016). Thus, stomatal spacing may be optimized not at the interstomatal level, but at a higher level, coordinating water transport and water loss. For example, the palisade mesophyll is more tightly packed than the spongy mesophyll as an adaptation to intercept light efficiently, so lateral diffusion may be more limiting in the adaxial portion of the leaf. This may explain why adaxial leaf surfaces have consistently higher dispersion indices than abaxial surfaces across all light treatments (Fig. 4). Future gas exchange models should incorporate heterogenous mesophyll structure and hydraulic traits such as veins.

We are not aware of a developmental pathway that ensures an idealized placement of stomata on the leaf surface. Rather, stomatal development is a dynamic process that must be plastic to environmental cues. Leaves develop based on short and long distance signalling pathways which relay information about incoming light, humidity, temperature, and surrounding stomata to developing leaf tissues (Pillitteri and Torii, 2012). Our results showing an intermediate level of dispersion in stomatal spacing may be best explained by these

developmental pathways which ensure the proper spacing of stomata, with an added random effect brought about by the necessity for plasticity in stomatal development (Fig. 4). However, deviations from ideal stomatal spacing may be compensated for by simultaneous and coordinated development of the IAS (Baillie and Fleming, 2020). The fact that stomata which supply a greater mesophyll volume tend to be larger suggests that plants may use coordinated development of multiple leaf anatomical features to compensate for nonideal stomatal spacing (Fig. 6).

In amphistomatous leaves, ideal stomatal spacing is complicated by a third dimension. Our gas exchange model demonstrates little photosynthetic gain from abaxial-adaxial stomatal coordination (Fig. 7). Even though lateral diffusion may limit photosynthesis (Morison et al., 2005), the marginal gain from optimally offsetting stomata is not sufficient to generate fitness differences relative to the strength of genetic drift (i.e. the drift-barrier). We can similarly extrapolate that an ideal, equilateral triangular stomatal spacing is only slightly better than a suboptimal pattern. Any benefit garnered by ideal stomatal spacing may be additionally offset by a cost to developmental flexibility in variable environments (Pillitteri and Torii, 2012; Baillie and Fleming, 2020). Explaining these observations as the result of weak selection is in tension with the finding that stomatal size and zone positively covary, which would suggest that small changes in lateral diffusion distance are significant. As described above, the positive correlation between stomatal size and zone may be explained by common developmental processes rather than as an adaptation to maximize CO₂ diffusion. In any case, there is no evidence for coordinated development of both leaf surfaces, and very little theoretical benefit to photosynthesis, except in marginal circumstances which are exceptionally rare in nature.

Our study corroborates previous studies which demonstrate that stomata are non-randomly distributed along the leaf surface as a result of developmental mechanisms such as spatially biased arrest of stomatal initials (Boetsch et al., 1995), oriented asymmetric cell division (Geisler et al., 2000), and cell cycle controls (Croxdale, 2000). We do not investigate the potential developmental pathways that influence stomatal dispersion in this study; however, they are important to consider as these pathways could limit plants from reaching a theoretical peak in the adaptive landscape: uniform stomatal patterning. Instead, as this study suggests, plants may simply compensate with higher stomatal density and by modulating stomatal size to the area that they supply with CO₂. To understand why stomata are not ideally dispersed, more modeling (with more realistic assumptions including vein density and IAS structure) should be done to estimate the photosynthetic properties of varying stomatal patterning. Additionally, genetic manipulation studies should attempt to create mutants with clustered and uniformly patterned stomata for a comparison of their photosynthetic traits. This could have important implications for maximizing assimilation rates in crops as most crop species are grown in high light where CO₂ is often limiting. In drought-prone environments, increased stomatal dispersion may increase water use efficiency by reducing the number of stomata needed to achieve the same internal CO₂ concentration, C_i . However, it would be necessary to account for many other differences between *A. thaliana* and crop leaves and canopies.

Our results suggest that after optimizing stomatal density and having developmental rules for spacing stomata relatively evenly, there may be limited gains to further optimization. Therefore, developmental constraints may be necessary to make sense of some features of stomatal spacing and distribution. The possibility that ideal stomatal spacing is not the “tallest” fitness peak must also be explored, as stomate size is demonstrated in this study to covary with mesophyll volume supplied with CO₂. This may be especially true in highly variable environments or in large tree species with sun and shade leaves where developmental cues may change rapidly. The temporal component, not considered here, could also have significant implications, as CO₂ may only be limiting to photosynthesis during short, relatively rare periods when all other conditions are ideal. In these cases, the theoretical benefits of ideal stomatal spacing are further diminished. Future exploration of these competing hypotheses would require more advanced modeling, additional exploration of IAS space development and its effects on gas exchange, both real and modeled, and knowledge about how often the species of interest is CO₂ limited across a range of natural settings. Despite these additional considerations, this study represents an important contribution to understanding the potential drivers of and limitations to stomatal anatomy in amphistomatous plants.

Data availability

Custom scripts are available on a GitHub repository (<https://github.com/cdmuir/stomata-spacing>) and archived on Zenodo: <https://doi.org/XXXX> Raw data are deposited on Dryad: <https://doi.org/10.5061/dryad.44j0zpcn6>

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Competing interests

The authors declare no competing interests.

Author contributions statement

JLW and CDM conceived of the project, analyzed data, and wrote the manuscript. GJD provided data. TNB contributed to model development and helped edit the manuscript.

