UNIVERSITY OF CALIFORNIA

Los Angeles

Causes and Consequences of Variation in Fern Leaf Form and Physiology

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Biology

by

Christine Fiona Czerniak

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ABSTRACT OF THE DISSERTATION

Causes and Consequences of Variation in Fern Leaf Form and Physiology

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Doctor of Philosophy in Biology

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Professor Lawren Sack, Chair

Understanding how ferns vary their leaf traits across species and environments will improve models of ecosystem response to environmental change because the habitat specificity of ferns makes them excellent indicator species. Identifying drivers of variation in fern leaf traits will also support construction of predictive models for species ecological tolerances, distributions, and evolutionary trajectories. The goal of this dissertation was to execute five studies of diverse species across variable environments from three global regions, combining meta-analyses, field research, and experimental manipulations to investigate the causes and consequences of variation in fern leaf form and physiology.

Size-scaling of plant and leaf parts to maintain functional proportions was a significant factor explaining diversity in fern anatomy and morphology across species and environments, from differences in the density of veins to the size and shape of leaves. Adherence to global trends for the leaf economics spectrum – investment in higher leaf mass per area (LMA) at a cost of lower photosynthesis – also explained variation in leaf form and physiology due to different resource allocation patterns in leaves; however, investment into photosynthetic and non-photosynthetic leaf tissues was optimized at different scales (leaf or leaflet) depending upon the species.

Ferns ranged widely across resource gradients, and differences in moisture and irradiance were strongly associated with variation in leaf form and performance at growth and evolutionary timescales. I found evidence that ferns can adapt by optimizing rates of resource capture, metabolism and growth, and/or by optimizing stress tolerance and survival. Fast rates of water transport and gas exchange were not consistently associated with high irradiance, as seen for angiosperms, suggesting that optimization of photosynthesis under high irradiance is mediated by moisture availability. Species also showed a wider range of stomatal responses than previously reported for ferns and angiosperms, indicating a complexity of control that would modulate adaptation and acclimation at a wide range of timescales. Finally, trends of acclimation and adaptation of leaf traits tending in different directions emphasize the importance of specific compromises between long-term adaptation versus short-term acclimation to irradiance and moisture availability, and are expected to strongly influence fern abundances, distributions, and evolutionary trajectories.

The dissertation of Christine Fiona Czerniak is approved.

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Phil Rundel

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University of California, Los Angeles

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EDUCATION

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ACADEMIC PUBLICATIONS

For forthcoming publications see Acknowledgements above; Creese is married name

Sack L, Bartlett M, Creese C, Guyot G, C. Scoffoni. 2011. Constructing and operating a hydraulics flow meter. Prometheus Wiki Protocol: http://shar.es/oWScg.

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PRESENTATIONS

2012 New insights into fern stomatal behavior based on leaf responses to light, CO_2, and VPD in a common garden. Ecological Society of America, *oral presentation*.

2011 Of leaves and ferns. La Selva Biological Station, invited *oral presentation*.

At the fern frontier: new insights into the causes and consequences of variation in fern leaf form and function across species and environments in Southern California, Hawaii and Costa Rica. International Botanical Congress, *oral presentation*.

The fern frontier: variation in form and function across species and environments. La Selva Organization for Tropical Studies Research Symposium, *oral presentation*.

2010 The coordination of structure and function in 15 fern species from Southern California. Botanical Society of America, *oral presentation*.

Disentangling drivers of morphological diversity in Hawaiian ferns: size-scaling, environment and growth Form. 13th Annual Biology Research Symposium, *poster*.

2009 Fern diversity and distributions in the Santa Monica Mountains. Resource Conservation District of the Santa Monica Mountains Education & Training Seminar, *invited talk*.

2009 Drivers of fern morphological diversity: an analysis of the Hawaiian pteridophyte trait database. Ecological Society of America, *oral presentation*.

2008 Adaptation to climate and correlated evolution in pines. Botanical Society of America, *oral presentation*.

From fern frond to flora: an analysis of the Hawaiian pteridophyte trait database. EcoLunch Series UCLA Ecology & Evolutionary Biology, *oral presentation*.

2007 Evolutionary significance of variation in pine structure and function. Graduate Seminar Series UCLA Ecology & Evolutionary Biology, *oral presentation*.

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CHAPTER 1

PREMISE OF THE DISSERTATION

Ferns are the oldest lineage of vascular plants and the second-most speciose after angiosperms with approximately 12,000 species. They also range widely in size, leaf form, and growth form, and span environments from semi-deserts to tropical rainforests. Their habitat specificity makes them excellent ecological indicators of environmental change, and models of ecosystem function can be improved by incorporating studies of fern responses. Yet we understand surprisingly little of how ferns have adapted to such diverse environments, or how they vary in their ability to plastically adjust their form and physiology to acclimate to changing conditions of irradiance, CO_2, and moisture availability – three of the most limiting factors for plant growth and survival. Because leaves are the nexus of resource acquisition and allocation in most plants, studies of leaf form and physiology in ferns are also critical to improve our understanding of the evolutionary past and future of plant diversity, and its ecological consequences. Nevertheless, studies of leaf traits have largely neglected the ferns. The goal of this dissertation program was to execute five studies of diverse fern species across variable environments from three global regions, combining meta-analyses, field research, and experimental manipulations to investigate the causes and consequences of variation in fern leaf form and physiology. The resulting data facilitate comparisons of leaf traits and their responses to environment between ferns and other plant groups to shed light on the evolution of plant diversity. These data also provide a new framework for exploring the universality and idiosyncrasy of mechanistic relationships among leaf form and physiology traits across all plants.

Chapter 2, an examination of morphological diversity across the Hawaiian fern flora, was designed to take advantage of the ‘natural laboratory’ of the Hawaiian Islands, host to the highest levels of fern endemism in the world, distributed across steep environmental gradients. After translating the most recent and comprehensive survey of Hawaiian ferns and lycophytes into a digital database that includes 109 nominal, ordinal and metric variables spanning all 200 fern taxa, I tested a priori hypotheses about possible drivers of trait × trait correlations due to adaptation, biophysical and genetic linkages and phyletic heritage, and trait × environment correlations due to adaptation to environment and ecological sorting. Further, I examined trait associations with the abundance and distribution of taxa, including differences between alien and native groups. This provided a foundation for developing hypotheses of how ferns may modify their leaf form and physiology in response to different environmental conditions over growth and evolutionary timescales, ideas that were tested using field research and common garden experiments in Chapters 3 – 6.

Chapters 3 and 4 present results of a field program in Southern California that investigated associations of plant size, growth form, leaf form, composition, and physiology with environment. This in-depth study focused on 15 diverse fern species in a mediterranean-type climate where light is typically saturating and water very limited, and included epipetric (growing on rock) and terrestrial species. I quantified variation in 223 leaf traits related to morphology, anatomy, and physiology across gradients in moisture, irradiance, temperature, and elevation. In Chapter 3, I used these data to test a priori hypotheses about the coordination of leaf traits due to an economy of scale (i.e., efficient production and mitigation of costs at greater size), and an economy of stress tolerance (i.e., greater durability given more expensive, long-lived leaves for survival under stressful conditions and limited resource supply). In Chapter 4, I tested a priori hypotheses about the association of leaf architecture and hydraulic and photosynthetic performance with differences in habitat and growth form. Given that water was typically the most limiting resource, I focused particularly on relationships between leaf hydraulic architecture (veins) and flux rates for hydraulic and gas exchange traits.

For Chapters 5 and 6, I constructed a common garden experiment in Costa Rica using 13 morphologically and phylogenetically diverse tropical rainforest species to examine fern stomatal and photosynthetic responses to different environmental factors under controlled conditions. With this level of environmental control, I was able to change light, CO_2, and vapor pressure deficit (air 'dryness') to monitor real-time stomatal responses of the different species for comparison with patterns reported for angiosperms (Chapter 5). By growing replicates of seven open habitat species and six shade habitat species under high and low irradiance treatments, I was also able to test a priori hypotheses about adaptive and plastic adjustments to leaf form, composition, and photosynthetic traits at growth and evolutionary timescales (Chapter 6). These leaf trait associations for tropical rainforest ferns where light is typically most limiting and water is abundant also provided an expanded perspective of physiological diversification from the Southern California ferns which specialized under conditions in which water is the more limited resource.

CHAPTER 2

DRIVERS OF MORPHOLOGICAL DIVERSITY AND DISTRIBUTION IN THE HAWAIIAN FERN FLORA: TRAIT ASSOCIATIONS WITH SIZE, GROWTH FORM, AND ENVIRONMENT

ABSTRACT

Premise of the study: Hawaii is home to 238 native and 35 alien fern and lycophyte taxa distributed across steep gradients in elevation and resource availability. The fern flora spans a wide range of growth forms, with extraordinary diversity in morphology and plant size. Yet the potential factors underlying this diversity have remained enigmatic.

Methods: We used a trait database generated from the most recent and comprehensive survey of Hawaiian ferns and lycophytes to test hypotheses of size-scaling and trait associations with environment and growth form as factors underlying this diversity. We also tested relationships among morphology, taxon abundance and distribution, and identified key differences between native and alien taxa.

Key results: Strong trait–trait relationships included geometric scaling of plant dimensions with a tendency for more divided fronds in larger ferns. Trait–environment relationships independent of size included more divided fronds at higher elevation, longer blades in shaded habitats, and fronds with shorter stipes and fewer pinnae in drier habitats. Growth forms differed in mean size with epiphytic and epipetric taxa smaller than terrestrial ferns. Plant size was independent of taxon abundance and distribution across islands, and native and alien ferns did not differ in mean size. Alien taxa were more abundant, especially at lower elevations, apparently due to human land use.

Conclusions: These relationships point to linkages of fern form and demography with biogeography and highlight potential flora-scale physiological and morphological adaptations in ferns across contrasting environments.

INTRODUCTION

The Hawaiian archipelago is the most isolated group of islands (Carson and Clague, 1995), a hotspot of biodiversity (Myers et al., 2000) with a flora assembled over tens of millions of years (Price and Clague, 2002). The flora has extremely high endemism (Sohmer and Gustafson, 1987; Wagner, Herbst, and Sohmer, 1990): 89% for angiosperm species (Wagner et al., 1999) and 77% for ferns and lycophytes (Geiger et al., 2007). Given long distance traveling of spores (Caulton et al., 2000; Ranker, Gemmill, and Trapp, 2000; Ranker and Haufler, 2008), the evolution of fern endemism in Hawaii is especially striking because it relies on barriers to gene flow, such as polyploidy or microallopatry due to sharp island topographies (Xiang et al., 2004; Kuehne et al., 2007; Wright and Ranker, 2010), and on rare conditions such as self-fertilization, apomixis, or dispersal of multiple individuals to establish a population (Flinn, 2006; Ranker and Geiger, 2008). Colonization and speciation events have led to the establishment of 273 fern and lycophyte taxa that comprise one-sixth of all Hawaiian vascular plant species (Geiger et al. 2007) and include keystone species in many wet forests (Durand and Goldstein, 2001a; Durand and Goldstein, 2001b; Arcand et al., 2008).

Hawaiian ferns are highly variable in size and form (Palmer, 2003; Schneider et al., 2005), but this variation has remained unexplained. Hawaiian ferns and lycophytes derive from at least 120 original colonizers from all major floristic regions (Fosberg, 1948; Geiger and Ranker, 2005; Schneider et al., 2005; Driscoll and Barrington, 2007; Geiger et al., 2007). Given colonization from such diverse origins, morphological diversity may persist because of evolutionary obstacles that drive phylogenetic and niche conservatism, or increase through novel adaptations. A critical step forward is to test hypothesized trait associations underlying current realized patterns. We tested for: 1) size-scaling of plant parts, and trait associations with 2) environment, 3) growth form, 4) abundance, 5) distribution across islands, and 6) differences between native and alien taxa.

Plant size and dimensions influence resource capture (Sinnott, 1921; Pearsall, 1927; Enquist, 2002; Sack, Grubb, and Marañón, 2003). The retention of a genetic-developmental “blueprint” that maintains proportionality and function ("bauplan" sensu von Goethe, 1790) may stabilize allometries among organ and body size. We hypothesized geometric scaling among frond parts (except pinnae number) based on reports for tree fern species *Cibotium glaucum* and *Cyathea caracasana* (Arens and Baracaldo, 2000; Arcand et al., 2008), and that frond dissectedness would increase with size to reduce the boundary layer for heat dissipation (Vogel, 2009) and/or to reduce investment in support tissues (e.g. vasculature, rachis) that scale disproportionately with leaf size at a cost to photosynthesis per mass (Niinemets et al., 2007).

Environmental pressures may have driven trait evolution or assembly of ferns and lycophytes along resource gradients, as found in several Hawaiian groups (Robichaux and Pearcy, 1984; Carlquist, Baldwin, and Carr (editors), 2003; Givnish, Montgomery, and Goldstein, 2004; Cornwell et al., 2007; Dunbar-Co, Sporck, and Sack, 2009). Since fern species can occupy a wide variety of habitats with respect to moisture and irradiance (Page, 2002; Aldasoro, Cabezas, and Aedo, 2004; Karst, Gilbert, and Lechowicz, 2005) we expected trait differences for taxa from open and dry sites (Hevly, 1963; Durand and Goldstein, 2001b; Watkins et al., 2006; Kessler and Siorak, 2007). Plants competing for light in wetter, shaded environments would be larger with larger frond parts to increase surface area for light capture per investment in support (Arens and Baracaldo, 2000) whereas plants from open and dry environments would be smaller with greater dissection to minimize the boundary layer for convective cooling (Vogel, 1968). At higher elevation, irradiance can cause photodamage and overheating, especially given low nutrient availability (Körner, 1999; Kluge and Kessler, 2007). We expected high elevation taxa to be smaller, and more dissected (Vogel, 1970, 1984), with lamina pubescence to reflect light and reduce heat load (Ehleringer and Mooney, 1978; Watkins et al., 2006).

We hypothesized that morphology would also vary with growth form. Plants with epipetric and epiphytic habits (dwelling on rocks and other plants, respectively) may benefit from small plant and frond sizes given on average poorer resource availability and access to substrate support (Watkins, Rundel, and Cardelus, 2007). By contrast, terrestrial taxa may be more vulnerable to mechanical damage, herbivory, and climbing plants and may benefit from protective scales (Page and Brownsey, 1986; Page, 2002).

Morphological variation may be associated with abundance and distribution. We hypothesized that larger taxa with larger parts, and presumably faster rates of resource uptake (Kessler et al., 2007), would have greater abundances and wider distributions across the Hawaiian Islands. We expected aliens to have larger plant and frond sizes, as found in angiosperms (Pysek et al., 2009; Kleunen, Weber, and Fischer, 2010), and to have achieved comparable abundances to natives, despite their recent introduction (Burney and Burney, 2003; Gillespie, Chu, and Pau, 2008). Given that many invasive species compete strongly in disturbed, resource-rich sites and have high dispersability (Daehler, 2003; Pysek et al., 2009), we expected aliens to be common in open, low elevation areas, and distributed across greater numbers of islands than native taxa. We expected that alien ferns would frequently exhibit the terrestrial growth form because, historically, terrestrial ferns have been cultivated more commonly in Hawaii than ferns from other habits (Wilson, 1996; Randall, 2002; Wilson, 2002).

As an approach complementary to the detailed comparison of sets of fern taxa (e.g. Durand and Goldstein 2001a,b, Amatangelo and Vitousek, 2008) we implemented a broad database analysis testing these predictions for 25 variables and 273 taxa on a flora-wide scale to determine trait associations contributing to Hawaiian fern morphological diversity and distributions.

MATERIALS AND METHODS

***Creating the database*—** We compiled information on Hawaiian fern and lycophyte morphological traits, growth form, abundance, distribution, habitat and environment from the most recent and comprehensive survey of Hawaii's ferns and lycophytes (Palmer 2003), based on detailed field observations as well as examination of specimens from 15 herbaria. We assembled this information into a trait database that we pruned to 25 variables with sufficient representation for analysis. The database contains information for 273 species, hybrid species, subspecies, varieties and forms. Given the strong variation among these taxa and the frequently poor species resolution of Hawaiian ferns and the likelihood of taxonomic revision (Geiger et al., 2007), we considered these as distinct taxa in the database. For continuous quantitative variables (i.e. plant dimensions such as frond width and length) we used mean taxon values or we averaged minimum and maximum values. We ordinalized descriptive data for abundance and plant size to create quantitative variables. Abundance classes aggregated descriptions from Palmer (2003), in which some taxa were given multiple descriptions because of variation across islands: 1 = extinct; 2 = endangered; 3 = rare; 4 = uncommon everywhere, or rare on one or more islands; 5 = common on one or more islands, uncommon elsewhere; 6 = not endangered or locally common; 7 = common. Plant size descriptions given by Palmer (2003) were: 1 = small; 2

= small–medium; 3 = medium, small–large, or small–medium–large; 4 = medium–large; 5 = large.

For nominal variables we sorted taxa into categorical groups. For growth form, we compared epiphytic vs. terrestrial and epipetric vs. non-epipetric taxa (insufficient data excluded arboreal and aquatic habits from analysis of trait-habit associations); for abundance, common vs. uncommon; we categorized plants as alien or native; for habitat and elevation as wet vs. dry, open vs. shade and high vs. low elevation range; and for morphology as simple vs. compound fronds, glabrous vs. pubescent lamina, and glabrous vs. scaly stipes and rhizomes. Taxa described as expressing more than one growth form were included as either epipetric or epiphytic to recognize the expression of these specialized habits (e.g. Watkins, Rundel, and Cardelús, 2007). Taxa were defined as "common" when their abundance class (see above) ranged from common to not-endangered, and as "uncommon" when this ranged from uncommon to extinct. Taxa were identified as "native" if indigenous or endemic; in our analysis, hybrids between aliens and natives were considered as aliens.

In the categorization of site environment and distribution, we aimed to capture fundamental differences in irradiance and moisture habitats and elevation range. By necessity, our environmental variables are coarse-grained according to the available information for this flora-scale analysis, and we therefore focused on patterns of species composition associated with coarse-grained environmental structure, rather than differences in distribution within similar habitat types (cf. Bell, Lechowicz, and Waterway, 2000; Cernusak, Winter, and Turner, 2010). Thus, "open" sites were exposed habitats lacking forest canopy, including exposed lava flows, rock outcrops and grass meadows, and "shade" sites included forests, sites beneath rock wall overhangs, and shaded slopes. "Wet" sites were those described as wet, moist or mesic, including stream areas, waterfalls, bogs and moist/rain-forests, and “dry” sites were those described as xeric, including lava flows, rocky outcrops, and dry forests. These habitat categories would likely also differ in soil composition and biota. Taxa that ranged across both open and shade or wet and dry environments were listed within the less common (and presumably more specialized) open and dry environment categories. For mean elevation range, “high” and “low” refer respectively to above and below the all-taxon mean elevation of 1012 m. Taxa with undivided (entire) leaves were categorized as “simple” and those with any number of pinnate divisions as “compound.” Taxa with and without lamina hairs were considered “pubescent” and “glabrous” respectively. Similarly, taxa with stipe or rhizome scales were included in the “scales” category, and those without as having “no scales.”

Datasets, statistical analyses, and meta-hypothesis testing— Trait information was derived from a database that included the most information available on Hawaiian ferns to highlight strong patterns in a highly diverse flora. However, we acknowledge several provisos and emphasize that limitations in this approach will require future study on smaller groups to fully confirm hypotheses. Most importantly, our analysis relied on taxon descriptions and these were sometimes incomplete. A key limitation of using a pre-compiled trait database (such as a flora) for comparative analysis is that not all taxa are represented across all variables, leading to unequal representation of groups in some comparisons; this is analogous to phylogenetic analyses in which larger datasets are inherently more sparse (Sanderson, 2007). However, the “total evidence” approach (Kluge, 1989) provides the best current knowledge by maximizing the number of taxa and characters to increase biological information without necessarily reducing resolution (e.g. Santini and Tyler, 2004). To minimize type I and type II error we omitted traits for which data were available for only few taxa (e.g. caudex dimensions, which only applied to tree ferns). Our minimum taxon representation for a given trait was 18; for traits that involved multiple character states, we required this level of taxon representation for each character state. We then generated trait dataframes for three groups: 1) ferns and lycophytes including natives and aliens, 2) only native ferns and lycophytes and, 3) only native ferns. Using these three dataframes, we tested trait-trait (T×T), trait-environment (T×E), trait-demography/distribution (T×D), environment-environment (E×E), environment-demography/distribution (E×D), and demography-demography (D×D) relationships. Our presentation of results focuses only on relationships for which we had a priori hypotheses. Additionally, we determined all possible variable relationships to investigate the percipience of our analyses (see below). We tested trait associations using Pearson and Spearman correlation coefficients on both untransformed and log-transformed data to accommodate linear and non-linear relationships for the three dataframes (all ferns and lycophytes; native ferns and lycophytes; and native ferns). We considered a correlation significant if Pearson and Spearman correlations were significant at $P \leq 0.05$.

We used analyses of variance (ANOVA) to test for variation in quantitative variables between categorical groups using dataframes 1 and 3 (all ferns and lycophytes and just native ferns, respectively). Relationships among categorical variables were tested using Chi-Square contingency tables and Fisher's Exact Tests for all taxa and only native ferns, respectively. Statistics were calculated using (SPSS 15.0, SPSS Inc., Chicago, Illinois).

For allometries between size traits we fitted standardized major axes (SMA using SMATR; Warton et al., 2006) to log-transformed data, equivalent to fitting power law curves; $\log (y) = \alpha + \beta \times \log (x)$ (Sokal and Rohlf, 1995; Sack et al., 2003). SMA is appropriate for determining functional relationships among two independent variables (Sokal and Rohlf, 1995;

Sack et al., 2003; Warton et al., 2006). We tested for deviation of SMA slopes from those expected by geometric scaling, i.e., the retention of proportional dimensions with increasing size (Niklas, 1994, Sack et al. 2003, Warton et al. 2006); for example, linear dimensions should scale with a β of 1, while a linear dimension should scale with an area dimension with a β of 0.5.

To quantify our ability to currently explain trait variation among native Hawaiian ferns, we evaluated the percipience of our analysis, and the predictive and explanatory success of our hypotheses, comparing results to those expected from random chance alone. We used a novel, simple multivariate approach based on probability theory (Tijms, 2007) to assess the strength of our overall analysis given multiple tests relative to random associations. We quantified overall “percipience” (i.e., the power of analyses to perceive differences) as the number of significant results out of the total number of tests: a value of 1 would indicate that hypotheses were targeting real variation in the data. This proportion was compared against the 0.05 expected from random chance. The “predictive power” of our hypotheses, i.e., the ability of the hypotheses to anticipate significant differences or correlations a priori, was calculated as the number of supported predictions out of the total number of tests and compared to the random chance model of 0.025 (the 0.05 probability of a significant result due to chance alone multiplied by the 0.5 probability of a correlation in the direction hypothesized). Finally, the “explanatory power” of hypotheses, i.e., the ability of a priori hypotheses to explain the observed significant differences post-hoc, was determined as the number of supported predictions divided by the total number of significant findings and compared to the random chance model of 0.5 (the probability that significant differences were in the expected direction by chance alone). A high explanatory power indicates that proposed mechanisms are sufficient to explain the significant trait associations.

Phylogenetic Analyses— Standard trait correlations can be used as evidence for true and realized association among plant traits, but are weak evidence of correlated evolution because they do not distinguish cases of pre-adaptation and ecological sorting from in situ evolution (Felsenstein, 1985). For instance, a strong bivariate trait correlation may indicate an evolutionary event and subsequent trait conservatism during diversification or repeated trait co-evolution. Thus, to ensure evolutionary correlations the common practice is to retest correlations using phylogenetically independent contrasts (PICs) that account for species' shared history (Harvey and Pagel, 1991; Maherali, Pockman, and Jackson, 2004). Using the best available phylogenetic data for Hawaiian fern lineages, the phylogenies for the endemic genus *Adenophorus* (Ranker et al., 2003) and *Dryopteris* (Geiger and Ranker, 2005), we calculated independent contrast correlations for traits represented by 7 or more taxa using the AOTF function in PHYLOCOM (Webb, Ackerly, and Kembel, 2008). We note that in the absence of more phylogenetic data, ahistorical analyses provide strong insight toward mechanistically grounded and ecologically important trait correlations but that further phylogenetic resolution and analysis are needed to tease apart patterns of sorting versus in situ adaptation.

RESULTS

Trait-trait relationships: scaling of size and morphology— Hawaiian ferns exhibit tremendous variation in plant size, from the small grammitids and filmy ferns (Polypodiales; Hymenophyllales) and aquatic ferns (Salviniales) to the large arborescent ferns (Cyatheales). Taxa also varied widely in frond size and shape (Table 2.2). Fern fronds ranged nearly 500-fold in length among both native (from *Gonocormus minutus* to *Cibotium glaucum*) and alien taxa (*Azolla filiculoides* to *Angiopteris evecta*). Native fern fronds also ranged from simple leaf types (e.g. *Adenophorus oahuensis*) up to 5-pinnate divisions (e.g. *Asplenium schizophyllum*), and alien fronds from 2 to 8 pinnate divisions (e.g. *Diplazium esculentum* and *Asplenium hispidulum*).

