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Amphistomy increases leaf photosynthesis more in coastal than montane plants of Hawaiian 'ilima (*Sida fallax*)

Genevieve Triplett[1] | Thomas N. Buckley[2] | Christopher D. Muir[1,3]

[1]School of Life Sciences, University of Hawai'i Mānoa, Honolulu, HI 96822, USA

[2]Department of Plant Sciences, University of California, Davis, CA 95616, USA

[3]Department of Botany, University of Wisconsin, Madison, WI 53706, USA

Correspondence

Christopher D. Muir, School of Life Sciences, University of Hawai'i Mānoa, Honolulu, HI 96822, USA.
Email: cdmuir@wisc.edu

Abstract

Premise: The adaptive significance of amphistomy (stomata on both upper and lower leaf surfaces) is unresolved. A widespread association between amphistomy and open, sunny habitats suggests the adaptive benefit of amphistomy may be greatest in these contexts, but this hypothesis has not been tested experimentally. Understanding amphistomy informs its potential as a target for crop improvement and paleoenvironment reconstruction.

Methods: We developed a method to quantify "amphistomy advantage" (AA) as the log-ratio of photosynthesis in an amphistomatous leaf to that of the same leaf but with gas exchange blocked through the upper surface (pseudohypostomy). Humidity modulated stomatal conductance and thus enabled comparing photosynthesis at the same total stomatal conductance. We estimated AA and leaf traits in six coastal (open, sunny) and six montane (closed, shaded) populations of the indigenous Hawaiian species 'ilima (*Sida fallax*).

Results: Coastal 'ilima leaves benefit 4.04 times more from amphistomy than montane leaves. Evidence was equivocal with respect to two hypotheses: (1) that coastal leaves benefit more because they are thicker and have lower CO_2 conductance through the internal airspace and (2) that they benefit more because they have similar conductance on each surface, as opposed to most conductance being through the lower surface.

Conclusions: This is the first direct experimental evidence that amphistomy increases photosynthesis, consistent with the hypothesis that parallel pathways through upper and lower mesophyll increase CO_2 supply to chloroplasts. The prevalence of amphistomatous leaves in open, sunny habitats can partially be explained by the increased benefit of amphistomy in "sun" leaves, but the mechanistic basis remains uncertain.

KEYWORDS

amphistomy, Hawai'i, leaf, light, Malvaceae, photosynthesis, Sida fallax, *stomata*

Amphistomy, the presence of stomata on both lower and upper surfaces of broad leaves, should increase carbon gain by reducing the average diffusion pathlength between stomata and chloroplasts, yet paradoxically, this seemingly simple adaptation is uncommon in nature, and we don't know why. Understanding variation in stomatal traits like amphistomy is imperative because these tiny pores play an outsized ecological role in the global carbon and water cycles (Hetherington and Woodward, 2003; Berry et al., 2010). A widely applicable, accurate representation of how stomata mediate the relationship between CO_2 gained through photosynthesis and water lost through transpiration is essential to predict future climate using earth systems models (Jarvis, 1976; Ball et al., 1987; Collatz et al., 1991; Leuning, 1995; Sellers et al., 1997). Optimality models accurately predict the major cause of water loss,

stomatal conductance (g_{sw}), by assuming plants maximize carbon gain minus a cost of water (Cowan and Farquhar, 1977; Givnish, 1986; Medlyn et al., 2011; Lin et al., 2015; Franks et al., 2018; Deans et al., 2020; Franklin et al., 2020; Wang et al., 2017, 2020; Harrison et al., 2021). Despite the success of optimality modeling in predicting g_{sw}, the same modeling approach has so far failed to explain the rarity of amphistomatous leaves (Muir, 2019). This gap between theory and observations strongly implies that we remain ignorant about some key benefits and costs associated with stomata.

Where are amphistomatous leaves found, and why aren't they more common? Among terrestrial flowering plants, amphistomatous leaves are rarely found on woody plants and shade-tolerant herbs, but they are common in annual and perennial herbs from sunny habitats (Salisbury, 1928; Parkhurst, 1978; Mott et al., 1982; Peat and Fitter, 1994; Gibson, 1996; Jordan et al., 2014; Bucher et al., 2017; Muir, 2015, 2018). Even in resupinate leaves where the abaxial surface faces the sky, stomata develop on the lower adaxial surface (Lyshede, 2002). Exceptions to this general pattern include some arid woody plants that typically have vertically oriented, isobilateral leaves (Wood, 1934; Jordan et al., 2014; de Boer et al., 2016; Drake et al., 2019) and floating/amphibious leaves of aquatic plants (Kaul, 1976; Doll et al., 2021). The dearth of amphistomatous leaves should be quite surprising and has been described as one of the most important unsolved problems in the study of leaf structure–function relations despite some recent progress (Grubb, 1977, 2020).

Amphistomatous leaves should be common because, all else being equal, a leaf with a given number of stomata per area could increase its photosynthetic rate simply by apportioning approximately half its stomata to each surface (Parkhurst, 1978; Gutschick, 1984a, b). The key difference between a hypo- and amphistomatous leaf, holding all other factors constant, is that an amphistomatous leaf has two parallel diffusion paths through the internal airspace to any given chloroplast. Those airspaces pose a resistance for CO_2 diffusion, so CO_2 concentration drops as it approaches chloroplasts. Shorter pathways mean a smaller drop in CO_2 concentration. Thus, chloroplasts in amphistomatous leaves experience higher CO_2 concentrations than in hypostomatous leaves, thereby increasing photosynthesis. The airspace resistance (or its inverse, the airspace conductance, g_{ias}) is rarely measured directly, and there is disagreement between empirical (Parkhurst and Mott, 1990; Morison et al., 2005; Evans et al., 2009; Tomás et al., 2013; Earles et al., 2018; Šantrůček et al., 2019; Nobel, 2020; Harwood et al., 2021; Márquez et al., 2023) and theoretical models (Tholen and Zhu, 2011; Ho et al., 2016; Théroux-Rancourt et al., 2021). The g_{ias} in thin, porous leaves may be so large as to be inconsequential given much lower conductances for other components of the diffusion pathway, whereas the g_{ias} of thick leaves with little airspace may greatly hinder CO_2 diffusion to chloroplasts. Amphistomy should confer the largest photosynthetic benefit in leaves with intrinsically low

g_{ias}. The airspace conductance is one component of the overall mesophyll conductance, g_m, which is often strongly influenced by the chloroplast surface area exposed to airspace and mesophyll cell wall thickness (Evans et al., 2009; Gago et al., 2020; Flexas et al., 2021). Hence, thicker leaves may compensate for lower g_{ias} through increased chloroplast surface area exposed to airspace (Terashima et al., 2006), but will still benefit from amphistomy as long as g_{ias} is finite.

Amphistomy should also enhance photosynthesis when leaf boundary layer resistance is high, because apportioning total flux between two boundary layers rather than one results in a smaller CO_2 concentration drop between the atmosphere and stomata. A similar effect has been validated with a computer model and measurements for transpiration: Amphistomatous leaves lose somewhat more water for the same vapor pressure deficit and total g_{sw} (Foster and Smith, 1986), but the additional carbon gain should be enough to offset this cost under most realistic conditions (Muir, 2019). However, if minimal stomatal conductance is related to stomatal density (Drake et al., 2013; Márquez et al., 2022) and the upper boundary layer conductance is higher, then amphistomy could cause additional, unavoidable water loss.

