

1 **Amphistomy increases leaf photosynthesis more in**
2 **coastal than montane plants of Hawaiian ‘ilima**
3 **(*Sida fallax*)**
4

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11 Running head: Amphistomy advantage in ‘ilima

12 ABSTRACT —

13 **Premise of the study** The adaptive significance of stomata on both upper and lower leaf surfaces, called
14 amphistomy, is unresolved. A widespread association between amphistomy and open, sunny habitats
15 suggests the adaptive benefit of amphistomy may be greatest in these contexts, but this hypothesis has
16 not been tested experimentally. Understanding why amphistomy evolves can inform its potential as a
17 target for crop improvement and paleoenvironment reconstruction.

18 **Methods** We developed a new method to quantify “amphistomy advantage”, AA, as the ratio of photo-
19 synthesis in an amphistomatous leaf to that of the same leaf with the same total stomatal conductance but
20 with gas exchange blocked through the upper (adaxial) surface, which we term ‘pseudohypostomy’. We
21 thus estimated AA and related physiological and anatomical traits in 12 populations, six coastal (open,
22 sunny) and six montane (closed, shaded), of the indigenous Hawaiian species ‘ilima (*Sida fallax*).

23 **Key results** Coastal ‘ilima leaves benefit 4.58 times more from amphistomy compared to their montane
24 counterparts. Our evidence was equivocal with respect to two hypotheses – that coastal leaves benefit
25 more because they are thicker and therefore have lower CO₂ conductance through the internal airspace,
26 and that leaves with more similar conductances at each surface benefit more than leaves with few
27 stomata on the upper surface.

28 **Conclusions** This is the first direct experimental evidence that amphistomy *per se* increases photo-
29 synthesis, consistent with the hypothesis that parallel pathways through upper and lower mesophyll
30 increase the supply of CO₂ to chloroplasts. The prevalence of amphistomatous leaves in open, sunny
31 habitats can partially be explained the increased benefit of amphistomy in ‘sun’ leaves, but the mecha-
32 nistic basis of this observation is an area for future research.

33 **Keywords:** amphstomy, leaf, light, Hawai‘i, leaf, *Sida fallax*, stomata

34 INTRODUCTION —

35 Amphistomy, the presence of stomata on both lower and upper surfaces of broad leaves, should increase
36 carbon gain by reducing the average diffusion pathlength between stomata and chloroplasts (??), yet
37 paradoxically this seemingly simple adaptation is uncommon in nature and we don't know why. Un-
38 derstanding variation in stomatal traits like amphistomy is imperative because these tiny pores play
39 an outsized ecological role in the global carbon and water cycles (Hetherington and Woodward, 2003;
40 Berry et al., 2010). A widely applicable, accurate representation of how stomata mediate the relation-
41 ship between CO₂ gained through photosynthesis and water lost through evapotranspiration is essential
42 to predict future climate using Earth Systems Models (Jarvis, 1976; Ball et al., 1987; Collatz et al., 1991;
43 Leuning, 1995; Sellers et al., 1997). Optimality models accurately predict the major cause of water loss,
44 stomatal conductance (g_{sw}), by assuming plants maximize carbon gain minus a cost of water (Cowan
45 and Farquhar, 1977; Givnish, 1986; Medlyn et al., 2011; Lin et al., 2015; Wang et al., 2017; Franks et
46 al., 2018; Deans et al., 2020; Franklin et al., 2020; Wang et al., 2020; Harrison et al., 2021). Despite
47 the success of optimality modeling in predicting g_{sw} , the same modeling approach has so far failed to
48 explain the rarity of amphistomatous leaves (Muir, 2019). **This gap between theory and observa-**
49 **tions strongly implies that we remain ignorant about some key benefits and costs associated with**
50 **stomata.**

51 Where are amphistomatous leaves found and why aren't they more common? Among terrestrial flower-
52 ing plants, amphistomatous leaves are rarely found on woody plants and shade-tolerant herbs, but they
53 are common in annual and perennial herbs from sunny habitats (Salisbury, 1928; Parkhurst, 1978; Mott
54 et al., 1982; Peat and Fitter, 1994; Gibson, 1996; Jordan et al., 2014; Muir, 2015, 2018; Bucher et al.,
55 2017). Even in resupinate leaves where the abaxial surface faces up toward the sky, stomata develop on
56 the lower adaxial surface (Lyshede, 2002). Exceptions to this general pattern include some arid woody
57 plants which typically have vertically oriented, isobilateral leaves (Wood, 1934; Jordan et al., 2014;
58 Boer et al., 2016; Drake et al., 2019) and floating/amphibious leaves of aquatic plants (Kaul, 1976;

59 Doll et al., 2021). The dearth of amphistomatous leaves should be quite surprising and has been de-
60 scribed as one of the most important unsolved problems in the study of leaf structure-function relations
61 despite some recent progress (Grubb, 1977, 2020).