References

- T. Aalto and E. Juurola. A three-dimensional model of CO₂ transport in airspaces and mesophyll cells of a silver birch leaf. *Plant, Cell & Environment*, 25(11):1399–1409, Nov. 2002. ISSN 01407791. doi: 10.1046/j.0016-8025.2002.00906.x. URL <http://doi.wiley.com/10.1046/j.0016-8025.2002.00906.x>.
- S. Bailey, R. G. Walters, S. Jansson, and P. Horton. Acclimation of *Arabidopsis thaliana* to the light environment: the existence of separate low light and high light responses. *Planta*, 213(5):794–801, Sept. 2001. ISSN 0032-0935. doi: 10.1007/s004250100556. URL <http://link.springer.com/10.1007/s004250100556>.
- A. L. Baillie and A. J. Fleming. The developmental relationship between stomata and mesophyll airspace. *New Phytologist*, 225(3):1120–1126, 2020. ISSN 1469-8137. doi: 10.1111/nph.16341. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/nph.16341>. [_eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/nph.16341](https://onlinelibrary.wiley.com/doi/pdf/10.1111/nph.16341).
- Y. Benjamini and Y. Hochberg. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1):289–300, 1995.
- J. Boetsch, J. Chin, and J. Croxdale. Arrest of Stomatal Initials in *Tradescantia* Is Linked to the Proximity of Neighboring Stomata and Results in the Arrested Initials Acquiring Properties of Epidermal Cells. *Developmental Biology*, 168(1):28–38, Mar. 1995. ISSN 00121606. doi: 10.1006/dbio.1995.1058. URL <https://linkinghub.elsevier.com/retrieve/pii/S0012160685710585>.
- A. M. Borsuk, A. B. Roddy, G. Th  roux-Rancourt, and C. R. Brodersen. Structural organization of the spongy mesophyll. *New Phytologist*, 234(3):946–960, May 2022. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.17971. URL <https://nph.onlinelibrary.wiley.com/doi/10.1111/nph.17971>.
- T. N. Buckley, L. Sack, and G. D. Farquhar. Optimal plant water economy. *Plant, Cell & Environment*, 40(6):881–896, June 2017. ISSN 0140-7791, 1365-3040. doi: 10.1111/pce.12823. URL <https://onlinelibrary.wiley.com/doi/10.1111/pce.12823>.
- P.-C. B  rkner. **brms** : An R Package for Bayesian Multilevel Models Using Stan. *Journal of Statistical Software*, 80(1), 2017. ISSN 1548-7660. doi: 10.18637/jss.v080.i01. URL <http://www.jstatsoft.org/v80/i01/>.
- P.-C. B  rkner. Advanced Bayesian Multilevel Modeling with the R Package brms. *The R Journal*, 10(1):395, 2018. ISSN 2073-4859. doi: 10.32614/RJ-2018-017. URL <https://journal.r-project.org/archive/2018/RJ-2018-017/index.html>.
- D. B  ssis, U. von Groll, J. Fisahn, and T. Altmann. Stomatal aperture can compensate altered stomatal density in *Arabidopsis thaliana* at growth light conditions. *Functional Plant Biology*, 33(11):1037, 2006. ISSN 1445-4408. doi: 10.1071/FP06078. URL <http://www.publish.csiro.au/?paper=FP06078>.
- H. S. Callahan and M. Pigliucci. Shade-induced plasticity and its ecological significance in wild populations of *Arabidopsis thaliana*. *Ecology*, 83(7):1965–1980, July 2002. ISSN 0012-9658. doi: 10.1890/0012-9658(2002)083[1965:SIPAIE]2.0.CO;2. URL [http://doi.wiley.com/10.1890/0012-9658\(2002\)083\[1965:SIPAIE\]2.0.CO;2](http://doi.wiley.com/10.1890/0012-9658(2002)083[1965:SIPAIE]2.0.CO;2).
- M. Carri  , H. M. Cabrera, M. . Conesa, R. E. Coopman, C. Douthe, J. Gago, A. Gall  , J. Galm  s, M. Ribas-Carbo, M. Tom  s, and J. Flexas. Diffusional limitations explain the lower photosynthetic capacity of ferns as compared with angiosperms in a common garden study: Photosynthetic comparison in ferns and angiosperms. *Plant, Cell & Environment*, 38(3):448–460, Mar. 2015. ISSN 01407791. doi: 10.1111/pce.12402. URL <https://onlinelibrary.wiley.com/doi/10.1111/pce.12402>.
- P. J. Clark and F. C. Evans. Distance to nearest neighbor as a measure of spatial relationships in populations. *Ecology*, 35(4):445–453, Oct. 1954. ISSN 00129658. doi: 10.2307/1931034. URL <http://doi.wiley.com/10.2307/1931034>.