The most promising adaptive hypothesis is that amphistomy is important for maximizing photosynthetic rate under high light. Mott et al. (1982, p. 455) proposed that "plants with a high photosynthetic capacity, living in full-sun environments, and experiencing rapidly fluctuating or continuously available soil water" would benefit most, in terms of increased carbon gain, from having amphistomatous leaves. As described above, herbs from sunny habitats are often amphistomatous. Most variation in stomatal density ratio (SR, the ratio of stomatal density between the upper and lower surfaces) among species is assumed to be genetic, but there is also putatively adaptive plasticity in response to light. Leaves of *Ambrosia cordifolia*, a desert perennial herb, are hypostomatous under low light (photosynthetic photon flux density, PPFD = 110 µmol m^{-2} s^{-1}) but develop ≈20% of their stomata on the upper surface under high light (1700 µmol m^{-2} s^{-1}) (Mott and Michaelson, 1991). Similarly, *Solanum lycopersicum* leaves are hypostomatous when grown in the shade but develop ≈20% of their stomata on the upper surface grown under high light-intensity (Gay and Hurd, 1975). Adult leaves of *Eucalyptus globulus* are amphistomatous, but the proportion of adaxial stomata increases from ≈10–20% under low light to ≈30–40% under high light (James and Bell, 2001). In summary, both genetic and plastic responses evince a widespread association between light and SR.
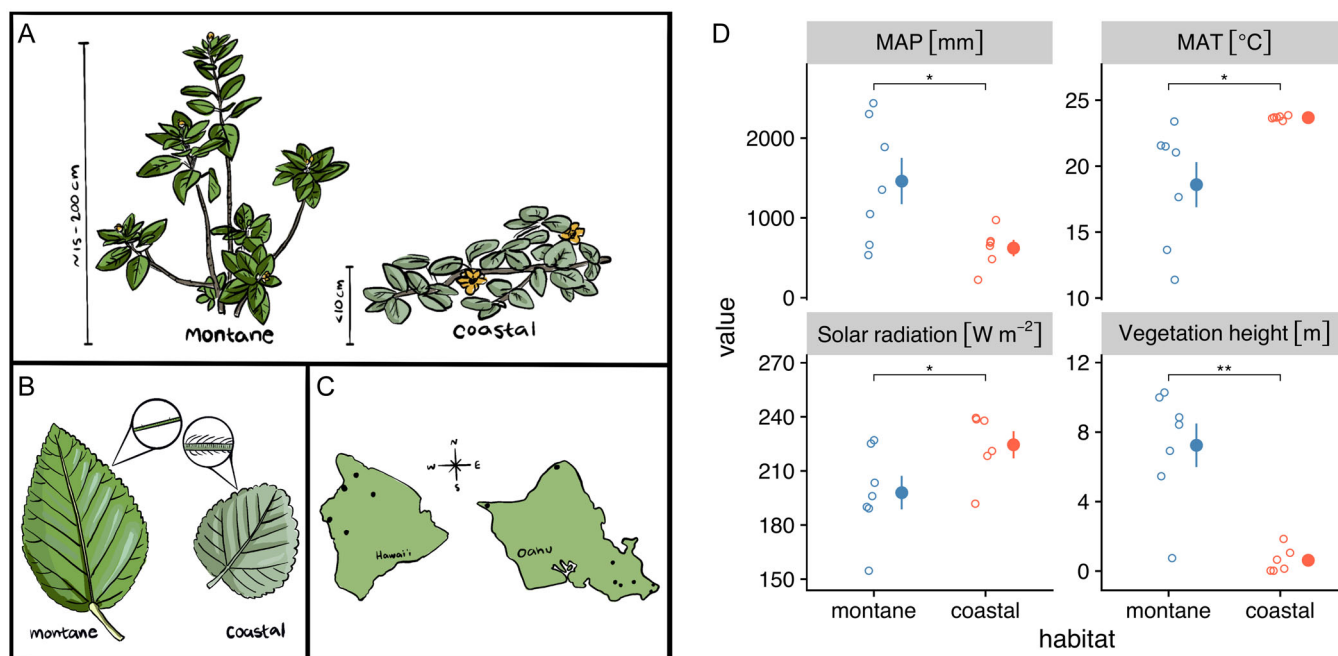
The association between high light and amphistomy suggests that "sun" leaves have the most to gain in terms of increased photosynthesis from having stomata on both surfaces, as Mott et al. (1982) hypothesized. Parkhurst (1978) proposed quantifying this benefit as "amphistomy advantage" (AA), which we adopt here with some modification (see Materials and Methods). This hypothesis has never been tested directly by comparing the photosynthetic rate of an amphistomatous leaf to that of an otherwise

identical hypostomatous leaf with the same total stomatal conductance under the same conditions. We proposed a straightforward method to do this comparison by experimentally creating a pseudohypostomatous leaf with gas exchange blocked through the upper surface (see Materials and Methods). We used humidity to modulate stomatal conductance so that amphi- and pseudohypostomatous leaves can be compared at the same total stomatal conductance. One reason that sun leaves might have greater AA is that they are usually thicker or denser (Poorter et al., 2019), which will often result in lower g_{ias} either by increasing the diffusion path length (Parkhurst, 1978) or making the airspace less porous. A nonmutually exclusive hypothesis is that if sun leaves have a stomatal density ratio closer to 0.5 (same density on each leaf surface), then they will have a greater advantage than amphistomatous leaves with most stomata on one surface. In other words, amphistomy doesn't make much difference if one leaf surface has few open stomata on it. We therefore predict that sun leaves will have greater AA possibly because they have thicker leaves and/or SR closer to 0.5. We actually report $g_{smax,ratio}$, which is similar to SR except that it accounts for differences in both stomatal density and size between surfaces.

The native flora of the Hawaiian archipelago is an excellent system to test the relationship between light habitat and AA. Many lineages have adapted to different light habitats after colonization, and leaf anatomical traits such as SR and thickness vary within and among closely related species.

Carlquist (1966) hypothesized that the common ancestor in many Hawaiian clades was a weedy species with high dispersal ability adapted to open habitats. Colonization was followed by adaptive radiation into higher elevation, montane, closed, forested habitats. Consequently, adaptation to sun and shade is a common axis of phenotypic variation among Hawaiian plants such as lobeliads (Montgomery and Givnish, 2008; Givnish et al., 2004, 2009; Givnish and Montgomery, 2014; Scoffoni et al., 2015), *Bidens* (Carlquist, 1966; Knope et al., 2020), *Scaevola* (Robichaux and Pearcy, 1984; McKown et al., 2016), *Euphorbia* (Sporck, 2011), and *Plantago* (Dunbar-Co et al., 2009).

Here we focused on variation within an indigenous plant species *Sida fallax* Walp. (Malvaceae), known in the Hawaiian language as 'ilima. 'Ilima is found from sea level to elevations >1000 m a.s.l. on multiple Hawaiian islands. Coastal populations are morphologically different from montane populations (Figure 1). Coastal regions of Hawai'i are characterized by high sun exposure, warmer temperatures, high winds, salinity, and variation in water availability. Coastal populations of 'ilima tend to be short and prostrate, which likely helps them to withstand the windy environment (Figure 1A). The leaves of these populations are covered on both surfaces in dense, soft hairs that give the leaves a silvery green appearance (Figure 1B) and help mitigate water loss by reflecting solar radiation, thereby lowering leaf temperature (Ehleringer and Björkman, 1978). Montane regions, on the other hand, provide very different challenges. Many other tall species grow on the slopes of these wet mountainous regions,

FIGURE 1 (A) Typical growth form of montane (left) and coastal (right) 'ilima plants and (B) leaves. (C) Map of the sites that were sampled on the islands of O'ahu and Hawai'i (aka Big Island). (D) Climatic, light, and vegetation height comparisons between montane (blue) and coastal (orange) habitats sampled in this study. Open circles are values for the midpoint of each site transect; closed circles and intervals are the mean ± 1 SE. The habitats differ significantly in mean annual precipitation (top left), solar radiation (bottom left), temperature (top right), and vegetation height (bottom right). MAP = mean annual precipitation; MAT = mean annual temperature; ns = not significant; *0.01 ≤ P < 0.05; **0.001 ≤ P < 0.01.

which makes light competition a factor that plants may need to adapt to. Possibly due to shading by other plants, montane populations are erect and shrub- or tree-like, capable of growing meters tall with strong, woody stems. These individuals have smooth, green foliage with serrated edges. Montane populations exhibit traits that may help them to compete for light availability. This montane morphology is not found in *S. fallax* populations on other Pacific Islands (Pejhanmehr et al., 2023).

Because of the contrasting habitat and leaf morphology, we treated leaves from coastal and montane plants as representatives of sun and shade leaves, respectively, for testing hypotheses about amphistomy advantage. Specifically, the objectives of our study were to test whether (1) sun leaves of coastal 'ilima plants have greater AA than shade leaves of montane plants; and if so, is this difference because (2a) coastal plants have thicker leaves than montane plants and/or (2b) coastal plants have a $g_{\mathrm{smax,ratio}}$ closer to 0.5?