62 Amphistomatous leaves should be common because, all else being equal, a leaf with a given number
63 of stomata per area could increase its photosynthetic rate simply by apportioning approximately half
64 its stomata to each surface (Parkhurst, 1978; Gutschick, 1984a, b). The key difference between a
65 hypo- and amphistomatous leaf, holding all other factors constant, is that an amphistomatous leaf has
66 two parallel diffusion paths through the internal airspace to any given chloroplast. Those airspaces
67 pose a resistance for CO₂ diffusion, so CO₂ concentration drops as it approaches chloroplasts. Shorter
68 pathways mean a smaller drop in CO₂ concentration. Thus, chloroplasts in amphistomatous leaves
69 experience higher CO₂ concentrations than in hypostomatous leaves, thereby increasing photosynthesis.
70 The airspace resistance (or its inverse, the airspace conductance, g_{ias}) is rarely measured directly and
71 there is disagreement between empirical (Parkhurst and Mott, 1990; Morison et al., 2005; Evans et al.,
72 2009; Tomás et al., 2013; Earles et al., 2018; Šantrůček et al., 2019; Nobel, 2020; Harwood et al., 2021;
73 Márquez et al., 2023) and theoretical models (Tholen and Zhu, 2011; Ho et al., 2016; Thérout-Rancourt
74 et al., 2021). The g_{ias} in thin, porous leaves may be so large as to be inconsequential given much lower
75 conductances for other components of the diffusion pathway, whereas the g_{ias} of thick leaves with
76 little airspace may greatly hinder CO₂ diffusion to chloroplasts. Amphistomy should confer the largest
77 photosynthetic benefit in leaves with intrinsically low g_{ias} .

78 Amphistomy should also enhance photosynthesis when leaf boundary layer resistance is high, because
79 apportioning total flux between two boundary layers rather than one results in a smaller CO₂ concen-
80 tration drop between the atmosphere and stomata. A similar effect has been validated with a computer
81 model and measurements for transpiration: amphistomatous leaves lose somewhat more water for the
82 same vapor pressure deficit and total g_{sw} (Foster and Smith, 1986), but the additional carbon gain should
83 be enough to offset this cost under most realistic conditions (Muir, 2019).

84 The most promising adaptive hypothesis is that amphistomy is important for maximizing photosynthetic
 85 rate under high light. Mott et al. (1982) proposed that “plants with a high photosynthetic capacity,
 86 living in full-sun environments, and experiencing rapidly fluctuating or continuously available soil
 87 water” would benefit most, in terms of increased carbon gain, from having amphistomatous leaves.
 88 As described above, herbs from sunny habitats are often amphistomatous. Most variation in stomatal
 89 density ratio (SR, the ratio of stomatal density between the upper and lower surfaces) among species
 90 is assumed to be genetic, but there is also putatively adaptive plasticity in response to light. Leaves of
 91 *Ambrosia cordifolia*, a desert perennial herb, are hypostomatous under low light (photosynthetic photon
 92 flux density, PPFD = $110 \mu\text{mol m}^{-2} \text{s}^{-1}$) but develop ~20% of their stomata on the upper surface
 93 under high light ($1700 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Mott and Michaelson, 1991). Similarly, *Solanum lycopersicum*
 94 leaves are hypostomatous when grown in the shade but develop ~20% of their stomata on the upper
 95 surface grown under high light-intensity (Gay and Hurd, 1975). Adult leaves of *Eucalyptus globulus*
 96 are amphistomatous, but the proportion of adaxial stomata increases from ~10-20% under low light to
 97 ~30-40% under high light (James and Bell, 2001). In summary, both genetic and plastic responses to
 98 evince a widespread association between light and SR.

99 The association between high light and amphistomy suggests that ‘sun’ leaves have the most to gain in
 100 terms of increased photosynthesis from having stomata on both surfaces, as Mott et al. (1982) hypoth-
 101 esized. Parkhurst (1978) proposed quantifying this benefit as ‘amphistomy advantage’ (AA), which
 102 we adopt here with some modification (see Materials and Methods). This hypothesis has never been
 103 tested directly by comparing the photosynthetic rate of an amphistomatous leaf to that of an otherwise
 104 identical hypostomatous leaf with the same total stomatal conductance under the same conditions. We
 105 propose a straightforward method to do this by experimentally creating a pseudohypostomatous leaf
 106 with gas exchange blocked through the upper surface (see Materials and Methods). One reason that
 107 sun leaves might have greater AA is that they are usually thicker or denser (Poorter et al., 2019), re-
 108 sulting in lower g_{ias} (Parkhurst, 1978). A nonmutually exclusive hypothesis is that if sun leaves have
 109 a stomatal density ratio closer to 0.5 (same density on each leaf surface), this will confer a greater ad-