- I. Cowan and G. Farquhar. Stomatal function in relation to leaf metabolism and environment. *Symposia of the Society for Experimental Biology*, 31:471–505, 1977.
- J. L. Croxdale. Stomatal patterning in angiosperms. *American Journal of Botany*, 87(8):1069–1080, Aug. 2000. ISSN 00029122. doi: 10.2307/2656643. URL <http://doi.wiley.com/10.2307/2656643>.
- H. J. de Boer, C. A. Price, F. Wagner-Cremer, S. C. Dekker, P. J. Franks, and E. J. Veneklaas. Optimal allocation of leaf epidermal area for gas exchange. *New Phytologist*, 210(4):1219–1228, 2016. ISSN 1469-8137. doi: 10.1111/nph.13929. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/nph.13929>. eprint: <https://nph.onlinelibrary.wiley.com/doi/pdf/10.1111/nph.13929>.
- R. M. Deans, T. J. Brodribb, F. A. Busch, and G. D. Farquhar. Optimization can provide the fundamental link between leaf photosynthesis, gas exchange and water relations. *Nature Plants*, 6(9):1116–1125, Sept. 2020. ISSN 2055-0278. doi: 10.1038/s41477-020-00760-6. URL <https://www.nature.com/articles/s41477-020-00760-6>.
- G. J. Dow, J. A. Berry, and D. C. Bergmann. The physiological importance of developmental mechanisms that enforce proper stomatal spacing in *rabidopsis thaliana*. *New Phytologist*, 201(4):1205–1217, Mar. 2014. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.12586. URL <https://onlinelibrary.wiley.com/doi/10.1111/nph.12586>.
- G. J. Dow, J. A. Berry, and D. C. Bergmann. Disruption of stomatal lineage signaling or transcriptional regulators has differential effects on mesophyll development, but maintains coordination of gas exchange. *New Phytologist*, 216(1):69–75, Oct. 2017. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.14746. URL <https://nph.onlinelibrary.wiley.com/doi/10.1111/nph.14746>.
- P. L. Drake, H. J. Boer, S. J. Schymanski, and E. J. Veneklaas. Two sides to every leaf: water and CO_2 transport in hypostomatous and amphistomatous leaves. *New Phytologist*, 222(3):1179–1187, May 2019. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.15652. URL <https://onlinelibrary.wiley.com/doi/10.1111/nph.15652>.
- J. M. Earles, G. Th  roux-Rancourt, M. E. Gilbert, A. J. McElrone, and C. R. Brodersen. Excess diffuse light absorption in upper mesophyll limits CO_2 drawdown and depresses photosynthesis. *Plant Physiology*, 174(2):1082–1096, June 2017. ISSN 0032-0889, 1532-2548. doi: 10.1104/pp.17.00223. URL <https://academic.oup.com/plphys/article/174/2/1082-1096/6117304>.
- J. M. Earles, G. Theroux-Rancourt, A. B. Roddy, M. E. Gilbert, A. J. McElrone, and C. R. Brodersen. Beyond Porosity: 3D Leaf Intercellular Airspace Traits That Impact Mesophyll Conductance. *Plant Physiology*, 178(1):148–162, Sept. 2018. ISSN 0032-0889, 1532-2548. doi: 10.1104/pp.18.00550. URL <https://academic.oup.com/plphys/article/178/1/148-162/6116629>.
- J. R. Evans, R. Kaldenhoff, B. Genty, and I. Terashima. Resistances along the CO_2 diffusion pathway inside leaves. *Journal of Experimental Botany*, 60(8):2235–2248, May 2009. ISSN 0022-0957, 1460-2431. doi: 10.1093/jxb/erp117. URL <https://academic.oup.com/jxb/article-lookup/doi/10.1093/jxb/erp117>.
- J. S. Evans and M. A. Murphy. *spatialEco*. 2023. URL <https://github.com/jeffreyevans/spatialEco>.
- G. D. Farquhar, S. von Caemmerer, and J. A. Berry. A biochemical model of photosynthetic CO_2 assimilation in leaves of C3 species. *Planta*, 149(1):78–90, June 1980. ISSN 0032-0935, 1432-2048. doi: 10.1007/BF00386231. URL <http://link.springer.com/10.1007/BF00386231>.
- L. Fiorin, T. J. Brodribb, and T. Anfodillo. Transport efficiency through uniformity: organization of veins and stomata in angiosperm leaves. *New Phytologist*, 209(1):216–227, 2016. ISSN 1469-8137. doi: 10.1111/nph.13577. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/nph.13577>. eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1111/nph.13577>.
- P. J. Franks, I. J. Leitch, E. M. Ruzs  la, A. M. Hetherington, and D. J. Beerling. Physiological framework for adaptation of stomata to CO_2 from glacial to future concentrations. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367(1588):537–546, Feb. 2012. ISSN 0962-8436, 1471-2970. doi: 10.1098/rstb.2011.0270. URL <https://royalsocietypublishing.org/doi/10.1098/rstb.2011.0270>.
- A. P. Gay and R. G. Hurd. The influence of light on stomatal density in the tomato. *New Phytologist*, 75(1):37–46, July 1975. ISSN 0028-646X, 1469-8137. doi: 10.1111/j.1469-8137.1975.tb01368.x. URL <https://onlinelibrary.wiley.com/doi/10.1111/j.1469-8137.1975.tb01368.x>.
- M. Geisler, J. Nadeau, and F. D. Sack. Oriented Asymmetric Divisions That Generate the Stomatal Spacing Pattern in Arabidopsis Are Disrupted by the *too many mouths* Mutation. *The Plant Cell*, 12(11):2075–2086, Nov. 2000. ISSN 1040-4651, 1532-298X. doi: 10.1105/tpc.12.11.2075. URL <https://academic.oup.com/plcell/article/12/11/2075-2086/6009369>.
- E. L. Harrison, L. Arce Cubas, J. E. Gray, and C. Hepworth. The influence of stomatal morphology and distribution on photosynthetic gas exchange. *The Plant Journal*, 101(4):768–779, Feb. 2020. ISSN 0960-7412, 1365-313X. doi: 10.1111/tpj.14560. URL <https://onlinelibrary.wiley.com/doi/10.1111/tpj.14560>.

- R. Harwood, G. Th  roux-Rancourt, and M. M. Barbour. Understanding airspace in leaves: 3D anatomy and directional tortuosity. *Plant, Cell & Environment*, page pce.14079, May 2021. ISSN 0140-7791, 1365-3040. doi: 10.1111/pce.14079. URL <https://onlinelibrary.wiley.com/doi/10.1111/pce.14079>.
- R. A. Hijmans. *raster: Geographic Data Analysis and Modeling*. 2023. URL <https://CRAN.R-project.org/package=raster>.
- G. J. Jordan, R. J. Carpenter, and T. J. Brodribb. Using fossil leaves as evidence for open vegetation. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 395:168–175, Feb. 2014. ISSN 00310182. doi: 10.1016/j.palaeo.2013.12.035. URL <https://linkinghub.elsevier.com/retrieve/pii/S0031018213005725>.
- G. J. Jordan, R. J. Carpenter, A. Koutoulis, A. Price, and T. J. Brodribb. Environmental adaptation in stomatal size independent of the effects of genome size. *New Phytologist*, 205(2):608–617, Jan. 2015. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.13076. URL <https://onlinelibrary.wiley.com/doi/10.1111/nph.13076>.
- E. Kaiser, A. Morales, J. Harbinson, E. Heuvelink, A. E. Prinzenberg, and L. F. M. Marcelis. Metabolic and diffusional limitations of photosynthesis in fluctuating irradiance in *Arabidopsis thaliana*. *Scientific Reports*, 6(1):31252, Aug. 2016. ISSN 2045-2322. doi: 10.1038/srep31252. URL <http://www.nature.com/articles/srep31252>.
- O. L. Lange, R. L. sch, E. D. Schulze, and L. Kappen. Responses of stomata to changes in humidity. *Planta*, 100(1):76–86, 1971. ISSN 0032-0935, 1432-2048. doi: 10.1007/BF00386887. URL <http://link.springer.com/10.1007/BF00386887>.
- T. Lawson and J. Weyers. Spatial and temporal variation in gas exchange over the lower surface of *Phaseolus vulgaris* L. primary leaves. *Journal of Experimental Botany*, 50(337):1381–1391, Aug. 1999. ISSN 0022-0957. doi: 10.1093/jxb/50.337.1381. URL <https://doi.org/10.1093/jxb/50.337.1381>.
- T. Lawson, J. Weyers, and R. A’Brook. The nature of heterogeneity in the stomatal behaviour of *Phaseolus vulgaris* L. primary leaves. *Journal of Experimental Botany*, 49(325):1387–1395, Aug. 1998. ISSN 0022-0957. doi: 10.1093/jxb/49.325.1387. URL <https://doi.org/10.1093/jxb/49.325.1387>.
- R. Lee and D. M. Gates. Diffusion resistance in leaves as related to their stomatal anatomy and micro-structure. *American Journal of Botany*, 51(9):963–975, Oct. 1964. ISSN 0002-9122, 1537-2197. doi: 10.1002/j.1537-2197.1964.tb06725.x. URL <https://onlinelibrary.wiley.com/doi/10.1002/j.1537-2197.1964.tb06725.x>.
- P. Lehmann and D. Or. Effects of stomata clustering on leaf gas exchange. *New Phytologist*, 207(4):1015–1025, Sept. 2015. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.13442. URL <https://onlinelibrary.wiley.com/doi/10.1111/nph.13442>.
- C. Lehmeier, R. Pajor, M. R. Lundgren, A. Mathers, J. Sloan, M. Bauch, A. Mitchell, C. Bellasio, A. Green, D. Bouyer, A. Schnittger, C. Sturrock, C. P. Osborne, S. Rolfe, S. Mooney, and A. J. Fleming. Cell density and airspace patterning in the leaf can be manipulated to increase leaf photosynthetic capacity. *The Plant Journal*, 92(6):981–994, Dec. 2017. ISSN 0960-7412, 1365-313X. doi: 10.1111/tpj.13727. URL <https://onlinelibrary.wiley.com/doi/10.1111/tpj.13727>.
- R. V. Lenth. *emmeans: Estimated Marginal Means, aka Least-Squares Means*. 2023. URL <https://CRAN.R-project.org/package=emmeans>.
- C. Liu, C. D. Muir, Y. Li, L. Xu, M. Li, J. Zhang, H. J. de Boer, L. Sack, X. Han, G. Yu, and N. He. Scaling between stomatal size and density in forest plants. preprint, *Plant Biology*, Apr. 2021. URL <http://biorxiv.org/lookup/doi/10.1101/2021.04.25.441252>.
- J. Males and H. Griffiths. Stomatal Biology of CAM Plants. *Plant Physiology*, 174(2):550–560, June 2017. ISSN 0032-0889, 1532-2548. doi: 10.1104/pp.17.00114. URL <https://academic.oup.com/plphys/article/174/2/550-560/6117326>.
- D. K. Manter. A/Ci curve analysis across a range of woody plant species: influence of regression analysis parameters and mesophyll conductance. *Journal of Experimental Botany*, 55(408):2581–2588, Sept. 2004. ISSN 1460-2431. doi: 10.1093/jxb/erh260. URL <https://academic.oup.com/jxb/article-lookup/doi/10.1093/jxb/erh260>.
- S. A. McAdam and T. J. Brodribb. Linking Turgor with ABA Biosynthesis: Implications for Stomatal Responses to Vapor Pressure Deficit across Land Plants. *Plant Physiology*, 171(3):2008–2016, July 2016. ISSN 1532-2548. doi: 10.1104/pp.16.00380. URL <https://academic.oup.com/plphys/article/171/3/2008/6115478>.
- J. C. McElwain, C. Yi  tis, and T. Lawson. Using modern plant trait relationships between observed and theoretical maximum stomatal conductance and vein density to examine patterns of plant macroevolution. *New Phytologist*, 209(1):94–103, 2016. ISSN 1469-8137. doi: 10.1111/nph.13579. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/nph.13579>. _eprint: <https://nph.onlinelibrary.wiley.com/doi/pdf/10.1111/nph.13579>.
- M. Melotto, L. Zhang, P. R. Oblessuc, and S. Y. He. Stomatal Defense a Decade Later. *Plant Physiology*, 174(2):561–571, June 2017. ISSN 0032-0889, 1532-2548. doi: 10.1104/pp.16.01853. URL <http://www.plantphysiol.org/lookup/doi/10.1104/pp.16.01853>.