MATERIALS AND METHODS

Plant sampling and climate

We identified seven suitable natural populations of 'ilima on O'ahu and five on Hawai'i Island by consulting Yorkston and Daehler (2006) and citizen scientist records on iNaturalist (Anonymous, 2022) (Figure 1C; Table 1). We avoided sites that appeared to be cultivated. We visited sites between August and November 2022. For logistical reasons, the sites on Hawai'i were sampled during one 3-day trip. We haphazardly sampled eight plants distributed evenly between the highest and lowest elevation plants along a transect at each site. For safety and conservation reasons, transects were along a trail or road. We did not sample

small individuals if there was risk removing leaves would cause mortality. From each plant, we collected two fully expanded leaves for trait measurements. We sampled stomatal traits on all leaves; leaf thickness on one leaf from three randomly selected plants per site; and, due to limited time, a single leaf from a single plant at the middle of each transect for gas exchange measurements. We downloaded climatic data on mean annual temperature, solar radiation, and vegetation height from the Climate and Solar Radiation of Hawai'i databases (Giambelluca et al., 2014) using the latitude and longitude at the middle of each transect. We also downloaded mean annual precipitation from 1978 to 2007 from the Rainfall Atlas of Hawai'i (Giambelluca et al., 2013). The spatial resolution is approximately 234×250 m. The temperature data are calibrated from networks of meteorological stations operating in the late 20th and 21st century; the solar radiation data are calibrated from satellite measurements collected between 2002 and 2009 (Giambelluca et al., 2014). We tested whether climatic variables differed among our coastal and montane populations using Welch's two-sample t-test.

Leaf traits

Stomata

We estimated the stomatal density and size on ab- and adaxial leaf surfaces from all leaves. For pubescent leaves (usually coastal), we dried and pressed leaves for ≈1 week (Hill et al., 2014), carefully scraped trichomes off with a razor blade, and rehydrated the leaf. Rehydration restores leaf area to its fresh value (Blonder et al., 2012). For glabrous leaves, we used fresh leaves. We applied clear nail polish to both leaf surfaces of fresh or rehydrated leaves in

TABLE 1 'Ilima study site location information.

Site	Island	Habitat	Latitude	Longitude	Elevation (m a.s.l.)
Kahuku Point	O'ahu	coastal	21.710	−157.982	4
Kaloko beach	O'ahu	coastal	21.293	−157.661	4
Kaloko-Honokōhau national historical park	Hawai'i	coastal	19.676	−156.024	6
Ka'ena Point	O'ahu	coastal	21.574	−158.278	4
Makapu'u beach	O'ahu	coastal	21.313	−157.661	3
Puakō petroglyph park	Hawai'i	coastal	19.957	−155.858	8
Hawai'i loa ridge	O'ahu	montane	21.294	−157.727	352
Hāloa ʻĀina	Hawai'i	montane	19.552	−155.793	1567
Ka'ohe game management area	Hawai'i	montane	19.817	−155.616	1946
Koai'a tree sanctuary	Hawai'i	montane	20.048	−155.737	970
Mau'umae ridge	O'ahu	montane	21.305	−157.779	248
Wa'ahila ridge	O'ahu	montane	21.314	−157.793	357

the middle of the lamina away from major veins. After the nail polish dried, we mounted impressions on a microscope slide using transparent tape (Mott and Michaelson, 1991). We digitized a portion of each leaf surface impression using a light microscope with bright-field optics (Leica DM2000, Wetzlar, Germany). We counted all stomata and divided by the visible leaf area (0.890 mm^2) to estimate density and measured guard cell length from five randomly chosen stomata per field using ImageJ (Schneider et al., 2012).

Leaf thickness

We cut thin sections using two razor blades taped together. We sectioned the leaf in a petri dish of water, wet-mounted sections onto a slide, and took digital micrographs using a brightfield microscope, as described above. Leaf thickness is measured as the length from upper cuticle to lower cuticle.

Gas exchange measurements

At each site, we selected one representative leaf from one plant near the middle of the transect for gas exchange measurements using a portable infrared gas analyzer (LI-6800PF, LI-COR Biosciences, Lincoln, NE, USA). We estimated the photosynthetic rate (A) and stomatal conductance to water vapor (g_{sw}) at saturating light (photosynthetic photon flux density [PPFD] = $2000 \, \mu\text{mol m}^{-2} \text{ s}^{-1}$), ambient CO_2 (415 ppm), and $T_{leaf} = 25.0–29.3°C$. The midday irradiance in coastal 'ilima typically meets or even exceeds a PPFD of $2000 \, \mu\text{mol m}^{-2} \text{ s}^{-1}$ and previous experiments with sun leaves revealed that $2000 \, \mu\text{mol m}^{-2} \text{ s}^{-1}$ is always at or near saturating irradiance. Even though lower irradiance may be saturating for montane leaves, we used this higher value for all leaves to standardize conditions.

We also estimated amphistomy advantage (AA) sensu Parkhurst (1978), but with a modification. For each leaf, we measured the photosynthetic rate of an untreated amphistomatous leaf (A_{amphi}) over a range of g_{sw} values. We refer to this as an $A–g_{sw}$ curve, which is described in more detail below. We compared the $A–g_{sw}$ curve of the untreated leaf to the photosynthetic rate of pseudohypostomatous leaf (A_{hypo}), which is the same leaf but with gas exchange through the upper surface blocked by a neutral density plastic (propafilm). Hypostomy refers to leaves with stomata only present on the lower, typically abaxial, surface. We refer to the untreated and partially blocked leaves as "amphi" and "pseudohypo", respectively. The AA was calculated as the log-response ratio of A compared at the same total g_{sw}: AA = log (A_{amphi}/A_{hypo}).

The log-response ratio is commonly used in social and biological sciences (e.g., Hedges et al. [1999]). It is straightforward to interpret because values above 0 indicate a photosynthetic advantage of amphistomy, whereas values less than 0 indicate a disadvantage. The log-response ratio is preferable to the absolute difference because it indicates a proportional change in A, which facilitates comparisons across leaves and environments with different baseline photosynthetic rates. The irradiance of the light source in the pseudohypo leaf was higher because the propafilm reduces transmission. To compensate for reduced transmission, we increased incident PPFD for pseudohypo leaves by a factor 1/0.91, the inverse of the measured transmissivity of the propafilm. We also set the stomatal conductance ratio, for purposes of calculating boundary layer conductance, to 0 for pseudohypo leaves following manufacturer directions.

Figure S1 in Appendix S1 illustrates our method for collecting $A–g_{sw}$ curves. We collected two curves per leaf, an amphi (untreated) curve and a pseudohypo (treated) curve. To control for order effects, we alternated between starting with amphi or pseudohypo leaf measurements, though we did not detect an effect of treatment order on AA (results not shown). In the field, we acclimated the focal leaf to saturating light and high relative humidity (RH = 70%), as described above, until A and g_{sw} reach their maximum. We used these data as our estimates of maximum A and g_{sw}. After that, we decreased RH to ≈10% to induce rapid stomatal closure without biochemical downregulation. Hence, A_{amphi} and A_{hypo} were both measured at low chamber humidity after the leaf had acclimated to high humidity. All other environmental conditions in the leaf chamber remained the same. We logged data until g_{sw} reached its nadir. We then repeated the process of acclimating the leaf to 70% RH and inducing stomatal closure with low RH with the other treatment (amphi or pseudohypo).

Data analyses

Objective 1: Do coastal leaves have greater amphistomy advantage than montane leaves?

It is not feasible to record A_{amphi} and A_{hypo} at the exact same g_{sw}. To overcome this limitation, we fit $A–g_{sw}$ curves using a linear regression of $\log(g_{sw})$ on A to interpolate modeled A for amphi and pseudohypo leaves at the same g_{sw}. Let $\hat{A}_{amphi}$ and $\hat{A}_{hypo}$ be the estimated A of the amphi and pseudohypo leaves, respectively. We estimated these quantities at the same g_{sw} using fitted parameters ($\hat{\beta}$'s):

$$\hat{A}_{amphi} = \hat{\beta}_{0,amphi} + \hat{\beta}_{1,amphi} \times \log(g_{sw})$$

$$\hat{A}_{hypo} = \hat{\beta}_{0,hypo} + \hat{\beta}_{1,hypo} \times \log(g_{sw}).$$

In 10 of 12 leaves, the minimum g_{sw} of the amphi curve was smaller than the maximum g_{sw} of the pseudohypo curve (i.e., the curves overlapped for a range of g_{sw} values). In those cases, we estimated $\hat{A}_{amphi}$ and $\hat{A}_{hypo}$ at the g_{sw} value in the middle of the range of overlap between the curves. In

2 of 12 leaves, the $A–g_{sw}$ curves did not quite overlap because the minimum g_{sw} of the amphi curve was slightly greater than the maximum g_{sw} of the pseudohypo curve. In those cases, we estimated AA by extrapolating slightly, 1.98×10^{-3} and 3.29×10^{-3} mol m^{-2} s^{-1}, beyond the measured curves to the g_{sw} value in between the curves. The vertical lines in Figure S2 (Appendix S1) show the g_{sw} for each leaf. We estimated AA from $\hat{A}_{amphi}$ and $\hat{A}_{hypo}$ for each leaf using the log-response ratio shown above.