We found strong scaling of the dimensions of fronds, frond components and rhizomes with plant size (Fig. 1; $r = 0.45 – 0.86$, $P < 0.05$, $N = 11 – 121$; SuppTables 2.1 to 2.3, see Supplemental Data with the online version of this article; relationships were significant for all dataframes unless otherwise specified and both r_p and r_s values are presented in SuppTables 2.1-2.3, with the highest value reported in the text). We also found tight coordination of length and width for fronds ($r = 0.98$, $P < 0.001$, $N = 58$), blades ($r = 0.61$, $P < 0.001$, $N = 7$), pinnae ($r = 0.95$, $P < 0.001$, $N = 29$) and ultimate segments ($r = 0.65$, $P = 0.012$, $N = 14$; see Fig. 2.1). As expected, pinnae number was independent of plant size ($r = -0.008$, $P = 0.945$, $N = 72$). Frond component dimensions were also positively correlated with one another: frond length was correlated with length and width of the blade ($r = 0.90\text{-}0.98$, $P <0.001$, $N = 7\text{-}10$), the pinna ($r = 0.94\text{-}0.95$, $P < 0.001$, $N = 28\text{-}47$), and the ultimate segment length ($r = 0.68$, $P = 0.002$, $N = 18$). Wider fronds had larger pinnae ($r = 0.91\text{-}0.99$, $P < 0.001$, $N = 15\text{-}18$), but fewer pinnae pairs ($r = -0.61$ $P < 0.001$, $N = 36$), and blade and pinna lengths were correlated ($r = 0.99$, $P < 0.001$, $N = 6$). We also found scaling of stipe diameter with laminar components, i.e., frond, blade and pinna lengths and ultimate segment width ($r = 0.61\text{-}0.82$, $P = 0.005\text{-}0.041$, $N = 9\text{-}20$), and greater rhizome diameter for taxa with longer fronds ($r = 0.76$, $P < 0.001$, $N = 28$).

Across the flora, size differences were linked with other morphological characters. Larger taxa were more commonly glabrous and smaller taxa pubescent ($P = 0.007$, $N = 136, 12$; SuppTable 2.4), with both size and pubescence associated with habit (see following section). Supporting our hypothesis for greater frond dissection in larger ferns, the fronds of larger taxa tended to have compound blades ($P < 0.001$, N = 101, 6; SuppTable 2.4), with more numerous pinnate divisions ($r = 0.48$, $P < 0.001$, $N = 95$; SuppTables 2.1-2.3), and greater variation in the number of these divisions ($r = 0.31$, $P = 0.002$, $N = 95$). Greater leaf dissection was also positively correlated with stipe diameter ($r = 0.54$, $P = 0.014$, $N = 20$), frond length (for native ferns only; $r = 0.270$, $P = 0.005$, $N = 109$; SuppTable 2.3), frond and blade widths ($r = 0.65$-0.88, $P \leq 0.02$, $N = 6$-48), blade length ($r = 0.704$, $P = 0.023$, $N = 10$), pinna size ($r = 0.48$-0.52, $P \leq 0.006$ $N = 27$-44), and the number of pinnate variations ($r = 0.63$, $P < 0.001$, $N = 136$; SuppTables 2.1-2.3). Taxa that expressed more variability in pinnate divisions also had on average wider fronds ($r = 0.30$, $P = 0.039$, $N = 38$) because of their longer pinnae ($r = 0.48$, $P = 0.004$, $N = 35$).

The significant relationships among size traits did not deviate from geometric scaling (95% confidence interval tests). The mean standard major axis slope (β) determined for 15 relationships among continuous (non-ordinal) traits was 1.09 ± 0.077 (N = 6 – 55; SuppTable 2.6).

***Trait-environment relationships: trends with elevation*—** Fern form varied with elevation. At higher elevations, ferns exhibited a greater number of pinnate divisions ($r = 0.20 – 0.21$, $P = 0.02 – 0.03$, $N = 113 – 127$) and pinnae pairs ($r = 0.34$, $P = 0.003$, $N = 76$), and reduced pinna length ($r = -0.38$, $P = 0.007$, $N = 48$), though no significant greater tendency for pubescent over glabrous laminae ($P > 0.05$, N = 21, 187; SuppTable 2.4). The trends of number and length of pinnae with elevation were apparently driven by the differences among native and alien taxa; the relationships were not supported when considering only native ferns ($r = -0.16$-0.20, $P = 0.1$-

0.33, *N* = 40-68, SuppTables 2.1-2.3). The trends were independent of plant size, which did not correlate with elevation (*r* = 0.011, *P* = 0.90, *N* = 135; SuppTables 2.1-2.3).

Trait-environment relationships: open versus shade and wet versus dry habitats— Plant traits correlated with irradiance and moisture habitat independently of plant size, which did not differ significantly between shade and open-habitat taxa (*P* = 0.39, *N* = 28, 134; SuppTable 2.4) or between wet and dry habitats (*P* = 0.60, *N* = 19, 129; SuppTable 2.4). Open fern sites also tended to be dry relative to shaded sites (*P* < 0.001, *N* = 194; SuppTable 2.5). Ferns that occupied open habitats tended to have shorter blades (*P* = 0.034, *N* = 8, 21; Fig. 2.2). Taxa from wetter sites tended to have blades with more pinnae pairs (*P* = 0.057, *N* = 57, 15 for all ferns; *P* = 0.046, N = 53, 11 for native ferns; Fig. 2.2).

Trait-demography relationships: associations with abundance and distribution— Across all native and alien taxa, and within the terrestrial, epiphytic and epipetric growth forms considered individually, abundance and distribution were independent of plant size (*r* = 0.079-0.092, *P* = 0.270-0.401, *N* = 116-146; SuppTable 2.1-2.3). However, frond traits varied with taxon abundance and island distribution. Abundant taxa tended to have larger pinnae (*r* = 0.36-0.48, *P* = 0.016, *N* = 25-44) with wider ultimate segments (*r* = 0.70, *P* < 0.001, *N* = 23) and were reported on a greater number of the Hawaiian Islands (*r* = 0.54, *P* < 0.001, *N* = 179, SuppTables 2.1-2.3). Taxa from open sites also tended to be more abundant (*P* = 0.054, *N* = 37, 122; SuppTable 2.5), an association apparently driven by alien ferns, as it was not apparent when only native ferns were considered (*P* = 0.21, *N* = 25, 107).

Taxa distributed across many islands tended to have longer fronds (r =0.20, $P = 0.01$, N = 157) composed of smaller blades (r = -0.44 to -0.47, P = 0.01-0.05, N = 18-31; SuppTables 2.1-2.3) and longer ultimate segments ($r = 0.43$, $P = 0.040$, $N = 23$). Considering only native ferns, taxa distributed across many islands tended to have greater frond widths ($r = 0.28$, $P = 0.04$, N = 55; SuppTable 2.3) and fewer pinnae pairs ($r = -0.27$, $P = 0.02$, $N = 71$), and were more common in dry sites ($P = 0.043$, N = 20, 136; SuppTable 2.4).

Differences in morphology and distribution across growth forms— The flora included terrestrial (e.g., *Ophioglossum nudicaule* and *Cibotium glaucum*), epiphytic (e.g., *Grammitis tenella* and *Asplenium nidum*) and epipetric taxa (e.g., *Christella wailele* and *Diellia erecta*), with the terrestrial growth form most common (see Table 2.1; 55.5 % of taxa were terrestrial, 35.3 % epiphytic and 9.2 % epipetric taxa). Epiphytes tended to be smaller plants with smaller parts (Fig. 2.3; SuppTable 2.4); terrestrial taxa were larger in plant size ($P = 0.008$, N = 75, 37; Fig. 2.3; SuppTable 2.4), rhizome diameter ($P = 0.003$, $N = 33$), frond length and width ($P <$ 0.001, N = 71, 29 and 28, 17 respectively; SuppTable 2.5), and number of pinnate divisions (P = 0.044, N = 66, 22). Considering only the native ferns, terrestrial taxa also exhibited greater pinna widths than epiphytes (P <0.05, N = 4, 19; SuppTable 2.4). Epiphytes more frequently occurred in shaded sites ($P < 0.001$; SuppTable 2.5) and possessed simple leaves ($P < 0.001$, N = 22, 67) with lamina pubescence ($P = 0.007$, N = 51, 93) and lower incidence of stipe scales ($P = 0.003$, N = 30, 75). Epipetric taxa also tended to be smaller in stature ($P < 0.05$, N = 10, 102; SuppTable 2.5; Fig. 2.3). Among native ferns, epipetric taxa had a lower mean elevation range than non-lithophytes ($P < 0.05$, N = 11, 95; the relationship was non-significant when including aliens and lycophytes; $P < 0.1$, N = 14, 117).

***Morphology, growth form and demography for aliens versus natives*—** Alien and native ferns did not differ in mean plant size ($P = 0.279$, $N = 19, 129$; SuppTable 2.4), but contrasted in frequency of habit and demography. As for native ferns, aliens were more commonly terrestrial than epiphytic ($P = 0.010$, $N = 51, 93$; SuppTable 2.5) with 80% terrestrial species with the rest divided across the epiphytic, epipetric and aquatic forms (data not shown). Aliens had a lower mean elevation range ($P < 0.001$, $N = 18, 189$; Fig. 2.4) and a higher proportional distribution in open ($P = 0.011$; SuppTable 2.5) and dry environments ($P = 0.051$) than native taxa. Although on average aliens were not more widely distributed across the Hawaiian Islands ($P > 0.1$, $N = 36, 237$; SuppTable 2.4), they were on average more abundant than native taxa ($P = 0.007$, $N = 16, 162$, SuppTable 2.5; $P = 0.020$; Fig. 2.4).

***Percipience, predictive and explanatory power*—** For native ferns, the percipience and the predictive and explanatory power of our hypotheses all exceeded expectations from chance (SuppTable 2.7). Percipience and predictive power were highest for tests relating to size-scaling (50%), followed by those related to growth form (30%), demography (20 – 40%) and environmental variables (4 – 20%). The explanatory power was lowest for hypotheses of distribution (57%), but very high for those based on size-scaling (91%) and other hypotheses for trait-trait and trait-environment correlations (100%).

***Phylogenetic Analyses*—** We hypothesized that larger plants with larger parts would also tend to be more abundant and widely distributed. However, in *Adenophorus*, frond length negatively correlated with abundance ($r = -0.65$, $P < 0.05$; $N = 10$; SuppTable 2.8). In *Dryopteris*, we found a strong scaling of frond length and plant size ($r = 0.86$, $P < 0.05$; $N = 11$) and an association of taxon abundance with a wide distribution across islands ($r = 0.72$, $P < 0.05$; $N = 8$; SuppTable 2.8).

DISCUSSION

This study highlighted novel, flora-scale relationships between structure and habitat and important differences between native and alien Hawaiian ferns. The trait linkages with plant size, growth form, demography and biogeography presented here are a first indicator of potential morphological and physiological adaptations to contrasting environments across the Hawaiian fern flora.

Size-scaling of Hawaiian fern form— We found a strong coordination of plant size and the dimensions of fronds, frond parts and rhizomes. These patterns indicated the maintenance of plant proportions with increasing size in Hawaiian ferns. This geometric scaling of plant parts extended to the flora scale, a trend previously documented for fronds within one species of tree fern (*Cibotium glaucum*; Arcand et al. 2008). The strength of size-scaling relationships is notable especially given that it applies across the very diverse species of the Hawaiian fern flora. Mechanisms for this pattern of scaling within a species and across an entire fern flora include genetic linkages and/or developmental constraints, which may result in a “bauplan” constraint on variation in gross morphology (e.g. the proportionality of organ dimensions). One genetic mechanism might be pleiotropy for size traits such that selection for increased plant size would affect all parts equally and lead to linear allometries (Bonduriansky, 2007), similar to ontogenetic constraints when matched relative growth rates of organs cause geometric scaling of their dimensions across a range of plant sizes (Pearsall, 1927; He et al., 2008). Notably, pinnae number appeared independent of plant and frond dimensions in our study and in a previous study of *Cyathea caracasana* tree ferns across light gradients where pinnae number was unrelated to stipe length (Arens and Baracaldo, 2000). As expected, frond dissectedness increased with plant size. This pattern may arise as pinnae become increasingly separated with increasing frond size as space becomes available for the addition of higher-order divisions. Further, there may be a functional advantage for larger plants with greater leaf sizes that avoid high temperatures by dividing the leaf into smaller parts to increase convective cooling (Vogel 1970, 1984). However, producing divided fronds may entail greater mass allocation to rachis support tissue instead of photosynthetic tissue (Niinemets et al. 2007).

Environmental associations of Hawaiian fern form— Our study is the first to our knowledge to show a linkage of frond form with light and water habitat and elevation independently of plant size. These patterns appear fundamental, as they are consistent with trends shown in other lineages of vascular plants and mosses, which evolved leaves independently (Givnish, 1987, 1988; Dunbar-Co, Sporck, and Sack, 2009; Waite and Sack, 2009). Dry environments and open environments with higher irradiance appear to have selected or assembled taxa with smaller fronds and fewer and shorter pinnae because a thinner boundary layer allows greater heat dissipation (Gibson, 1998; Sack, Grubb, and Marañón, 2003). Additionally, small leaves may possess a hydraulic advantage for water transport from veins to mesophyll, all else being equal (McKown, Cochard, and Sack, 2009). The tendency for longer blades in shaded environments would facilitate light capture relative to mass investment (Givnish, 1987).

The variation among taxa in frond traits was also associated with elevation. Our analysis showed a greater abundance of alien taxa with simple leaves at low elevation. The positive association of frond dividedness with elevation may additionally be influenced by water availability, as higher elevation taxa tended to occur in wetter sites and wet-site taxa had more divided fronds. This finding contrasted with the trend towards greater representation of simple leaves at high elevation in Bolivia and Borneo (Kessler, Parris, and Kessler, 2001) and suggests a potential for distinct but functionally equivalent types of adaptation to high elevation. By reducing overall leaf size, simple leaves may reduce their boundary layer, like dissected leaves, to prevent overheating under high irradiance. Across floras, differences in the relationship between leaf dividedness and elevation may be driven by unique phylogenetic history and assembly processes. We did not find support for greater frond pubescence in high elevation ferns, though such a mechanism to reduce photodamage and heat load under higher irradiance has been shown in angiosperms (e.g. Ehleringer and Mooney, 1978). Notably, a lack of pubescence at higher elevation was also found in ferns of the Bolivian Andes where greater precipitation at high elevation was believed to ameliorate the need for greater reflectance (Kessler et al., 2007). A strength of this flora-wide analysis is its canonical perspective on trait variation across environments, showing general trends by aggregating communities with different species that are assembled across many local resource and climate gradients. There is a clear need in future studies to consider specific elevation gradients, and how traits are optimized to the particular ways that elevation, light and water availability co-vary along those gradients, to improve understanding of variation in trait patterns within and across floras.

Trait associations with growth form— Plant size and morphological heterogeneity were strongly associated with growth form. Epiphytic and epipetric taxa were on average smaller, with smaller component parts than terrestrial ferns that were larger and had more divided fronds. Epiphytes exhibited simple leaves with lamina pubescence but often lacked stipe scales and were more likely to occur in shaded sites and at lower elevations. This finding suggests that scales may help to protect developing crosiers and stave off climbing plant competitors (Page and Brownsey 1986, Page 2002). In taxa from exposed habitats, scales may protect the photosynthetic apparatus from photodamage during desiccation (Farrant et al. 2009); however, we did not find a trend for greater scale representation in dry or open habitats, or among epipetric taxa. As found for angiosperms, the trend for greater lamina pubescence in epiphytes may reflect allocation to mechanical and herbivore defense (Gruner, Taylor, and Forkner, 2005) or to longer lifespan in low resource environments (Joel, Aplet, and Vitousek, 1994; Cordell et al., 1998; Hoof et al., 2008), given that fern epiphytes persist in water and nutrient-limited aerial habitats (Cardelús, Colwell, and Watkins, 2006; Watkins, Rundel, and Cardelús, 2007). Pubescence might also reduce transpiration rates through effects on boundary layer and/or by reflecting radiation, improving water-use efficiency (Watkins et al. 2007).

Morphologies conducive to greater abundance and distribution across islands and habitats— With the ability to proliferate and disperse very long distances via spores, fern abundance and distribution may be limited by environmental filters on acceptable forms and physiologies in given habitats (Tuomisto et al., 2002; Tuomisto, Ruokolainen, and Yli-Halla, 2003; Jones et al., 2006). Across vascular plant floras, larger plants have been found to be more competitive and to have greater abundances and wider distributions (Cadotte, Murray, and Lovett-Doust, 2006;

Pyšek and Richardson, 2007; Pysek et al., 2009). Notably, our analysis indicated that across Hawaiian ferns, abundance and distribution were independent of plant size overall and within the terrestrial, epiphytic and epipetric growth forms. Additionally, abundant taxa were distributed across more islands. One reason for this linkage may be that greater abundance provides greater dispersal source strength. This trend for Hawaiian ferns contrasts with the weak negative correlation of range size and local abundance found for pteridophytes along elevation gradients in Bolivia; in that study, species abundances were reduced for wider ranging species sampled at their geographic range limits (Kessler, 2002). The trend found here for Hawaiian ferns is consistent with the more commonly reported positive correlation of species' abundance and range size for angiosperms and animals (Hengeveld, 1992; Lawton et al., 1994; Brown, 1995). Whether the trend in Hawaiian ferns arose due to pre-adapation and sorting—i.e., the success of larger and large-fronded colonizers in dispersal and competition—or due to the evolution of such competitors in multiple colonizing lineages, requires further investigation when more phylogenetic information becomes available.

We expected that shade and wet site taxa would be most abundant because the literature has emphasized that many fern species are commonly associated with moist, shady environments (Page, 2002; Aldasoro, Cabezas, and Aedo, 2004; Karst, Gilbert, and Lechowicz, 2005). However, we found that open-site taxa were typically more abundant when aliens were included, but among native ferns considered alone, taxa of all habitats were similar in their mean abundance. Also in contrast to expectation, dry-site taxa were more widely distributed across the Hawaiian Islands. One possible explanation may be the greater availability of dry site habitat across the islands before human settlement (Juvik and Juvik, 1998). Biological mechanisms may also play a role, e.g., the presence of apogamy (development of embryo without fertilization) may facilitate wider establishment of xeric-adapted ferns by reducing reliance on external water availability for reproduction, and by producing, on average, faster rates of gametophyte and sporophyte growth and maturation (Tryon, 1972; Moran, 2004; Townsend et al., 2007).

Key differences in alien and native fern growth form, abundance and distribution— Aliens differed significantly from natives in their typical growth form, abundances and distributions. Contrary to the expectation that aliens would be larger than natives, we found similar ranges in the sizes of plants and organs. Whereas native ferns exhibited a wider and more even distribution of growth forms, 80% of alien fern species were terrestrial. This pattern may result from the more common cultivation of terrestrial ferns (Wilson, 1996; Randall, 2002; Wilson, 2002), or reflect a greater ability of terrestrial ferns to naturalize. Future studies are needed to test for whether given traits (such as growth form) correlate with rates of escape for cultivated ferns, and to inform weed assessment programs (Daehler, 2009).

Overall, given spore dispersal, we expected that alien ferns would also be widely distributed and abundant in Hawaii, in spite of their relatively recent introductions. Indeed, on average alien and native fern taxa occurred across a similar number of islands. However, among native ferns, those common on dry sites tended to have widest distributions, whereas among aliens, distribution was independent of habitat. We were surprised to find that aliens were more abundant on average than native ferns, due to 34 of 35 alien taxa being common, whereas nearly 25% of the 163 native taxa were rare, endangered or extinct. Although we note that this trend may not account for some additional recently escaped alien species with low abundances, the finding of so many alien species more abundant than natives draws attention to an important phenomenon. As expected, ranges for alien taxa were centered at lower elevation than natives and aliens were commonly found in open sites where concentrated human activity may have generated disturbed habitats preferred by alien ferns and lycophytes.

Success of hypotheses and future directions— The power of our hypotheses for trait associations was very high for explaining morphological variation in Hawaiian ferns (near 100% of hypotheses were successful in explaining differences in all cases except for island distribution, 57%). This finding demonstrates the strength of fundamental premises to explain key flora-wide patterns. Explanatory power was in our definition the success of the hypotheses to point the direction a significant difference was found. The *predictive power* is lower given its definition as the success of hypotheses to anticipate all differences correctly and in the right direction; thus, predictive power decreases when no significant differences are found. Still, the predictive power of our hypotheses was substantially greater than that expected from random chance (45% for size-scaling, 27-33% for growth form, 4-20% for environment and 21-22% for demography). Despite these substantial proportions, that a minority of our hypotheses were predictive points to the need for further research to elucidate additional factors determining variation in form and distribution. Additional trait relationships might be supported given finer scale information of trait values and resource gradients, and data for all taxa, such that we might identify trends with greater robustness, as well as interesting outliers to general trends. Studies of ferns in common garden and field conditions would also allow assessment of plastic responses to environmental variation, and the extent to which intraspecific plasticity might contribute to the observed trends. We note that linking single traits with habitat and distributions does not account for multiple 'optimal designs' for adaptation to particular habitats (Marks and Lechowicz, 2006). Thus, different clusters of traits may convey functional equivalence and a similar ability to persist, disperse and become abundant in given environments. As more data become available, future studies may consider the linkage among trait clusters with environment simultaneously.

The trait relationships elucidated here suggest potential adaptation or ecological sorting of pre-adapted taxa (Gould and Lewontin, 1979), and indicate that morphological traits would be important in resource capture and acquisition and in determining differences in plant fitness across environments. Still, we note that the traits available in the flora, though valuable, are limited. Many other features of form and physiology are highly diverse across ferns and would be critical to determine performance and habitat specialization. For example, further work is needed to resolve how variation in nutrient composition and stoichiometry, leaf venation and structural support tissue allocation patterns influence water and gas flux rates, and consequently, growth and reproduction in ferns (Brodribb et al., 2005; Amatangelo and Vitousek, 2008; Brodribb, Feild, and Sack, 2010). Additionally, the identification of ancestral colonists will enable determination of the persistence of traits and ecology. For example, the three Hawaiian *Polystichum* species, all typically of high elevations, radiated from an Asian ancestor of high elevation (e.g. Driscoll and Barrington, 2007). Further resolution of phylogenetic relationships among fern taxa will also clarify the historical patterns that influenced trait evolution and current distributions (Hennequin et al., 2010), the degree to which variation evolved in situ, and the emergence of adaptive trait complexes (cf. Dunbar-Co, Sporck, and Sack, 2009). Future work on flora-scale databases should open up exciting avenues for such important investigations.

Table 2.1. Number of fern and lycophyte taxa (in bold), including endemic, indigenous and alien taxa, as well as, in parentheses, families, genera and species respectively represented for growth form, environmental, demographic and morphological variables (data from Palmer 2003).

	Ferns			*Lycophytes*		
Variables	**Endemic**	**Indigenous**	**Alien**	**Endemic**	**Indigenous**	**Alien**
Database						
All Taxa	**173** (21,46,99)	**41** (15,20,35)	**33** (13,23,30)	**15** (3, 4, 9)	**8** (1, 3, 7)	**3** (1, 1, 3)
Growth Form						
Terrestrial	**50** (11,20,41)	**16** (6,8,16)	**15** (8,10,15)	**3** (2, 2, 3)	**4** (1, 2, 4)	**3** (1, 1, 3)
Epiphytic	**35** (6,11,31)	**7** (6,6,7)	**2** (1,2,2)	**4** (1, 1, 4)	**2** (1, 1, 2)	**0** (0, 0, 0)
Epipetric	**5** (5,5,5)	**6** (5,5,6)	**3** (2,3,3)	**0** (0, 0, 0)	**0** (0, 0, 0)	**0** (0, 0, 0)
Environment						
Open	**21** (13,15,19)	**10** (7,8,10)	**10** (5,8,10)	**5** (3, 4, 4)	**4** (1, 3, 4)	**1** (1, 1, 1)
Shade	**109** (16,38,86)	**21** (12,13,20)	**13** (8,12,13)	**5** (2, 2, 4)	**3** (1, 1, 3)	**0** (0, 0, 0)
Wet	**115** (18,41,89)	**20** (12,13,19)	**14** (10,13,14)	**7** (3, 3, 6)	**6** (1, 2, 6)	**1** (1, 1, 1)
Dry	**11** (6,8,10)	**9** (6,7,9)	**6** (2,5,6)	**1** (1, 1, 1)	**1** (1, 1, 1)	**0** (0, 0, 0)
High Elevation	**63** (15,25,46)	**13** (9,9,13)	**11** (8,10,11)	**3** (1, 1, 2)	**1** (1, 1, 1)	**0** (0, 0, 0)
Low Elevation	**67** (13,26,51)	**18** (9,12,16)	**12** (8,11,12)	**2** (1, 1, 1)	**0** (0, 0, 0)	**0** (0, 0, 0)
Demography						
Common	**68** (13,26,60)	**19** (10,11,19)	**13** (7,11,13)	**3** (3, 3, 2)	**3** (1, 2, 3)	**1** (1, 1, 1)
Uncommon	**52** (17,26,40)	**7** (5,6,7)	**2** (2,2,2)	**8** (2, 2, 4)	**1** (1, 1, 1)	**0** (0, 0, 0)
Morphology						
Simple Fronds	**4** (1,2,4)	**2** (2,2,2)	**0** (0,0,0)	**n/a**	**n/a**	**n/a**
Compound Fronds	**92** (12,29,73)	**20** (7,11,19)	**19** (8,14,19)	**n/a**	**n/a**	**n/a**
Pubescent Lamina	**18** (7,12,16)	**1** (1,1,1)	**3** (3,3,3)	**0** (0, 0, 0)	**0** (0, 0, 0)	**0** (0, 0, 0)
Glabrous Lamina	**140** (20,42,93)	**37** (14,19,33)	**27** (12,20,27)	**15** (3, 4, 9)	**8** (1, 3, 7)	**3** (1, 1, 3)
Stipe Scales	**60** (10,20,52)	**12** (7,8,12)	**10** (7,9,10)	**n/a**	**n/a**	**n/a**
Stipe No Scales	**54** (16,28,49)	**13** (6,8,13)	**14** (7,10,14)	**n/a**	**n/a**	**n/a**
Rhizome Scales	**16** (7,8,15)	**5** (3,3,5)	**5** (2,5,5)	**n/a**	**n/a**	**n/a**
Rhizome No Scales	**87** (17,34,81)	**24** (11,13,23)	**15** (7,10,15)	**n/a**	**n/a**	**n/a**

Table 2.2. Ranges of trait values for size- and shape-related traits, and demographic and environment traits for Hawaiian native ferns and all taxa (minimum, mean, maximum).