- J. I. Morison, E. Gallouët, T. Lawson, G. Cornic, R. Herbin, and N. R. Baker. Lateral diffusion of CO₂ in leaves is not sufficient to support photosynthesis. *Plant Physiology*, 139(1):254–266, Sept. 2005. ISSN 1532-2548, 0032-0889. doi: 10.1104/pp.105.062950. URL <https://academic.oup.com/plphys/article/139/1/254/6113368>.
- J. I. L. Morison and T. Lawson. Does lateral gas diffusion in leaves matter? *Plant, Cell & Environment*, 30(9):1072–1085, Sept. 2007. ISSN 01407791, 13653040. doi: 10.1111/j.1365-3040.2007.01685.x. URL <https://onlinelibrary.wiley.com/doi/10.1111/j.1365-3040.2007.01685.x>.
- K. A. Mott and O. Michaelson. Amphistomy as an adaptation to high light intensity in *Ambrosia cordifolia* (Compositae). *American Journal of Botany*, 78(1):76–79, Jan. 1991. ISSN 0002-9122, 1537-2197. doi: 10.1002/j.1537-2197.1991.tb12573.x. URL <https://onlinelibrary.wiley.com/doi/10.1002/j.1537-2197.1991.tb12573.x>.
- K. A. Mott, A. C. Gibson, and J. W. O’Leary. The adaptive significance of amphistomatic leaves. *Plant, Cell & Environment*, 5(6):455–460, Dec. 1982. ISSN 01407791. doi: 10.1111/1365-3040.ep11611750. URL <https://onlinelibrary.wiley.com/doi/10.1111/1365-3040.ep11611750>.
- C. D. Muir. Light and growth form interact to shape stomatal ratio among British angiosperms. *New Phytologist*, 218(1):242–252, Apr. 2018. ISSN 0028646X. doi: 10.1111/nph.14956. URL <https://onlinelibrary.wiley.com/doi/10.1111/nph.14956>.
- C. D. Muir. Is Amphistomy an Adaptation to High Light? Optimality Models of Stomatal Traits along Light Gradients. *Integrative and Comparative Biology*, 59(3):571–584, Sept. 2019. ISSN 1540-7063, 1557-7023. doi: 10.1093/icb/icz085. URL <https://academic.oup.com/icb/article/59/3/571/5505428>.
- C. D. Muir, M. . Conesa, J. Galmés, V. S. Pathare, P. Rivera, R. López Rodríguez, T. Terrazas, and D. Xiong. How important are functional and developmental constraints on phenotypic evolution? An empirical test with the stomatal anatomy of flowering plants. *The American Naturalist*, 201(6):794–812, June 2023. ISSN 0003-0147, 1537-5323. doi: 10.1086/723780. URL <https://www.journals.uchicago.edu/doi/10.1086/723780>.
- M. Murray, W. K. Soh, C. Yiotis, R. A. Spicer, T. Lawson, and J. C. McElwain. Consistent relationship between field-measured stomatal conductance and theoretical maximum stomatal conductance in C₃ woody angiosperms in four major biomes. *International Journal of Plant Sciences*, 181(1):142–154, Jan. 2020. ISSN 1058-5893, 1537-5315. doi: 10.1086/706260. URL <https://www.journals.uchicago.edu/doi/10.1086/706260>.
- M. Papanatsiou, A. Amtmann, and M. R. Blatt. Stomatal clustering in *Begonia* associates with the kinetics of leaf gaseous exchange and influences water use efficiency. *Journal of Experimental Botany*, 68(9):2309–2315, Apr. 2017. ISSN 0022-0957, 1460-2431. doi: 10.1093/jxb/erx072. URL <https://academic.oup.com/jxb/article-lookup/doi/10.1093/jxb/erx072>.
- D. F. Parkhurst. The Adaptive Significance of Stomatal Occurrence on One or Both Surfaces of Leaves. *Journal of Ecology*, 66(2):367–383, 1978. ISSN 0022-0477. doi: 10.2307/2259142. URL <https://www.jstor.org/stable/2259142>.
- D. F. Parkhurst. Diffusion of CO₂ and other gases inside leaves. *New Phytologist*, 126(3):449–479, 1994. URL <http://www.jstor.org/stable/2557929>.
- D. F. Parkhurst and K. A. Mott. Intercellular Diffusion Limits to CO₂ Uptake in Leaves: Studies in Air and Helox. *Plant Physiology*, 94(3):1024–1032, Nov. 1990. ISSN 0032-0889, 1532-2548. doi: 10.1104/pp.94.3.1024. URL <https://academic.oup.com/plphys/article/94/3/1024-1032/6088593>.
- R. Pieruschka. Lateral gas diffusion inside leaves. *Journal of Experimental Botany*, 56(413):857–864, Jan. 2005. ISSN 1460-2431. doi: 10.1093/jxb/eri072. URL <https://academic.oup.com/jxb/article-lookup/doi/10.1093/jxb/eri072>.
- R. Pieruschka, U. Schurr, M. Jensen, W. F. Wolff, and S. Jahnke. Lateral diffusion of CO₂ from shaded to illuminated leaf parts affects photosynthesis inside homobaric leaves. *New Phytologist*, 169(4):779–788, Feb. 2006. ISSN 0028-646X, 1469-8137. doi: 10.1111/j.1469-8137.2005.01605.x. URL <https://onlinelibrary.wiley.com/doi/10.1111/j.1469-8137.2005.01605.x>.
- L. J. Pillitteri and K. U. Torii. Mechanisms of Stomatal Development. *Annual Review of Plant Biology*, 63(1):591–614, 2012. doi: 10.1146/annurev-arplant-042811-105451. URL <https://doi.org/10.1146/annurev-arplant-042811-105451>. [_eprint: https://doi.org/10.1146/annurev-arplant-042811-105451](https://doi.org/10.1146/annurev-arplant-042811-105451).
- K. S. Pollard, S. Dudoit, and M. J. Van Der Laan. Multiple Testing Procedures: the multtest Package and Applications to Genomics. In W. Wong, M. Gail, K. Krickeberg, A. Tsiatis, J. Samet, R. Gentleman, V. J. Carey, W. Huber, R. A. Irizarry, and S. Dudoit, editors, *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*, pages 249–271. Springer New York, New York, NY, 2005. ISBN 978-0-387-25146-2 978-0-387-29362-2. doi: 10.1007/0-387-29362-0_15. URL http://link.springer.com/10.1007/0-387-29362-0_15. Series Title: Statistics for Biology and Health.