110 vantage than an amphistomatous leaf with most stomata on one surface. In other words, amphistomy
111 doesn't make much difference if one leaf surface has very open stomata on it. We therefore predict
112 that sun leaves will have greater AA possibly because they have thicker leaves and/or SR closer to 0.5.
113 We actually report $g_{\text{smax, ratio}}$, which is similar to SR except that takes into account differences in both
114 stomatal density and size between surfaces.

115 The native flora of the Hawaiian archipelago is an excellent system to test the relationship between
116 light habitat and AA. Many lineages have adapted to different light habitats after colonization and
117 leaf anatomical traits such as SR and thickness vary within and among closely related species. It is
118 hypothesized that the common ancestor in many Hawaiian clades was a "weedy" species with high
119 dispersal ability adapted to open habitats (Carlquist, 1966). Colonization was followed by adaptive
120 radiation into higher elevation, montane, closed, forested habitats. Consequently, adaptation to sun and
121 shade is a common axis of phenotypic variation among Hawaiian plants such as lobeliads (Givnish
122 et al., 2004; Montgomery and Givnish, 2008; Givnish et al., 2009; Givnish and Montgomery, 2014;
123 Scoffoni et al., 2015), *Bidens* (Carlquist, 1966; Knope et al., 2020), *Scaevola* (Robichaux and Pearcy,
124 1984; McKown et al., 2016), *Euphorbia* (Sporck, 2011), and *Plantago* (Dunbar-Co et al., 2009).

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

135 which makes light competition a factor that plants may need to adapt to. Possibly due to this, montane
136 populations are erect and shrub- or tree-like, capable of growing meters tall with strong, woody stems.
137 These individuals have smooth, green foliage with serrated edges. Montane populations exhibit traits
138 that help them to compete for light availability (Raunch et al. 1997). This montane morphology is
139 not found in *S. fallax* populations on other Pacific Islands (Pejhanmehr, 2022). The variable morphol-
140 ogy between coastal and montane populations has even led some scientists to argue that it makes up a
141 species complex, rather than a single species (Raunch et al. 1997).

142 The objectives of our study are to test whether 1) ‘sun’ leaves of coastal ‘ilima plants will have greater
143 AA than ‘shade’ leaves of montane plants; and if so, is this because 2a) coastal plants have thicker
144 leaves than montane plants and/or 2b) coastal plants have a $g_{\text{max, ratio}}$ closer to 0.5?