Trait Category	Trait	Native Ferns Min, Mean, Max (N)	All Taxa Min, Mean, Max (N)
Size-Related	Species Plant Size	1, 2.73, 5 (123)	1, 2.76, 5 (148)
	Rhizome Diameter	0.3, 6.59, 37.5 (37)	0.3, 6.53, 37.5 (40)
	Stipe Diameter	0.3, 1.79, 4.5 (24)	0.3, 1.79, 4.5 (24)
	Stipe Length	0.1, 17.27, 100 (17)	0.1, 19.09, 100 (18)
	Frond Width	0.25, 9.69, 40 (55)	0.25, 15.66, 300 (58)
	Frond Length	1.5, 77.98, 700 (136)	1.5, 81.66, 700 (157)
	Blade Width	0.3, 11.87, 57.5 (16)	0.3, 11.77, 57.5 (18)
	Blade Length	0.75, 30.53, 110 (29)	0.75, 30.01, 110 (31)
	USEG Width	0.2, 3.63, 10 (34)	0.2, 4.62, 20 (37)
	USEG Length	0.5, 11.6, 37.5 (20)	0.5, 13.22, 55 (23)
	Pinna Width	0.95, 30.32, 180 (29)	0.95, 36.96, 260 (31)
	Pinna Length	1.5, 127.6, 850 (43)	1, 168.62, 1500 (54)
	Number Pinnae Pairs	1.5, 20.099, 40 (71)	1.5, 19, 40 (80)
Shape	Number of Pinnate Divisions	0, 1.78, 4.5 (118)	0, 1.81, 5 (136)
	Number of Pinnate Variations	1, 1.52, 4 (118)	1, 1.55, 7 (136)
Demography	Abundance	1, 5.29, 7 (146)	1, 5.36, 7 (179)
	Distribution (#Islands)	0, 5.06, 8 (211)	0, 4.99, 8 (273)
Environment	Elevation	81, 1042.33, 2470 (170)	81, 1012.69, 2470 (208)

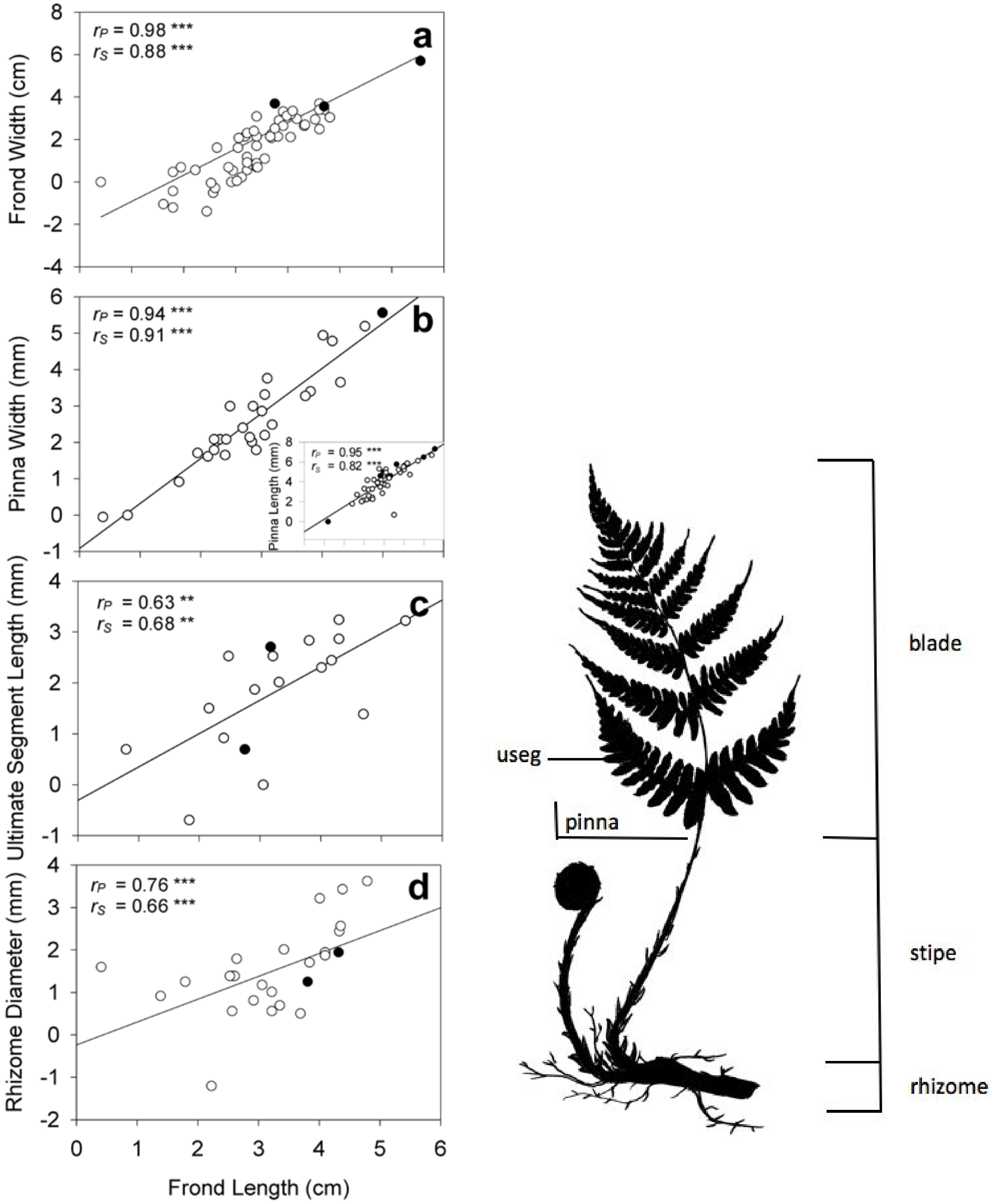
FIGURE CAPTIONS

Fig 2.1 *Left:* Size scaling in Hawaiian ferns as seen by positive correlations (Spearman, r_s, and Pearson, r_p) between frond length and: a) frond width, b) pinna width (inset: pinna length), c) ultimate segment length, and d) rhizome diameter. Open symbols, native fern taxa; filled symbols, aliens. Lycophytes were excluded because they lack the frond morphology of ferns. * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$. *Right:* Schematic of fern indicating the frond aboveground and rhizome belowground. The frond is separated into a stipe (stem-like) and blade (lamina). The first order division of the blade is termed "pinna" and the pinnae may be further divided, with the highest order division termed an "ultimate segment" (useg).

Fig 2.2 Association of frond form with habitat moisture and irradiance in Hawaiian ferns (ANOVAs, log-transformed data): a) Mean number of pinnae pairs is greater for wet-site taxa (WET), b) Mean blade length is shorter in open sites (OPEN). * $P \leq 0.05$

Fig 2.3 Association of plant size with growth form in Hawaiian ferns (ANOVAs, log-transformed data): a) epipetric taxa (EPE) have a smaller mean plant size than non-epipetric taxa (NOT_EPE), b) epiphytic taxa (EPH) have a smaller mean plant size than terrestrial taxa (TER). * $P \leq 0.05$ ** $P \leq 0.01$

Fig 2.4 Contrasting abundance and distribution of alien and native Hawaiian ferns (ANOVAs, log-transformed data): a) aliens are on average more abundant than native taxa and, b) occur at a lower elevations. * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$



a
r_P = 0.98 ***
r_S = 0.88 ***
Frond Width (cm)
8
6
4
2
0
-2
-4
b
r_P = 0.94 ***
r_S = 0.91 ***
Pinna Width (mm)
6
5
4
3
2
1
0
-1
r_P = 0.95 ***
r_S = 0.82 ***
Pinna Length (mm)
8
6
4
2
0
c
r_P = 0.63 **
r_S = 0.68 **
Ultimate Segment Length (mm)
4
3
2
1
0
-1
d
r_P = 0.76 ***
r_S = 0.66 ***
Rhizome Diameter (mm)
4
3
2
1
0
-1
-2
0
1
2
3
4
5
6
Frond Length (cm)
blade
useg
pinna
stipe
rhizome

Fig 2.1

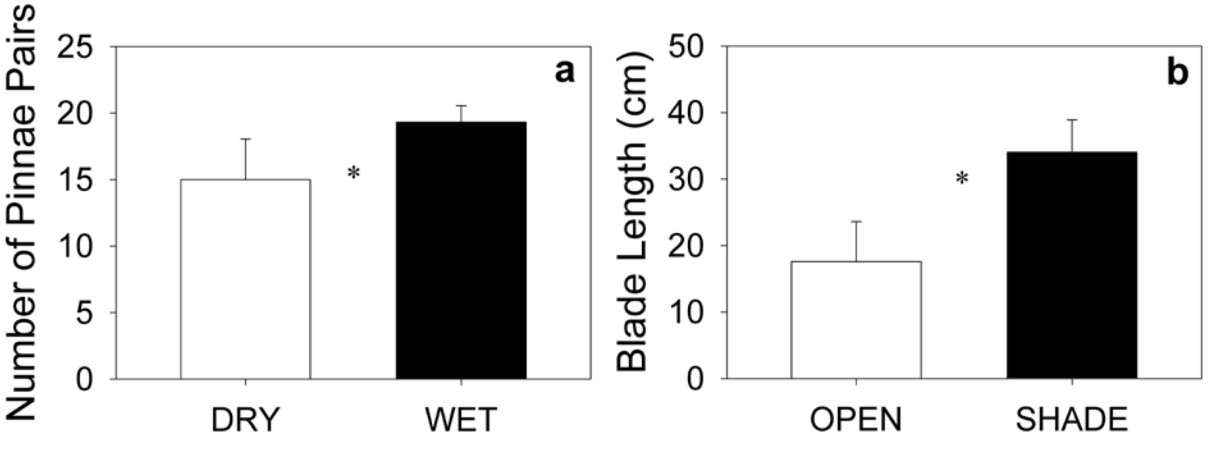

Number of Pinnae Pairs
25
20
15
10
5
0
a
*
DRY
WET
Blade Length (cm)
50
40
30
20
10
0
b
*
OPEN
SHADE

Fig 2.2

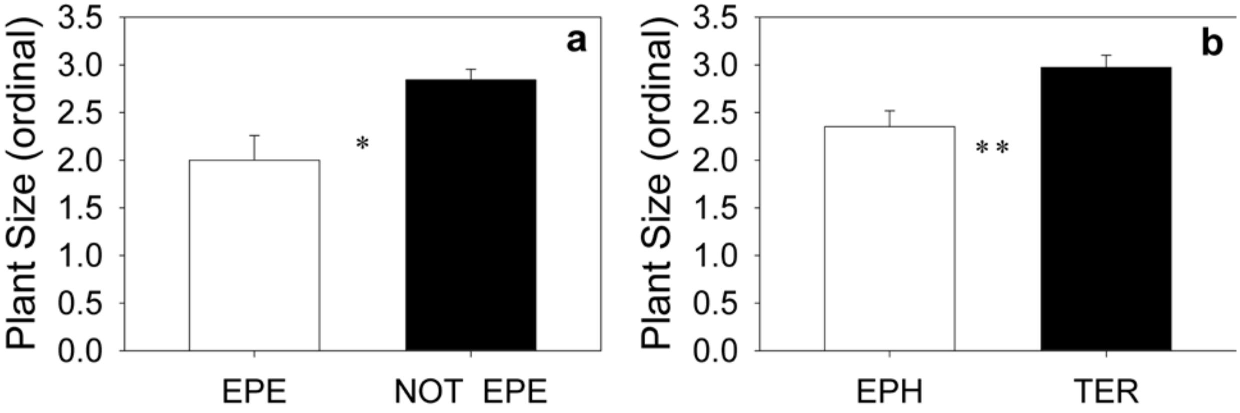

Plant Size (ordinal)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
*
EPE
NOT EPE
Plant Size (ordinal)
b
**
EPH
TER

Fig 2.3

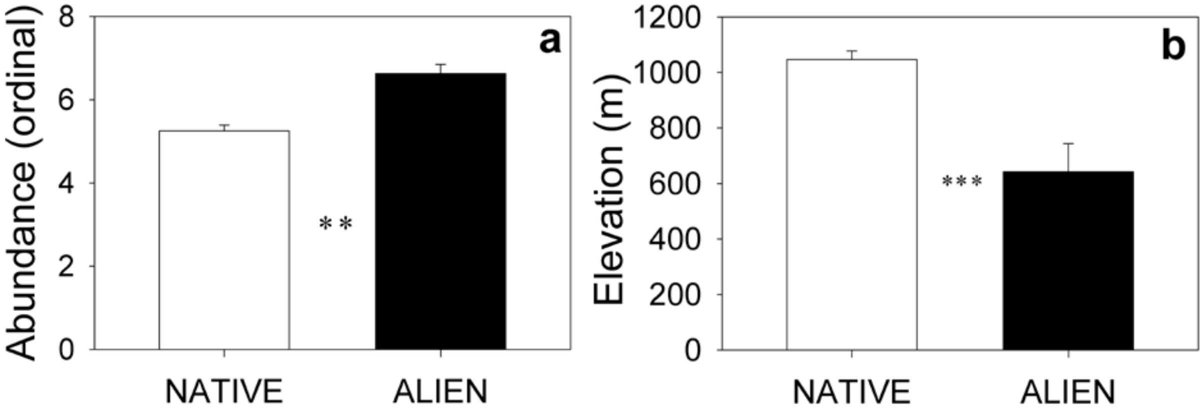

Abundance (ordinal)
8
6
4
2
0
a
**
NATIVE
ALIEN
Elevation (m)
1200
1000
800
600
400
200
0
b
***
NATIVE
ALIEN

Fig 2.4

SUPPLEMENTAL MATERIAL

SUPPLEMENTARY TABLE CAPTIONS

SuppTable 2.1 Correlation matrix for all taxa with Pearson and Spearman above and below the diagonal, respectively. Bold values represent significant correlations for both Pearson (r_p) and Spearman (r_s) tests and when italicized indicate log-transformation (used when more significant than untransformed data). When underlined, the Pearson correlation was significant at $P < 0.1$ (Spearman at $P < 0.05$). *P*-value and N below correlation coefficients.

SuppTable 2.2 Correlation matrix for native ferns and lycophytes with Pearson (r_p) and Spearman (r_s) above and below the diagonal, respectively. Spearman below diagonal. Bold values represent significant correlations for both Pearson and Spearman tests and when italicized indicate log-transformation (used when more significant than untransformed data). When underlined, the Pearson correlation was significant at $P < 0.1$ (Spearman at $P < 0.05$). *P*-value and N below correlation coefficients.

SuppTable 2.3 Correlation matrix for means of numeric variables for native ferns with Pearson (r_p) and Spearman (r_s) above and below the diagonal, respectively. *P*-value and *N* below correlation coefficients. Bold values represent significant correlations for both Pearson and Spearman tests and when italicized indicate log-transformation (used when more significant than untransformed data). When underlined, the Pearson correlation was significant at $P \leq 0.1$ (Spearman at $P \leq 0.05$). *P*-value and N below correlation coefficients.

SuppTable 2.4 Trait ranges for taxa (minimum, mean, maximum) and mean log-transformed trait values for ANOVA grouping variables for all taxa (above) and native ferns only (below in grey). Blank cells indicate a sample size below 5 for at least one group. Bold values are significant at $P \leq 0.05$ and when italicized indicate a low sample size in one or more groups ($N < 5$). Asterisks represent significant difference of means across taxa groups. ($P \leq {}^{+}0.10$ *0.05 **0.01 ***0.001) Blank cells indicate < 3 taxa/group, <5 taxa/group and not significant, or N/A (not testable).

SuppTable 2.5 Summary of Chi-Square test results for all taxa below the diagonal, for native ferns above the diagonal. Sample sizes for category rows (x) and columns (y) appear as x1y1, x2y1, x1y2, x2y2 with the Pearson's Chi-Square statistic in parentheses. One degree of freedom for all tests. $P \leq {}^{+}0.10$ *0.05 **0.01 ***0.001.

SuppTable 2.6 Size-related traits significantly correlated with one another demonstrate geometric scaling to maintain proportionality based on Standardized Major Axis regression curve slope analysis. Ordinal traits, including plant size, number of pinnate divisions and variations, and number of pinnae pairs, may not exhibit linear relationships and did not scale isometrically.

SuppTable 2.7 Summary of approach percipience for native ferns as well as predictive and explanatory power of hypotheses regarding conservation of proportions (positive size-scaling), growth form constraints (epiphytic vs. terrestrial; epipetric vs. non-epipetric), environmental pressures (open vs. shade, wet vs. dry, high vs. low elevation) and demographic trait associations (distribution; abundance).

SuppTable 2.8 Summary of Pearson correlation coefficients (R) and taxa sample size (N) for phylogenetically independent contrast correlations in *Adenophorus* and *Dryopteris*. Values in bold and underlined are significant at $P \leq 0.05$.

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CHAPTER 3

INFLUENCE OF ECONOMIES OF SCALE AND STRESS TOLERANCE ON FERN SIZE, LEAF FORM AND COMPOSITION ACROSS HABITATS IN SOUTHERN CALIFORNIA

ABSTRACT

Ferns exhibit exceptional diversity of leaf form with some of the largest and smallest leafed species in the world. To elucidate the underlying sources of this variation, we tested hypotheses for coordination of leaf traits, and for associations of leaf traits with environment for 15 diverse fern species from dry exposed and epipetric vs. moist shaded and terrestrial habitats in Southern California. We hypothesized that species of large size may be explained by a coordination of plant traits consistent with an economy of scale, i.e., high capacity and efficient production and mitigation of costs at greater size, and that these species would be associated with resource-rich environments favorable to ferns. By contrast, we hypothesized that species of small size may be explained by plant traits conferring stress tolerance, i.e., greater durability via more expensive, long-lived leaves for survival under stressful conditions and limited resource supply. As expected, larger species were associated with moist shaded and terrestrial habitats. We found trait relationships consistent with an economy of scale: isometric scaling of plant and leaf size indicating the ability to maintain functional proportions; an absence of diminishing returns for leaf mass per area (LMA) and lamina thickness at the leaf level; and further mitigation of costs of greater size via narrower leaflets, which would reduce support costs and self-shading. We also found evidence for an economy of stress tolerance: species from dry exposed and epipetric habitats had smaller plants, allocated less leaf mass to support tissues, and showed stress

tolerance traits including greater LMA, blade dissection to avoid overheating, higher lifetime integrated water use efficiency and water mass per area to avoid desiccation. This diverse optimization of fern leaf economics across species and conditions helps to explain the persistence of ferns in competitive angiosperm-dominated communities.

INTRODUCTION

Ferns vary dramatically in their size, leaf architecture, growth form, and range widely across moisture and light gradients (Raven et al. 1999; Moran 2004). We examined 15 diverse Southern California fern species to test a series of hypotheses for a large set of leaf traits, and their association with different habitats (Tables 3.1-3.4). There are considerable advantages to larger size, such as greater capacity for faster rates of overall resource capture and thus potential competitive advantage, all else being equal (Thomas and Bazzaz 1999; Zotz et al. 2002; Testo and Watkins 2012). However, greater size also increases ‘overhead costs’ given internal competition and structural support costs (e.g., Givnish 1982; Chazdon 1985; Niinemets et al. 2007). We hypothesized that species of large size would be associated with resource-rich and terrestrial habitats favorable to ferns, and would exhibit traits for size, shape, allocation, nutrient concentration, stoichiometric and isotopic ratios (Table 3.1) that would be conducive to an economy of scale, i.e., efficient production and mitigation of costs at greater size (Table 3.3). Further, we hypothesized that more resource-limited environments and rock-dwelling habitats would be associated with small species with plant traits conducive to an economy of stress tolerance, i.e., greater durability via more expensive, long-lived leaves for survival under stressful conditions (Table 3.4). Note these hypotheses are not mutually exclusive, as the first relates to changes of traits with plant size independently of habitat, and the second relates to shifts in traits between habitats. Across species we expected these economic principles of scale and stress tolerance to influence the coordination of leaf traits to improve performance, maintain functional proportions, and reduce costs of size and expensive leaf construction.

Based on an economy of scale, we made predictions for traits that increase capacity and efficiency for overall productivity, and help mitigate costs of larger plant size. Thus, we predicted that species with larger plants would have higher leaf N per area or per mass, an indicator of greater investment in rubisco for faster photosynthetic rates (Ripullone et al. 2003; Niinemets and Anten 2009; Wyka et al. 2012). Increases in leaf size have also been associated with allometric increases in leaf thickness and dry mass per area (LMA; Milla and Reich 2007; Niklas et al. 2007); however, we expected larger species to mitigate these diminishing returns at the entire leaf level, and/or to compensate for these diminishing returns by otherwise maintaining functional proportions via isometric scaling of leaves and leaf parts as seen for Hawaiian ferns (Arens and Baracaldo 2000; Arcand et al. 2008; Creese et al. 2011). We also predicted that large and divided leaves would incur costs from greater allocation of biomass to support tissues (Niinemets et al. 2007), with the potential for greater C investment in cell walls leading to higher C, C:N and C:P, and lower N and P concentrations (Elser et al. 2010). We expected ferns to offset these costs with large species adjusting proportions of nutrients to maintain C:N and C:P ratios similar to small sized species and/or to construct fewer leaves per plant (Yang et al. 2008; Whitman and Aarssen 2010). Since large leaves are prone to overheating, we expected greater dissection of leaf blades to improve convective heat transfer (Vogel 1968; Vogel 1970; Vogel 2009). Because plants with large leaflets or “ultimate segments” (USEGs; highest order division of a compound leaf) may experience greater internal competition for sunlight, we predicted narrowing of USEG shape via higher length-to-width (L:W) and square perimeter-to-area (P^2:A) ratios to reduce self-shading (Zotz et al. 2002; Kern et al. 2004).

Based on an economy of stress tolerance, we predicted that species with high LMA, conferring longer leaf lifespans, would exhibit higher lifetime integrated water use efficiency (WUE, indicated by $\partial^{13}C$; see Methods) to help avoid desiccation (Ni and Pallardy 1991; Edwards et al. 2012), and would exhibit low N_{mass}, P_{mass}, and high N_{area} given global leaf economic trends (Wright et al. 2004; Shipley et al. 2006; Karst and Lechowicz 2007; Waite and Sack 2011).

We hypothesized that costs of living in different environments would further contribute to variation in leaf traits. In moist shaded habitats favorable to ferns, we expected that abundant resources and competition for light would favor large species. Thus we predicted that species of moist shaded habitats would be large, with large leaves and narrow USEGs, a high chlorophyll concentration (Chl_{area}, Chl_{mass}), leaf water content, and mass allocation to support tissues, as well as low LMA and diluted C, N, P nutrient concentrations (Wright et al. 2004; Reich et al. 2007; Poorter et al. 2009; Pasquet-Kok et al. 2010). By contrast, dry exposed habitats with high temperatures, irradiance, and vapor pressure deficits (VPD) should favor smaller species with more numerous stress tolerant leaves. Thus we predicted for dry exposed habitats, small plants with high LMA, lamina density, thickness, C, N, P, C:N, C:P and N:P associated with slower growth, and a relatively greater allocation of leaf P to lamina tissue to expedite replacement of photodamaged proteins, RNA, and DNA (Thompson et al. 1997; Wright et al. 2001; Niinemets and Sack 2006; Kessler et al. 2007; Veneklaas et al. 2012). We also expected a low chlorophyll concentration given high light, higher leaf water mass per area (LWMA) and WUE to help avoid desiccation, and greater pinnate divisions (PDIV) to help avoid overheating. To offset costs of constructing expensive stress tolerant leaves, we predicted greater mass allocation to lamina than to support tissues. We additionally tested for significant correlations of these stress tolerance-related traits with continuous climate variables for dryness, irradiance and temperature (Tables 3.1, 3.4).

The most low-resource environments occupied by our fern species were located on rock substrate, i.e., epipetric habitat (Watkins et al. 2007). Thus, similar to our hypotheses for dry exposed species relative to moist shaded species, for epipetric relative to terrestrial species we predicted: small plants, more numerous leaves, and highly dissected leaves with high LMA, lamina thickness, density, broad USEGs with low P^2:A, and high N_{area}, C_{area}, and P_{area}. To help balance the costs of expensive leaf construction, we expected reduced allocation of leaf nutrients to rachis tissue and mass allocation to support tissues. To help avoid desiccation, we predicted higher LWMA and WUE to buffer leaf water potential. Because our epipetric species tended to occur on exposed rocky outcrops under high irradiance, we additionally tested for reduced investment in photosynthetic apparatus via low chlorophyll concentration, Chl_{mass}:N_{mass}, and a high C:N consistent with exposure to high irradiance in angiosperms (Kull and Niinemets 1998; Evans and Poorter 2001; Hogewoning et al. 2010).

MATERIALS AND METHODS

Plant material

We selected 15 diverse species located in the Santa Monica and San Gabriel Mountain ranges to maximize diversity in size and form (Table 3.2). A minimum of five leaves per species from different individuals representative of species adult size were collected from native habitats April to June 2008 for morphology and composition measurements, and again between April and June

2009 for ultimate segment shape parameters. Whole leaves included stipe, rachis, and lamina; USEGs were defined as the highest order division of the leaf supplied by its own midvein, which included the discrete pinnatifications of *Polypodium californicum* and *Woodwardia fimbriata*. Leaf 'blades' are defined as the whole leaf with stipe subtracted, i.e., the laminate portion of the leaf. Leaves were excised from plants in the field and immediately recut under water, then transferred to the lab where they were recut again and bagged overnight with stipe submerged to ensure hydration. Leaves were then separated into stipe, rachis and lamina parts for saturated and dry measurements.