To estimate $\hat{\beta}$'s from the $A–g_{sw}$ curve for each leaf, we fit Bayesian regressions using the R package brms version 2.20.4 (Bürkner, 2017) with Markov chain Monte Carlo sampling in Stan (Stan Development Team, 2023). We used CmdStan version 2.33.1 and cmdstanr version 0.6.1 (Gabry et al., 2023) to interface with R version 4.3.1 (R Core Team, 2023). We sampled the posterior distribution from four chains with 1000 iterations each after 1000 warmup iterations per chain. We estimated parameters and confidence intervals as the median and 95% quantile intervals of the posterior, respectively. The key prediction is that $AA_{coastal} > AA_{montane}$, meaning the 95% confidence intervals of $AA_{coastal} − AA_{montane}$ should be positive and not encompass 0.

Objective 2a: Are coastal leaves thicker than montane leaves?

We tested whether leaf thickness (log-transformed) varied between coastal and montane populations and among individuals within populations using a Bayesian mixed-effects model with habitat as a fixed effect and individual plant and site as random effects. We used the R package brms version 2.20.4 (Bürkner, 2017) to fit the model in Stan (Stan Development Team, 2023) with CmdStan version 2.33.1 and cmdstanr version 0.6.1 (Gabry et al., 2023). We sampled the posterior distribution from four chains with 1000 iterations each after 1000 warmup iterations per chain. We estimated the relationship between population average leaf thickness and AA measured from a single individual per population. We used this approach because most of the variation in leaf thickness was among sites and the plant selected for gas exchange measurements was not always among the plants randomly selected for leaf thickness, precluding individual level correlation. We propagated uncertainty about in AA and leaf thickness estimates by integrating over the entire posterior distribution sample for each variable. The key prediction is that the effect of leaf thickness on AA is positive, meaning the 95% confidence interval of the slope should be positive and not encompass 0.

Objective 2b: Is $g_{smax,ratio}$ closer to 0.5 in coastal leaves than montane leaves?

We tested whether $g_{smax,ratio}$ varied between coastal and montane populations and among individuals within populations using a Bayesian multiresponse, mixed-effects model. The modeled response variables are stomatal count and guard cell length on each surface. Counts were modeled as negative binomially distributed variable from a latent stomatal density and a parameter ϕ to estimate over-dispersion in counts relative to a Poisson model. For all traits, the explanatory variables were habitat as a fixed effect and leaf within individual plant, individual plant, and site as random effects. We used the R package brms version 2.20.4 (Bürkner, 2017) to fit the model in Stan (Stan Development Team, 2023) with CmdStan version 2.33.1 and cmdstanr version 0.6.1 (Gabry et al., 2023). We interpolated missing adaxial guard cell lengths from six of 185 samples with zero adaxial stomata using the mi function in the brms package. We sampled the posterior distribution from four chains with 1000 iterations each after 1000 warmup iterations per chain. From each posterior sample, we calculated $g_{smax,ratio}$ as

$$g_{smax,ratio} = \frac{g_{smax,upper}}{g_{smax,lower} + g_{smax,upper}},$$

where $g_{smax,lower}$ and $g_{smax,upper}$ are the maximum stomatal conductance to water vapor at $T_{leaf} = 25°C$ on the lower and upper surface, respectively. The maximum stomatal conductance was calculated from stomatal density and length, assuming that stomata are fully open, following Sack and Buckley (2016) as $g_{smax} = bmds^{0.5}$, where b is a biophysical constant, m is a morphological constant, d is the stomatal density, and s is the stomatal complex area. We assume that b, which is determined by the molecular species, temperature, and air pressure, is the same for both surfaces; we assume that m, which is determined by guard cell allometry is also the same for both surfaces. Hence, constants b and m cancel out of $g_{smax,ratio}$, and only density and length (l), which is proportional to the square root of area, affect the ratio: $g_{smax} \propto dl$.

We estimated the relationship between leaf $g_{smax,ratio}$ and AA measured from a single leaf per population. We propagated uncertainty about AA and $g_{smax,ratio}$ by integrating over the entire posterior distribution sample for each variable. The key prediction is that the effect of $g_{smax,ratio}$ on AA is positive until $g_{smax,ratio} < 0.5$, meaning the 95% confidence interval of the slope should be positive and not encompass 0 in the domain $g_{smax,ratio} < 0.5$.
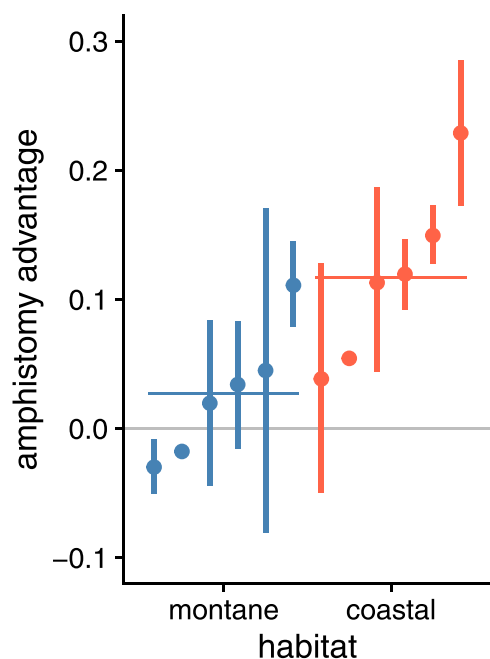
RESULTS

Coastal 'ilima are surrounded by shorter vegetation than their montane counterparts (Figure 1D; Welch two-sample t-test, $t_{6.67} = 5.13$, $P = 0.002$). The montane site with the lowest vegetation height is a remnant dry forest (Koai'a tree sanctuary) in a matrix of cattle pasture; hence, the satellite derived vegetation height may be lower than what existed prior to human disturbance. Coastal sites receive greater average

solar radiation at the top of the canopy (Figure 1D; Welch two-sample t-test, $t_{10.86} = -2.22$, $P = 0.049$); coastal sites are significantly warmer (Figure 1D; Welch two-sample t-test, $t_{6.01} = -2.96$, $P = 0.025$); and coastal sites receive less precipitation (Figure 1D; Welch two-sample t-test, $t_{7.45} = 2.73$, $P = 0.028$).

Amphistomy advantage is greater in coastal leaves

Amphistomy increases photosynthesis in leaves of coastal 'ilima plants more than in those of montane plants. Amphistomy advantage was significantly greater than 0 (95% confidence intervals did not overlap 0) in five of six coastal leaves, but only one of six montane leaves (Figure 2; see Appendix S1 [Figure S2] for individual curves). Overall, the average AA among coastal and montane leaves is 0.12 (95% CI: 0.077–0.15) and 0.027 (95% CI: –0.0034 to 0.057), respectively; the difference in average AA between habitat types is $AA_{coastal} - AA_{montane} = 0.09$ (95% CI: 0.039–0.14). Posterior predictions closely match observed values of A (Appendix S1, Figure S3), indicating an adequate model fit from which we can interpolate between measurements reliably. It also suggests that slight extrapolation beyond the data should be reliable. When

we removed two leaves where we extrapolated slightly beyond fitted $A-g_{sw}$ curves, we estimated that $AA_{coastal}$ is still positive, 0.081 (95% CI: 0.023–0.13), but the difference between coastal and montane leaves is smaller, 0.053 (95% CI: –0.012 to 0.12), and confidence intervals slightly overlap 0. Maximum photosynthetic rate was slightly, but not significantly higher in coastal leaves (Welch two-sample t-test, $t_{9.65} = 1.60$, $P = 0.14$); total stomatal conductance was similar (Welch two-sample t-test, $t_{9.71} = -0.09$, $P = 0.93$) in coastal and montane leaves (Appendix S1, Figure S4). Water-use efficiency (A/g_{sw}) was significantly higher in coastal leaves (Welch two-sample t-test, $t_{9.99} = 2.54$, $P = 0.03$).