- H. Poorter, . Niinemets, N. Ntagkas, A. Siebenkäs, M. Mäenpää, S. Matsubara, and T. L. Pons. A meta-analysis of plant responses to light intensity for 70 traits ranging from molecules to whole plant performance. *New Phytologist*, 223(3):1073–1105, 2019. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.15754. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/nph.15754>.
- A. B. Roddy, G. Thérroux-Rancourt, T. Abbo, J. W. Benedetti, C. R. Brodersen, M. Castro, S. Castro, A. B. Gilbride, B. Jensen, G.-F. Jiang, J. A. Perkins, S. D. Perkins, J. Loureiro, Z. Syed, R. A. Thompson, S. E. Kuebbing, and K. A. Simonin. The Scaling of Genome Size and Cell Size Limits Maximum Rates of Photosynthesis with Implications for Ecological Strategies. *International Journal of Plant Sciences*, 181(1):75–87, Jan. 2020. ISSN 1058-5893, 1537-5315. doi: 10.1086/706186. URL <https://www.journals.uchicago.edu/doi/10.1086/706186>.
- D. Royer. Stomatal density and stomatal index as indicators of paleoatmospheric CO₂ concentration. *Review of Palaeobotany and Palynology*, 114(1-2):1–28, Mar. 2001. ISSN 00346667. doi: 10.1016/S0034-6667(00)00074-9. URL <https://linkinghub.elsevier.com/retrieve/pii/S0034666700000749>.
- T. Sachs. The Developmental Origin of Stomata Pattern in Crinum. *Botanical Gazette*, 135(4):314–318, Dec. 1974. ISSN 0006-8071. doi: 10.1086/336767. URL <https://www.journals.uchicago.edu/doi/10.1086/336767>.
- L. Sack and T. N. Buckley. The Developmental Basis of Stomatal Density and Flux. *Plant Physiology*, 171(4):2358–2363, Aug. 2016. ISSN 1532-2548. doi: 10.1104/pp.16.00476. URL <https://academic.oup.com/plphys/article/171/4/2358/6115373>.
- C. A. Schneider, W. S. Rasband, and K. W. Eliceiri. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7):671–675, July 2012. ISSN 1548-7091, 1548-7105. doi: 10.1038/nmeth.2089. URL <http://www.nature.com/articles/nmeth.2089>.
- P.-G. Schoch, C. Zinsou, and M. Sibi. Dependence of the Stomatal Index on Environmental Factors during Stomatal Differentiation in Leaves of *Vigna sinensis* L.: 1. EFFECT OF LIGHT INTENSITY. *Journal of Experimental Botany*, 31(5):1211–1216, 1980. ISSN 0022-0957, 1460-2431. doi: 10.1093/jxb/31.5.1211. URL <https://academic.oup.com/jxb/article-lookup/doi/10.1093/jxb/31.5.1211>.
- J. S. Sperry, M. D. Venturas, W. R. L. Anderegg, M. Mencuccini, D. S. Mackay, Y. Wang, and D. M. Love. Predicting stomatal responses to the environment from the optimization of photosynthetic gain and hydraulic cost: A stomatal optimization model. *Plant, Cell & Environment*, 40(6):816–830, June 2017. ISSN 01407791. doi: 10.1111/pce.12852. URL <https://onlinelibrary.wiley.com/doi/10.1111/pce.12852>.
- Stan Development Team. *Stan Modeling Language Users Guide and Reference Manual*. 2023. URL <https://mc-stan.org>. version 2.31.0.
- W. Sung, M. S. Ackerman, S. F. Miller, T. G. Doak, and M. Lynch. Drift-barrier hypothesis and mutation-rate evolution. *Proceedings of the National Academy of Sciences*, 109(45):18488–18492, Nov. 2012. ISSN 0027-8424, 1091-6490. doi: 10.1073/pnas.1216223109. URL <https://pnas.org/doi/full/10.1073/pnas.1216223109>.
- G. Thérroux-Rancourt, J. M. Earles, M. E. Gilbert, M. A. Zwieniecki, C. K. Boyce, A. J. McElrone, and C. R. Brodersen. The bias of a two-dimensional view: comparing two-dimensional and three-dimensional mesophyll surface area estimates using noninvasive imaging. *New Phytologist*, 215(4):1609–1622, Sept. 2017. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.14687. URL <https://onlinelibrary.wiley.com/doi/10.1111/nph.14687>.
- A. Vehtari, A. Gelman, D. Simpson, B. Carpenter, and P.-C. Bürkner. Rank-Normalization, Folding, and Localization: An Improved R² for Assessing Convergence of MCMC (with Discussion). *Bayesian Analysis*, 16(2), June 2021. ISSN 1936-0975. doi: 10.1214/20-BA1221. URL <https://projecteuclid.org/journals/bayesian-analysis/volume-16/issue-2/Rank-Normalization-Folding-and-Localization--An-Improved-R2-for/10.1214/20-BA1221.full>.
- S. Wall, S. Vialet-Chabrand, P. Davey, J. Van Rie, A. Galle, J. Cockram, and T. Lawson. Stomata on the abaxial and adaxial leaf surfaces contribute differently to leaf gas exchange and photosynthesis in wheat. *New Phytologist*, 235(5):1743–1756, 2022. ISSN 1469-8137. doi: 10.1111/nph.18257. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/nph.18257>. eprint: <https://nph.onlinelibrary.wiley.com/doi/pdf/10.1111/nph.18257>.
- Y. Wang, J. S. Sperry, W. R. L. Anderegg, M. D. Venturas, and A. T. Trugman. A theoretical and empirical assessment of stomatal optimization modeling. *New Phytologist*, 227(2):311–325, July 2020. ISSN 0028-646X, 1469-8137. doi: 10.1111/nph.16572. URL <https://onlinelibrary.wiley.com/doi/10.1111/nph.16572>.
- J. D. Weyers and T. Lawson. Heterogeneity in Stomatal Characteristics. In *Advances in Botanical Research*, volume 26, pages 317–352. Elsevier, 1997. ISBN 978-0-12-005926-3. doi: 10.1016/S0065-2296(08)60124-X. URL <https://linkinghub.elsevier.com/retrieve/pii/S006522960860124X>.