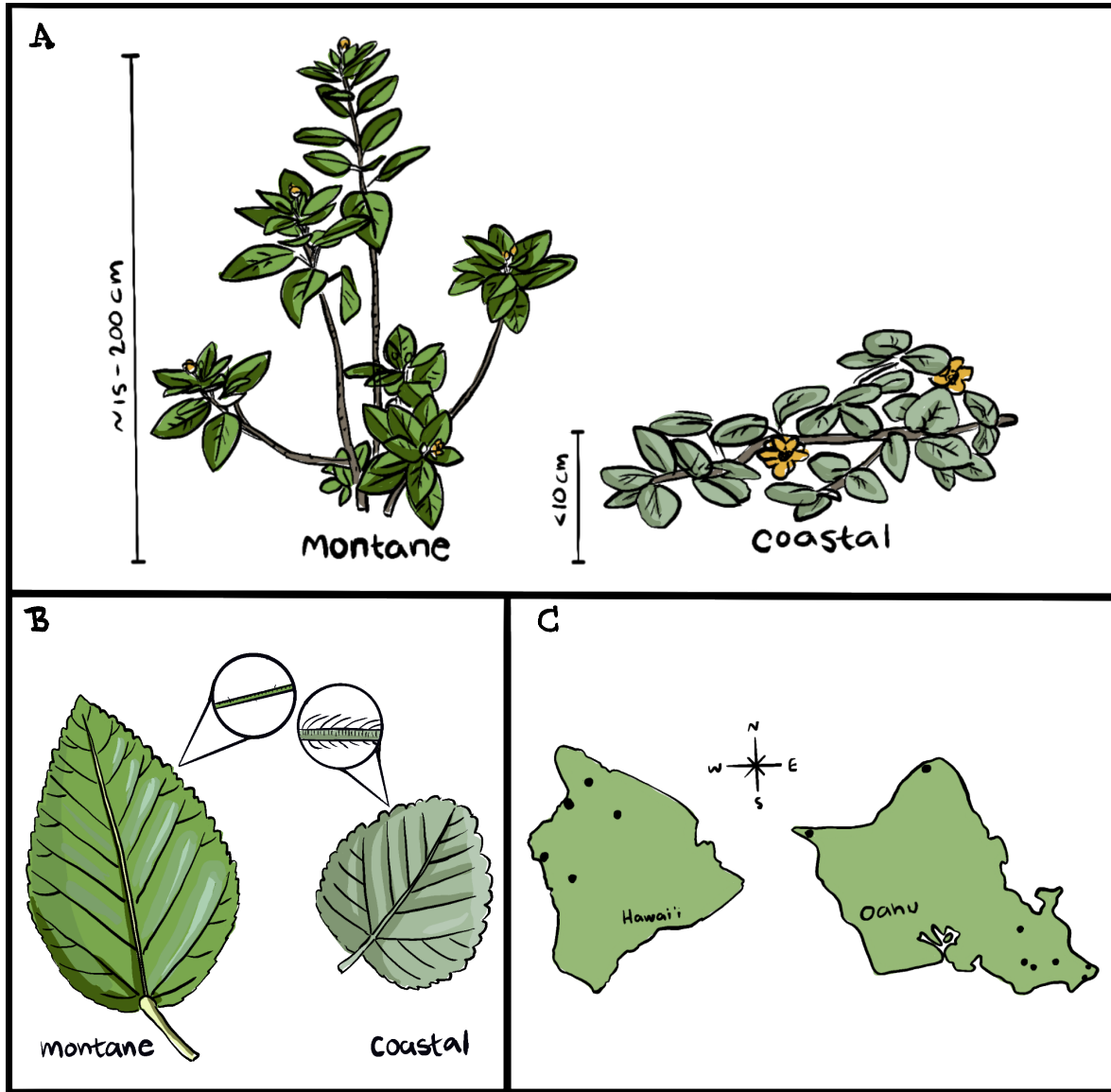


Figure 1: (A) Typical growth form of montane (left) and coastal (right) 'ilima plants and (B) leaves. (C) Map of the sites that were sampled on the islands of O'ahu and Hawai'i (aka Big Island).

Table 1: 'Ilima study sites for this study.

Site	Island	Habitat	Latitude	Longitude	Elevation (mas)
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 'Āina	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

MATERIALS AND METHODS —

Plant sampling —

We identified 8 suitable natural populations of 'ilima on O'ahu and 5 on Hawai'i Island by consulting (Yorkston and Daehler, 2006) and citizen scientist records on iNaturalist (Anon, 2022) (Fig. 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 in one three-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 traits 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.

157 **Leaf traits —**

158 *Stomata —*

159 We estimated the stomatal density and size on ab- and adaxial leaf surfaces from all leaves. For
160 pubescent leaves (usually coastal), we dried and pressed leaves for ≈ 1 week (Hill et al., 2014), care-
161 fully scraped trichomes off with a razor blade, and rehydrated the leaf. For glabrous leaves, we used
162 fresh leaves. We applied clear nail polish to both leaf surfaces of fresh or rehydrated leaves in the
163 middle of the lamina away from major veins. After nail polish dried, we mounted impressions on a mi-
164 croscope slide using transparent tape (Mott and Michaelson, 1991). We digitized a portion of each leaf
165 surface impression using a brightfield microscope (Leica DM2000, Wetzlar, Germany). We counted
166 all stomata and divided by the visible leaf area (0.890 mm^2) to estimate density and measured guard
167 cell length from five randomly chosen stomata per field using ImageJ (Schneider et al., 2012).

168 *Leaf thickness —*

169 We cut thin sections using two razor blades taped together and cutting the leaf in a petri dish of water,
170 wet-mounted sections onto a slide, and took digital micrographs using a brightfield microscope, as
171 described above.

172 *Gas exchange measurements —*

173 At each site, we selected one representative leaf from one plant near the middle of the transect for gas
174 exchange measurements using a portable infrared gas analyzer LI-6800PF, LI-COR Biosciences, Lin-
175 coln, NE, USA). We estimated the photosynthetic rate (A) and stomatal conductance to water vapor
176 (g_{sw}) at saturating light (photosynthetic photon flux density (PPFD) = $2000 \mu\text{mol m}^{-2} \text{ s}^{-1}$), ambient
177 CO_2 (415 ppm), and $T_{\text{leaf}} = 25.0\text{--}29.3^\circ\text{C}$. We also estimated ‘amphistomy advantage’ (AA) *sensu*