Leaf morphology and composition

Plant height was measured in the field vertically from the ground or rock wall attachment site to the apex of the longest leaf, and number of leaves per plant and number of pinnae per leaf were counted. In the lab, leaf dimensions were quantified using hydrated leaves. Leaf area was measured with a leaf area meter (LI-COR 3100; LI-COR Biosciences, Lincoln, NE, USA) and USEG area was measured in Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA) using leaf scans (Epson Perfection 4490 flatbed scanner, Epson America Inc., Long Beach, CA, USA) or light microscope photos of the small cheilanthoid species (DMRB, Leica Microsystems microscope at 5x objective with 14.2 Color Mosaic digital camera, Diagnostic Instruments). The thickness of the lamina (T_{lam}) and length and width dimensions for leaf, blade, USEG, rachis, and stipe were measured by tape and digital calipers (Mitutoyo 500-752-10 Coolant Proof Digital Caliper, City of Industry, California, USA); lamina thickness was an average of three measurements from the most central USEG, and widths were determined from stipe, rachis and leaf midpoints. Leaf dissectedness was quantified as number of pinnate divisions (PDIV) and pinnae pairs (PP), and shape of USEGs was quantified by the length-to-width (L:W) and squared perimeter-to-area (P^2:A) ratios, such that elongate USEGs would have relatively greater length than width and relatively greater perimeter than area compared to circular USEGs. Since small USEGs would necessarily have a greater perimeter-to-area ratio than large USEGs, we used P^2:A because of its independence from USEG size. Chlorophyll concentrations were averaged from at least three measurements taken from the central lamina (excluding the primary vein) of each leaf using a chlorophyll meter (model SPAD-502, Konica Minolta, New Jersey, USA). Following fresh measurements, leaf parts were oven dried at 70 ºC for at least one week before mass measurements with an analytical balance (XS205; Mettler Toledo, Columbus, OH, USA).

Biomass allocation determined from dry mass values included the percent allocation of whole leaf mass to lamina (%L), rachis (%R), and stipe (%S) tissues, lamina mass per area (LaMA) and leaf mass per area (LMA), as well as lamina density (ρ_{lam} = LaMA / T_{lam}; g m^{-3}). We determined water mass at full hydration for stipe, rachis and lamina (Arcand et al. 2008); water content was calculated as (saturated mass – dry mass) / dry mass (g g^{-1}) for lamina (LaWC) and whole leaves (LWC), and water mass per area was calculated as water content / leaf area (kg m^{-2}) for lamina (LaWMA) and whole leaves (LWMA). Leaf C, N, P concentrations, stoichiometric ratios (C:N, N:P, C:P, Chl:N), and isotope ratios ($\delta^{13}C$) were measured via mass spectrometry (Costech Elemental Analyzer & Thermo-Finnegan Delta V IRMS) on subsamples of dried tissue at the Analytical Laboratory, University of Hawaii, Hilo. We analyzed $\delta^{13}C$ to reflect a lifetime integrated water-use-efficiency (WUE) as smaller stomatal apertures and persistently lower stomatal conductance discriminate against the influx of heavier ^{13}C isotopes (Farquhar and Richards 1984) to provide a more reliable indicator of species' tendencies for stomatal behavior. Nutrient allocation to leaf blade tissues was calculated as the percent of blade C, N, and P allocated to lamina versus rachis tissues. For biomass allocation, nutrient concentrations, stoichiometric and isotopic ratios we considered leaf tissues together and separately (Table 3.1) to control for potential differences in rachis and lamina composition. Because traits of different leaf tissues were often correlated (SuppTable 3.2), reported results apply for all leaf tissues unless otherwise noted.

Habitat and climate

Individual habitats were categorized as epipetric for ferns growing on rocks without soil, or terrestrial if growing in soil (Table 3.2). We further quantified species environments using discrete microsite habitat variables and continuous climate variables. Given the association of moisture and light across sites (Table 3.2, SuppTable 3.2), riparian and understory microsites were classified as moist and shaded compared to dry and exposed microsites. Microsite wetness was quantified using an ordinal scale of 1 – 4 from driest to wettest. To compare climate data for species collection sites, we determined mean annual temperature (MAT) and precipitation (MAP), as well as seasonal variation in temperature and precipitation from GPS coordinates using WorldClim and Prism climate databases (Hijmans et al. 2005; Prism Climate Group, http://prism.oregonstate.edu) using DIVA GIS (http://www.diva-gis.org). Variation in air moisture, i.e. vapor pressure deficit (VPD; units kPa), was calculated as the difference between saturated vapor pressure (e_S) and ambient water vapor pressure (e_a), determined using the average temperature dewpoint and MAT from Prism 2003-2007 (Pearcy et al. 1989). We used percent open sky (%OS) as an index of habitat light availability, estimated at the plant level as the proportion of sky unobstructed by canopy and topography (Waite and Sack 2011).

Statistical analyses

Trait-trait and trait-environment correlations were assessed using Spearman and Pearson correlation coefficients on raw and log-transformed data (R Development Core Team, 2010, R Foundation for Statistical Computing, Vienna, Austria). We present a correlation matrix of all variables (SuppTable 3.2) so that readers may examine the intercorrelative structure among variables. Because we tested only previously hypothesized relationships, we did not use statistical correction for multiple correlation tests, and do not endorse any significant trait relationships that were not previously hypothesized, and would recommend statistical correction for any “mining” of relationships. To determine the slopes of relationships between traits, we fitted standardized major axes (SMA) to log-transformed data using SMATR (Falster et al. 2006), equivalent to fitting power law curves $\log(y) = \alpha + \beta \times \log(x)$ (Sokal and Rohlf 1995; Sack et al. 2003). We tested for deviation of these SMA slopes from those expected by isometric scaling to determine whether plants maintained functional proportions (Niklas 1994, Sack et al. 2003, Warton et al. 2006; Creese et al. 2011). Finally, we used nested ANOVAs (Minitab 16 Statistical Software 2010, Minitab, Inc., State College, Pennsylvania, USA) to test for significant trait differences across species grouped by dry exposed versus moist shaded environments, and by epipetric versus terrestrial habitats.

RESULTS

Variation in plant traits and environment

The 15 fern species from Southern California varied substantially in size, leaf form and composition (Fig 3.1, SuppTable 3.2). Leaf size parameters and number of pinnae pairs varied most strongly, 20-500 fold, followed by plant height (18-fold); leaf thickness, which may be more tightly controlled for a given leaf size due to biophysical constraints, still showed 10-fold variation for species averages, with individual leaves ranging from 70 µm thick (*Adiantum capillus-veneris*) up to 900 µm thick (*Cheilanthes covillei*). Leaf number also varied substantially, ranging on average from seven leaves per plant (*Pityrogramma triangularis*) up to 50 leaves per plant (*Pellaea andromedifolia*). Species varied in leaf dissectedness from zero divisions, with only lamina pinnatifications (*Polypodium californicum)*, up to four orders of division (e.g. *Notholaena californica*), and USEG P^2:A varied 7-fold.

Species also exhibited strong differences in leaf composition. Leaf mass allocation to lamina varied twice as much as mass allocation to rachis and stipe (14-fold). Lamina density varied 60-fold, and LMA and allocation of leaf mass to water mass (per mass and per area) varied 4 to 340-fold; when lamina tissue was considered separately from rachis tissue, variation increased from 35 to 2735-fold. Nutrient concentrations were more tightly constrained across species. With the exception of Chl_{mass}, which varied 32-fold, mass-based concentrations were less variable than area-based concentrations: leaf N_{mass}, C_{mass}, and P_{mass} varied up to 4-fold whereas N_{area}, C_{area}, P_{area} and Chl_{area} varied 5-22 fold. The allocation of blade nutrients to rachis tissue was most variable with 10-15 fold differences in within-blade allocation of N and P. Thus the allocation of nutrients associated with metabolism may be more tightly controlled for lamina tissue than for support tissues. Species varied 3-16 fold in C:N, C:P and N:P ratios. Most variation in stoichiometric and isotopic ratios was associated with N, including 10-24 fold differences in Chl_{mas}:N_{mass} and blade N:P. Because nutrient concentration, allocation, stoichiometric and isotopic traits calculated respectively for the blade, lamina, and rachis were intercorrelated (SuppTable 3.2), the following results were significant for all tissue types unless otherwise noted.

Fern species ranged across moist shaded and dry exposed microhabitats that varied 2-fold in MAP and MAT and 10-fold in %OS (Table 3.2, SuppTable 3.1).

Correlations of size and shape traits

We observed strong coordination among leaf and plant dimensions. We found positive scaling of leaf, blade and stipe lengths and widths, and leaf area dimensions with plant height ($P \leq 0.001$, Fig 3.2; SuppTable 3.2), and scaling among leaf dimensions, i.e., USEG area and blade length, leaf length, and leaf area ($P \leq 0.05$, SuppTable 3.2). Notably, leaf number and thickness were independent of the other size traits (Fig 3.2; SuppTable 3.2). Slope analyses revealed that leaves and leaf parts scaled isometrically with one another and with plant size (Slope = 0.81 to 2.16, $P \leq 0.05$, SuppTable 3.3), and although leaf thickness and LMA scaled allometrically with USEG area (Slope = -0.38 to -0.29, $P \leq 0.05$, SuppTable 3.3), they did not significantly scale with leaf area (SuppTable 3.3). Differences in size dimensions were also coordinated with leaf dissectedness and USEG shape: large plants with large leaves and leaf parts had narrower USEGs with a high L:W ($P \leq 0.05$, Fig 3.3, SuppTable 3.2). Although PDIV was not significantly correlated with plant or leaf size, leaves with large USEG area had significantly fewer pinnate divisions ($P \leq 0.01$, Fig 3.3, SuppTable 3.2) and high USEG P^2:A ($P \leq 0.05$, SuppTable 3.2).

Correlations of size, shape and composition traits

Differences in size and shape were significantly correlated with leaf composition traits. As expected, larger leaf, blade and stipe dimensions scaled with greater percent allocation of leaf mass to rachis (%R) and stipe (%S; $P \leq 0.05$, SuppTable 3.2). Differences in C, N, P, C:N, C:P and leaf number with leaf size were not significant (SuppTable 3.2), but greater leaf dissectedness was linked to greater investment in support with higher C_{mass} in the rachis and higher lamina C:P ($P \leq 0.05$, SuppTable 3.2). Contrary to expectation, for our fern species, greater plant height was not significantly correlated with greater leaf N per area or per mass (SuppTable 3.2); rather, greater investment in blade N_{area} was strongly associated with plant size as quantified by a higher number of leaves per plant ($P \leq 0.05$, SuppTable 3.2). As predicted, LMA was positively correlated with $\delta^{13}C$ ($P \leq 0.05$, SuppTable 3.2), and high LMA was correlated with greater blade and lamina N_{area} and lower lamina N_{mass}, but was not significantly associated with P_{mass} ($P \leq 0.001$, SuppTable 3.2).

Associations of leaf traits with moist shaded versus dry exposed habitats

Species from dry exposed habitats and moist shaded habitats varied in their size, shape, and composition traits as predicted (SuppTable 3.1). Dry exposed site species were commonly epipetric, growing on rocky outcrops ($P \leq 0.001$ Chi-square test, data not shown), though *Pellaea andromedifolia* and *Pityrogramma triangularis* were two exceptions with the terrestrial habitat growth form. As hypothesized, dry exposed site species were on average smaller plants with a greater number of leaves that were more highly dissected, smaller and thicker, and included smaller blades, stipes and USEGs with lower L:W and P^2:A ratios in comparison with moist shaded site species ($P \leq 0.01$, Fig 3.4, SuppTable 3.1). Dry exposed site species also differed significantly in their biomass allocation, with lower investment of leaf mass into rachis and stipe tissues ($P \leq 0.05$, SuppTable 3.1), and higher LMA ($P \leq 0.001$, Fig 3.4, SuppTable 3.1), ρ_{lam} ($P \leq 0.05$), and LWMA ($P \leq 0.001$, SuppTable 3.1). Nutrient concentrations significantly differed on a per mass and per area basis (SuppTable 3.1), with dry exposed site species showing higher C_{mass}, C_{area}, and N_{area} ($P \leq 0.05$, Fig 3.4), but lower Chl_{mass}, Chl_{area}, lamina P_{mass}, and lower P_{mass} and P_{area} in the rachis ($P \leq 0.01$, SuppTable 3.1). Dry exposed site species also allocated a higher percent of total blade P to the lamina tissue ($P \leq 0.01$, Fig 3.5, SuppTable 3.1). And the lamina and rachis tissues of dry exposed species showed significantly higher C:N, N:P and C:P, as well as higher $\delta^{13}C$ ($P \leq 0.05$, Fig 3.4, SuppTable 3.1).

Correlations of leaf traits with habitat conditions

Species from dry exposed sites experienced more seasonally variable temperatures and precipitation than moist shaded site species, including warmer MAT but cooler minimum temperatures, and lower MAP with less precipitation in wettest and driest quarters, as well as higher VPD and %OS ($P \leq 0.001$, SuppTable 3.1). Overall, environmental variables were uncorrelated except for a negative relationship between MAP and %OS (SuppTable 3.2). Across species, leaf size, shape, and composition traits were strongly related to moisture, irradiance, and temperature gradients. Although microsite wetness and MAP were not significantly correlated, they overlapped in their associations with other plant traits. Higher MAP and wetter microsites yielded larger leaves and USEGs with higher P^2:A ($P \leq 0.05$, Fig 3.6, SuppTable 3.2). By contrast, lower MAP was associated with greater LMA, LWMA, and more leaves per plant ($P \leq 0.05$, Fig 3.6, SuppTable 3.2). High %OS was significantly associated with smaller USEG area, low Chl_{mass}, and high $\delta^{13}C$ ($P \leq 0.05$, Fig 3.6, SuppTable 3.2). High MAT was significantly associated with greater leaf dissectedness ($P \leq 0.05$, Fig 3.6, SuppTable 3.2).

Associations of leaf traits with epipetric versus terrestrial habitats

Leaf form and composition varied strongly with plant habitat (SuppTable 3.1). Epipetric species experienced more stressful conditions in microsites that were drier with greater seasonal variation in precipitation, and high VPD, MAT, and %OS ($P \leq 0.001$, SuppTable 3.1). Although there were no significant differences in LMA, T_{lam} or ρ_{lam} ($P > 0.05$, SuppTable 3.1), as expected based on an economy of stress tolerance, epipetric species tended to be smaller than terrestrial species, with smaller leaves and leaf parts, broad USEGs with low P^2:A, and exhibited more leaves per plant and more pinnate divisions (though fewer total pinnae pairs; $P \leq 0.01$, SuppTable 3.1). Epipetric species showed high LWMA, reduced investment of leaf mass in support tissues given lower %R and %S ($P \leq 0.01$), and allocated less leaf C and N to rachis tissue ($P \leq 0.001$, Fig 3.5, SuppTable 3.1). Epipetric species also tended to have higher nutrient concentrations, given high blade and lamina C_{area}, N_{area}, P_{area}, and higher Chl_{mass}:N_{mass}, and lower C:N for lamina and blades ($P \leq 0.001$, SuppTable 3.1). Isotopic ratios differed significantly as well, with epipetric species showing higher $\delta^{13}C$ for all leaf tissues ($P \leq 0.05$, SuppTable 3.1).

DISCUSSION

Diversity in Southern California fern leaf size, shape and composition

Fern species showed remarkable diversity in size and leaf form, ranging from the very small and divided cheilanthoid species (*Cheilanthes covillei*, *Cheilanthes newberryi*) to the large and less divided bracken and giant chain ferns (*Pteridium aquilinum*, *Woodwardia fimbriata*). Fern species ranged 7 to 500-fold in size and shape traits and 1 to 2735-fold in composition traits. This variation was strongly associated with variation in other traits, and with differences in habitat, climate conditions, and habit in accordance with economies of scale and stress tolerance.

Differences in fern size and the coordination of leaf size, shape, and composition are related to an economy of scale

We hypothesized that large species would have traits consistent with greater capacity and efficiency for overall faster rates of resource capture, and would have to mitigate costs of greater size to preserve an economy of scale. Across species, we expected larger plants to show higher concentrations of leaf N as it is related to greater investment in rubisco for faster photosynthetic rates (Ripullone et al. 2003; Niinemets and Anten 2009; Wyka et al. 2012). Notably, taller plants did not show greater leaf N concentrations per area or per mass; however, higher N_{area} was strongly associated with a greater number of leaves per plant. This is especially interesting given that angiosperms tend to exhibit decreasing nutrient concentrations with increasing plant mass based on a meta-analysis of woody and herbaceous species (Elser et al. 2010). This suggests that ferns are able to maintain nutrient concentrations across a range of plant heights, and couple their investment in a larger number of leaves with investment into greater photosynthetic capacity for these leaves. This would provide a considerable advantage, especially under shaded conditions, as these fern species would be able to capitalize on the morphology of creeping rhizomes or stolons to horizontally expand their plant size via construction of new high capacity leaves in search of light gaps to maximize light capture at the whole-plant level.

Larger species also showed costs of greater size. Though we observed diminishing returns of allometric increases in leaf thickness and mass per area with USEG area, allometric increases in leaf thickness and LMA disappeared at the leaf level (Milla and Reich 2007; Niklas et al. 2007). Notably, diminishing returns at the leaf level were reported in an analysis of ferns and angiosperms (Niklas et al. 2007); however, data for the entire leaves and USEGs of ferns were analysed together, thus we expect that the reported leaf-level relationships were in fact driven by relationships at the USEG-level. Leaf thickness, LMA, and leaf and USEG size were not significantly correlated in our study, suggesting that larger fern LMA can be decoupled from leaf size due to the allocation among rachis and lamina, and thus, large fern leaves do not necessarily entail a higher LMA. These results suggest that ferns help mitigate costs of greater size and increase their efficiency by avoiding diminishing returns of large leaf size. Additionally and as predicted, isometric scaling among plant size and dimensions of leaves and leaf parts maintained functional proportions to support efficient function at greater size. Given that positive size-scaling has been shown to hold across distinct growth forms, across environmental gradients in moisture, temperature and irradiance, and has been observed across distinct fern floras (Arens and Baracaldo 2000; Arcand et al. 2008; Creese et al. 2011), there may be universal developmental linkages responsible for maintaining functional proportions (Pearsall 1927; Strauss 1993; Bonduriansky 2007).

We expected that larger size would also incur greater structural support costs that would influence allocation of leaf biomass and nutrients and potentially leaf number. For ferns, in contrast with woody and herbaceous angiosperms (Kleiman and Aarssen 2007; Yang et al. 2008; Whitman and Aarssen 2010), number of leaves and pinnae pairs per leaf were actually independent of plant and organ size, though dry exposed and epipetric species did show smaller and more numerous leaves. We hypothesize that the development of fern leaves from terrestrial rhizomes may help ameliorate biophysical limitations of leaf construction by providing greater space and support for leaf construction than aerial woody stems and branches, thus decoupling leaf size from leaf number, except under circumstances of extreme resource limitation. As expected, larger species made greater investments of leaf mass into the rachis and stipe support tissues, and greater dissection of the leaf was associated with higher C_{mass} in the rachis and higher C:P in the lamina, which may reflect greater investment in cell walls. However, larger species were able to adjust nutrient concentrations to maintain C concentrations, C:N, and C:P similar to smaller species.

To further minimize costs of greater size, ferns modified their shape. We expected positive scaling of plant size with leaf dissectedness, as seen for Hawaiian ferns (Creese et al. 2011), to help reduce overheating in large leaves; however, number of pinnate divisions was not correlated with plant size, and negatively correlated with USEG area. Even though they are found in the mediterranean-type climate of Southern California, tall and large leafed ferns tended to occur in moist shaded sites, and thus there may be no advantage to further dividing leaves, whereas the small ferns from dry exposed habitats would benefit from greater dissectedness, thinner boundary layer, and relatively greater perimeter for faster latent heat transfer (Vogel 1968; Vogel 1970; Vogel 2009). As hypothesized, USEG shape did vary significantly with size, as large plants with large leaves and USEGs exhibited a higher L:W ratio, which can importantly reduce self-shading (Zotz et al. 2002; Kern et al. 2004).

Differences in leaf composition are related to an economy of stress tolerance

To maximize benefits in stress tolerance, we hypothesized that fern species would have high LMA and exhibit conservative water use to maximize longevity, durability, and survival under stressful conditions, and would follow global trends for the leaf economics spectrum (Wright et al. 2004; Shipley et al. 2006). We expected that expensive high LMA fern leaves should have longer leaf lifespans (Wright et al. 2004; Shipley et al. 2006; Karst and Lechowicz 2007). Consistent with conservative resource use, high LMA leaves showed greater WUE (∂^{13}C). Also in accordance with the leaf economics spectrum, our fern species with high LMA showed high blade and lamina N_{area} and low lamina N_{mass}, but P_{mass} did not vary significantly. It may be that ferns are less variable in their P uptake generally: in contrast to other plant groups, ferns showed no significant relationship between leaf N and P in a survey of 753 terrestrial species in China (Han et al. 2005), and fern species from very different calcifugous and calcicolous soils showed no significant differences in leaf P (Hou 1950).

Fern size, leaf form and composition vary distinctly with habitat conditions in accordance with economies of scale and stress tolerance

We hypothesized differences in leaf form and composition to correspond with different environments. We expected that moist shaded habitats favorable to ferns would favor large species in accordance with an economy of scale, and that dry exposed habitats stressful to ferns would favor small species in accordance with an economy of stress tolerance. As expected, moist shaded site species built larger plants and leaves, and as expected to mitigate costs of greater size, possessed narrower USEGs with high L:W, P^2:A, and chlorophyll concentration to reduce self-shading and improve light capture. Greater investment of leaf mass into rachis and support tissues, lower LMA and lamina thickness may also help to ameliorate biomechanical stress associated with greater size (Niklas 1994; Dunbar-Co et al. 2009). By contrast, dry exposed site species built smaller plants and leaves, with more leaves per plant for greater redundancy, and exhibited traits associated with expensive construction, greater stress tolerance, slower growth and longer lifespan: higher LMA, ρ_{lam}, T_{lam}, C_{mass}, C_{area}, N_{area}, C:N, C:P, and N:P (Wright et al. 2004; Shipley et al. 2006; Waite and Sack 2011). Higher leaf N and N:P of dry exposed species may also reflect compensation for reduced photosynthetic activity under drought (Galmés et al.

2011), and reduced mobility of P in dry soils (Cernusak et al. 2010). To avoid overheating and desiccation, leaves of dry exposed site species were more dissected to improve convective cooling (Vogel 1968; Vogel 1970; Vogel 2009), and exhibited higher WUE and LWMA, which should increase leaf capacitance to help buffer leaf water potential (Sack et al. 2003; Bartlett et al. 2012). In addition to reduced allocation of leaf mass to rachis and stipe tissues, higher LWMA may provide a cheaper form of structural support in already expensive leaf tissues. Even at lower P tissue concentrations overall, species of dry exposed sites preferentially allocated more leaf P to lamina than to rachis tissue. Because the lamina is more important than rachis for leaf metabolism and may be more vulnerable to light and heat damage, greater allocation of leaf P to lamina suggests it is the priority for growth and replacement of photodamaged proteins, RNA, and DNA (Thompson et al. 1997; Kessler et al. 2007).

Stress tolerance traits were further correlated with stressful environmental conditions. Lower MAP and drier microsites were correlated with smaller durable leaves with high LMA, more leaves for plant for greater redundancy, and greater water mass stored per area to buffer against drought. High irradiance sites with high %OS were correlated with smaller USEGs with low $\mathrm{Chl}_{\mathrm{mass}}$ and higher WUE to help avoid desiccation. Finally, sites with high MAT were associated with greater leaf dissectedness for convective cooling.

Fern size, leaf form and composition vary distinctly with terrestrial versus epipetric habitat according to an economy of stress tolerance

In accordance with an economy of stress tolerance, we predicted that many of the traits advantageous in dry exposed habitats would also provide an advantage for epipetric species given limited resource and substrate availability (Watkins et al. 2007). Similar to dry exposed site species, epipetric species tended to occur in dry environments with high VPD, MAT, %OS, and accordingly exhibited lower chlorophyll concentrations, greater leaf dissection to improve convective cooling, and greater leaf water mass per area and higher WUE (∂^{13}C) to help buffer leaf water potential against desiccation. By contrast with dry exposed site species, although epipetric species showed broader USEGs with low P^2:A, LMA, T_{lam} and ρ_{lam} were not significantly different from terrestrial species. This suggests that LMA and leaf turnover rates would not be adjusted in response to terrestrial and epipetric habitats at growth or evolutionary timescales independent of differences in habitat light and moisture availability. Similar to dry exposed site species, however, and consistent with an economy of stress tolerance, epipetric species had smaller sized plants and leaves, reduced investment of leaf mass into support tissues, and showed high C, N, P concentrations per area, which may also be accounted for by their thicker lamina tissue. We additionally expected lower Chl_{mass}:N_{mass} and higher C:N in epipetric species consistent with exposure to high irradiance as seen for angiosperms (Kull and Niinemets 1998; Evans and Poorter 2001; Hogewoning et al. 2010) because our epipetric species tended to occur on exposed rocky outcrops in sites with higher %OS; however, epipetric species showed higher Chl_{mass}:N_{mass} and lower C:N. A higher Chl_{mass}:N_{mass} has been associated with a greater capacity to optimize maximum photosynthesis (A_{max}) under changing irradiance (Brooks et al. 1996). We thus hypothesize that similar to cases of xeric adapted angiopserms (Gibson 1998), high Chl_{mass}:N_{mass} may be important for optimizing A_{max} under greater extremes of radiation associated with a rock substrate habitat compared with a more buffered terrestrial habitat. The lower C:N in epipetric species may also indicate cost-savings due to reduced investment in defensive compounds (e.g. polyphenols) compared with species from terrestrial habitats where greater plant densities have been correlated with greater herbivory (Angulo-Sandoval and Aide

2000). Reciprocal transplants of fern species that grow facultatively in either the terrestrial or epipetric habits would be especially informative to test for significant differences in vulnerability to herbivory inherent to growth form. In addition to a lower C:N, epipetric species showed more conservative within-blade nutrient allocation than terrestrial species, investing less leaf C and N into rachis tissue, likely due to greater nutrient limitation.

