

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  
20 but with gas exchange blocked through the upper (adaxial) surface, which we term “pseudohypos-  
21 tomy”. We thus estimated AA and related physiological and anatomical traits in 12 populations, six  
22 coastal (open, sunny) and six montane (closed, shaded), of the indigenous Hawaiian species ‘ilima (*Sida*  
23 *fallax*).

24 **Key results** Coastal ‘ilima leaves benefit 4 times more from amphistomy compared to their montane  
25 counterparts. Our evidence was equivocal with respect to two hypotheses – that coastal leaves benefit  
26 more because they are thicker and therefore have lower CO<sub>2</sub> conductance through the internal airspace,  
27 and that leaves with more similar conductances at each surface benefit more than leaves with few  
28 stomata on the upper surface.

29 **Conclusions** This is the first direct experimental evidence that amphistomy *per se* increases photo-  
30 synthesis, consistent with the hypothesis that parallel pathways through upper and lower mesophyll  
31 increase the supply of CO<sub>2</sub> to chloroplasts. The prevalence of amphistomatous leaves in open, sunny  
32 habitats can partially be explained the increased benefit of amphistomy in ‘sun’ leaves, but the mecha-  
33 nistic basis of this observation is an area for future research.

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

## 35 INTRODUCTION —

36 Amphistomy, the presence of stomata on both lower and upper surfaces of broad leaves, should in-  
37 crease carbon gain by reducing the average diffusion pathlength between stomata and chloroplasts, yet  
38 paradoxically this seemingly simple adaptation is uncommon in nature and we don't know why. Un-  
39 derstanding variation in stomatal traits like amphistomy is imperative because these tiny pores play  
40 an outsized ecological role in the global carbon and water cycles (Hetherington and Woodward, 2003;  
41 Berry et al., 2010). A widely applicable, accurate representation of how stomata mediate the relation-  
42 ship between CO<sub>2</sub> gained through photosynthesis and water lost through evapotranspiration is essential  
43 to predict future climate using Earth Systems Models (Jarvis, 1976; Ball et al., 1987; Collatz et al., 1991;  
44 Leuning, 1995; Sellers et al., 1997). Optimality models accurately predict the major cause of water loss,  
45 stomatal conductance ( $g_{sw}$ ), by assuming plants maximize carbon gain minus a cost of water (Cowan  
46 and Farquhar, 1977; Givnish, 1986; Medlyn et al., 2011; Lin et al., 2015; Wang et al., 2017; Franks et  
47 al., 2018; Deans et al., 2020; Franklin et al., 2020; Wang et al., 2020; Harrison et al., 2021). Despite  
48 the success of optimality modeling in predicting  $g_{sw}$ , the same modeling approach has so far failed to  
49 explain the rarity of amphistomatous leaves (Muir, 2019). **This gap between theory and observa-**  
50 **tions strongly implies that we remain ignorant about some key benefits and costs associated with**  
51 **stomata.**

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

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

63 Amphistomatous leaves should be common because, all else being equal, a leaf with a given number  
64 of stomata per area could increase its photosynthetic rate simply by apportioning approximately half  
65 its stomata to each surface (Parkhurst, 1978; Gutschick, 1984a, b). The key difference between a  
66 hypo- and amphistomatous leaf, holding all other factors constant, is that an amphistomatous leaf has  
67 two parallel diffusion paths through the internal airspace to any given chloroplast. Those airspaces  
68 pose a resistance for CO<sub>2</sub> diffusion, so CO<sub>2</sub> concentration drops as it approaches chloroplasts. Shorter  
69 pathways mean a smaller drop in CO<sub>2</sub> concentration. Thus, chloroplasts in amphistomatous leaves  
70 experience higher CO<sub>2</sub> concentrations than in hypostomatous leaves, thereby increasing photosynthesis.  
71 The airspace resistance (or its inverse, the airspace conductance,  $g_{ias}$ ) is rarely measured directly and  
72 there is disagreement between empirical (Parkhurst and Mott, 1990; Morison et al., 2005; Evans et al.,  
73 2009; Tomás et al., 2013; Earles et al., 2018; Šantrůček et al., 2019; Nobel, 2020; Harwood et al., 2021;  
74 Márquez et al., 2023) and theoretical models (Tholen and Zhu, 2011; Ho et al., 2016; Thérout-Rancourt  
75 et al., 2021). The  $g_{ias}$  in thin, porous leaves may be so large as to be inconsequential given much lower  
76 conductances for other components of the diffusion pathway, whereas the  $g_{ias}$  of thick leaves with  
77 little airspace may greatly hinder CO<sub>2</sub> diffusion to chloroplasts. Amphistomy should confer the largest  
78 photosynthetic benefit in leaves with intrinsically low  $g_{ias}$ .