178 Parkhurst (1978), but with modification. For each leaf, we measured the photosynthetic rate of an un-
 179 treated amphistomatous leaf (A_{amphi}) over a range of g_{sw} values, holding other environmental variables
 180 constant. We refer to this as an A - g_{sw} curve. We compared the A - g_{sw} curve of the untreated leaf to
 181 the photosynthetic rate of “pseudohypostomatous” leaf (A_{hypo}), which is the same leaf but with gas
 182 exchange through the upper surface blocked by a neutral density plastic (propafilm). We refer to the
 183 untreated and partially blocked leaves as “amphi” and “pseudohypo”, respectively. AA is calculated
 184 as the log-response ratio of A compared at the same total g_{sw} :

$$\text{AA} = \log(A_{\text{amphi}}/A_{\text{hypo}})$$

185 The log-response ratio is easy to interpret because values above 0 indicate a photosynthetic advantage of
 186 amphistomy, whereas values less than 0 indicate a disadvantage. The response ratio is also better than
 187 the absolute difference because it indicates a proportional change in A , which facilitates comparisons
 188 across leaves and environments with different baseline photosynthetic rates. The irradiance of the
 189 light source in the pseudohypo leaf is higher because the propafilm reduces transmission. Because the
 190 propafilm reduces transmission, we used a higher incident PPFD for pseudohypo leaves (higher by a
 191 factor $1/0.91$, the inverse of the measured transmissivity of the propafilm). We also set the stomatal
 192 conductance ratio, for purposes of calculating boundary layer conductance, to 0 for pseudohypo leaves
 193 following manufacturer directions.

194 To control for order effects, we alternated between starting with amphi (untreated) or pseu-
 195 dohypo (treated) leaf measurements. In the field, we acclimated the focal leaf to high light
 196 ($\text{PPFD} = 2000 \mu\text{mol m}^{-2} \text{s}^{-1}$), high relative humidity ($\text{RH} = 70\%$), $T_{\text{leaf}} = 25 - 30^\circ\text{C}$, depending
 197 on ambient temperature, until A and g_{sw} reach their maximum. We used these data as our estimates
 198 of maximum A and g_{sw} . After that, we decreased RH to $\approx 10\%$ to induce rapid stomatal closure
 199 without photosynthetic downregulation. We logged data until g_{sw} reached its nadir. We then repeated

200 the process with the other treatment (amphi or pseudohypo).

201 **Data analysis —**

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

203 It is not feasible to record A_{amphi} and A_{hypo} at the exact same g_{sw} . To overcome this, we fit A - g_{sw}
204 curves using a flexible generalized additive model (GAM) to interpolate modeled A for amphi and
205 pseudohypo leaves at the same g_{sw} . In 10 of 12 leaves, the minimum g_{sw} of the amphi curve was
206 smaller than the maximum g_{sw} of the pseudohypo curve (i.e. the curves overlapped for a range of g_{sw}
207 values). In those cases, we estimated AA at the g_{sw} value in the middle of the range of overlap. In 2
208 of 12 leaves, the A - g_{sw} curves did not quite overlap because the minimum g_{sw} of the amphi curve was
209 slightly greater than the maximum g_{sw} of the pseudohypo curve. In those cases, we estimated AA by
210 extrapolating slightly, 1.98×10^{-3} and $3.29 \times 10^{-3} \text{ mol m}^{-2} \text{ s}^{-1}$, beyond the measured curves to
211 the g_{sw} value in between the curves. We fit GAMs using the *R* package **brms** version 2.20.1 (Bürkner,
212 2017) for Bayesian statistical inference with MCMC sampling in *Stan* (Stan Development Team, 2023).
213 We used CmdStan version 2.33.0 and **cmdstanr** version 0.6.1 (Gabry and Češnovar, 2023) to interface
214 with *R* version 4.3.1 (R Core Team, 2023). We sampled from posterior distribution from 4 chains with
215 1000 iterations each after 1000 warmup iterations. We estimated parameters and confidence intervals
216 as the median and 95% quantile intervals of the posterior, respectively. The key prediction is that
217 $AA_{\text{coastal}} > AA_{\text{montane}}$.

218 *Objective 2a: Are coastal leaves thicker than montane leaves? —*

219 We tested whether leaf thickness (log-transformed) varied between coastal and montane populations
220 and among individuals within populations using a Bayesian mixed-effects model with site-type as a
221 fixed effect and individual plant and site as random effects. We used the *R* package **brms** version

222 2.20.1 (Bürkner, 2017) to fit the model in *Stan* (Stan Development Team, 2023) with CmdStan version
 223 2.32.2 and **cmdstanr** version 0.5.3 (Gabry and Češnovar, 2023). We sampled the posterior distribution
 224 from 4 chains with 1000 iterations each after 1000 warmup iterations. We estimated the relationship
 225 between population average leaf thickness and AA measured from a single individual per population.
 226 We used this approach most of the variation in leaf thickness was among sites (see Results) and the
 227 plant selected for gas exchange measurements was not always among the plants randomly selected for
 228 leaf thickness, precluding individual level correlation. We propagated uncertainty about in AA and
 229 leaf thickness by integrating over the entire posterior distribution sample for each variable. The key
 230 prediction is that the effect of leaf thickness on AA is positive.