CONCLUSIONS

Ferns exhibit remarkable diversity of leaf form and composition and include the world's smallest and largest leaves. The underlying sources of this variation may be explained by economies of scale and stress tolerance that influence the coordination of leaf traits and associations of species with particular habitats. Size-scaling of plant parts appears vital to efficient function across species and environments. Moist shaded environments and the terrestrial habitat with higher resource availability favorable to ferns were associated with large size and adjustments to leaflet shape to reduce internal competition by self-shading. By contrast, stressful conditions of the dry exposed and epipetric habitats favored construction of more expensive leaves with higher LMA and nutrient concentrations that should be associated with slower growth and longer retention, as well as reductions in size and support tissues, and greater water use efficiency. This diverse optimization of fern leaf form and composition across habitats should yield strong differences in physiology that influence species range sizes (Elser et al. 2010) and responses to environmental change. Thus, studies examining coordination of fern leaf architecture with hydraulic and photosynthetic physiology are needed.

ACKNOWLEDGEMENTS

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Table 3.1 Variable symbols, units, descriptions; P = Prism Climate Model; WC = Worldclim Climate Model.

Traits	Units	Trait Description
Environment		
Microsite wetness	#	Dry to wet (1 – 4 ordinal scale)
MAP_WC	mm	Mean annual precipitation (1950-2000 WC; 1971-2000 P)
MAPSEAS	mm	Mean annual precipitation seasonality; standard deviation (1950-2000 WC)
PWQ	mm	Mean precipitation wettest quarter (1950-2000 WC)
PDQ	mm	Mean precipitation of driest quarter (1950-2000 WC)
VPD	kPa	Vapour pressure deficit (1971-2000 P)
MAT_WC	°C	Mean annual temperature (1950-2000 WC; 1971-2000 P)
MATSEAS	°C	Mean annual temperature seasonality (SD;1950-2000WC)
TMAX	°C	Maximum annual temperature (1971-2000 P)
TMIN	°C	Minimum annual temperature (1971-2000 P)
%OS	%	Percent open sky
Size & Shape		
PH	cm	Plant height
LN	#	Leaf number per plant
LL	cm	Leaf length
BL	cm	Blade length
BW	cm	Blade width
SL	cm	Stipe length
SD	cm	Maximum stipe diameter
LA	cm^2	Leaf area
USEG A	mm^2	Ultimate segment area
T_{lam}	mm	Lamina thickness
PDIV	#	Maximum number of pinnate divisions (0-4)
PP	#	Number of pinnae pairs
USEG L:W	$mm\ mm^{-1}$	Ultimate segment length width ratio
USEG P^2:A	$mm^2\ mm^{-2}$	Ultimate segment squared perimeter to area ratio

Traits	**Units**	**Trait Description**
Biomass Allocation		
LaMA	g m^{-2}	Lamina mass per area
LMA	g m^{-2}	Leaf mass per area
ρ_{lam}	g m^{-3}	Lamina density
LaWC	g g^{-1}	Lamina water content per dry mass
LWC	g g^{-1}	Leaf water content per dry mass
LaWMA	kg m^{-2}	Lamina water mass per area
LWMA	kg m^{-2}	Leaf water mass per area
%S	%	Percent of total leaf mass allocated to stipe
%R	%	Percent of total leaf mass allocated to rachis
%L	%	Percent of total leaf mass allocated to lamina
Nutrient Concentrations Per Mass		
Chl_{mass}	SPAD g^{-1}	Chlorophyll concentration per unit mass for leaf blade or lamina
C_{mass}	g 100 g^{-1}	Carbon concentration per unit mass for leaf blade, lamina or 1^o Rachis
N_{mass}	g 100 g^{-1}	Nitrogen concentration per unit mass for leaf blade, lamina or 1^o Rachis
P_{mass}	g 100 g^{-1}	Phosphorus concentration per unit mass for leaf blade, lamina or 1^o Rachis
Nutrient Concentrations Per Area		
Chl_{area}	SPAD units	Chlorophyll concentration per unit area for leaf blade or lamina
C_{area}	mg m^{-2}	Carbon concentration per unit area for leaf blade, lamina or 1^o Rachis
N_{area}	mg m^{-2}	Nitrogen concentration per unit area for leaf blade, lamina or 1^o Rachis
P_{area}	mg m^{-2}	Phosphorus concentration per unit area for leaf blade, lamina or 1^o Rachis
Within-Blade Nutrient Allocation		
%C	%	Percent of blade Carbon allocated away from lamina to rachis
%N	%	Percent of blade Nitrogen allocated away from lamina to rachis
%P	%	Percent of blade Phosphorus allocated away from lamina to rachis

Traits	Units	Trait Description
Stoichiometry		
C:N		Carbon to Nitrogen concentration ratio for leaf blade, lamina or 1° Rachis
N:P		Nitrogen to Phosphorus concentration ratio for leaf blade, lamina or 1° Rachis
C:P		Carbon to Phosphorus concentration ratio for leaf blade, lamina or 1° Rachis
Chlmass:Nmass		Carbon to Phosphorus concentration ratio for leaf blade or lamina
Isotope Ratios		
$\delta^{13}C$	‰	^{13}C to ^{12}C ratio for leaf blade

Table 3.2 Study plants family, genus and species with habitat and microhabitat types, as well as species average mean annual temperature (MAT), mean annual precipitation (MAP), and irradiance by percent open sky (%OS; Waite and Sack 2011).

Family	**Genus**	**Species**	**Habitat**	**Microhabitat**	**MAT** °C	**MAP** mm	**%OS**
Sinopteridaceae	*Adiantum*	*capillus-veneris*	epipetric	moist shaded	17.77	570.69	13
Sinopteridaceae	*Adiantum*	*jordanii*	terrestrial	moist shaded	18.13	680.95	5
Sinopteridaceae	*Aspidotis*	*californica*	terrestrial	moist shaded	18.17	563.91	30.6
Sinopteridaceae	*Cheilanthes*	*covillei*	epipetric	dry exposed	17.405	462.49	50
Sinopteridaceae	*Cheilanthes*	*newberryi*	epipetric	dry exposed	16.14	385.3	57
Dryopteridaceae	*Dryopteris*	*arguta*	terrestrial	moist shaded	16.13	680.95	22.5
Sinopteridaceae	*Notholaena*	*californica*	epipetric	dry exposed	18.13	494.49	38
Sinopteridaceae	*Pellaea*	*andromedifolia*	terrestrial	dry exposed/shaded*	18.15	590.97	5
Sinopteridaceae	*Pellaea*	*mucronata*	epipetric	dry exposed	17.92	590.97	60
Sinopteridaceae	*Pityrogramma*	*triangularis*	terrestrial	dry exposed	17.92	458.48	46
Polypodiaceae	*Polypodium*	*californicum*	epipetric	moist shaded	17.34	570.69	5
Dennstaedtiaceae	*Polystichum*	*munitum*	terrestrial	moist shaded	13.66	1074.08	5
Dryopteridaceae	*Pteridium*	*aquilinum*	terrestrial	moist shaded/exposed*	16.97	574.21	45
Thelypteridaceae	*Thelypteris*	*puberula*	terrestrial	moist shaded	17.78	464.46	8
Blechnaceae	*Woodwardia*	*fimbriata*	terrestrial	moist shaded	17.34	694.91	21

MAT, mean annual temperature, MAP, mean annual precipitation, %OS, percent open sky.

**Pellaea andromedifolia* occurred in dry exposed habitats but co-occurred with larger plants that obstructed the majority of open sky

**Pteridium aquilinum* occurred in moist forested areas that were generally shaded but leaves often recruited into forest gaps with more open sky

Table 3.3 Expectations for shifts in plant traits associated with increasing size given an economy of scale, i.e., high capacity and efficient production, as well as mitigation of costs at greater size. Arrow direction indicates whether a trait is expected to increase or decrease with increasing plant size; ***** denotes traits that should be maintained despite changes in size; **§** denotes traits that typically show diminishing returns with increased size, but were not expected to shift with fern size. Note that leaf N and LMA are expected to additionally shift in opposite directions from one another given the leaf economics spectrum (e.g., Wright et al. 2004; Shipley et al. 2006). Values in bold indicate relationships that were statistically significant or invariable with size as predicted (SuppTables 3.1-3.3).

Patterns	**Traits**
Size-scaling for proportionality	↑ **PH**, **LA**, **USEG A**, **BL**, **BW**, **SL**, **SD**
Increasing capacity	↑ **N**, N:P
Increasing 'overhead'	↑ **C**, **%S**, **%R**
Increasing efficiency/ reducing costs of size	↑ PDIV, **USEG L:W**, **USEG P^2:A** ↓ LN, PP * **C**, **P**, **C:N**, **C:P** § **T$_{lam}$**, **LaMA**, **LMA**

Table 3.4 Expectations for shifts in plant traits across gradients of increasing moisture (mean annual precipitation; MAP), irradiance (percent open sky; %OS), and temperature (mean annual temperature; MAT), as well as for shifts across moist shaded and terrestrial habitats versus dry exposed and epipetric habitats based on an economy of stress tolerance for protection against extremes of irradiance, temperature, vapor pressure deficit, and drought; values in bold indicate relationships that were statistically significant as predicted (SuppTables 3.1, 3.2).

Habitat	**Traits**
Moist Shaded & Terrestrial **vs.** **Dry Exposed & Epipetric**	↑ **PH**, **LA**, **USEG A**, **USEG L:W**, **USEG P^2:A**, **Chl**, **%S**, **%R** ↓ **LN**, **PDIV**, **%L**, **LMA**, LaWC, LWC, **LaWMA**, **LWMA**, **T_{lam}**, ρ_{lam}, **C**, **N**, **P**, **C:N**, **C:P**, **N:P**, Lamina %C, %N, **%P**, δ^{13}**C** ↑ **LN**, **PDIV**, **%L**, **LMA**, LaWC, LWC, **LaWMA**, **LWMA**, **T_{lam}**, ρ_{lam}, **C**, **N**, **P**, **C:N**, **C:P**, **N:P**, Lamina %C, %N, **%P**, δ^{13}**C** ↓ **PH**, **LA**, **USEG A**, **USEG L:W**, **USEG P^2:A**, **Chl**, **%S**, **%R**
Climate: MAP	↓ **LMA**, **LN**, **LaWC**, LWC, **LaWMA**, LWMA
Microclimate: %OS	↓ PH, LA, **USEG A**, **Chl**, δ^{13}**C**
Climate: MAT	↑ **PDIV**

FIGURE CAPTIONS

Fig 3.1 Representative scanned leaves ("frond") or leaf parts (denoted as first or second order pinna for too large species) and average leaf area for wetter site species (top) versus drier site species (below). Leaf size varied 500-fold and leaf dissectedness ranged from zero to four pinnate divisions, with a tendency for smaller, more divided fronds in drier habitats.

Fig 3.2 Isometric scaling of plants, leaves and leaf parts scaled (SuppTable 3.4) included positive scaling of plant height with: a) blade length and blade width (inset), b) stipe length and diameter (inset), and c) leaf area and leaf number (inset; uncorrelated with size traits). Wetter habitats indicated by circles, drier habitats by triangles; open and shaded symbols represent open and shaded microsites, respectively; ***$P \leq 0.001$, **$P \leq 0.01$, * $P \leq 0.05$.

Fig 3.3 Coordination of size and shape: a) taller plants exhibited narrower ultimate segments (USEGs) given higher length-to-width ratio (L:W); b) leaves with larger leaf parts, i.e. USEG area, were also more dissected given a higher number of pinnate divisions; and c) large plants with large leaves exhibited greater allocation of leaf mass to stipe and rachis (inset) support tissues. Wetter habitats indicated by circles, drier habitats by triangles; open and shaded symbols represent open and shaded microsites, respectively; ***$P \leq 0.001$, **$P \leq 0.01$, * $P \leq 0.05$.

Fig 3.4 Species from dry exposed habitats (open bar) exhibited key differences in leaf size, shape and composition compared to moist shaded habitats (black bar), including: a) smaller plants (and leaf size; SuppTable 3.2); b) more leaves per plant; c) more dissected leaves given a higher average number of pinnate divisions; d) less elongate or more rounded ultimate segments given a lower squared perimeter-to-area (P^2:A) ratio; e) greater leaf mass per area (LMA); f) thicker lamina (T_{lam}); g) higher instantaneous water-use efficiency given higher $\partial^{13}C$; and h) higher lamina N per area (N_{area}); all associations were significant at $P \leq 0.001$ based on nested ANOVA.

Fig 3.5 Average percent mass of leaf blade N, C and P invested into the lamina (grey) versus primary rachis (black) tissues across all species (left), and significant differences in allocation for species grouped by epipetric versus terrestrial habitats, and by dry exposed and moist shaded habitats; values in pies represent respectively average percent mass of the given blade nutrient allocated to lamina tissue, the average nutrient concentration per mass, and the average percent leaf mass that was allocated to lamina tissue (rather than to rachis and stipe). Based on nested ANOVAs, dry exposed site species invested relatively more leaf mass into lamina tissue rather than support tissue and showed higher C_{mass}, but were more P-limited given lower P_{mass} in lamina and rachis compared to moist shaded site species; epipetric species invested relatively more leaf mass into lamina tissue and showed higher blade N_{mass} and lamina C_{mass} than terrestrial species ($P \leq 0.05$; SuppTable 3.1).

Fig 3.6 Size, shape and composition traits were significantly correlated with moisture and light gradients (SuppTable 3.3): a) ultimate segment size and perimeter-to-area ratio (P^2:A; inset) increased with mean annual precipitation (MAP); b) lamina water mass per area decreased with increasing MAP; c) lamina $\partial^{13}C$ as lifetime integrated water-use efficiency increased with increasing irradiance quantified as percent open sky; and d) chlorophyll concentration per mass decreased with increasing percent open sky; $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*} P \leq 0.05$

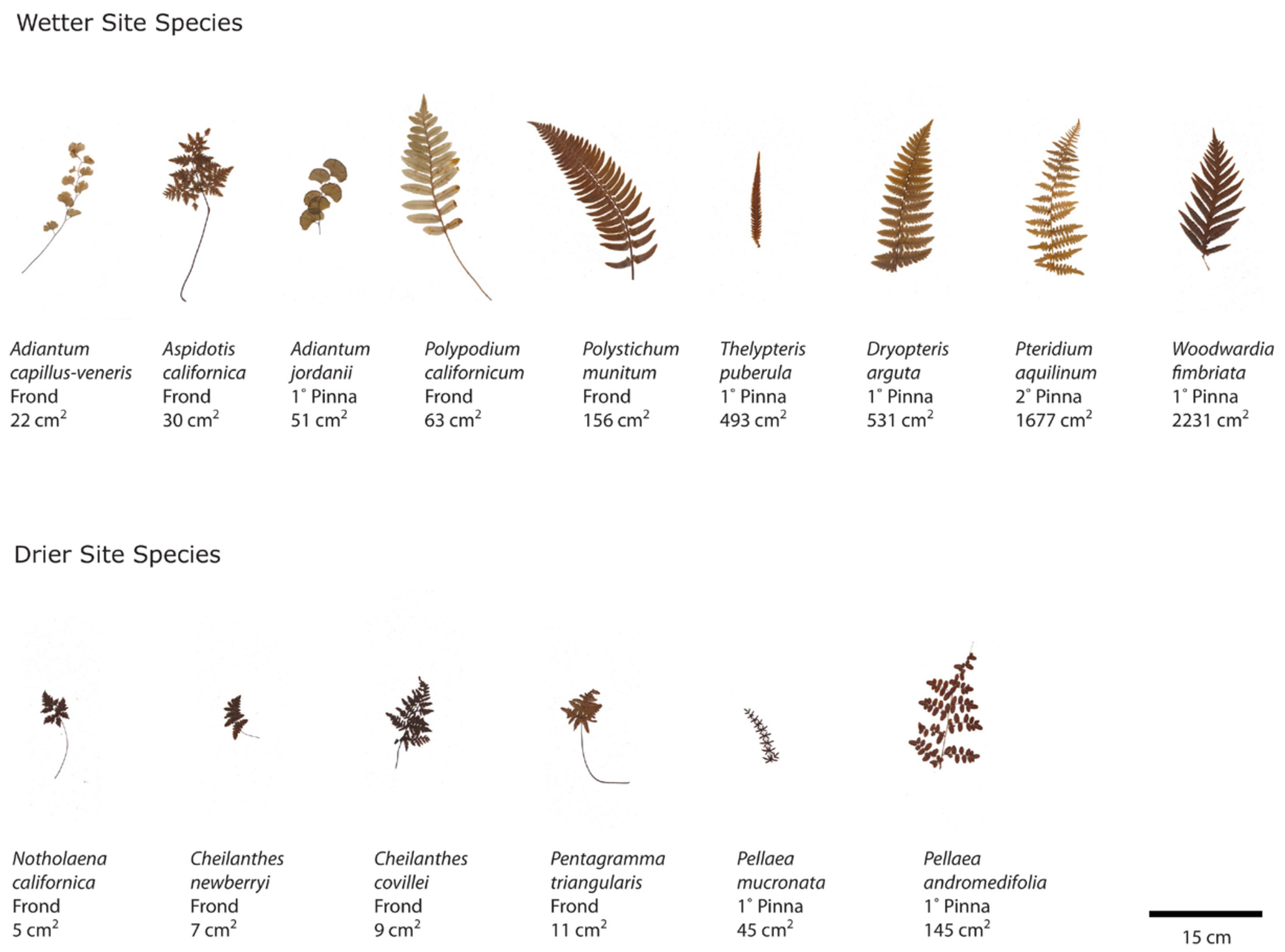
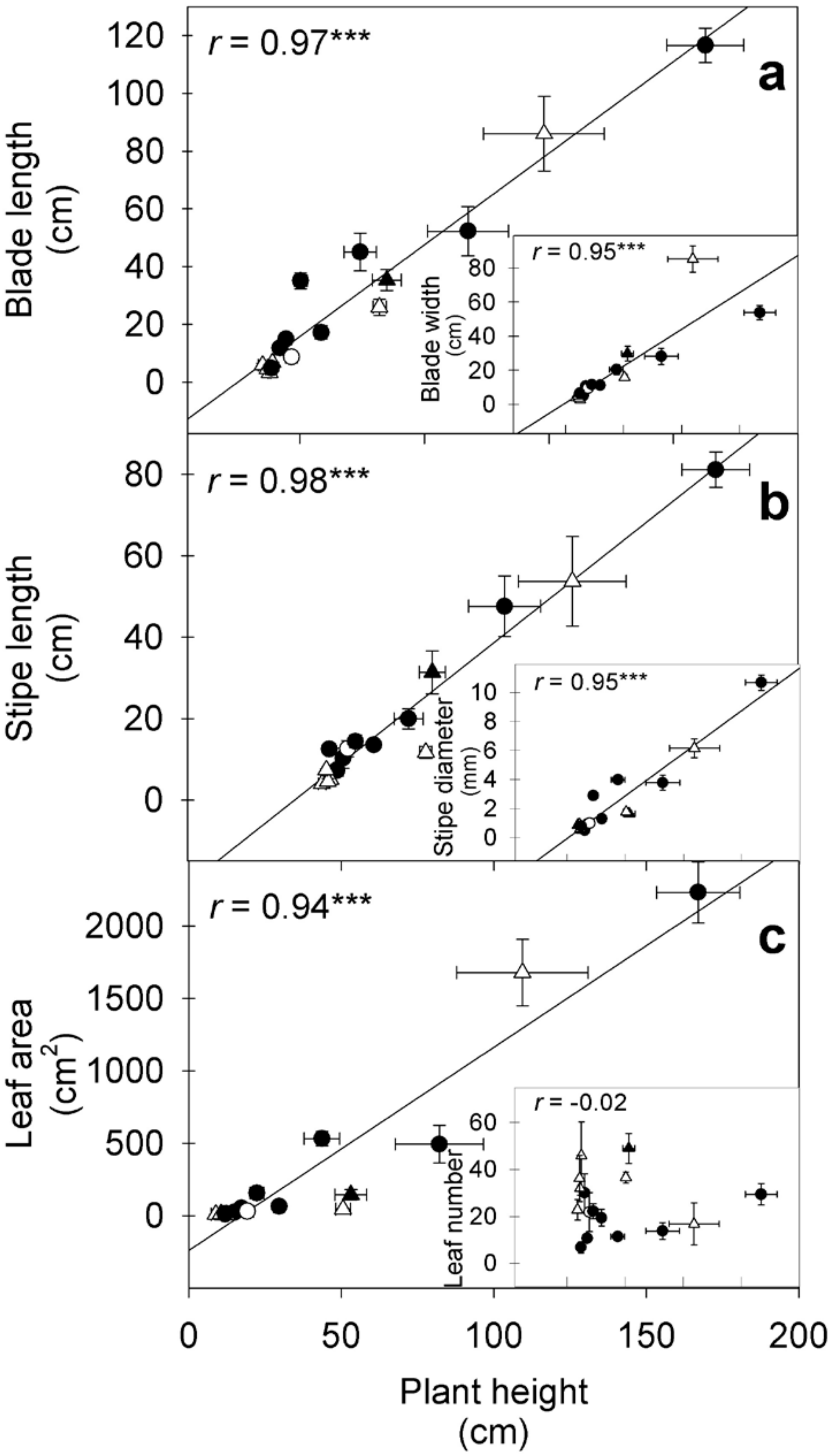

Wetter Site Species
Adiantum capillus-veneris Frond 22 cm^2
Aspidotis californica Frond 30 cm^2
Adiantum jordanii 1° Pinna 51 cm^2
Polypodium californicum Frond 63 cm^2
Polystichum munitum Frond 156 cm^2
Thelypteris puberula 1° Pinna 493 cm^2
Dryopteris arguta 1° Pinna 531 cm^2
Pteridium aquilinum 2° Pinna 1677 cm^2
Woodwardia fimbriata 1° Pinna 2231 cm^2
Drier Site Species
Notholaena californica Frond 5 cm^2
Cheilanthes newberryi Frond 7 cm^2
Cheilanthes covillei Frond 9 cm^2
Pentagramma triangularis Frond 11 cm^2
Pellaea mucronata 1° Pinna 45 cm^2
Pellaea andromedifolia 1° Pinna 145 cm^2
15 cm

Fig 3.1



r = 0.97***
a
Blade length (cm)
120
100
80
60
40
20
0
r = 0.95***
Blade width (cm)
80
60
40
20
0
r = 0.98***
b
Stipe length (cm)
80
60
40
20
0
r = 0.95***
Stipe diameter (mm)
10
8
6
4
2
0
r = 0.94***
c
Leaf area (cm²)
2000
1500
1000
500
0
r = -0.02
Leaf number
60
40
20
0
0
50
100
150
200
Plant height (cm)

Fig 3.2

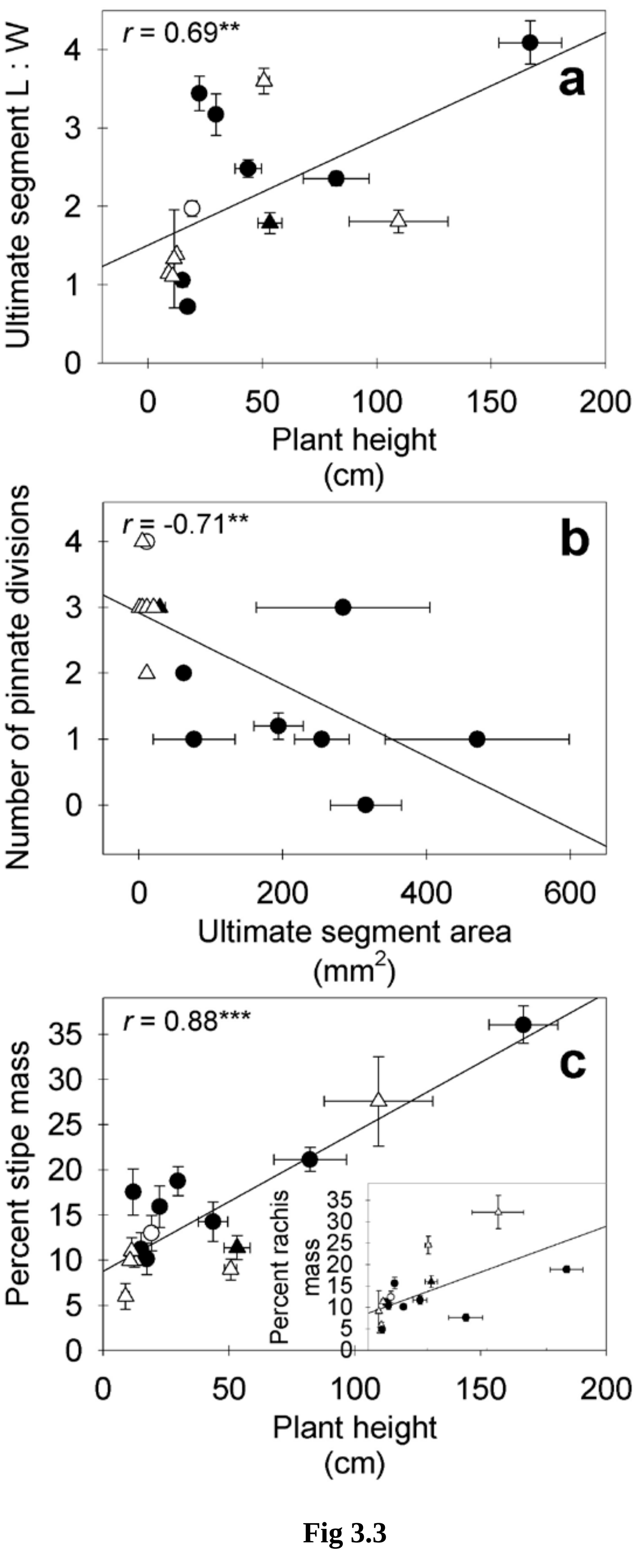

r = 0.69**
a
Ultimate segment L : W
Plant height (cm)
0
1
2
3
4
0
50
100
150
200
r = -0.71**
b
Number of pinnate divisions
Ultimate segment area (mm2)
0
200
400
600
r = 0.88***
c
Percent stipe mass
Percent rachis mass
0
5
10
15
20
25
30
35
Plant height (cm)