-
- 547 F. I. Woodward. Stomatal numbers are sensitive to increases in CO₂ from pre-industrial levels. *Nature*, 327(6123):617–618, June 1987.
548 ISSN 0028-0836, 1476-4687. doi: 10.1038/327617a0. URL <http://www.nature.com/articles/327617a0>.
- 549 N. Wuyts, J.-C. Palauqui, G. Conejero, J.-L. Verdeil, C. Granier, and C. Massonnet. High-contrast three-dimensional imaging of the
550 *Arabidopsis* leaf enables the analysis of cell dimensions in the epidermis and mesophyll. *Plant Methods*, 6(1):17, Dec. 2010. ISSN
551 1746-4811. doi: 10.1186/1746-4811-6-17. URL <https://plantmethods.biomedcentral.com/articles/10.1186/1746-4811-6-17>.
- 552 Yi Gan, Lei Zhou, Zhong-Ji Shen, Zhu-Xia Shen, Yi-Qiong Zhang, and Gen-Xuan Wang. Stomatal clustering, a new marker for
553 environmental perception and adaptation in terrestrial plants. *Botanical Studies*, 51(3):325–336, July 2010. ISSN 1817406X. URL
554 <https://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=60102322&site=ehost-live>.

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Ideal Stomatal Patterning

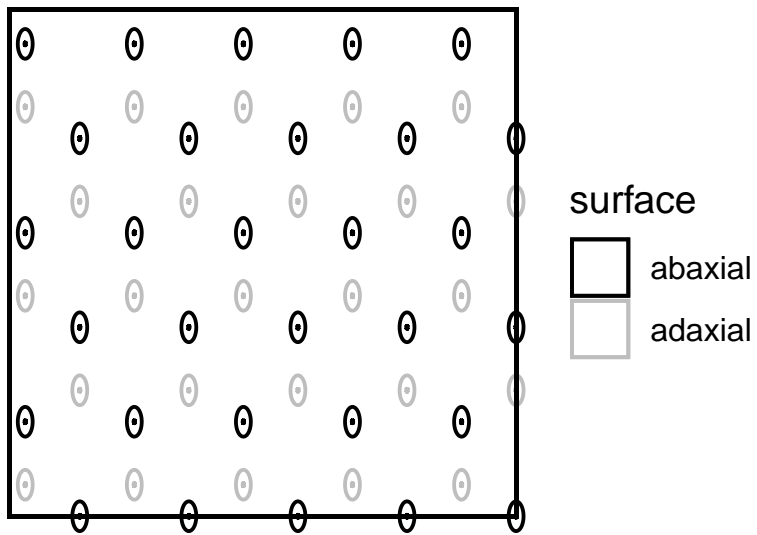


Fig. 1: Idealized amphistomatous stomatal grid with uniform stomatal patterning and perfect abaxial-adaxial coordination.