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

232 We tested whether $g_{\text{smax, ratio}}$ varied between coastal and montane populations and among individuals
 233 within populations using a Bayesian multiresponse, mixed-effects model. The modeled response vari-
 234 ables are stomatal count and guard cell length on each surface. Counts were modeled as negative
 235 binomially distributed variable from a latent stomatal density and a parameter ϕ to estimate overdispersion
 236 in counts relative to a Poisson model. For all traits, the explanatory variables were site-type as a
 237 fixed effect and leaf within individual plant, individual plant, and site as random effects. We used the *R*
 238 package **brms** version 2.20.1 (Bürkner, 2017) to fit the model in *Stan* (Stan Development Team, 2023)
 239 with CmdStan version 2.32.2 and **cmdstanr** version 0.5.3 (Gabry and Češnovar, 2023). We interpolated
 240 missing adaxial guard cell lengths from 6 out of 185 samples with zero adaxial stomata using the “mi”
 241 function in **brms** package. We sampled the posterior distribution from 4 chains with 1000 iterations
 242 each after 1000 warmup iterations. From each posterior sample, we calculated $g_{\text{smax, ratio}}$ as:

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

where $g_{\text{smax,lower}}$ and $g_{\text{smax,upper}}$ are maximum stomatal conductance to water vapor at $T_{\text{leaf}} = 25^\circ \text{C}$ on the lower and upper surface, respectively. The maximum stomatal conductance is calculated from stomatal density and length, assuming that stomata are fully open, following Sack and Buckley (2016).

We estimated the relationship between leaf $g_{\text{smax,ratio}}$ and AA measured from a single leaf per population. We propagated uncertainty about in AA and $g_{\text{smax,ratio}}$ by integrating over the entire posterior distribution sample for each variable. The key prediction is that the effect of $g_{\text{smax,ratio}}$ on AA is positive until $g_{\text{smax,ratio}} < 0.5$.

RESULTS —

Amphistomy advantage is greater in coastal leaves —

Amphistomy increases photosynthesis in leaves of coastal ‘ilima plants more than that of montane plants. AA was significantly greater than 0 (95% confidence intervals did not overlap 0) in 5 of 6 coastal leaves, but only 1 of 6 montane leaves (Fig. 2; see Fig. S1 for individual curves). Overall, the average AA among coastal and montane leaves is 0.12 [0.078–0.15] and 0.027 [–0.0033–0.057], respectively; the difference in average AA between habitat types is $AA_{\text{coastal}} - AA_{\text{montane}} = 0.09$ [0.04–0.14]. The difference between coastal and montane leaves is lower, 0.054 [–0.012–0.11], but still greater than 0 if we remove two sites where we extrapolated slightly beyond fitted $A-g_{\text{sw}}$ curves. Maximum photosynthetic rate was slightly, but not significantly higher in coastal leaves (Welch Two Sample t -test, $t_{9,65} = 1.6$, $P = 0.14$); total stomatal conductance was similar (Welch Two Sample t -test, $t_{9,71} = -0.09$, $P = 0.93$) in coastal and montane leaves (Fig. S2).

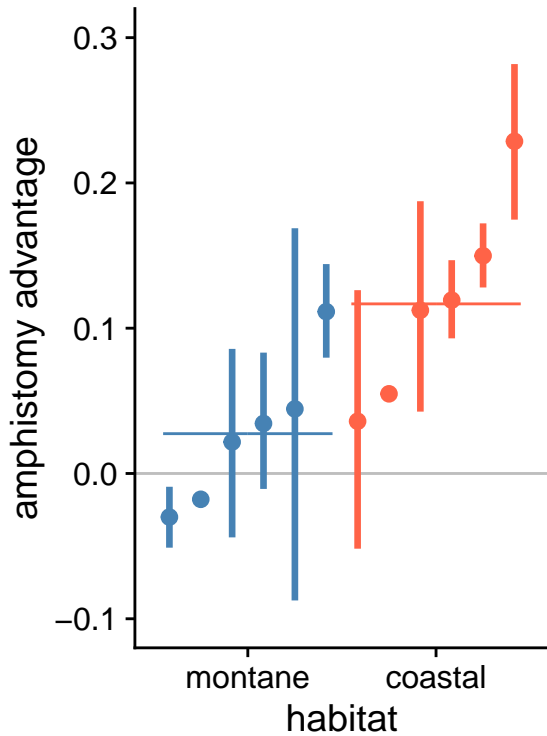


Figure 2: Coastal leaves benefit more amphistomy than montane leaves. A positive amphistomy advantage (y -axis) means that the photosynthetic rate of an amphistomatous leaf is greater than that of an identical pseudohypostomatous leaf at the same overall g_{sw} . Each point-interval is the median posterior 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.