Fig 3.3

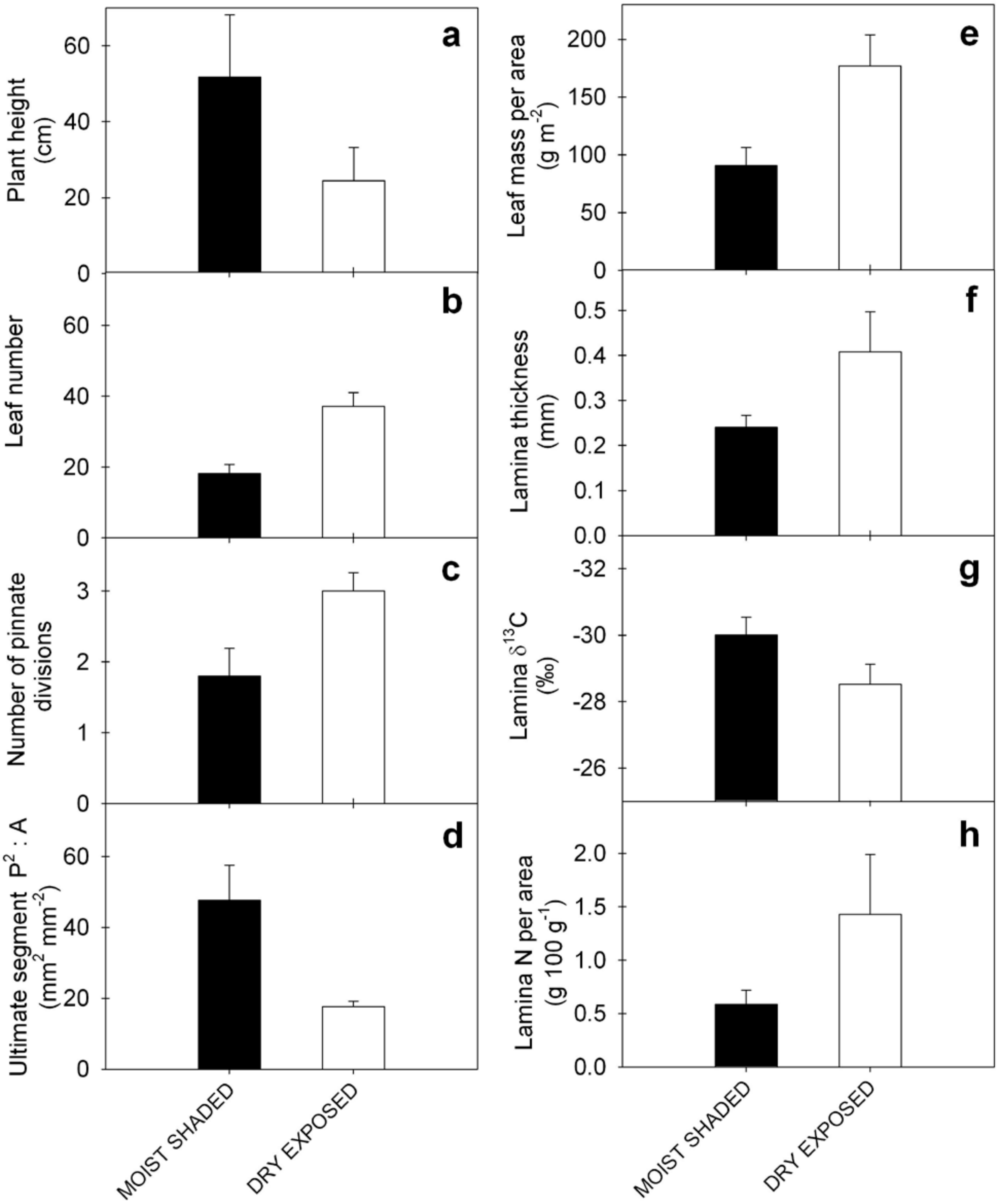

a
Plant height (cm)
0
20
40
60
b
Leaf number
0
20
40
60
c
Number of pinnate divisions
0
1
2
3
d
Ultimate segment P^2 : A (mm^2 mm^{-2})
0
20
40
60
e
Leaf mass per area (g m^{-2})
0
50
100
150
200
f
Lamina thickness (mm)
0.0
0.1
0.2
0.3
0.4
0.5
g
Lamina δ^{13}C (‰)
-26
-28
-30
-32
h
Lamina N per area (g 100 g^{-1})
0.0
0.5
1.0
1.5
2.0
MOIST SHADED
DRY EXPOSED
MOIST SHADED
DRY EXPOSED

Fig 3.4

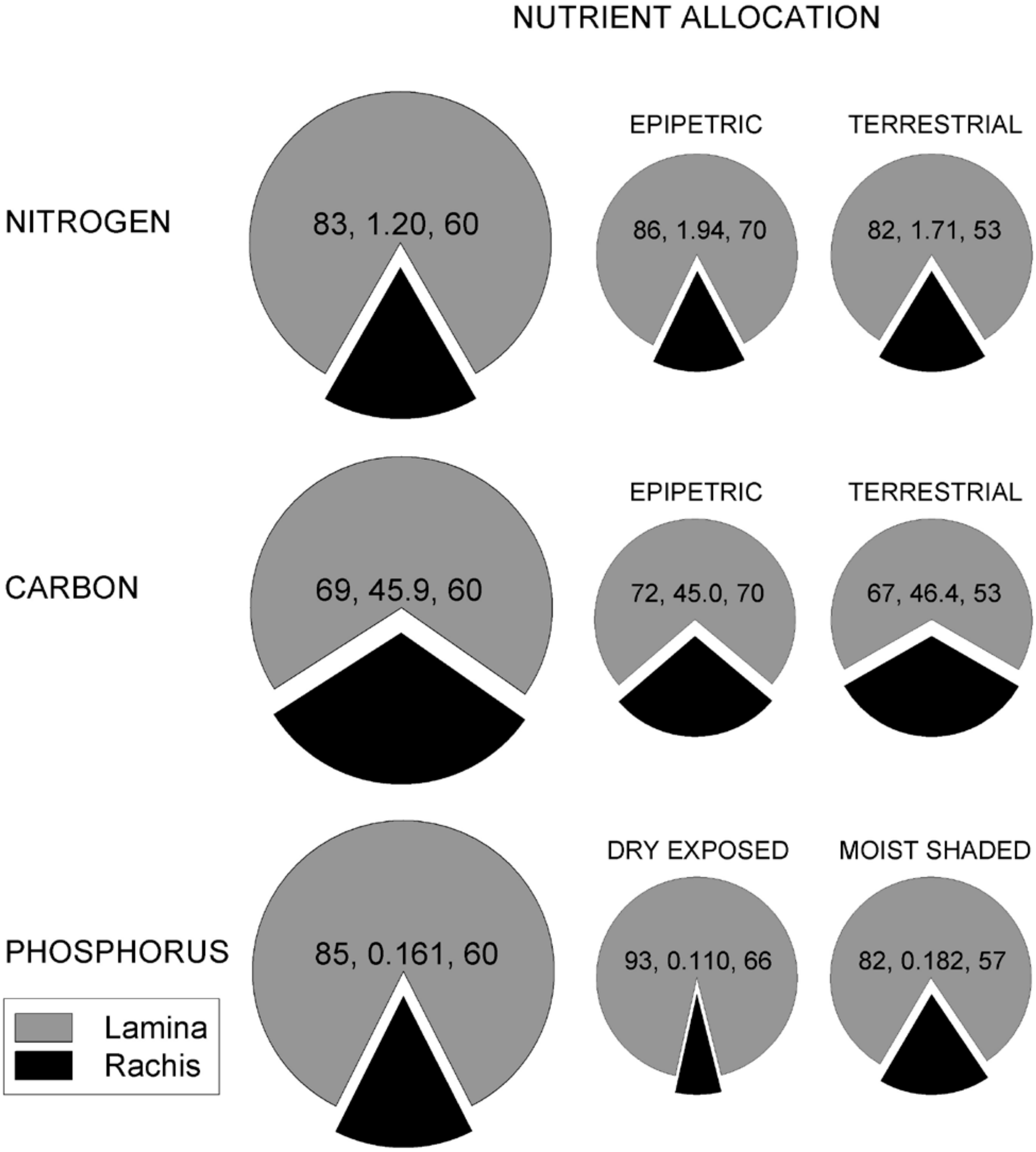

NUTRIENT ALLOCATION
EPIPETRIC
TERRESTRIAL
NITROGEN
83, 1.20, 60
86, 1.94, 70
82, 1.71, 53
EPIPETRIC
TERRESTRIAL
CARBON
69, 45.9, 60
72, 45.0, 70
67, 46.4, 53
DRY EXPOSED
MOIST SHADED
PHOSPHORUS
85, 0.161, 60
93, 0.110, 66
82, 0.182, 57
Lamina
Rachis

Fig 3.5

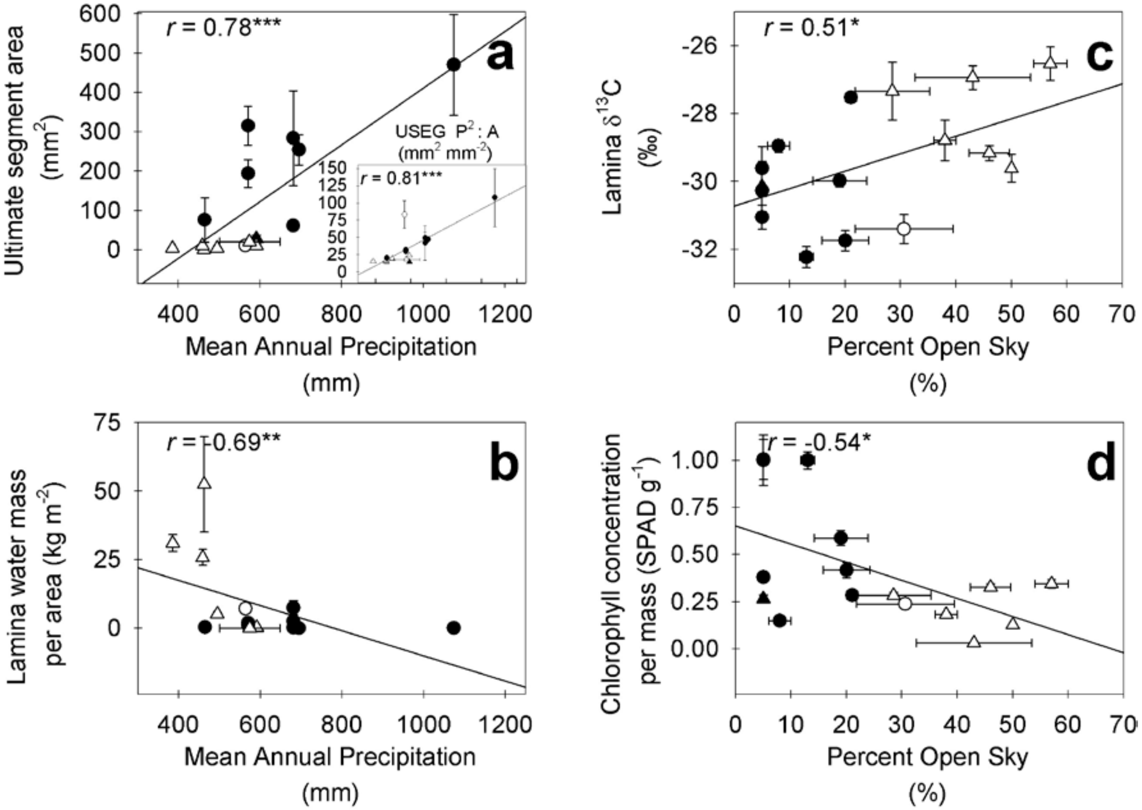

r = 0.78***
a
Ultimate segment area (mm^2)
600
500
400
300
200
100
0
400
600
800
1000
1200
Mean Annual Precipitation (mm)
USEG P^2 : A (mm^2 mm^{-2})
r = 0.81***
150
125
100
75
50
25
0
r = 0.51*
c
Lamina δ^{13}C (‰)
-26
-28
-30
-32
0
10
20
30
40
50
60
70
Percent Open Sky (%)
r = -0.69**
b
Lamina water mass per area (kg m^{-2})
75
50
25
0
400
600
800
1000
1200
Mean Annual Precipitation (mm)
r = -0.54*
d
Chlorophyll concentration per mass (SPAD g^{-1})
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
60
70
Percent Open Sky (%)

Fig 3.6

SUPPLEMENTAL MATERIAL

SUPPLEMENTARY TABLE CAPTIONS

SuppTable 3.1 Minimum, mean and maximum trait values for all taxa, as well as trait means and P-values for taxa grouped by dry exposed versus moist shaded habitats, and grouped by epipetric versus terrestrial habitats for three-level nested ANOVA significance tests; ns = not significant $^{+}P \leq 0.1$ $^{*}P \leq 0.05$ $^{**}P \leq 0.01$ $^{***}P \leq 0.001$.

SuppTable 3.2 Correlation matrix with Spearman (r_s) and Pearson (r_p) coefficients and P-values for respective ranked, raw (untransformed), and log-transformed datasets where highlighted cells represent relationships significant for at least Spearman and one of the two Pearson tests; $^{*}P \leq 0.05$ $^{**}P \leq 0.01$ $^{***}P \leq 0.001$.

SuppTable 3.3 Results of Standardized Major Axis (SMA) regression slope analysis show isometric scaling among size-related leaf traits and allometric scaling of lamina thickness and leaf mass per area with ultimate segment area in Southern California ferns.

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CHAPTER 4

SHIFTS IN FERN LEAF ARCHITECTURE AND PERFORMANCE ACROSS MOISTURE AND LIGHT GRADIENTS IN SOUTHERN CALIFORNIA

ABSTRACT

Ferns span large gradients of moisture and irradiance and range widely in leaf size and form, but the underlying sources of variation in their performance are poorly understood. We examined 15 diverse species in Southern California to test hypotheses of leaf trait associations with dry and exposed versus moist and shaded habitats. We also tested for physiological and structural trait coordination to compare with global patterns previously reported for angiosperms. In angiosperms, major vein length per area (major VLA) increases with smaller leaf size, conferring drought tolerance; in ferns, which lack minor veins, that should be true of total vein length per area (VLA; also known as "vein density"). Further, VLA is expected to scale positively with leaf hydraulic conductance (K_{leaf}) and rates of gas exchange for plants adapted to a given climate. In the California ferns, species from dry exposed habitats had higher VLA but lower K_{leaf} and maximum rates of gas exchange, than the species from moist shaded habitats. Further, K_{leaf} was decoupled from *VLA* across species, except when variation in irradiance was accounted for via partial correlation. The dry exposed species had higher *VLA* in smaller leaves, but a high *LMA* associated with greater structural protection. These dry exposed species did not achieve high K_{leaf} or photosynthesis per area apparently due to adaptation and acclimation to high irradiance conditions. This unique coordination of leaf form and performance across environments in Southern California helps to explain the habitat specificity of fern species.

INTRODUCTION

Studies of variation in leaf form and function have focused mainly on angiosperms (e.g., Ackerly and Reich 1999; Scoffoni et al. 2011; Sack et al. 2012). Discovering how ferns vary their leaf architecture and physiology across environmental gradients will better inform models of ecosystem response to environmental change, especially as the habitat specificity of ferns makes them excellent indicator species (e.g., Andama et al. 2003; Chang et al. 2009). Despite species habitat specificity, ferns as a group range from semi-deserts to tropical rainforests, and structurally diverse leptosporangiate ferns have evolved in more dry and peripheral environments (Scott and Galtier 1985; DiMichele and Hook 1992). Thus to test hypotheses of fern adaptation in different environments, and of coordination among leaf traits, we examined whole plant and leaf form and physiology, including vein and stomatal densities, and flux-related traits per mass and per area (Table 4.1) for 15 species selected from moist shaded and dry exposed habitats in Southern California (Table 4.2). We additionally tested whether relationships among fern leaf traits followed global trends.

We hypothesized that species from dry exposed habitats would exhibit leaf traits conducive to stress tolerance and longer lifespan for survival under drought and high irradiance (Creese and Sack *in prep-b*), and that species from more congenial moist shaded habitats would exhibit leaf traits conducive to shade tolerance. Specifically we predicted, with respect to form, that plants of dry exposed species would have a greater number of smaller leaves for redundancy, and each with greater dissection for convective cooling (Nobel 1976; Vogel 2009), and with higher leaf mass per area (*LMA*), which would contribute to longer leaf lifespan (Reich et al. 1992; Wright et al. 2004; Wright et al. 2010). We also predicted that plants of dry exposed species would have higher vein length per area (*VLA*; also known as "vein density") to confer greater drought tolerance, higher stomatal densities, and faster rates of gas exchange during periods when water is available (Wylie 1951; Brodribb et al. 2007; Baroli et al. 2008; Simonin et al. 2012). In angiosperms, a greater major vein length per area (major *VLA*), typical in smaller leaves, is associated with drought tolerance by providing redundancy in the water supply system (Scoffoni et al. 2011; Sack et al. 2012), and in ferns, which typically lack minor veins as defined in angiosperms, that principle should apply to the entire vein system. Additionally, because angiosperms under high irradiance show higher flux rates (Givnish 1988; Sack et al. 2005; Markesteijn et al. 2011), we expected that species of dry exposed sites may have higher leaf hydraulic conductance (K_{leaf}), stomatal conductance (g_{s}), and rates of photosynthesis (A), rubisco carboxylase activity ($V_{\mathrm{c,max}}$), electron transport (J_{max}), dark respiration (R_{d}), and a greater quantum yield of photosystem II (ϕ_{PSII}). Alternatively, we expected that the stress of high irradiance coupled with low moisture availability might favor species with conservative flux rates (Domingues et al. 2007; but see Manzoni et al. 2011), and low carbon isotope ratio values (∂^{13}C, typically related to high water-use-efficiency, *WUE*; see *Methods*).

By contrast, given competition for light, we predicted that species in moist shaded habitats would be large with low *LMA,* high chlorophyll concentration with greater investment of leaf N into chlorophyll (high Chl_{mass}:N_{mass}), and low *VLA* to reduce the proportion of non-photosynthetic tissues (Villar and Merino 2001; Poorter et al. 2006; Feng et al. 2008). As a result, moist shaded site species might have lower flux rates because a low *VLA* typically reduces K_{leaf}, g_{s}, and A (Aasamaa et al. 2001; Sack and Holbrook 2006; Brodribb et al. 2007; Brodribb et al. 2010). However, in angiosperms, a greater *VLA* of minor veins may increase maximum K_{leaf} because of more parallel xylem flow paths and shorter pathways for water to travel from veins to stomata (Brodribb et al. 2010; McKown et al. 2010). Although ferns typically lack minor veins, we expected that moist shaded site species may compensate for a low total *VLA* by increasing the number of vein branching orders (*B*) to help reduce hydraulic resistance by increasing the number of parallel flow pathways for a given *VLA*. We additionally tested whether ferns would show positive correlations of *VLA* and D_{stom}, K_{leaf} and *VLA*, and positive correlations of K_{leaf} with other flux traits across species (Sack and Frole 2006; Brodribb et al. 2007; McKown et al. 2010; Brodribb and Jordan 2011; Zhang et al. 2012). To further assess performance-related trait associations with environment across species, we tested whether *VLA*, *B*, stomatal density (D_{stom}) and flux traits decrease with decreasing mean annual precipitation (MAP) and whether *VLA* and flux traits increase with irradiance.

The unique development of fern leaves and their venation compared with other plant groups (Pray 1960; Boyce 2005; Nicotra et al. 2011; Boyce and Zwieniecki 2012) may distinguish ferns from global trends for the leaf economics spectrum (Wright et al. 2004; Shipley et al. 2006), and for the scaling of *VLA* and *B* with leaf size (Sack et al. 2012). Since vein branching is accomplished in ferns as the margin grows during development, greater lamina expansion would allow more branching orders to form, and higher vein densities in the outer lamina regions (Sack et al. 2012). Across angiosperms and gymnosperms, greater *LMA* and leaf lifespan is correlated with lower chlorophyll per mass (Chl_{mass}), light-saturated photosynthesis per mass (A_{mass}) and K_{leaf} per mass ($K_{leaf,mass}$) (Wright et al. 2004; Simonin et al. 2012). This low $K_{leaf,mass}$ is believed to result from the normalizing of area-based measures by high *LMA*, which is typically determined by cell packing and thick cell walls (Sack and Holbrook 2006; Zwieniecki et al. 2007; McKown et al. 2010; Simonin et al. 2012). All else being equal, we expected high *LMA* leaves with low $K_{leaf,mass}$ to have greater stomatal limitation resulting in higher *WUE* (∂^{13}C) and lower rates of g_s, A, $V_{c,max}$, J_{max}, and R_d (Brodribb et al. 2005). To help reduce this extra-xylem resistance, we expected high *LMA* leaves to show greater *VLA* to shorten extra-xylem water transport pathways (Brodribb et al. 2010; McKown et al. 2010). Southern California ferns with their extraordinary variation in size and wide distributions across moisture and irradiance gradients are thus an excellent system to investigate how these traits of leaf form and physiology vary with one another and with environment.

MATERIALS AND METHODS

Species and plant material

We selected 15 diverse species located in dry exposed and moist shaded habitats from the Santa Monica and San Gabriel Mountain ranges to maximize diversity in size and form (Table 4.2, SuppTable 4.1). At least five leaves per species from different individuals were collected from native habitats April to June 2008 for morphology and composition measurements, and again between April and June 2009 to measure flux traits. Fern leaves included stipe, rachis, and lamina, with ultimate segments (USEGs) defined as the highest order division of the leaf plumbed by its own midvein, which included the discrete pinnatifications of *Polypodium californicum* and *Woodwardia fimbriata*. Leaves were excised from plants in the field and immediately recut under water, then transferred to clean water and bagged to maintain hydration during transport to the lab where they were recut in clean water and bagged overnight with stipe under water to ensure hydration. Leaves were then separated into stipe, rachis and lamina parts for saturated and dry measurements, or used in their entirety for flux trait measurements.

Leaf form and composition traits

In the field, plant height (*PH*) was measured vertically from the ground or rock wall attachment to the apex of the longest leaf, and number of leaves per plant (*LN*) were counted. In the lab, leaf area (*LA*) was measured with a leaf area meter (LI-COR 3100; LI-COR Biosciences, Lincoln, NE, USA), and USEG area (*USEGA*) was measured in Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA) using leaf scans (Epson Perfection 4490 flatbed scanner, Epson America Inc., Long Beach, CA, USA), or light microscope photos of the small cheilanthoid species (DMRB, Leica Microsystems microscope at 5× objective with 14.2 Color Mosaic digital camera, Diagnostic Instruments). Leaf dissectedness was quantified as number of pinnate divisions (*PDIV*). Chlorophyll concentration (*Chl*) was averaged from at least three measurements taken from the central lamina (excluding the primary vein) of each leaf using a chlorophyll meter (model SPAD-502, Konica Minolta, New Jersey, USA), and divided by leaf mass per area (*LMA*) to obtain concentration per mass (Chl_{mass}); SPAD measurements are linearly related to total chlorophyll (a + b) per unit area (Marquard and Tipton 1987; Singha and Townsend 1989; Fanizza et al. 1991; Manetas et al. 1998). To determine leaf mass per area (*LMA*), leaves were oven dried at 70 ºC for at least one week before mass measurements with a microbalance (XS205; Mettler Toledo, Columbus, OH, USA). Dried lamina tissue was then analysed by isotope ratio mass spectrometry (Thermo-Finnegan Delta V IRMS; Analytical Laboratory, University of Hawaii, Hilo) to determine ∂^{13}C, a measure of lifetime integrated *WUE*. Although we measured instantaneous water-use-efficiency (*WUE* = *A* / transpiration), we preferentially analyzed ∂^{13}C to reflect a longer-term integrated *WUE* as smaller stomatal apertures and persistently lower stomatal conductance discriminate against the influx of heavier

^{13}C isotopes (Farquhar and Richards 1984) to provide a more reliable indicator of species' tendencies for stomatal behavior.

Stomatal density

We quantified stomatal density (D_{stom}) for abaxial leaf surfaces as number of stomata per leaf area using microscopy photos of three to six cleared leaves per species prepared as above for vein traits in image J (U. S. National Institutes of Health, Bethesda, Maryland, USA).