Leaf thickness is associated with amphistomy advantage between but not within habitats

Coastal 'ilima leaves were an average 91 (95% CI: 26–164) µm thicker than their montane counterparts. Although coastal leaves were thicker and had greater AA, there was little relationship between leaf thickness and AA within habitats (Figure 3A; slope= –0.11; 95% CI: –0.28 to 0.035).

$g_{smax,ratio}$ is not associated with amphistomy advantage

Coastal and montane leaves had similar average $g_{smax,ratio}$, the ratio of adaxial (upper) to total g_{smax}, the anatomical maximum stomatal conductance to water vapor (Appendix S1, Figure S5); coastal leaves had 0.059 (95% CI: –0.14 to 0.28) higher $g_{smax,ratio}$ than montane leaves, but the 95%

FIGURE 2 Coastal leaves benefit more from amphistomy than montane leaves. A positive amphistomy advantage (y-axis) means that the photosynthetic rate of an amphistomatous leaf is greater than that of an identical pseudohypostomatous leaf at the same overall g_{sw}. Each point-interval is the median posterior estimate plus 95% confidence interval of amphistomy advantage for that leaf. Each leaf is from a different montane (blue) or coastal (orange) site, arranged by habitat and ascending amphistomy advantage within habitat. The longer horizontal bars are the average amphistomy advantage for montane and coastal leaves. g_{sw}, stomatal conductance to water vapor.
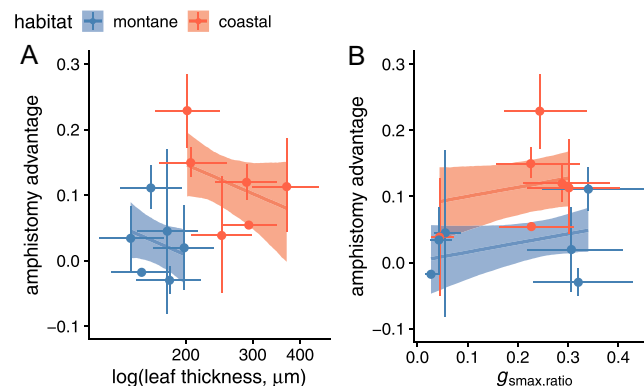

FIGURE 3 Relationships between leaf amphistomy advantage, (A) leaf thickness and (B) $g_{smax,ratio}$ among 'ilima (*Sida fallax*) plants from montane (blue) and coastal (orange) habitats in Hawai'i. A positive amphistomy advantage (y-axis) means that the photosynthetic rate of an amphistomatous leaf is greater than that of an identical pseudohypostomatous leaf at the same overall g_{sw}. Each point-interval is the median posterior estimate plus 95% confidence interval of the trait value. Each leaf is from a different montane (blue) or coastal (orange) site. Lines are the estimated linear regression of (A) log(leaf thickness) and (B) $g_{smax,ratio}$ on amphistomy advantage; ribbons are the 95% confident bands of the regression. $g_{smax,ratio}$, anatomical maximum stomatal conductance ratio; g_{sw}, stomatal conductance to water vapor.

confidence intervals overlap 0 difference. The $g_{smax,ratio}$ was somewhat bimodal among sites. Some sites in both habitats had leaves with $g_{smax,ratio} < 0.07$ and others with $g_{smax,ratio} > 0.2$ (Appendix S1, Figure S5). For example, montane sites on the Big Island of Hawai'i all had low $g_{smax,ratio}$, whereas montane sites on O'ahu had relatively high $g_{smax,ratio}$. There was no relationship between $g_{smax,ratio}$ and AA in either habitat (Figure 3B; slope = 0.14; 95% CI: −0.057 to 0.34) in our sample.

DISCUSSION

Amphistomy is a seemingly simple way that leaves can increase carbon gain without significant additional water loss, yet it is rare in nature, and we do not know why. The strong association between amphistomy and sunny, open habitats suggests that amphistomy may benefit sun leaves more than shade leaves, but progress has been limited by the lack of evidence that amphistomy per se improves photosynthesis in a given leaf. By experimentally blocking gas exchange through the upper surface in a controlled environment, we directly compared an amphistomatous leaf to an otherwise identical pseudohypostomatous leaf, which allowed us to quantify the amphistomy advantage (AA) holding all else constant. Taking advantage of the steep climatic gradients in the Hawaiian archipelago, we applied this new method to show for the first time that sun leaves benefited 4.04 times more from amphistomy than shade leaves on 'ilima (*Sida fallax*) plants (AA_coastal = 0.12 vs. AA_montane = 0.027). Coastal and montane 'ilima leaves are likely good representatives of classic sun and shade leaf syndromes because (1) they vary in traits like reflective pubescence (Ehleringer and Björkman, 1978) and leaf thickness (Terashima et al., 2001) that typically characterize sun–shade adaptations, and (2) since 'ilima shrubs are typically <1 m tall, they are shaded by trees in montane, but not coastal habitats (Figure 1D). While this result has not yet been validated in other species, our results indicate that part of the reason amphistomatous leaves are found most commonly in high-light habitats is that the adaptive benefit is greater in such environments.

If AA is typically greater in sun leaves than shade leaves, then this fact could partially explain the distribution of amphi- and hypostomatous leaves, but the precise mechanism(s) require further study. One hypothesis is that the internal airspace conductance, g_{ias}, from stomata to mesophyll cell walls is lower in thicker sun leaves (Parkhurst, 1978). All else being equal, a leaf with lower g_{ias} will benefit more from amphistomy. Our results partially support this hypothesis. Coastal 'ilima leaves with high AA (Figure 2) are thicker than montane leaves, but the relationship between AA and leaf thickness within habitats is actually slightly negative (Figure 3A), the opposite of our prediction. Since coastal and montane leaves differ in many respects besides thickness, we do not have enough data to conclude that leaf thickness explains the variation in AA

between habitats. Alternatively, other biochemical or anatomical differences between coastal and montane leaves may explain why AA is greater in coastal leaves. The negative relationship, albeit nonsignificant in that 95% confidence intervals encompassed 0, between leaf thickness and AA could be explained if thicker leaves compensated by having a more porous mesophyll and/or less tortuous airspaces (Théroux-Rancourt et al., 2021).

A second natural hypothesis is that amphistomatous leaves with few adaxial (upper) stomata benefit less than those with similar densities on both surfaces. We predicted that leaves with $g_{smax,ratio}$ closer to 0.5 would have higher AA based on biophysical models (Gutschick, 1984a). The logic is that a small number of stomata on the upper surface are insufficient to supply the entire upper mesophyll due to limited lateral diffusion (Morison et al., 2005). Our results do not support this hypothesis. Montane leaves from Big Island sites had low $g_{smax,ratio}$ and low AA, whereas low montane leaves on O'ahu had high $g_{smax,ratio}$, but similarly low AA (Figure 3B). Among coastal sites, the site with the lowest $g_{smax,ratio}$ had the lowest AA, but there was little variation in $g_{smax,ratio}$ among coastal leaves in our sample. We therefore cannot rule out that a larger sample of coastal leaves with greater variance in $g_{smax,ratio}$ might support this hypothesis.

Two major implications from our study are that (1) photosynthesis in hypostomatous leaves is likely limited by CO_2 concentration drawdown within leaf airspaces; and (2) amphistomy per se contributes to, but is not wholly responsible for, higher photosynthetic rates among amphistomatous leaves. The amphistomy advantage we observe in 'ilima leaves implies decreased CO_2 supply in pseudohypostomatous leaves because of concentration drawdowns in the leaf airspace. Limited diffusion through the airspace has long been hypothesized to depress photosynthesis in hypostomatous leaves (Parkhurst, 1994), with empirical support from helox studies (Parkhurst and Mott, 1990). However, these studies relied on interspecific comparisons of amphi- and hypostomatous leaves that differ systematically in many traits that affect gas exchange and photosynthesis (Xiong and Flexas, 2020). Our experimental approach overcomes this limitation and implies that the drop in CO_2 concentration from substomatal cavities to the upper surface depresses photosynthesis.