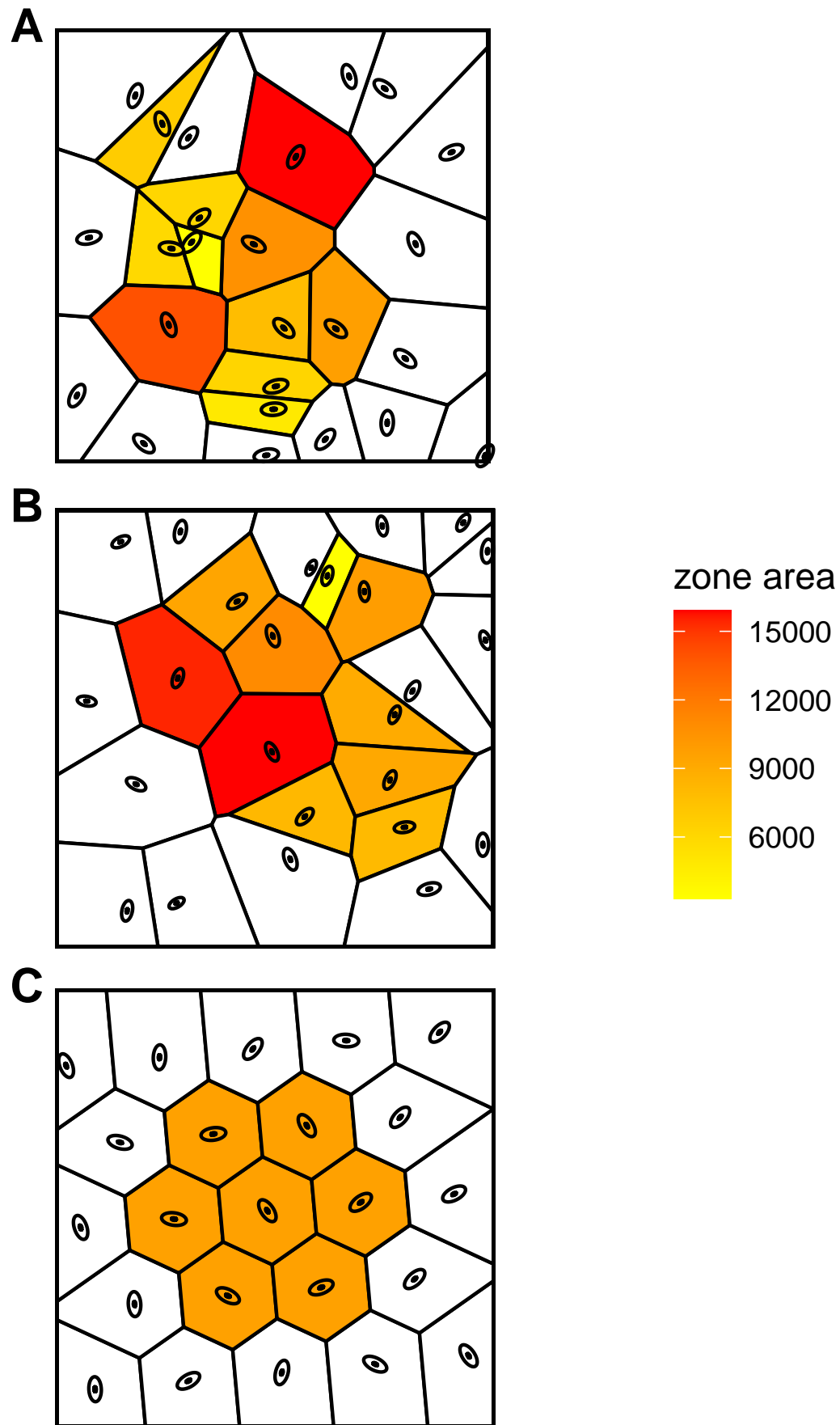


Fig. 2: Examples of synthetic and real leaf surfaces. A) Uniform random synthetic leaf surface; B) Example of real leaf surface; C) Uniformly distributed synthetic leaf surface. The zone defined by each stomate was calculated with voronoi tessellation and correlated with stomatal length in real leaves.

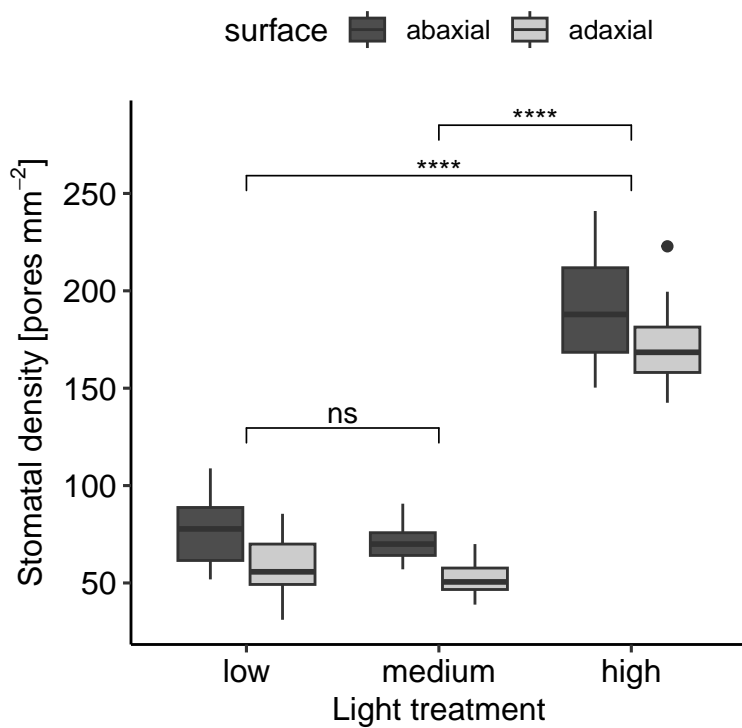


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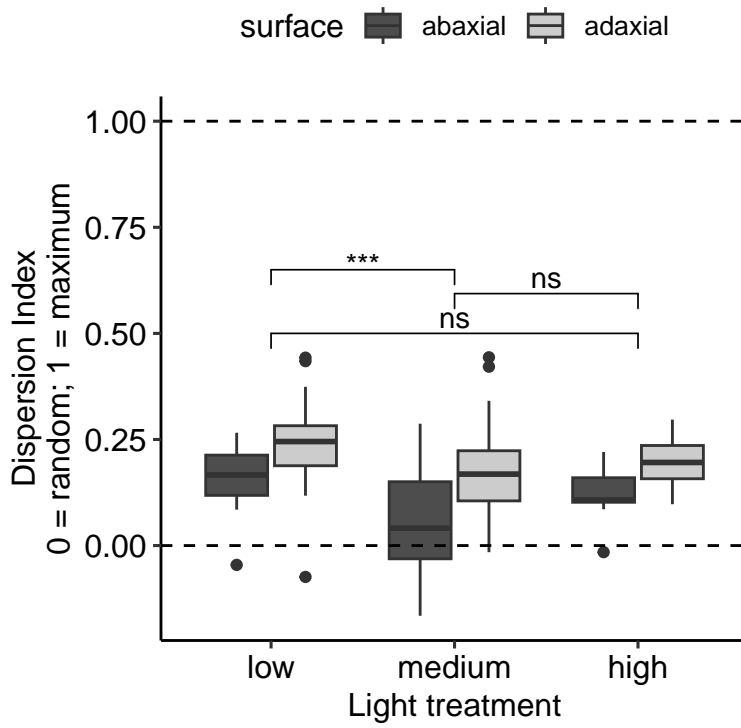


Fig. 4: Stomata are more dispersed than expected under the null model of random patterning (dispersion index = 0) but far from a distribution that maximizes distance between stomata (dispersion index = 1; uniform patterning). We determined statistical significance between light treatments using Tukey post-hoc tests. * $0.05 > P \geq 0.01$; ** $0.01 > P \geq 0.001$; *** $0.0001 > P \geq 0.0001$; *** $P < 0.0001$.