Vein density and hierarchy

Fern venation varies from single-veined leaves, to simple dichotomous branching, to fully reticulate patterning, though in our sample, just two species, *Polypodium californicum* and *Woodwardia fimbriata*, showed a degree of reticulation via repeating anastomoses or "vein loops". For each species, leaf vein lengths were measured using middle USEGs of three to six cleared and stained leaves from different individuals as per Scoffoni and Sack (2011) from field collected material that was fixed in 70% formalin-acetic acid-alcohol (FAA; 10% formalin: 5% glacial acetic acid: 48% ethanol: 37% water). Leaves were cleared in 5% sodium hydroxide in ethanol for 7 – 125 hours, followed by a 70%–50%–30% ethanol-water rehydration series, a rinse in 50% sodium hypochlorite bleach, then a 30%–50%–70%–100% ethanol-water dehydration series before staining in 1% safranin and 1% fast green. Vein lengths were measured using Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA) for 1200 dpi resolution scans (Epson Perfection 4490 flatbed scanner, Epson America Inc., Long Beach, CA, USA), and light microscope photos (DMRB, Leica Microsystems microscope at 5× and 10× objectives with 14.2 Color Mosaic digital camera, Diagnostic Instruments) for the very small

Cheilanthes covillei leaves. Because number of vein branching orders varied across the USEG, we measured all veins for each USEG, and because ferns typically lack minor veins as defined for angiosperms (Hickey 1973; Ellis et al. 2009; Sack et al. 2012), *VLA* reflects measurements of all vein lengths per area. To quantify variation in the hierarchy of a vein system, the classical approach developed for dicotyledenous angiosperms with highly reticulate vein networks, rather than for ferns with simpler dichotomously branching veins, typically defines vein orders by their diameter. However, distinguishing vein orders by diameter is problematical when differences in diameter are slight, such as when separating higher order major veins from lower order minor veins (c.f. Bohn et al. 2002; Sack et al. 2012), and for ferns with different vein patterning. Because all our fern species exhibited dichotomous branching, it was most practicable and reliable to use branching order as a standardized definition of vein order, starting with the primary (1^o) vein as the midrib, secondary (2^o) veins as branching off the midrib, tertiary (3^o) veins as branching from the 2^o veins, quaternary (4^o) veins as branching from the 3^o veins, and so on, up to the highest vein branching order for each leaf (i.e., ranging from a maximum of 3^o veins for *Cheilanthes covillei*, up to a maximum of 12^o veins for *Polystichum munitum*). We then used the maximum number of vein orders averaged for each species to quantify differences in vein branching order (*B*).

Flux traits

Given substantial differences in microsite moisture availability, we excised leaves in the field and transported them to the lab for rehydration overnight (see *Species and plant material*) to standardize physiology measurements by using fully hydrated leaves. Measurements of gas exchange and leaf hydraulic conductance were made within 24 hours for at least five leaves per species from different individuals.

Responses of photosynthetic assimilation to intercellular CO_2 concentration ($A - c_i$) response curves were measured with a LI-6400 XT with 6400-40 leaf chamber fluorometer (LI-COR, Lincoln, NE, USA). We quantified standard stomatal conductance (g_s) and photosynthesis (A) under atmospheric CO_2 concentration (40 Pa), as well as maximum stomatal conductance (g_{max}), light-saturated photosynthesis (A_{max}), rubisco carboyxlation ($V_{c,max}$), electron transport (J_{max}) and dark respiration (R_d) rates. We solved for $V_{c,max}$ and J_{max} at 25°C (using PS-FIT_7.3; Farquhar, Caemmerer & Berry 1980, Bernacchi, Pimentel & Long 2003, Long & Bernacchi 2003), and all area-based flux traits were additionally divided by *LMA* for expression per unit mass. We also determined maximum quantum yield of photosystem II ($\Phi_{PSIIMAX}$).

Leaf hydraulic conductance (K_{leaf}) was measured for at least ten leaves per species from at least five different individuals using the evaporative flux method (Sack et al. 2002); calculated as steady-state transpirational flow rate (E, mmol m^{-2} s^{-1}) divided by the water potential driving force ($\Delta\Psi_{leaf} = -\Psi_{leaf}$, MPa), normalized by leaf area. Measurements were made on whole leaves, or, on the mid-most pinnae pair for large-leafed species as necessary (i.e., *Dryopteris arguta*, *Pteridium aquilinum*, and *Woodwardia fimbriata*); resistance of the rachis in the large-leafed species was expected to be small, justifying this approach (e.g., Brodribb and Holbrook 2004). For these large species, the apex of cut stipe for the middle pinnae pair was sealed with Loctite (409 with accelerator 712, Loctite Corporation, Hartford, CT, USA). We divided K_{leaf} by *LMA* to determine leaf hydraulic conductance per mass ($K_{leaf,mass}$).

Habitat and microsite environment

We quantified species environments using both discrete microsite habitat variables and continuous climate variables. Given the association of moisture and light across sites (Table 4.2, SuppTable 4.2), riparian and understory microsites were classified as moist and shaded habitats versus dry and exposed habitats. To compare climate data for species collection sites, we determined mean annual precipitation (MAP) from GPS coordinates using WorldClim and Prism climate databases (Hijmans et al. 2005; Prism Climate Group, http://prism.oregonstate.edu) using DIVA GIS (http://www.diva-gis.org). Variation in air moisture, i.e. vapor pressure deficit (VPD), was calculated as the difference between saturated vapor pressure (e_s) and ambient water vapor pressure (e_a), determined using the average temperature dewpoint and mean annual temperature from Prism 2003-2007 (Pearcy et al. 1989). We used percent open sky (%OS) as an index of habitat light availability, measured at the plant level as the proportion of sky unobstructed by canopy and topography (Waite and Sack 2011).

Statistical analyses

We tested for significant trait differences across species grouped by dry exposed versus moist shaded habitats using nested ANOVAs (Minitab 16 Statistical Software 2010, Minitab, Inc., State College, Pennsylvania, USA). Trait-trait and trait-environment correlations were assessed using Spearman and Pearson correlation coefficients on raw and log-transformed data (R Development Core Team, 2010, R Foundation for Statistical Computing, Vienna, Austria). The association of K_{leaf} with *VLA* was further tested using Pearson partial correlation coefficients (Sokal and Rohlf, 1995) to control for associations with MAP, VPD and %OS by testing the relationship between the two plant traits while holding a third environmental variable constant (corpcor package in R; Schaefer et al. 2007). We present a correlation matrix of all variables (SuppTable 4.2) so that readers may examine the intercorrelative structure among types of variables. We tested only previously hypothesized relationships, do not endorse any significant trait relationships that were not previously hypothesized, and would recommend statistical correction for any such “mining” of relationships. Correlations were considered significant when Spearman plus one of the Pearson tests were significant, and partial correlation results were considered significant when one or both of the Pearson tests were significant.

RESULTS

Variation in fern leaf traits and species’ environments

Variation in fern leaf traits was strongly associated with differences in environment and other leaf traits (Figs 4.1-4.7, SuppTables 4.1-4.3). The fern species in our study ranged across moist shaded and dry exposed microhabitats with 2 to 3-fold variation in MAP and VPD, and 10-fold variation in %OS. These ferns also exhibited extraordinary variation in leaf form and physiology across species and environments, including 40 to 1300-fold variation in plant and leaf size parameters, 20-fold variation in LMA, 6-fold variation in D_{stom}, and 4 to 90-fold variation in VLA and B. With respect to chlorophyll and flux traits, species showed greater variation in leaf traits per mass than per area, with 7-fold variation in chlorophyll concentration per area versus 52-fold variation in Chl_{mass}, 56-fold variation in Chl_{mass}:N_{mass}, and 5 to 230-fold variation in hydraulic and gas exchange traits per area, versus 28 to 390-fold variation in these flux traits per mass.

Leaf form and performance varied with dry exposed versus moist shaded habitats

Across species, plant form and performance differed significantly between dry and exposed versus moist and shaded habitats (SuppTable 4.1). With respect to form, dry exposed site species had smaller plants, leaves and leaf parts, with more pinnate divisions, and number of leaves per plant ($P < 0.01$, Figs 4.1, 4.2). Dry exposed site species also showed greater *LMA* and lower chlorophyll concentrations per area and per mass ($P < 0.05$, Fig 4.2), as well as significantly higher D_{stom} ($P < 0.001$, Fig 2). Dry exposed species showed simpler venation architecture; these species lacked reticulation, i.e., no anastomoses or "vein loops", and had significantly fewer vein orders, i.e., lower *B* ($P < 0.001$, Figs 4.1, 4.2). As hypothesized, dry exposed species exhibited greater *VLA* ($P < 0.001$). Notably, K_{leaf} and photosynthetic traits per unit area did not significantly differ across habitats except for a lower A_{max} measured at ambient CO_2 for dry exposed species ($P < 0.05$, SuppTable 4.1). However, flux rates per mass were strongly associated with habitat type: $K_{\mathrm{leaf,mass}}$, $A_{\mathrm{max,mass}}$, $V_{\mathrm{c,max,mass}}$, $J_{\mathrm{max,mass}}$, and $R_{\mathrm{d,mass}}$ were significantly lower for dry exposed species, due to the division by their higher *LMA* ($P < 0.05$, Fig 4.3). As expected, *WUE* (∂^{13}C) was higher for dry exposed species, and Φ_{PSIIMAX} was unexpectedly higher as well ($P < 0.001$, Fig 4.3). By contrast, chlorophyll concentrations, Chl_{mass}:N_{mass}, and flux rates per mass were significantly higher for moist shaded site species.

Correlation of leaf performance-related traits with environment

Variation in leaf performance was strongly correlated with environmental gradients in moisture and irradiance (Figs 4.4, 4.5, SuppTable 4.2). These gradients themselves were associated; MAP and %OS were negatively correlated ($r = -0.58$, $P < 0.05$, SuppTable 4.2). With decreasing MAP, D_{stom} significantly increased ($r = -0.79$, $P < 0.01$, SuppTable 4.2), and *B*, K_{leaf}, $V_{\mathrm{c,max}}$, and

$J_{\max}$ significantly decreased (r = 0.70-0.83, $P < 0.05$, Fig 4.4). With increasing %OS, *WUE* and *VLA* increased (r = 0.53-0.68, $P < 0.05$), but chlorophyll concentration, K_{leaf}, $A_{\max}$, $V_{c,\max}$, and $J_{\max}$ per mass decreased (r = -0.54 to -0.81, $P < 0.05$, Fig 4.5); on a per area basis, only $V_{c,\max}$, and $J_{\max}$ were significantly correlated with %OS (SuppTable 4.2).

Correlation of leaf traits with LMA

Leaf mass per area was significantly associated with variation in vein and photosynthetic traits (Fig 4.6, SuppTable 4.2). As expected, species with high *LMA* exhibited lower Chl_{mass} (r = -0.88, $P < 0.001$, Fig 4.6) and higher *VLA* and *WUE* (∂^{13}C; r = 0.64-0.77, $P < 0.01$, Fig 4.6). Despite this greater density of veins, species with high *LMA* exhibited lower K_{leaf}, $A_{\max}$, $V_{c,\max}$, $J_{\max}$ and R_d (less negative) per mass (r = -0.78 to -0.85, $P < 0.01$, Fig 4.6); relationships with flux traits per area were not significant (SuppTable 4.2).

Correlations of VLA with D_{stom}, and of K_{leaf} with VLA and gas exchange traits

Leaf photosynthetic and hydraulic traits per mass (not area) were strongly correlated with one another, and the correlation of K_{leaf} and *VLA* relied on controlling for differences in environment across species via partial correlation (SuppTables 4.2, 4.3). As expected, a high $K_{\text{leaf,mass}}$ was correlated with low *WUE* (∂^{13}C; r = -0.69, $P < 0.05$), and high A, $A_{\max}$, $V_{c,\max}$, $J_{\max}$ and R_d (more negative) per mass (r = 0.81-0.90, $P < 0.01$, SuppTable 4.2); however, K_{leaf} and $K_{\text{leaf,mass}}$ were not significantly correlated with vein density traits (SuppTable 4.2). K_{leaf} was only correlated with *VLA* when %OS was partialled out (SuppTable 4.3), and the correlation of *VLA* with D_{stom} was not quite significant (r = 0.51, $P > 0.05$, SuppTable 4.2).

Correlations of VLA and B with leaf size

Vein densities appeared independent of total leaf area, but were significantly related to USEG area (SuppTable 4.2). As expected given global trends, *VLA* was negatively correlated with size, measured as USEG area (r = -0.76 to -0.85, $P < 0.001$, Fig 4.7). The *B* was also positively correlated with USEG area ($r = 0.82$, $P < 0.001$, Fig 4.7).

DISCUSSION

Diversity in fern leaf architecture and physiology

Ferns showed strong variation in size, leaf design, and performance across species and environments, with 4 to 1300-fold variation in leaf traits, and ranged from the very small and dissected cheilanthoid species (*Cheilanthes covillei*, *C. newberryi*, *Notholaena californica*) adapted in dry exposed habitats, to the very large bracken and chain ferns (*Pteridium aquilinum* and *Woodwardia fimbriata*) adapted in moist, congenial habitats. This variation in leaf architecture and performance was strongly associated with differences in moisture and irradiance, and was coordinated with other leaf traits in accordance with global patterns established for other plant groups, and in ways unique to ferns.

Leaf architecture and performance vary with moisture and irradiance across species and habitats

The strong associations of leaf traits with habitat type supported the hypothesis that fern species of dry exposed sites have evolved traits conducive to stress tolerance and longer lifespan for survival under drought and high irradiance, whereas species of moist shaded sites have evolved traits conducive to shade tolerance and faster rates of resource capture. The smaller plants and leaves of dry exposed species, and greater leaf dissectedness would improve convective cooling in sites with extreme highs of irradiance and temperature (Nobel 1976; Vogel 2009). The high *LMA* of dry exposed species reflects greater investment in durable leaves that should have slower growth but longer leaf lifespan (Reich et al. 1992; Wright et al. 2004; Wright et al. 2010). Further, dry exposed species had higher *VLA* and D_{stom}, which would increase the supply of water for transpiration, potentially maintaining gas exchange as leaf water potential falls, and also allowing greater maximum rates of photosynthesis during periods when water is available (Brodribb et al. 2007; Scoffoni et al. 2011; Sack and Scoffoni 2013). Species of dry exposed habitat showed much simpler venation patterns with no reticulation, and fewer vein orders (lower *B*). Although species adapted in environments prone to drought would benefit from higher vein reticulation, the simpler vein patterning and lower *B* of dry exposed site species may be driven by selection for smaller leaf size, which tends to increase *VLA* and reduce hydraulic vulnerability to cavitation (Scoffoni et al. 2011). The developmental program for veins of dry exposed species may further limit their potential to develop branching and studies comparing leaf size and plasticity in leaf vein hierarchy for reciprocally transplanted dry exposed and moist shaded site species would be valuable to identify potential developmental limitations (c.f. Carins Murphy et al. 2012).

Notably, ϕ_{PSII} was higher for dry exposed species, contrary to our expectation, and to a previous study's finding that tropical ferns showed a positive correlation of K_{leaf} with ϕ_{PSII} (Brodribb et al. 2005); in our study, species with high K_{leaf} had low ϕ_{PSII}. This decoupling of K_{leaf} and ϕ_{PSII} may be due to diverging selection pressures on resource allocation to PSII in dry exposed versus moist shaded environments. Moist shaded site species exhibited high K_{leaf} but may have allocated proportionally less leaf N and chlorophyll to PSII resulting in lower ϕ_{PSII}

than dry exposed species; this would provide a cost savings for moist shaded species under light limited conditions because the N cost per mole of chlorophyll is three times higher for PSII than for the light harvesting complexes (Hikosaka and Terashima 1996). Another possible explanation is that despite a lower K_{leaf} in dry exposed species, they preferentially allocate more leaf chlorophyll to PSII resulting in a higher ϕ_{PSII} to help compensate for a reduction in quantum efficiency of PSII during periods of drought stress (e.g., Albert et al. 2011). Notably, reductions in $V_{\mathrm{c,max}}$ and J_{max} without reductions in ϕ_{PSII} have also been reported for other plants (Nogués and Baker 1995; Baker et al. 1997).

By contrast with dry exposed species, species from moist shaded habitats exhibited leaf traits conducive to shade tolerance and faster rates of resource capture. As expected, moist shaded species had higher chlorophyll concentrations per area and per mass, and allocated more of their leaf N to chlorophyll to optimize light capture in the shaded understory (Sack et al. 2003). Given strong competition for light, as expected, moist shaded species showed lower *VLA*, which would reduce encroachment on photosynthetic tissues (Villar and Merino 2001; Poorter et al. 2006; Feng et al. 2008). We hypothesize that this lower *VLA*, all else being equal, may limit photosynthetic physiology (Brodribb et al. 2007; Brodribb et al. 2010; McKown et al. 2010). However, the species of dry exposed habitats may have had decreased photosynthetic pigments and proteins, and increased photoprotective compounds. This may explain why moist shaded species, despite having lower *VLA* had greater $K_{\mathrm{leaf,mass}}$, $A_{\mathrm{max,mass}}$, $V_{\mathrm{c,max,mass}}$, $J_{\mathrm{max,mass}}$, and faster respiration rates than dry exposed species.

Variation in leaf performance was also strongly correlated with measured environmental gradients of moisture and irradiance. Following trait associations with habitat type, stressful conditions of decreasing MAP and increasing irradiance (%OS) were associated with lower flux rates, chlorophyll concentration, and greater *WUE* (∂^{13}C). Notably, *B* was negatively correlated with MAP, and *VLA* was strongly positively correlated with irradiance. This suggests that in light limited environments there may be stronger selection for reductions in non-photosynthetic tissues via lower vein densities, versus stronger selection on vein hierarchy in environments with limited moisture. Thus, differences in light and moisture availability in fern habitats may lead to selection on different aspects of venation architecture. Studies examining closely related species or ecotypes in environments that vary in moisture and light availability would be helpful to further investigate modifications to different vein traits, and examination of potential differences in the lability of different vein traits would also be informative.

Coordination of VLA with D_{stom} *across fern species*

Given strong correlations of *VLA* with D_{stom} across angiosperm species, independent of and with respect to phylogenetic relationships (Brodribb and Jordan 2011; Zhang et al. 2012), we tested whether these traits would also be coordinated in ferns – a case of independent leaf evolution. Although the positive correlation was not quite statistically significant, we expect that if the *VLA* is measured centrally in the USEG where stomata are most concentrated, the relationship would be robust. As a preliminary test of this hypothesis, a correlation of *VLA* for the first three branching orders (typically most central for our fern species) with D_{stom} was indeed significant ($r = 0.66$, $P < 0.05$). Further examination of this relationship for species grown under variable irradiance and moisture availability treatments will help determine whether the coordination of vein and stomatal density in ferns is maintained across species and with plastic shifts in leaf dimensions.

Coordination of K_{leaf} with gas exchange and the decoupling of K_{leaf} and VLA across fern species

We examined whether ferns would show strong positive correlations of K_{leaf} with *VLA* and rates of gas exchange as seen for other plant groups, or whether these traits might tend in different directions because of diverging selection pressures from different environments. Theoretically a higher K_{leaf} should support greater stomatal conductance, and consequently faster photosynthesis (Brodribb et al. 2005; Sack and Holbrook 2006). Indeed, our fern species showed strong positive correlations of $K_{\mathrm{leaf,mass}}$ with A and A_{max}, and additionally with $V_{\mathrm{c,max}}$, J_{max} and faster dark respiration. These correlations suggest that investment in faster hydraulic supply is coordinated with greater investment in the photosynthetic apparatus to optimize rates of rubisco carboxylase activity and electron transport, and is associated with faster respiration and possibly faster growth and leaf turnover (Reich et al. 1992; Wright et al. 2010). All else being equal, an increase in *VLA* will increase hydraulic supply rate (Sack and Frole 2006; Brodribb et al. 2007; McKown et al. 2010). However, high K_{leaf} and $K_{\mathrm{leaf,mass}}$ were only significantly correlated with higher *VLA* when variation in irradiance was accounted for via partial correlation. Thus, differences in irradiance can effectively decouple the positive association of K_{leaf} and *VLA*, which helps to explain why species grouped by habitat showed either high *VLA* but lower flux rates, or the reverse. Detailed examination of tracheid anatomical traits and cavitation resistance of species within the same habitat and across habitats are needed.

Scaling of leaf vein and performance traits with LMA: insights into the leaf economics spectrum

Ferns exhibit unique leaf and vein development compared to other plant groups (Pray 1960; Nicotra et al. 2011; Boyce 2005; and Zwieniecki 2012), but ferns have not been well represented in studies of global patterns for the leaf economics spectrum (Wright et al. 2004; Shipley et al.

2006; but see Karst and Lechowicz 2007). Thus we examined whether variation in vein density and photosynthetic and respiration rates for a diverse group of ferns from distinct habitats would follow this spectrum of increased investment into *LMA* at the expense of carbon assimilation rates per mass. Our fern species showed the same trend of decreasing A_{mass} with increasing *LMA* (Wright et al. 2004; Shipley et al. 2006) – previously unsupported in temperate ferns (Karst and Lechowicz 2007). Our fern species additionally showed slower respiration rates and lower $K_{\mathrm{leaf,mass}}$, and thus are expected to have longer leaf lifespans (Simonin et al. 2012). Strong associations of vein and flux traits with *LMA* also shed light on the respective roles of hydraulic, stomatal, and biochemical limitations on photosynthesis in high *LMA* leaves. For instance, the greater investment into *VLA* may contribute to higher *LMA* and lower photosynthetic rates by reducing the proportion of photosynthetic tissue (Villar and Merino 2001; Poorter et al. 2006; Feng et al. 2008). The high *WUE* (∂^{13}C) and low $g_{\mathrm{max,mass}}$ of leaves with high *LMA* also suggest that maximum photosynthetic rates are strongly limited by stomatal conductance. Finally, the slow rates of $V_{c,max}$ and J_{max} per mass in high *LMA* leaves suggest that photosynthesis is further limited by reduced optimization of rubisco carboxylase activity and electron transport rates.

Ferns follow global trends for scaling of vein density with size

We hypothesized that variation in fern vein patterning would be associated with differences in size as well as environment and leaf composition, thus we examined the scaling of fern *VLA* and *B* with leaf size. For angiosperms, increasing leaf size is associated with decreasing total and major vein densities, but minor vein density appears independent of leaf size (Sack et al. 2012). Relationships of *VLA* and *B* with leaf size were significant for our fern species when using ultimate segment area rather than leaf area. This may be because fern leaves are more highly dissected on average than angiosperm leaves (Raven et al. 1999), so fern ultimate segments connected by rachis to the main axis may be the functional equivalent of entire angiosperm leaves connected by petiole to the stem. As a result, ferns may be able to plastically adjust the size, vein branching orders and vein densities of their USEGs during the leaf development process, rather than relying solely on initiation of new (different) leaves in response to changing environmental conditions. This may be especially important because the marginal growth of fern veins (Pray 1960) may preclude adjustments to vein density during leaf expansion. Studies examining intra- and inter-leaf vein plasticity in response to environmental stimuli under controlled conditions are needed.

CONCLUSIONS

Southern California ferns showed strong variation in size, leaf design, and performance associated with differences in moisture, irradiance, and other leaf traits. Species from dry exposed habitats had leaf traits conducive to stress tolerance and longer lifespan, whereas species from congenial moist shaded habitats had leaf traits conducive to shade tolerance and optimization of leaf hydraulic conductance and rates of exchange. The exception to this was a greater ϕ_{PSII} in dry exposed species, which may compensate for periods of drought-stress that lower the quantum efficiency of PSII. The lower ϕ_{PSII} in moist shaded species may also be a cost savings mechanism to optimize positive carbon gain under limited light. Notably, the positive relationship between VLA and K_{leaf} was decoupled by the effects of these different habitats, becoming significant only when variation in %OS was controlled. This result, in tandem with the greater B and of moist shaded species shows that differences in light availability in fern habitats may lead to selection on different aspects of venation architecture.

The higher *VLA* and lower flux rates of dry exposed species were further explained by strong associations with *LMA* and leaf size traits. Across fern species, investment in high *LMA* was associated with greater *VLA* and *WUE* (∂^{13}C), but lower K_{leaf}, g_{max}, A_{max}, $V_{\mathrm{c,max}}$, J_{max} and slower respiration rates per mass. This illustrates combined roles of hydraulic, stomatal and biochemical limitations in generating the leaf economics spectrum in ferns. Finally, the smaller size of leaves from dry exposed species further explains the observed increase in *VLA* and lower *B* in accordance with global patterns of vein density scaling with leaf size. Taken together these results demonstrate diverse optimization of leaf traits for greater stress tolerance versus faster resource capture across environmental gradients of moisture and irradiance and help to explain the habitat specificity of fern species.

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Table 4.1 Variable symbols, units, descriptions; P = Prism Climate Model; WC = Worldclim Climate Model.

Traits	Units	Trait Description
Environment		
MAP_WC; MAP_P	mm	Mean annual precipitation (1950-2000 WC; 1971-2000 P)
VPD	kPa	Vapour pressure deficit (1971-2000 P)
%OS	%	Percent open sky
Leaf form & composition		
PH	cm	Plant height
LN	#	Leaf number per plant
LA	cm^2	Leaf area
USEGA	mm^2	Ultimate segment area
PDIV	#	Maximum number of pinnate divisions (0-4)
LMA	g m^{-2}	Leaf mass per area
Chl	SPAD units	Chlorophyll concentration per unit area
Chl_{mass}	SPAD g^{-1}	Chlorophyll concentration per unit mass
Chl_{mass}:N_{mass}		Ratio of chlorophyll to leaf N per mass
Stomatal & vein traits		
D_{stom}	mm^2	Stomatal density on abaxial leaf surface
VLA	mm^{-1}	Vein length per area (‘density’) for all veins
B	#	Hierarchy of veins, i.e. max number of vein orders
Flux traits		
K_{leaf}	mmol m^{-2} s^{-1} MPa^{-1}	Leaf hydraulic conductance
g_{s}	mol m^{-2} s^{-1}	Stomatal conductance per area at 40 Pa CO$_2$
g_{max}	mol m^{-2} s^{-1}	Maximum stomatal conductance per area at 40 Pa CO$_2$
A	µmol m^{-2} s^{-1}	Photosynthesis per area at 40 Pa CO$_2$
A_{max}	µmol m^{-2} s^{-1}	Maximum photosynthetc rate per area at 40 Pa CO$_2$
$A_{\mathrm{max,mass}}$	µmol g^{-1} s^{-1}	Maximum photosynthetic rate per unit mass
V_{cmax}	µmol m^{-2} s^{-1}	Maximum rate of rubisco carboxylase activity per area
J_{max}	µmol m^{-2} s^{-1}	Maximum photosynthetic electron transport rate per area
R_{d}	µmol m^{-2} s^{-1}	Leaf dark respiration rate per unit area
$K_{\mathrm{leaf,mass}}$	mmol g^{-1} s^{-1} MPa^{-1}	Leaf hydraulic conductance per mass
g_{mass}	mol g^{-1} s^{-1}	Stomatal conductance per mass at 40 Pa CO$_2$
$g_{\mathrm{max,mass}}$	mol g^{-1} s^{-1}	Maximum stomatal conductance per unit mass
A_{mass}	µmol g^{-1} s^{-1}	Photosynthesis per mass at 40 Pa CO$_2$
$A_{\mathrm{max,mass}}$	µmol g^{-1} s^{-1}	Maximum photosynthesis per mass at 40 Pa CO$_2$
$V_{\mathrm{cmax,mass}}$	µmol g^{-1} s^{-1}	Maximum rate of rubisco carboxylase activity per mass
$J_{\mathrm{max,mass}}$	µmol g^{-1} s^{-1}	Maximum photosynthetic electron transport rate per mass
$R_{\mathrm{d,mass}}$	µmol g^{-1} s^{-1}	Leaf dark respiration rate per unit mass
WUE (∂^{13}C)	‰	Lifetime integrated water-use-efficiency as ^{13}C : ^{12}C ratio
φ_{PSII}		Maximum quantum yield of photosystem II

Table 4.2 Family, genus, species, and habitat type, as well as mean annual precipitation (MAP), vapor pressure deficit (VPD), and irradiance quantified as percent open sky (%OS; Waite and Sack 2011) for species collection sites.