79 Amphistomy should also enhance photosynthesis when leaf boundary layer resistance is high, because  
80 apportioning total flux between two boundary layers rather than one results in a smaller CO<sub>2</sub> concen-  
81 tration drop between the atmosphere and stomata. A similar effect has been validated with a computer  
82 model and measurements for transpiration: amphistomatous leaves lose somewhat more water for the  
83 same vapor pressure deficit and total  $g_{sw}$  (Foster and Smith, 1986), but the additional carbon gain should  
84 be enough to offset this cost under most realistic conditions (Muir, 2019).

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

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

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

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

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

136 which makes light competition a factor that plants may need to adapt to. Possibly due to this, montane  
137 populations are erect and shrub- or tree-like, capable of growing meters tall with strong, woody stems.  
138 These individuals have smooth, green foliage with serrated edges. Montane populations exhibit traits  
139 that may help them to compete for light availability. This montane morphology is not found in *S. fallax*  
140 populations on other Pacific Islands (Pejhanmehr, 2022).

141 Because of their contrasting habitat and morphology, we treat leaves from coastal and montane plants as  
142 representatives of sun and shade leaves, respectively, for testing hypotheses about amphistomy advantage.  
143 Specifically, the objectives of our study are to test whether 1) sun leaves of coastal 'ilima plants  
144 will have greater AA than shade leaves of montane plants; and if so, is this because 2a) coastal plants  
145 have thicker leaves than montane plants and/or 2b) coastal plants have a  $g_{\text{smax, ratio}}$  closer to 0.5?