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

263 Coastal ‘ilima leaves are 91 [26–164] μm thicker than their montane counterparts. Although coastal
 264 leaves are thicker and have greater AA, the relationship between leaf thickness and AA within habitats
 265 is actually slightly negative (Fig. 3A; slope = $-0.11 [-0.28-0.033]$), the opposite direction our
 266 hypothesis predicts.

267 $g_{\text{smax, ratio}}$ **is not associated with amphistomy advantage** —

268 Coastal and montane leaves have similar average $g_{\text{smax, ratio}}$, the ratio of adaxial (upper) to total
269 g_{smax} , the anatomical maximum stomatal conductance to water vapor (Fig. S3); coastal leaves have
270 0.057 [−0.16–0.28] higher $g_{\text{smax, ratio}}$ than montane leaves, but the 95% confidence intervals overlap 0
271 difference. The $g_{\text{smax, ratio}}$ is somewhat bimodal among sites. Some sites in both habitats have leaves
272 with $g_{\text{smax, ratio}} < 0.07$ and others with $g_{\text{smax, ratio}} > 0.2$ (Fig. S3). This is particularly noticeable in
273 montane sites where those on the Big Island of Hawai‘i all have low $g_{\text{smax, ratio}}$ whereas those on O‘ahu
274 have relatively high $g_{\text{smax, ratio}}$. There is no relationship between $g_{\text{smax, ratio}}$ and AA in either habitat
275 (Fig. 3B; slope = 0.11 [−0.032–0.25]) in our sample.

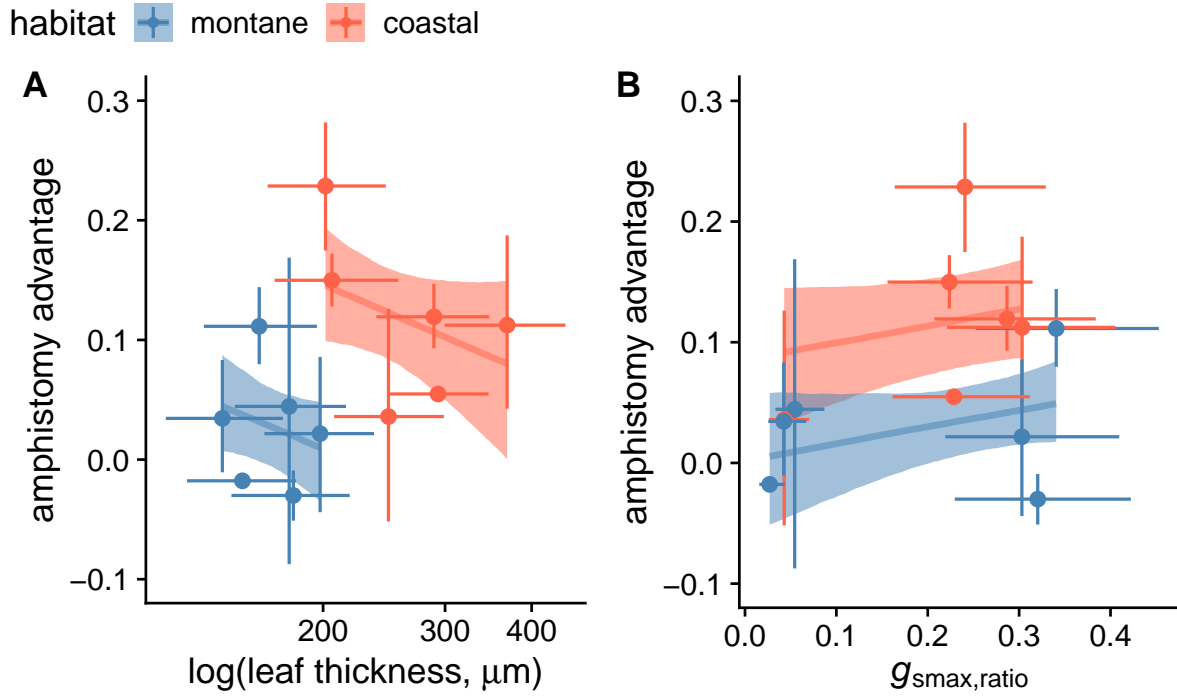


Figure 3: Relationships between leaf amphistomy advantage, (A) $g_{smax,ratio}$ and (B) leaf thickness among ‘ilima (*Sida fallax*) plants from montane (blue) and coastal (orange) habitats in Hawai‘i, and amphistomy advantage. A positive amphistomy advantage (y -axis) means that the photosynthetic rate 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) $\log(\text{leaf thickness})$ 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.