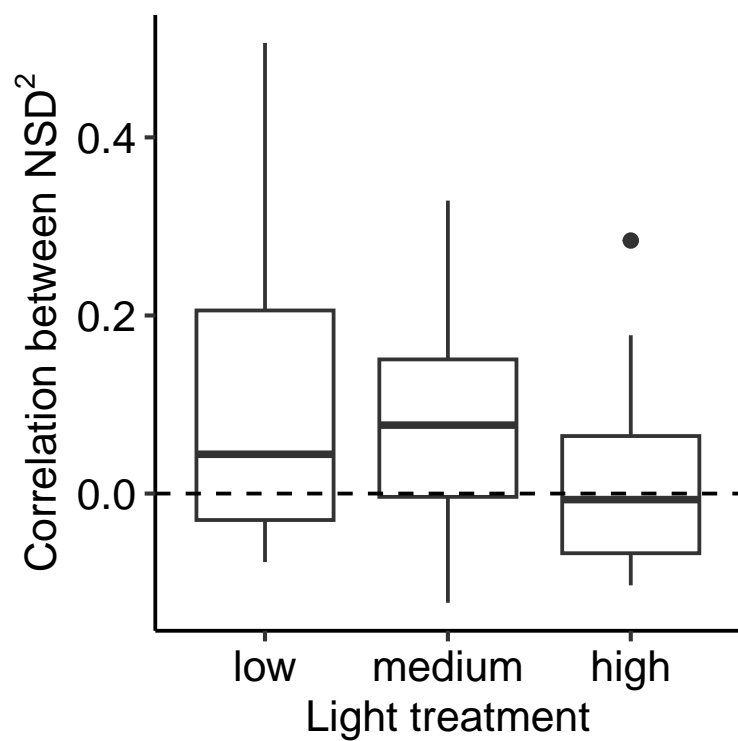


Fig. 5: Pixel-wise correlation between near stomatal distance (NSD) squared on paired abaxial and adaxial leaf surfaces. Dashed line indicates zero correlation. Weak positive correlations are not significantly different from zero after correcting for multiple comparisons. The correlation does not differ among light treatments.

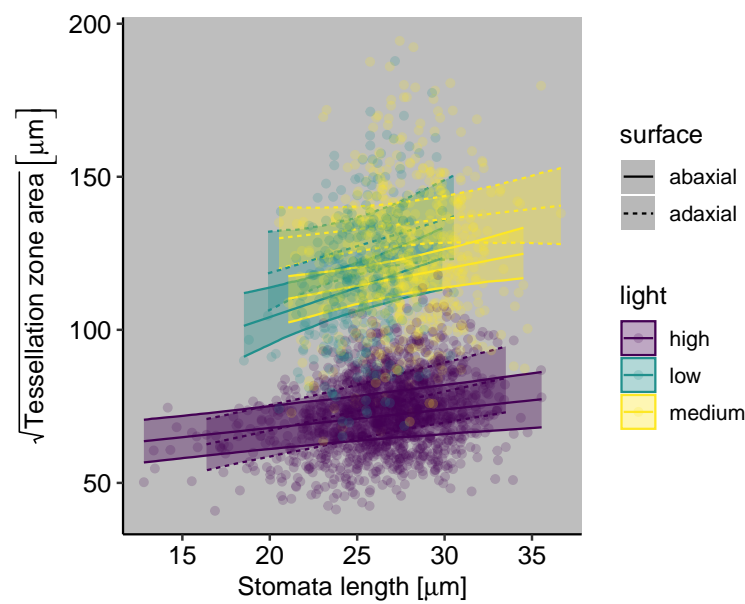


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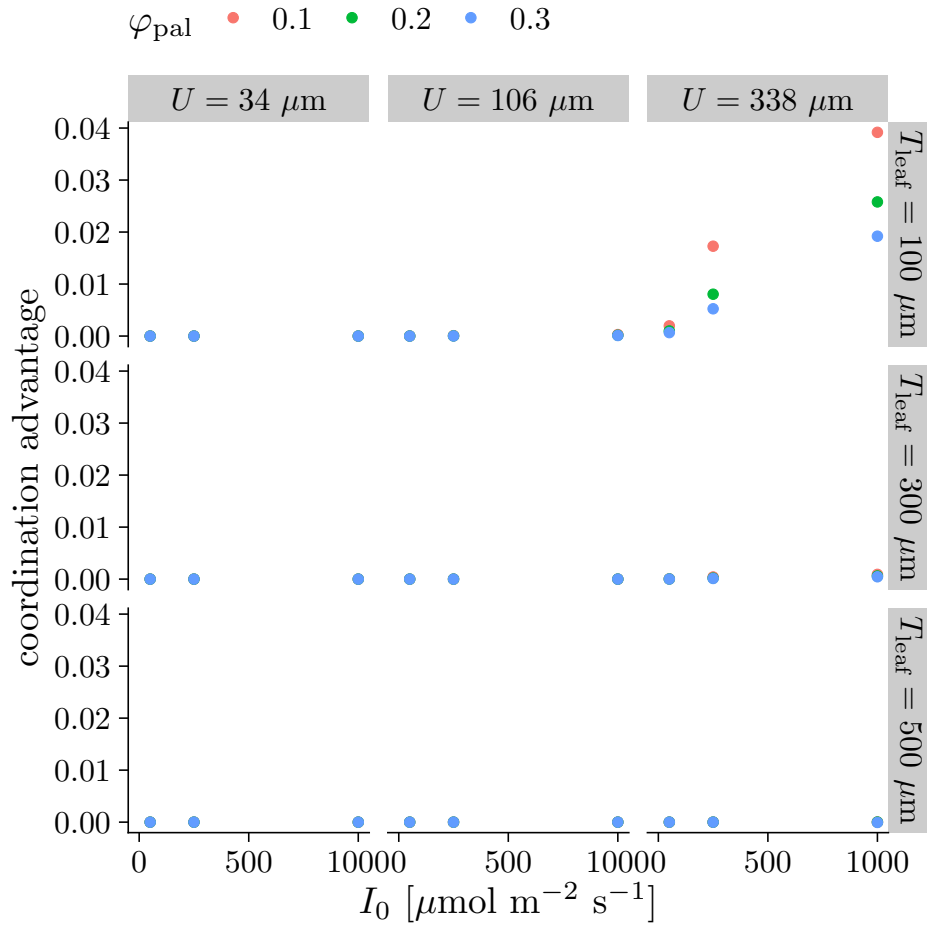


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