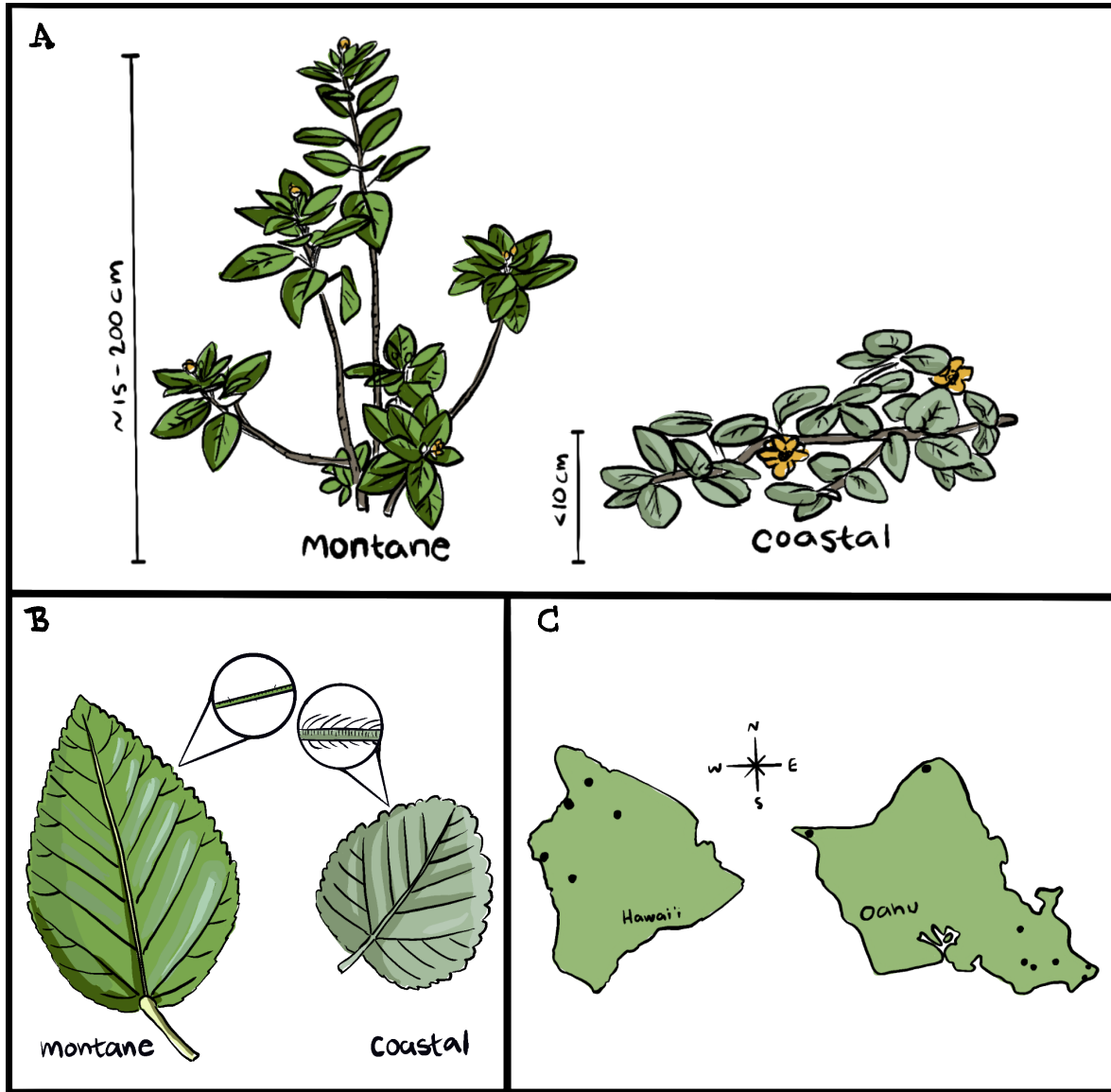


Figure 1: (A) Typical growth form of montane (left) and coastal (right) 'ilima plants and (B) leaves. (C) Map of the sites that were sampled on the islands of O'ahu and Hawai'i (aka Big Island).



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 7 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 during 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 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.

## 158 **Leaf traits** —

### 159 *Stomata* —

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

### 169 *Leaf thickness* —

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

### 173 *Gas exchange measurements* —

174 At each site, we selected one representative leaf from one plant near the middle of the transect for gas  
175 exchange measurements using a portable infrared gas analyzer (LI-6800PF, LI-COR Biosciences, Lin-  
176 coln, NE, USA). We estimated the photosynthetic rate ( $A$ ) and stomatal conductance to water vapor  
177 ( $g_{sw}$ ) at saturating light (photosynthetic photon flux density (PPFD) =  $2000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ), ambient  
178  $\text{CO}_2$  (415 ppm), and  $T_{\text{leaf}} = 25.0\text{--}29.3^\circ\text{C}$ . We also estimated ‘amphistomy advantage’ (AA) *sensu*

179 Parkhurst (1978), but with modification. For each leaf, we measured the photosynthetic rate of an un-  
 180 treated amphistomatous leaf ( $A_{\text{amphi}}$ ) over a range of  $g_{\text{sw}}$  values. We refer to this as an  $A$ - $g_{\text{sw}}$  curve.  
 181 We compared the  $A$ - $g_{\text{sw}}$  curve of the untreated leaf to the photosynthetic rate of pseudohypostoma-  
 182 tous leaf ( $A_{\text{hypo}}$ ), which is the same leaf but with gas exchange through the upper surface blocked by  
 183 a neutral density plastic (propafilm). Hypostomy refers to leaves with stomata only present on the  
 184 lower, typically abaxial, surface. We refer to the untreated and partially blocked leaves as “amphi” and  
 185 “pseudohypo”, respectively. AA is calculated as the log-response ratio of  $A$  compared at the same total  
 186  $g_{\text{sw}}$ .