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

280 shade leaves, but progress has been limited by the lack of evidence that amphistomy *per se* affects
281 improves photosynthesis in a given leaf. By experimentally blocking gas exchange through the upper
282 surface in a controlled environment, we directly compared an amphistomatous leaf to an otherwise iden-
283 tical pseudohypostomatous leaf. This allows us to quantify the ‘amphistomy advantage’ (AA) holding
284 all else constant. Taking advantage of the of the steep climatic gradients in the Hawaiian archipelago,
285 we applied this new method to show for the first time that sun leaves benefit more from amphistomy
286 than shade leaves in ‘ilima (*Sida fallax*). While this result has not yet been validated in other speci-
287 esthere are limits to how much we can generalize to all land plants, our results indicate that part of
288 the reason amphistomatous leaves are found most commonly in high light habitats is that the adaptive
289 benefit is greater in this environment.

290 If AA is typically greater in sun leaves than shade leaves, it could partially explain the distribution of
291 amphi- and hypostomatous leaves, but the precise mechanism(s) require further study. One hypothesis
292 is that the internal airspace conductance, g_{ias} , from stomata to mesophyll cell walls is lower in thicker
293 sun leaves (Parkhurst, 1978). All else being equal, a leaf with lower g_{ias} will benefit more from am-
294 phistomy. Our results partially support this hypothesis. Coastal ‘ilima leaves with high AA (Fig. 2)
295 are thicker than montane leaves, but the relationship between AA and leaf thickness within habitats is
296 actually slightly negative (Fig. 3A), opposite our prediction. Since coastal and montane leaves differ in
297 many respects besides thickness, we do not have enough data to conclude that leaf thickness explains the
298 variation in AA between habitats. Alternatively, other biochemical or anatomical differences between
299 coastal and montane leaves may explain why AA is greater in coastal leaves. The negative relationship
300 between leaf thickness and AA could be explained if thicker leaves compensated by having a more
301 porous mesophyll and/or less tortuous airspaces (Th  roux-Rancourt et al., 2021).

302 A second natural hypothesis is that amphistomatous leaves with few adaxial (upper) stomata benefit
303 less than those with similar densities on both surfaces. We predicted that leaves with $g_{smax, ratio}$ closer to
304 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 $g_{\text{smax, ratio}}$ and low AA whereas low montane leaves on O'ahu had high $g_{\text{smax, ratio}}$, but similarly low AA (??B). Among coastal sites, the site with the lowest $g_{\text{smax, ratio}}$ had the lowest AA, but there was little variation in $g_{\text{smax, ratio}}$ among coastal leaves in our sample. We therefore cannot rule out that a larger sample of coastal leaves with greater variance in $g_{\text{smax, ratio}}$ 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 $2\times$ higher area-based photosynthetic rates (Xiong and Flexas, 2020), naively implying an $\text{AA} \approx \log 2 = 0.69$. This 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 limita-

tion 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 crops leaves tend to increase stomatal density ratio during domestication (Milla et al., 2013).

Two limitations of this study are the small sample sizes and experimental design that precludes distinguishing genetic from environmental differences in leaf traits. 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 exciting avenues 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, it would be useful understand genetic and environmental contributions to trait variation. A reciprocal transplant would be able to determine the genetic and environmental contributions, as well their interaction, to trait variance in nature.

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 straightfor-

ward gas exchange 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*) enjoyed a greater photosynthetic benefit from amphistomy than nearby montane leaves living in more closed forest. We cannot yet ascribe the difference in amphistomy advantage between coastal and montane leaves to particular physiological or anatomical variation, but this is a promising area for future research.

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Author Contributions —

GT and CDM contributed equally to all stages of this project; TNB contributed to development of the method and helped edit the manuscript.

Data Availability Statement —

Custom scripts are available on a GitHub repository (<https://github.com/cdmuir/stomata-ilima>) and will be archived on Zenodo with a DOI and stable URL upon publication. Raw data will be deposited on Dryad with a DOI and stable URL upon publication.

Supporting Information —

Additional supporting information may be found online in the Supporting Information section at the end of the article

- Appendix S1: Supplemental figures and table

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