Among land plants grown in a common garden, amphistomatous leaves have on average nearly 2 × higher area-based photosynthetic rates (Xiong and Flexas, 2020), naively implying an AA≈log 2 = 0.69, which is much higher than our estimate of 0.12 among coastal 'ilima leaves. The most likely explanation is that amphistomy is not the only cause of high photosynthetic rate. Indeed, species adapted to open, high light habitats with amphistomatous leaves also have higher concentrations of Rubisco, overall stomatal conductance, and photosynthetic capacity (Smith et al., 1997; Xiong and Flexas, 2020). For a leaf with high photosynthetic capacity that is well illuminated and hydrated, the major limitation becomes CO_2. Under these conditions,

amphistomy may substantially increase photosynthesis, as we observe in coastal 'ilima leaves. Selection on increased photosynthesis under similar conditions may explain why crop leaves tend to increase stomatal density ratio during domestication (Milla et al., 2013).

Three limitations of this study are the small sample size, experimental design that precludes distinguishing genetic from environmental differences in leaf traits, and potentially confounding effects of other environmental differences besides light environment. Understanding the mechanistic basis of higher AA in sun leaves would require much larger sample sizes. Sun leaves tend to be thicker, more densely packed with mesophyll cells, and have greater photosynthetic capacity and higher stomatal conductance, among other traits (Lambers et al., 2008). Each of these factors and others potentially modulate AA. Quantifying the contribution of all these factors requires larger samples and additional measurements that are beyond the scope of this study, but is an exciting avenue for future research on leaf structure–function relations. Although many morphological traits that distinguish coastal and montane 'ilima populations persist in a common environment (Yorkston and Daehler, 2006), we cannot distinguish between genetic effects and plastic responses to habitat as causes of difference in AA because we measured naturally occurring plants in situ. While disentangling genetic and plastic contributions is not necessarily important for understanding the distribution of amphistomatous leaves, knowing the genetic and environmental contributions to trait variation would provide useful insights. A reciprocal transplant experiment would help us determine the contributions of genetics and environment, and their interactions, to trait variance in nature. However, reciprocal transplants cannot control for other differences between coastal and montane habitats besides vegetation height, such as temperature and precipitation. Experimental studies in controlled environments will be necessary to isolate the effects of light quantity and quality on AA.

CONCLUSIONS

This study reports the first direct experimental evidence that having stomata open on both leaf surfaces, amphistomy, increases photosynthesis for a given total stomatal conductance, particularly in leaves from the type of open, sunny habitats where this trait is most common. By developing a straightforward experimental method to block gas exchange through the upper surface, we directly compared the photosynthetic rate of a leaf with gas exchange through both surfaces or just one, holding all other factors constant. In doing so, we found that coastal leaves of the indigenous Hawaiian 'ilima (Sida fallax) received a greater photosynthetic benefit from amphistomy than did nearby montane leaves in a more-closed forest. This difference is not because amphistomatous leaves necessarily have greater

leaf surface available for stomata, although that likely influences realized photosynthetic rates in natural populations. Rather, our experiments show that coastal amphistomatous leaves with the same total leaf stomatal conductance photosynthesize more than identical hypostomatous leaves. We cannot yet ascribe the difference in amphistomy advantage between coastal and montane leaves to a particular physiological or anatomical variation, but uncovering these mechanisms is a promising area for future research.

DATA AVAILABILITY STATEMENT
Custom scripts are available on a GitHub repository (https://github.com/cdmuir/stomata-ilima) and archived on Zenodo: https://doi.org/10.5281/zenodo.10369114 (Muir, 2023). Raw data are deposited on Dryad: https://doi.org/10.5061/dryad.rxwdbrvfw (Triplett et al., 2024).

ORCID
Thomas N. Buckley [iD] http://orcid.org/0000-0001-7610-7136
Christopher D. Muir [iD] http://orcid.org/0000-0003-2555-3878

REFERENCES

Anonymous. 2022. Yellow 'ilima (Sida fallax). https://www.inaturalist.org/taxa/54995-Sida-fallax. iNaturalist.

Ball, J. T., I. E. Woodrow, and J. A. Berry. 1987. A model predicting stomatal conductance and its contribution to the control of photosynthesis under different environmental conditions. In J. Biggins [ed.], Progress in photosynthesis research, 221–224. Springer, Dordrecht, Netherlands.

Berry, J. A., D. J. Beerling, and P. J. Franks. 2010. Stomata: key players in the earth system, past and present. Current Opinion in Plant Biology 13: 232–239.

Blonder, B., V. Buzzard, I. Simova, L. Sloat, B. Boyle, R. Lipson, B. Aguilar-Beaucage, et al. 2012. The leaf-area shrinkage effect can bias paleoclimate and ecology research. American Journal of Botany 99: 1756–1763.

Bucher, S. F., K. Auerswald, C. Grün-Wenzel, S. I. Higgins, J. Garcia Jorge, and C. Römermann. 2017. Stomatal traits relate to habitat preferences of herbaceous species in a temperate climate. Flora 229: 107–115.

Bürkner, P.-C. 2017. brms: An R package for Bayesian multilevel models using Stan. Journal of Statistical Software 80: 1–28.

Carlquist, S. 1966. The biota of long-distance dispersal. II. Loss of dispersibility in Pacific Compositae. *Evolution* 20: 30–48.

Collatz, G. J., J. T. Ball, C. Grivet, and J. A. Berry. 1991. Physiological and environmental regulation of stomatal conductance, photosynthesis and transpiration: a model that includes a laminar boundary layer. *Agricultural and Forest Meteorology* 54: 107–136.

Cowan, I. R., and G. D. Farquhar. 1977. Stomatal function in relation to leaf metabolism and environment. *Symposia of the Society for Experimental Biology* 31: 471–505.

de Boer, H. J., P. L. Drake, E. Wendt, C. A. Price, E.-D. Schulze, N. C. Turner, D. Nicolle, and E. J. Veneklaas. 2016. Apparent overinvestment in leaf venation relaxes leaf morphological constraints on photosynthesis in arid habitats. *Plant Physiology* 172: 2286–2299.

Deans, R. M., T. J. Brodribb, F. A. Busch, and G. D. Farquhar. 2020. Optimization can provide the fundamental link between leaf photosynthesis, gas exchange and water relations. *Nature Plants* 6: 1116–1125.

Doll, Y., H. Koga, and H. Tsukaya. 2021. *Callitriche* as a potential model system for evolutionary studies on the dorsiventral distribution of stomata. *Plant Signaling & Behavior* 16: 1978201.

Drake, P. L., H. J. de Boer, S. J. Schymanski, and E. J. Veneklaas. 2019. Two sides to every leaf: Water and CO_2 transport in hypostomatous and amphistomatous leaves. *New Phytologist* 222: 1179–1187.

Drake, P. L., R. H. Froend, and P. J. Franks. 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *Journal of Experimental Botany* 64: 495–505.

Dunbar-Co, S., M. J. Sporck, and L. Sack. 2009. Leaf trait diversification and design in seven rare taxa of the Hawaiian *Plantago* radiation. *International Journal of Plant Sciences* 170: 61–75.

Earles, J. M., G. Theroux-Rancourt, A. B. Roddy, M. E. Gilbert, A. J. McElrone, and C. R. Brodersen. 2018. Beyond porosity: 3D leaf intercellular airspace traits that impact mesophyll conductance. *Plant Physiology* 178: 148–162.

Ehleringer, J. R., and O. Björkman. 1978. Pubescence and leaf spectral characteristics in a desert shrub, *Encelia farinosa*. *Oecologia* 36: 151–162.

Evans, J. R., R. Kaldenhoff, B. Genty, and I. Terashima. 2009. Resistances along the CO_2 diffusion pathway inside leaves. *Journal of Experimental Botany* 60: 2235–2248.

Flexas, J., M. J. Clemente-Moreno, J. Bota, T. J. Brodribb, J. Gago, Y. Mizokami, M. Nadal, et al. 2021. Cell wall thickness and composition are involved in photosynthetic limitation. *Journal of Experimental Botany* 72: 3971–3986.