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

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

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

201 photosynthetic downregulation. All other environmental conditions in the leaf chamber remained the  
202 same. We logged data until  $g_{sw}$  reached its nadir. We then repeated the process with the other treatment  
203 (amphi or pseudohypo).

## 204 **Data analysis —**

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

206 It is not feasible to record  $A_{amphi}$  and  $A_{hypo}$  at the exact same  $g_{sw}$ . To overcome this, we fit  $A-g_{sw}$   
207 curves using a linear regression of  $\log(g_{sw})$  on  $A$  to interpolate modeled  $A$  for amphi and pseudohypo  
208 leaves at the same  $g_{sw}$ . In 10 of 12 leaves, the minimum  $g_{sw}$  of the amphi curve was smaller than the  
209 maximum  $g_{sw}$  of the pseudohypo curve (i.e. the curves overlapped for a range of  $g_{sw}$  values). In those  
210 cases, we estimated AA at the  $g_{sw}$  value in the middle of the range of overlap. In 2 of 12 leaves, the  
211  $A-g_{sw}$  curves did not quite overlap because the minimum  $g_{sw}$  of the amphi curve was slightly greater  
212 than the maximum  $g_{sw}$  of the pseudohypo curve. In those cases, we estimated AA by extrapolating  
213 slightly,  $1.98 \times 10^{-3}$  and  $3.29 \times 10^{-3} \text{ mol m}^{-2} \text{ s}^{-1}$ , beyond the measured curves to the  $g_{sw}$  value in  
214 between the curves. We fit Bayesian regressions using the *R* package **brms** version 2.20.1 (Bürkner,  
215 2017) with MCMC sampling in *Stan* (Stan Development Team, 2023). We used CmdStan version  
216 2.33.0 and **cmdstanr** version 0.6.1 (Gabry and Češnovar, 2023) to interface with *R* version 4.3.1 (R  
217 Core Team, 2023). We sampled the posterior distribution from 4 chains with 1000 iterations each after  
218 1000 warmup iterations. We estimated parameters and confidence intervals as the median and 95%  
219 quantile intervals of the posterior, respectively. The key prediction is that  $AA_{coastal} > AA_{montane}$ .

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

221 We tested whether leaf thickness (log-transformed) varied between coastal and montane populations  
222 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 2.20.1 (Bürkner, 2017) to fit the model in *Stan* (Stan Development Team, 2023) with CmdStan version 2.33.0 and **cmdstanr** version 0.6.1 (Gabry and Češnovar, 2023). We sampled the posterior distribution from 4 chains with 1000 iterations each after 1000 warmup iterations. We estimated the relationship between population average leaf thickness and AA measured from a single individual per population. We used this approach 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 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.

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

We tested whether  $g_{\text{smax, ratio}}$  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  $\phi$  to estimate overdispersion 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 2.20.1 (Bürkner, 2017) to fit the model in *Stan* (Stan Development Team, 2023) with CmdStan version 2.33.0 and **cmdstanr** version 0.6.1 (Gabry and Češnovar, 2023). We interpolated missing adaxial guard cell lengths from 6 out of 185 samples with zero adaxial stomata using the “mi” function in **brms** package. We sampled the posterior distribution from 4 chains with 1000 iterations 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 [0.077–0.15] and 0.027 [−0.0044–0.057], respectively; the difference in average AA between habitat types is  $\text{AA}_{\text{coastal}} - \text{AA}_{\text{montane}} = 0.089$  [0.04–0.14]. Posterior predictions closely match observed values of  $A$  (Fig. S2), 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, but this is less certain. When we remove two leaves where extrapolated slightly beyond fitted  $A-g_{\text{sw}}$  curves, we estimate that  $\text{AA}_{\text{coastal}}$  is still positive, 0.081 [0.022–0.14], but the difference between coastal and montane leaves is smaller, 0.053 [−0.011–0.12], and confidence intervals slightly overlap 0. Maximum photosynthetic rate was slightly, but not significantly higher in coastal leaves (Welch Two Sample  $t$ -test,  $t_{9,65} = 1.6$ ,  $P = 0.14$ ); total stomatal conductance was similar (Welch Two Sample  $t$ -test,  $t_{9,71} = -0.09$ ,  $P = 0.93$ ) in coastal and montane leaves (Fig. S3).

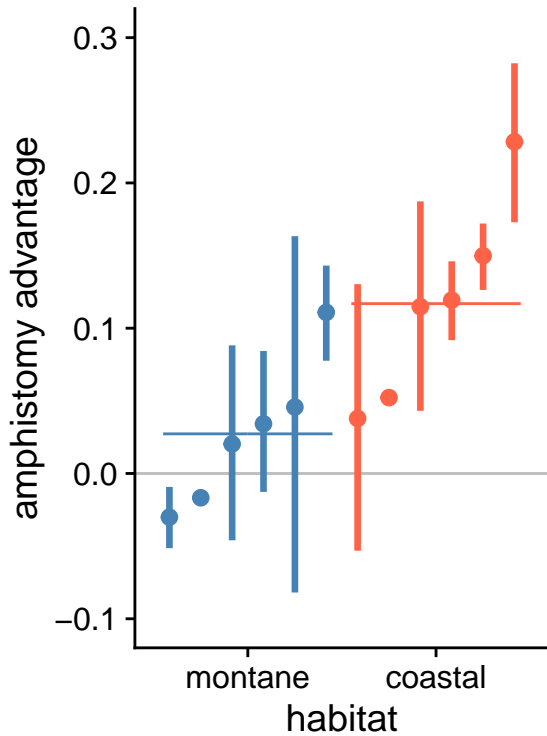


Figure 2: Coastal leaves benefit more amphistomy than montane leaves. A positive amphistomy advantage ( $y$ -axis) means that the photosynthetic rate of an amphistomatous leaf is greater than that of an identical pseudohypostomatous leaf at the same overall  $g_{sw}$ . Each point-interval is the median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

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

269 Coastal ‘ilima leaves are 91 [26–164]  $\mu\text{m}$  thicker than their montane counterparts. Although coastal  
 270 leaves are thicker and have greater AA, there is little relationship between leaf thickness and AA within  
 271 habitats (Fig. 3A; slope =  $-0.11$  [ $-0.27$ – $0.036$ ]).

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

273 Coastal and montane leaves have similar average  $g_{\text{smax, ratio}}$ , the ratio of adaxial (upper) to total  
274  $g_{\text{smax}}$ , the anatomical maximum stomatal conductance to water vapor (Fig. S4); coastal leaves have  
275 0.059 [−0.14–0.28] higher  $g_{\text{smax, ratio}}$  than montane leaves, but the 95% confidence intervals overlap 0  
276 difference. The  $g_{\text{smax, ratio}}$  is somewhat bimodal among sites. Some sites in both habitats have leaves  
277 with  $g_{\text{smax, ratio}} < 0.07$  and others with  $g_{\text{smax, ratio}} > 0.2$  (Fig. S4). This is particularly noticeable in  
278 montane sites where those on the Big Island of Hawai‘i all have low  $g_{\text{smax, ratio}}$  whereas those on O‘ahu  
279 have relatively high  $g_{\text{smax, ratio}}$ . There is no relationship between  $g_{\text{smax, ratio}}$  and AA in either habitat  
280 (Fig. 3B; slope = 0.14 [−0.06–0.35]) in our sample.