Foster, J. R., and W. K. Smith. 1986. Influence of stomatal distribution on transpiration in low-wind environments. *Plant, Cell & Environment* 9: 751–759.

Franklin, O., S. P. Harrison, R. Dewar, C. E. Farrior, Å. Brännström, U. Dieckmann, S. Pietsch, et al. 2020. Organizing principles for vegetation dynamics. *Nature Plants* 6: 444–453.

Franks, P. J., G. B. Bonan, J. A. Berry, D. L. Lombardozzi, N. M. Holbrook, N. Herold, and K. W. Oleson. 2018. Comparing optimal and empirical stomatal conductance models for application in Earth system models. *Global Change Biology* 24: 5708–5723.

Gabry, J., R. Češnovar, and A. Johnson. 2023. cmdstanr: R interface to 'CmdStan'. Website: https://mc-stan.org/cmdstanr/

Gago, J., D. M. Daloso, M. Carriquí, M. Nadal, M. Morales, W. L. Araújo, A. Nunes-Nesi, et al. 2020. The photosynthesis game is in the "interplay": mechanisms underlying CO_2 diffusion in leaves. *Environmental and Experimental Botany* 178: 104174.

Gay, A. P., and R. G. Hurd. 1975. The influence of light on stomatal density in the tomato. *New Phytologist* 75: 37–46.

Giambelluca, T. W., Q. Chen, A. G. Frazier, J. P. Price, Y.-L. Chen, P.-S. Chu, J. K. Eischeid, and D. M. Delparte. 2013. Online rainfall atlas of Hawai'i. *Bulletin of the American Meteorological Society* 94: 313–316.

Giambelluca, T. W., X. Shuai, M. L. Barnes, R. J. Alliss, R. J. Longman, T. Miura, Q. Chen, et al. 2014. Evapotranspiration of Hawai'i. Final report submitted to the U.S. Army Corps of Engineers—Honolulu District, and the Commission on Water Resource Management, Mānoa, State of Hawai'i USA.

Gibson, A. C. 1996. Structure–function relations of warm desert plants. Springer, Berlin, Germany.

Givnish, T. J. [ed.]. 1986. On the economy of plant form and function. Cambridge University Press, Cambridge, UK.

Givnish, T. J., K. C. Millam, A. R. Mast, T. B. Paterson, T. J. Theim, A. L. Hipp, J. M. Henss, et al. 2009. Origin, adaptive radiation and diversification of the Hawaiian lobeliads (Asterales: Campanulaceae). *Proceedings of the Royal Society, B, Biological Sciences* 276: 407–416.

Givnish, T. J., and R. A. Montgomery. 2014. Common-garden studies on adaptive radiation of photosynthetic physiology among Hawaiian lobeliads. *Proceedings of the Royal Society, B, Biological Sciences* 281: 20132944.

Givnish, T. J., R. A. Montgomery, and G. Goldstein. 2004. Adaptive radiation of photosynthetic physiology in the Hawaiian lobeliads: light regimes, static light responses, and whole-plant compensation points. *American Journal of Botany* 91: 228–246.

Grubb, P. J. 2020. Leaf structure and function. *In* A. Dobson, D. Tilman, and R. D. Holt [eds.], Unsolved problems in ecology, 124–144. Princeton University Press, Princeton, NJ, USA.

Grubb, P. J. 1977. Leaf structure and function. *In* R. Duncan and M. Weston-Smith [eds.], The encyclopedia of ignorance, 317–330. Pergamon, Oxford, UK.

Gutschick, V. P. 1984a. Photosynthesis model for C_3 leaves incorporating CO_2 transport, propagation of radiation, and biochemistry 1. Kinetics and their parameterization. *Photosynthetica* 18: 549–568.

Gutschick, V. P. 1984b. Photosynthesis model for C_3 leaves incorporating CO_2 transport, propagation of radiation, and biochemistry 2. Ecological and agricultural utility. *Photosynthetica* 18: 569–595.

Harrison, S. P., W. Cramer, O. Franklin, I. C. Prentice, H. Wang, Å. Brännström, H. Boer, et al. 2021. Eco-evolutionary optimality as a means to improve vegetation and land-surface models. *New Phytologist* 231: 2125–2141.

Harwood, R., G. Théroux-Rancourt, and M. M. Barbour. 2021. Understanding airspace in leaves: 3D anatomy and directional tortuosity. *Plant, Cell & Environment* 44: 2455–2465.

Hedges, L. V., J. Gurevitch, and P. S. Curtis. 1999. The meta-analysis of response ratios in experimental ecology. *Ecology* 80: 1150–1156.

Hetherington, A. M., and F. I. Woodward. 2003. The role of stomata in sensing and driving environmental change. *Nature* 424: 901–908.

Hill, K. E., G. R. Guerin, R. S. Hill, and J. R. Watling. 2014. Temperature influences stomatal density and maximum potential water loss through stomata of *Dodonaea viscosa* subsp. *Angustissima* along a latitude gradient in southern Australia. *Australian Journal of Botany* 62: 657.

Ho, Q. T., H. N. C. Berghuijs, R. Watté, P. Verboven, E. Herremans, X. Yin, M. A. Retta, et al. 2016. Three-dimensional microscale modelling of CO_2 transport and light propagation in tomato leaves enlightens photosynthesis: 3-D modelling of photosynthesis in leaves. *Plant, Cell & Environment* 39: 50–61.

James, S. A., and D. T. Bell. 2001. Leaf morphological and anatomical characteristics of heteroblastic *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *Australian Journal of Botany* 49: 259.

Jarvis, P. G. 1976. The interpretation of the variations in leaf water potential and stomatal conductance found in canopies in the field. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 273: 593–610.

Jordan, G. J., R. J. Carpenter, and T. J. Brodribb. 2014. Using fossil leaves as evidence for open vegetation. *Palaeogeography, Palaeoclimatology, Palaeoecology* 395: 168–175.

Kaul, R. B. 1976. Anatomical observations on floating leaves. *Aquatic Botany* 2: 215–234.

Knope, M. L., V. A. Funk, M. A. Johnson, W. L. Wagner, E. M. Datlof, G. Johnson, D. J. Crawford, et al. 2020. Dispersal and adaptive

radiation of *Bidens* (Compositae) across the remote archipelagoes of Polynesia. *Journal of Systematics and Evolution* 58: 805–822.

Lambers, H., F. S. Chapin, and T. L. Pons. 2008. Plant physiological ecology. Springer, NY, NY, USA.

Leuning, R. 1995. A critical appraisal of a combined stomatal–photosynthesis model for C_3 plants. *Plant, Cell and Environment* 18: 339–355.

Lin, Y.-S., B. E. Medlyn, R. A. Duursma, I. C. Prentice, H. Wang, S. Baig, D. Eamus, et al. 2015. Optimal stomatal behaviour around the world. *Nature Climate Change* 5: 459–464.

Lyshede, O. B. 2002. Comparative and functional leaf anatomy of selected Alstroemeriaceae of mainly Chilean origin. *Botanical Journal of the Linnean Society* 140: 261–272.

Márquez, D. A., H. Stuart-Williams, L. A. Cernusak, and G. D. Farquhar. 2023. Assessing the CO_2 concentration at the surface of photosynthetic mesophyll cells. *New Phytologist* 238: 1446–1460.

Márquez, D. A., H. Stuart-Williams, G. D. Farquhar, and F. A. Busch. 2022. Cuticular conductance of adaxial and abaxial leaf surfaces and its relation to minimum leaf surface conductance. *New Phytologist* 233: 156–168.

McKown, A. D., M. E. Akamine, and L. Sack. 2016. Trait convergence and diversification arising from a complex evolutionary history in Hawaiian species of Scaevola. *Oecologia* 181: 1083–1100.

Medlyn, B. E., R. A. Duursma, D. Eamus, D. S. Ellsworth, I. C. Prentice, C. V. M. Barton, K. Y. Crous, et al. 2011. Reconciling the optimal and empirical approaches to modelling stomatal conductance. *Global Change Biology* 17: 2134–2144.

Milla, R., N. de Diego-Vico, and N. Martín-Robles. 2013. Shifts in stomatal traits following the domestication of plant species. *Journal of Experimental Botany* 64: 3137–3146.