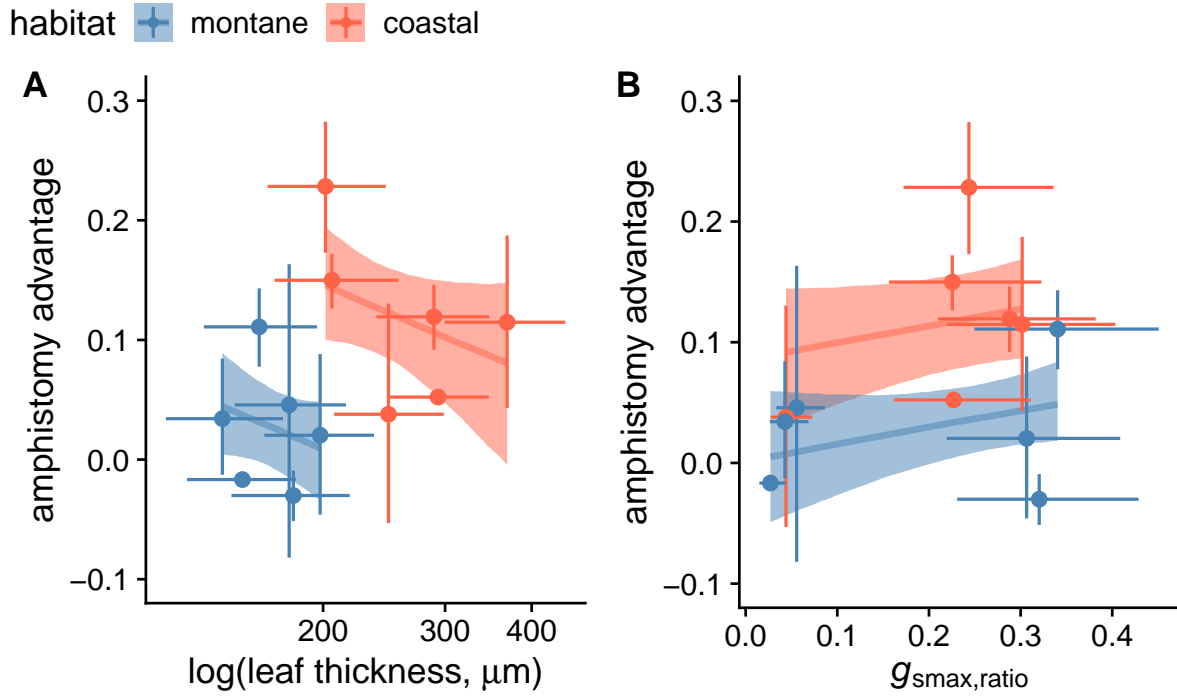


Figure 3: Relationships between leaf amphistomy advantage, (A) leaf thickness and (B)  $g_{\text{smax,ratio}}$  among 'ilima (*Sida fallax*) plants from montane (blue) and coastal (orange) habitats in Hawai'i. A positive amphistomy advantage ( $y$ -axis) means that the photosynthetic rate of an amphistomatous leaf is greater than that of an identical pseudohypostomatous leaf at the same overall  $g_{\text{sw}}$ . 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)  $g_{\text{smax,ratio}}$  on amphistomy advantage; ribbons are the 95% confident bands of the regression. Symbols:  $g_{\text{smax,ratio}}$ , anatomical maximum stomatal conductance ratio;  $g_{\text{sw}}$ , 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 shade leaves, but progress has been limited by the lack of evidence that amphistomy *per se* affects

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. This allows 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 benefit 4 times more from amphistomy than shade leaves on 'ilima (*Sida fallax*) plants ( $AA_{\text{coastal}} = 0.12$  vs.  $AA_{\text{montane}} = 0.027$ ). 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 this environment.

If AA is typically greater in sun leaves than shade leaves, it 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,  $g_{\text{ias}}$ , from stomata to mesophyll cell walls is lower in thicker sun leaves (Parkhurst, 1978). All else being equal, a leaf with lower  $g_{\text{ias}}$  will benefit more from amphistomy. Our results partially support this hypothesis. Coastal 'ilima leaves with high AA (Fig. 2) are thicker than montane leaves, but the relationship between AA and leaf thickness within habitats is actually slightly negative (Fig. 3A), opposite 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 between leaf thickness and AA could be explained if thicker leaves compensated by having a more porous mesophyll and/or less tortuous airspaces (Th  roux-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  $g_{\text{max, ratio}}$  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

311 limited lateral diffusion (Morison et al., 2005). Our results do not support this hypothesis. Montane  
312 leaves from Big Island sites had low  $g_{\text{smax, ratio}}$  and low AA whereas low montane leaves on O‘ahu had  
313 high  $g_{\text{smax, ratio}}$ , but similarly low AA (Fig. 3B). Among coastal sites, the site with the lowest  $g_{\text{smax, ratio}}$   
314 had the lowest AA, but there was little variation in  $g_{\text{smax, ratio}}$  among coastal leaves in our sample. We  
315 therefore cannot rule out that a larger sample of coastal leaves with greater variance in  $g_{\text{smax, ratio}}$  might  
316 support this hypothesis.

317 Two major implications from our study are that 1) photosynthesis in hypostomatous leaves is likely  
318 limited by CO<sub>2</sub> concentration drawdown within leaf airspaces; and 2) amphistomy *per se* contributes  
319 to, but is not wholly responsible for, higher photosynthetic rates among amphistomatous leaves. The  
320 amphistomy advantage we observe in ‘ilima leaves implies decreased CO<sub>2</sub> supply in pseudohypostom-  
321 atous leaves because of concentration drawdowns in the leaf airspace. Limited diffusion through the  
322 airspace has long been hypothesized to depress photosynthesis in hypostomatous leaves (Parkhurst,  
323 1994), with empirical support from helox studies (Parkhurst and Mott, 1990). However, these studies  
324 relied on interspecific comparisons of amphi- and hypostomatous leaves that differ systematically in  
325 many traits that affect gas exchange and photosynthesis (Xiong and Flexas, 2020). Our experimental  
326 approach overcomes this limitation and implies that the drop in CO<sub>2</sub> concentration from substomatal  
327 cavities to the upper surface depresses photosynthesis.

328 Among land plants grown in a common garden, amphistomatous leaves have on average nearly 2×  
329 higher area-based photosynthetic rates (Xiong and Flexas, 2020), naively implying an AA  $\approx \log 2 =$   
330 0.69. This is much higher than our estimate of 0.12 among coastal ‘ilima leaves. The most likely expla-  
331 nation is that amphistomy is not the only cause of high photosynthetic rate. Indeed, species adapted to  
332 open, high light habitats with amphistomatous leaves also have higher concentrations of Rubisco, over-  
333 all stomatal conductance, and photosynthetic capacity (Smith et al., 1997; Xiong and Flexas, 2020).  
334 For a leaf with high photosynthetic capacity that is well illuminated and hydrated, the major limita-  
335 tion becomes CO<sub>2</sub>. Under these conditions, amphistomy may substantially increase photosynthesis,

336 as we observe in coastal 'ilima leaves. Selection on increased photosynthesis under similar conditions  
337 may explain why crops leaves tend to increase stomatal density ratio during domestication (Milla et al.,  
338 2013).

339 Two limitations of this study are the small sample size and experimental design that precludes distin-  
340 guishing genetic from environmental differences in leaf traits. Understanding the mechanistic basis of  
341 higher AA in sun leaves would require much larger sample sizes. Sun leaves tend to be thicker, more  
342 densely packed with mesophyll cells, and have greater photosynthetic capacity and higher stomatal  
343 conductance, among other traits (Lambers et al., 2008). Each of these factors and others potentially  
344 modulate AA. Quantifying the contribution of all these factors requires larger samples and additional  
345 measurements that are beyond the scope of this study, but exciting avenues for future research on leaf  
346 structure-function relations. Although many morphological traits that distinguish coastal and montane  
347 'ilima populations persist in a common environment (Yorkston and Daehler, 2006), we cannot distin-  
348 guish between genetic effects and plastic responses to habitat as causes of difference in AA because  
349 we measured naturally occurring plants *in situ*. While disentangling genetic and plastic contributions  
350 is not necessarily important for understanding the distribution of amphistomatous leaves, it would be  
351 insightful to know about genetic and environmental contributions to trait variation. A reciprocal trans-  
352 plant would be able to determine the genetic and environmental contributions, as well their interaction,  
353 to trait variance in nature.

## 354 CONCLUSIONS —

355 This study reports the first direct experimental evidence that having stomata open on both leaf surfaces,  
356 amphistomy, increases photosynthesis for a given total stomatal conductance, particularly in leaves  
357 from the type of open, sunny habitats where this trait is most common. By developing a straightfor-  
358 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. [THE GITHUB REPO AND DRYAD DATA ARE AVAILABLE TO REVIEWERS]

## 377 Supporting Information —

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

- 380 • Appendix S1: Supplemental figures and table

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