Montgomery, R. A., and T. J. Givnish. 2008. Adaptive radiation of photosynthetic physiology in the Hawaiian lobeliads: dynamic photosynthetic responses. *Oecologia* 155: 455–467.

Morison, J. I. L., E. Gallouët, T. Lawson, G. Cornic, R. Herbin, and N. R. Baker. 2005. Lateral diffusion of CO_2 in leaves is not sufficient to support photosynthesis. *Plant Physiology* 139: 254–266.

Mott, K. A., A. C. Gibson, and J. W. O'Leary. 1982. The adaptive significance of amphistomatic leaves. *Plant, Cell & Environment* 5: 455–460.

Mott, K. A., and O. Michaelson. 1991. Amphistomy as an adaptation to high light intensity in *Ambrosia cordifolia* (Compositae). *American Journal of Botany* 78: 76–79.

Muir, C.. 2023. cdmuir/stomata-ilima: v1.0 (Version v1.0) [Computer software]. Zenodo. https://doi.org/10.5281/ZENODO.10369114

Muir, C. D. 2019. Is amphistomy an adaptation to high light? Optimality models of stomatal traits along light gradients. *Integrative and Comparative Biology* 59: 571–584.

Muir, C. D. 2018. Light and growth form interact to shape stomatal ratio among British angiosperms. *New Phytologist* 218: 242–252.

Muir, C. D. 2015. Making pore choices: repeated regime shifts in stomatal ratio. *Proceedings of the Royal Society, B, Biological Sciences* 282: 20151498.

Nobel, P. S. 2020. Physicochemical and environmental plant physiology, 5th ed. Academic Press, London, UK.

Parkhurst, D. F. 1994. Diffusion of CO_2 and other gases inside leaves. *New Phytologist* 126: 449–479.

Parkhurst, D. F. 1978. The adaptive significance of stomatal occurrence on one or both surfaces of leaves. *Journal of Ecology* 66: 367–383.

Parkhurst, D. F., and K. A. Mott. 1990. Intercellular diffusion limits to CO_2 uptake in leaves: Studies in air and helox. *Plant Physiology* 94: 1024–1032.

Peat, H. J., and A. H. Fitter. 1994. A comparative study of the distribution and density of stomata in the British flora. *Biological Journal of the Linnean Society* 52: 377–393.

Pejhanmehr, M., M. Yorkston, and C. W. Morden. 2023. Low genetic diversity in the highly morphologically diverse *Sida fallax* Walp. (Malvaceae) throughout the Pacific. *Pacific Science* 76: 361–376.

Poorter, H., Ü. Niinemets, N. Ntagkas, A. Siebenkäs, M. Mäenpää, S. Matsubara, and T. L. Pons. 2019. A meta-analysis of plant responses to light intensity for 70 traits ranging from molecules to whole plant performance. *New Phytologist*: 223: 1073–1105.

R Core Team. 2023. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website: https://R-project.org

Robichaux, R. H., and R. W. Pearcy. 1984. Evolution of C_3 and C_4 plants along an environmental moisture gradient: patterns of photosynthetic differentiation in Hawaiian *Scaevola* and *Euphorbia* species. *American Journal of Botany* 71: 121–129.

Sack, L., and T. N. Buckley. 2016. The developmental basis of stomatal density and flux. *Plant Physiology* 171: 2358–2363.

Salisbury, E. J. 1928. I. On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. *Philosophical Transactions of the Royal Society of London, B, Containing Papers of a Biological Character* 216: 1–65.

Šantrůček, J., L. Schreiber, J. Macková, M. Vráblová, J. Květoň, P. Macek, and J. Neuwirthová. 2019. Partitioning of mesophyll conductance for CO_2 into intercellular and cellular components using carbon isotope composition of cuticles from opposite leaf sides. *Photosynthesis Research*: 141: 33–51.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9: 671–675.

Scoffoni, C., J. Kunkle, J. Pasquet-Kok, C. Vuong, A. J. Patel, R. A. Montgomery, T. J. Givnish, and L. Sack. 2015. Light-induced plasticity in leaf hydraulics, venation, anatomy, and gas exchange in ecologically diverse Hawaiian lobeliads. *New Phytologist* 207: 43–58.

Sellers, P. J., R. E. Dickinson, D. A. Randall, A. K. Betts, F. G. Hall, J. A. Berry, G. J. Collatz, et al. 1997. Modeling the exchanges of energy, water, and carbon between continents and the atmosphere. *Science* 275: 502–509.

Smith, W. K., T. C. Vogelmann, E. H. DeLucia, D. T. Bell, and K. A. Shepherd. 1997. Leaf form and photosynthesis. *BioScience* 11: 785–793.

Sporck, M. J. 2011. The Hawaiian C_4 *Euphorbia* adaptive radiation: an ecophysiological approach to understanding leaf trait variation. Ph.D. dissertation, University of Hawai'i at Mānoa, Honolulu, HI, USA.

Stan Development Team. 2023. Stan modeling language users guide and reference manual. Website: https://mc-stan.org/

Terashima, I., Y. T. Hanba, Y. Tazoe, P. Vyas, and S. Yano. 2006. Irradiance and phenotype: comparative eco-development of sun and shade leaves in relation to photosynthetic CO_2 diffusion. *Journal of Experimental Botany* 57: 343–354.

Terashima, I., S.-I. Miyazawa, and Y. T. Hanba. 2001. Why are sun leaves thicker than shade leaves?—Consideration based on analyses of CO_2 diffusion in the leaf. *Journal of Plant Research* 114: 93–105.

Théroux-Rancourt, G., A. B. Roddy, J. M. Earles, M. E. Gilbert, M. A. Zwieniecki, C. K. Boyce, D. Tholen, et al. 2021. Maximum CO_2 diffusion inside leaves is limited by the scaling of cell size and genome size. *Proceedings of the Royal Society, B, Biological Sciences* 288: 20203145.

Tholen, D., and X.-G. Zhu. 2011. The mechanistic basis of internal conductance: theoretical analysis of mesophyll cell photosynthesis and CO_2 diffusion. *Plant Physiology* 156: 90–105.

Tomás, M., J. Flexas, L. Copolovici, J. Galmés, L. Hallik, H. Medrano, M. Ribas-Carbó, et al. 2013. Importance of leaf anatomy in determining mesophyll diffusion conductance to CO_2 across species: quantitative limitations and scaling up by models. *Journal of Experimental Botany* 64: 2269–2281.

Triplett, G., T. Buckley, and C. Muir. 2024. Amphistomy increases leaf photosynthesis more in coastal than montane plants of Hawaiian 'ilima (Version 4) [Data set]. Dryad. https://doi.org/10.5061/DRYAD.RXWDBRVFW

Wang, H., I. C. Prentice, T. F. Keenan, T. W. Davis, I. J. Wright, W. K. Cornwell, B. J. Evans, and C. Peng. 2017. Towards a universal model for carbon dioxide uptake by plants. *Nature Plants* 3: 734–741.

Wang, Y., J. S. Sperry, W. R. L. Anderegg, M. D. Venturas, and A. T. Trugman. 2020. A theoretical and empirical assessment of stomatal optimization modeling. *New Phytologist* 227: 311–325.

Wood, J. G. 1934. The physiology of xerophytism in Australian plants: the stomatal frequencies, transpiration and osmotic pressures of sclerophyll and tomentose-succulent leaved plants. *Journal of Ecology* 22: 69–87.

Xiong, D., and J. Flexas. 2020. From one side to two sides: the effects of stomatal distribution on photosynthesis. *New Phytologist* 228: 1754–1766.

Yorkston, M., and C. C. Daehler. 2006. Interfertility between Hawaiian ecotypes of *Sida fallax* (Malvaceae) and evidence of a hybrid disadvantage. *International Journal of Plant Sciences* 167: 221–230.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Supplemental figures and table.
Table S1. Average traits values among 'ilima leaves at each site.
Figure S1. Idealized method for collecting A-gsw curves on either amphi or pseudohypo leaves.

Figure S2. Individual -Agsw curves used to estimate AA.
Figure S3. Posterior predictions from fitted -Agsw curves closely match the observed distribution, indicating the the statistical model adequately captures variation in the response variable over the measured range.
Figure S4. The photosynthetic rate and stomatal conductance to water vapor of montane and coastal 'ilima leaves.
Figure S5. The gsmax, ratio (-yaxis) of montane and coastal 'ilima leaves.