Family	**Genus**	**Species**	**Habitat type**	**MAP** mm	**VPD** kPa	**%OS** %
Sinopteridaceae	*Adiantum*	*capillus-veneris*	moist shaded	570.69	1.07	13
Sinopteridaceae	*Adiantum*	*jordanii*	moist shaded	680.95	1.12	5
Sinopteridaceae	*Aspidotis*	*californica*	moist shaded	563.91	1.09	30.6
Sinopteridaceae	*Cheilanthes*	*covillei*	dry exposed	462.49	1.26	50
Sinopteridaceae	*Cheilanthes*	*newberryi*	dry exposed	385.3	0.85	57
Dryopteridaceae	*Dryopteris*	*arguta*	moist shaded	680.95	1.12	22.5
Sinopteridaceae	*Notholaena*	*californica*	dry exposed	494.49	1.28	38
Sinopteridaceae	*Pellaea*	*andromedifolia*	dry exposed/shaded*	590.97	1.08	5
Sinopteridaceae	*Pellaea*	*mucronata*	dry exposed	590.97	1.09	60
Sinopteridaceae	*Pityrogramma*	*triangularis*	dry exposed	458.48	1.19	46
Polypodiaceae	*Polypodium*	*californicum*	moist shaded	570.69	1.07	5
Dennstaedtiaceae	*Polystichum*	*munitum*	moist shaded	1074.08	1.25	5
Dryopteridaceae	*Pteridium*	*aquilinum*	moist shaded/exposed*	574.21	1.04	45
Thelypteridaceae	*Thelypteris*	*puberula*	moist shaded	464.46	0.86	8
Blechnaceae	*Woodwardia*	*fimbriata*	moist shaded	694.91	1.11	21

* *Pellaea andromedifolia* occurred in dry exposed habitats but co-occurred with larger plants that obstructed the majority of open sky

* *Pteridium aquilinum* occurred in moist forested areas that were generally shaded but leaves often recruited into forest gaps with more open sky

FIGURE CAPTIONS

Fig 4.1 Leaf silhouettes and magnified photos of leaf veins for 15 Southern California fern species arranged by wetter (left) versus drier (right) sites and from smallest to largest leaves illustrating that species from dry exposed habitats were smaller with greater density of all vein orders combined and greater density of the major veins (first three vein orders), whereas species from moist shaded habitats were larger with lower total vein density, but showed greater vein hierarchy (higher maximum number of vein orders) and greater density of the minor veins (fourth order and higher; SuppTable 4.1). Silhouettes are to scale and represent entire leaves (leaf), or a pinna of the first order (1^{o} pinna) or second order (2^{o} pinna) for large-leafed species (see species captions for mean leaf areas in cm^2). Leaf vein images are close-ups of ultimate segments; scale bars = 2 mm.

Fig 4.2 Species from dry exposed habitats (open bar) exhibited key differences in size and leaf form compared with species from moist shaded habitats (black bar), including: a) smaller plants; b) more leaves per plant; c) more dissected leaves given a higher average number of pinnate divisions; d) smaller ultimate segments (and leaf size; SuppTable 4.2); e) greater leaf mass per area (*LMA*); f) greater vein length per area of all vein orders (*VLA*; SuppTable 4.1); g) lower maximum number of vein orders, i.e., lower number of vein branching orders (*B*; SuppTabel 1); and h) greater stomatal density (D_{stom}); all associations were significant at $P \leq 0.05$ based on nested ANOVAs.

Fig 4.3 Species from dry exposed habitats (open bar) exhibited key differences in leaf flux-related traits per mass compared with species from moist shaded habitats (black bar), including:

a) lower leaf hydraulic conductance per mass ($K_{\mathrm{leaf,mass}}$); b) higher lifetime-integrated water-use-efficiency (*WUE*; indicated by ∂^{13}C, see *Methods*); c) lower chlorophyll concentration (Chl_{mass}; and lower ratio of Chl_{mass}:N_{mass}, SuppTable 4.1); d) lower light-saturated photosynthetic rate ($A_{\mathrm{max,mass}}$); e) lower rates of maximum carboxylase activity of rubisco ($V_{\mathrm{cmax,mass}}$); f) lower rates of photosynthetic electron transport ($J_{\mathrm{max,mass}}$); g) lower maximum quantum yield efficiency of photosystem II (ϕ_{PSII}); and h) slower rates of dark respiration ($\mathrm{R}_{\mathrm{d,mass}}$); all associations were significant at $P \leq 0.05$ based on nested ANOVAs.

Fig 4.4 Leaf flux-related traits were significantly correlated with mean annual precipitation (MAP; SuppTable 4.2): low MAP was associated with a) higher stomatal density (D_{stom}); b) lower leaf hydraulic conductance (K_{leaf}); c) lower rates of maximum carboxylase activity of rubisco (V_{cmax}); and d) lower rates of photosynthetic electron transport (J_{max}); ***$P \leq 0.001$, **$P \leq 0.01$, * $P \leq 0.05$

Fig 4.5 Leaf flux-related traits per mass were significantly correlated with irradiance quantified as percent open sky (%OS, see *Methods*; SuppTable 4.2): high %OS was associated with a) higher lifetime-integrated water-use-efficiency (*WUE*; indicated by ∂^{13}C, see *Methods*); b) lower leaf hydraulic conductance ($K_{\mathrm{leaf,mass}}$); c) lower chlorophyll concentration (Chl_{mass}); d) lower light-saturated photosynthetic rate ($A_{\mathrm{max,mass}}$); e) lower rates of maximum carboxylase activity of rubisco ($V_{\mathrm{cmax,mass}}$); and f) lower rates of photosynthetic electron transport ($J_{\mathrm{max,mass}}$); ***$P \leq 0.001$, **$P \leq 0.01$, * $P \leq 0.05$

Fig 4.6 Leaf flux-related traits per mass were significantly correlated with leaf mass per area (*LMA*; SuppTable 4.2): high *LMA* was associated with a) lower chlorophyll concentration per mass (Chl_{mass}); b) greater vein length per area (‘density’) of all vein orders (*VLA*); c) lower leaf hydraulic conductance ($K_{leaf,mass}$); d) higher lifetime-integrated water-use-efficiency (*WUE*; indicated by ∂^{13}C, see *Methods*); e) lower maximum stomatal conductance ($g_{max,mass}$); f) lower light-saturated photosynthetic rate ($A_{max,mass}$); g) lower rates of maximum carboxylase activity of rubisco ($V_{cmax,mass}$); and h) lower rates of photosynthetic electron transport ($J_{max,mass}$); and i) slower rates of dark respiration ($R_{d,mass}$); $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$

Fig 4.7 Southern California fern species follow the global trend observed for angiosperms of decreasing vein length per area (*VLA*) with increasing leaf size (Sack et al. 2012), and additionally show a reduction in vein branching order, but at the level of the fern leaf ultimate segment (USEG, highest order division of a compound leaf): a) number of vein branching orders (*B*) is strongly negatively correlated with USEG area, b) length per area of all vein orders (*VLA*) decreases with increasing USEG area (SuppTable 4.2); $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$

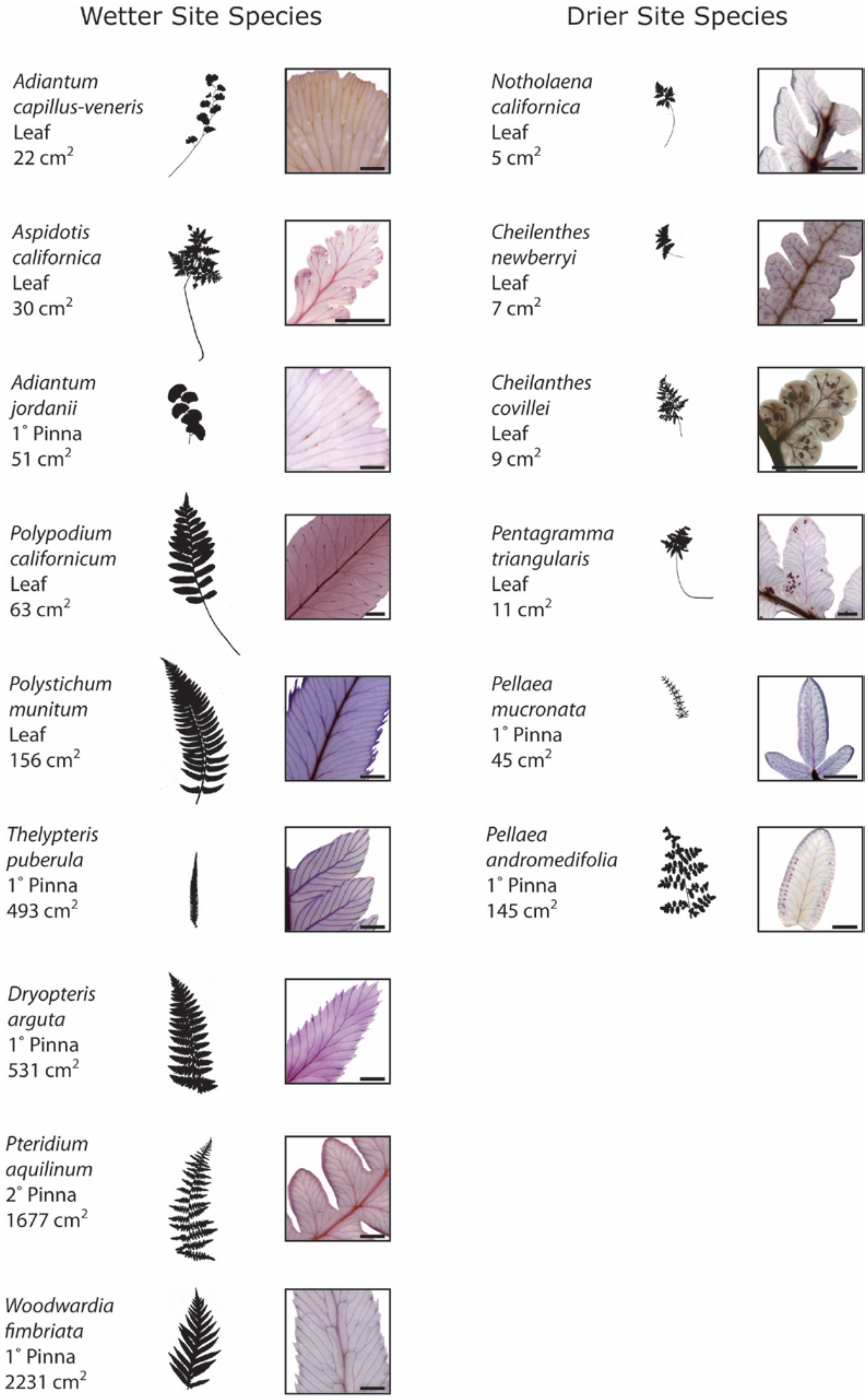

Wetter Site Species
Adiantum capillus-veneris
Leaf
22 cm^2
Aspidotis californica
Leaf
30 cm^2
Adiantum jordanii
1° Pinna
51 cm^2
Polypodium californicum
Leaf
63 cm^2
Polystichum munitum
Leaf
156 cm^2
Thelypteris puberula
1° Pinna
493 cm^2
Dryopteris arguta
1° Pinna
531 cm^2
Pteridium aquilinum
2° Pinna
1677 cm^2
Woodwardia fimbriata
1° Pinna
2231 cm^2
Drier Site Species
Notholaena californica
Leaf
5 cm^2
Cheilenthes newberryi
Leaf
7 cm^2
Cheilanthes covillei
Leaf
9 cm^2
Pentagramma triangularis
Leaf
11 cm^2
Pellaea mucronata
1° Pinna
45 cm^2
Pellaea andromedifolia
1° Pinna
145 cm^2

Fig 4.1

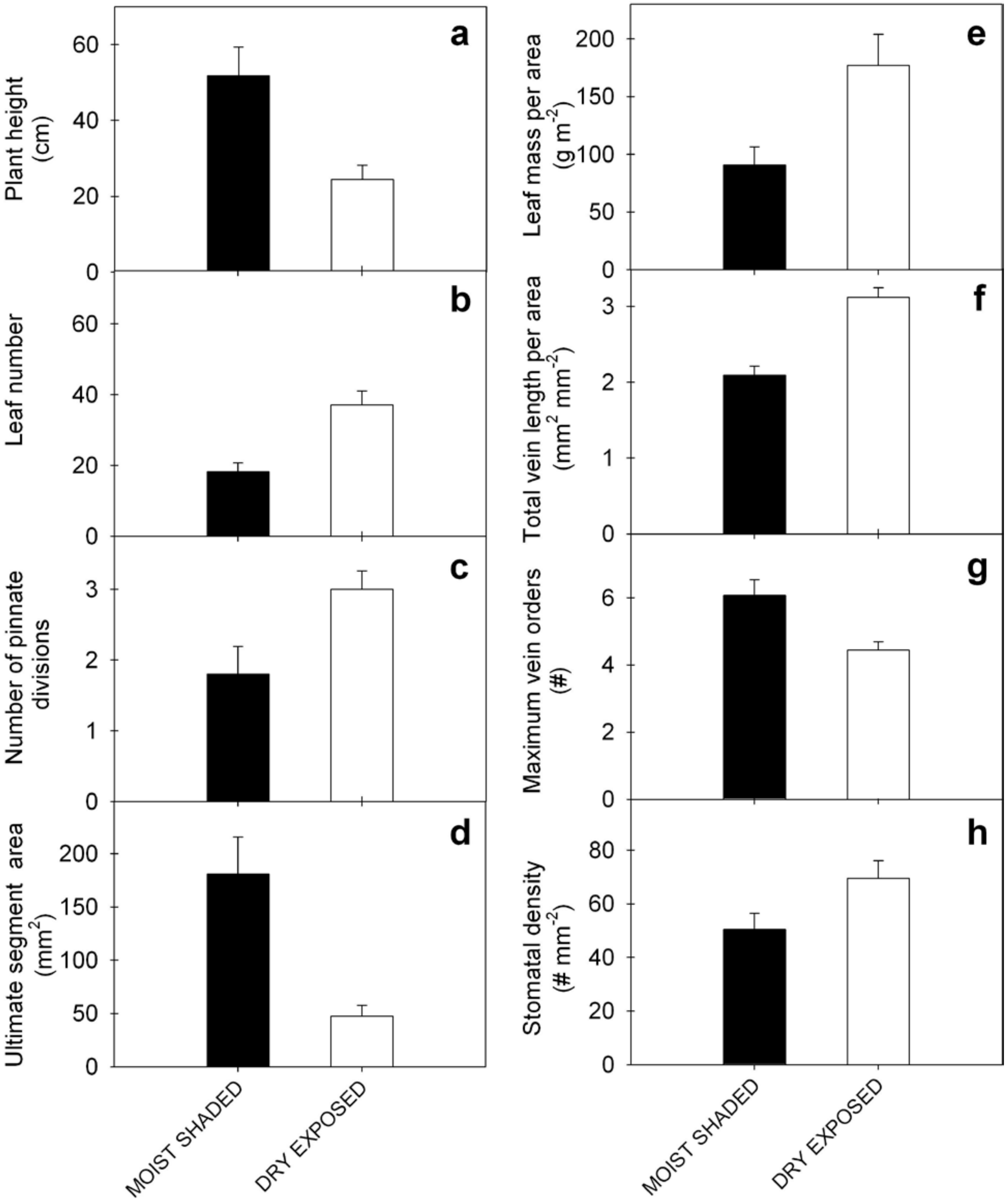

a
Plant height (cm)
0
20
40
60
b
Leaf number
0
20
40
60
c
Number of pinnate divisions
0
1
2
3
d
Ultimate segment area (mm^2)
0
50
100
150
200
e
Leaf mass per area (g m^{-2})
0
50
100
150
200
f
Total vein length per area (mm^2 mm^{-2})
0
1
2
3
g
Maximum vein orders (#)
0
2
4
6
h
Stomatal density (# mm^{-2})
0
20
40
60
80
MOIST SHADED
DRY EXPOSED
MOIST SHADED
DRY EXPOSED

Fig 4.2

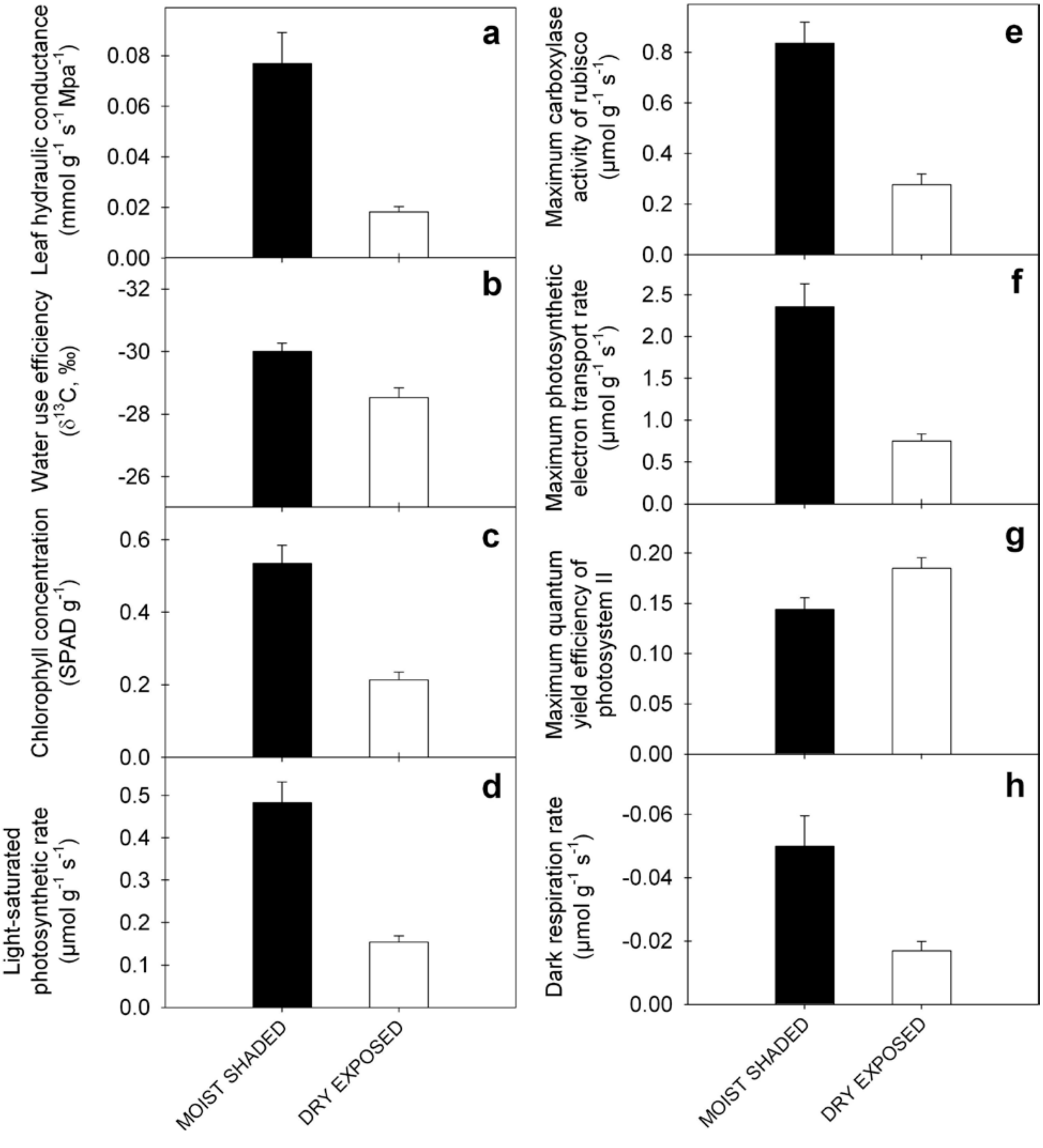

a
Leaf hydraulic conductance (mmol g^{-1} s^{-1} Mpa^{-1})
0.00
0.02
0.04
0.06
0.08
b
Water use efficiency (δ^{13}C, ‰)
-26
-28
-30
-32
c
Chlorophyll concentration (SPAD g^{-1})
0.0
0.2
0.4
0.6
d
Light-saturated photosynthetic rate (µmol g^{-1} s^{-1})
0.0
0.1
0.2
0.3
0.4
0.5
e
Maximum carboxylase activity of rubisco (µmol g^{-1} s^{-1})
0.0
0.2
0.4
0.6
0.8
f
Maximum photosynthetic electron transport rate (µmol g^{-1} s^{-1})
0.0
0.5
1.0
1.5
2.0
2.5
g
Maximum quantum yield efficiency of photosystem II
0.00
0.05
0.10
0.15
0.20
h
Dark respiration rate (µmol g^{-1} s^{-1})
0.00
-0.02
-0.04
-0.06
MOIST SHADED
DRY EXPOSED
MOIST SHADED
DRY EXPOSED

Fig 4.3

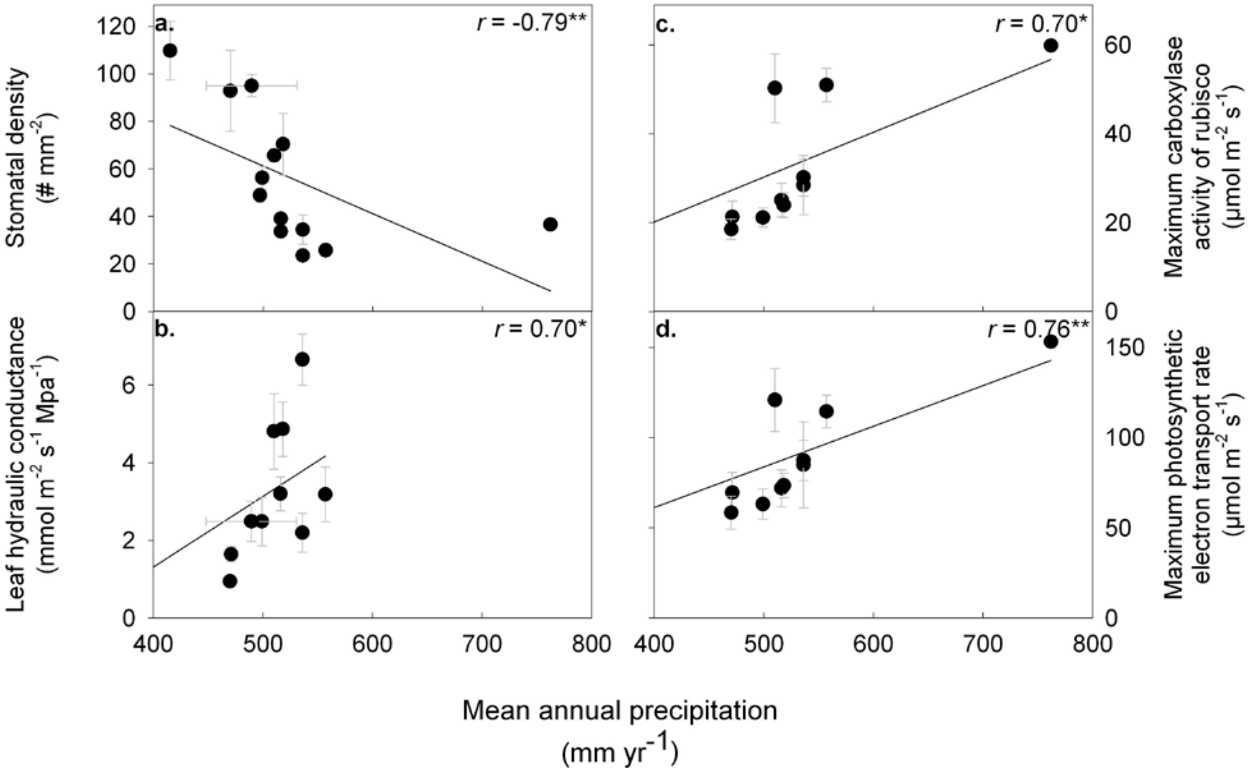

a.
r = -0.79**
Stomatal density (# mm-2)
120
100
80
60
40
20
0
b.
r = 0.70*
Leaf hydraulic conductance (mmol m-2 s-1 Mpa-1)
6
4
2
0
c.
r = 0.70*
Maximum carboxylase activity of rubisco (µmol m-2 s-1)
60
40
20
0
d.
r = 0.76**
Maximum photosynthetic electron transport rate (µmol m-2 s-1)
150
100
50
0
400
500
600
700
800
Mean annual precipitation (mm yr-1)

Fig 4.4

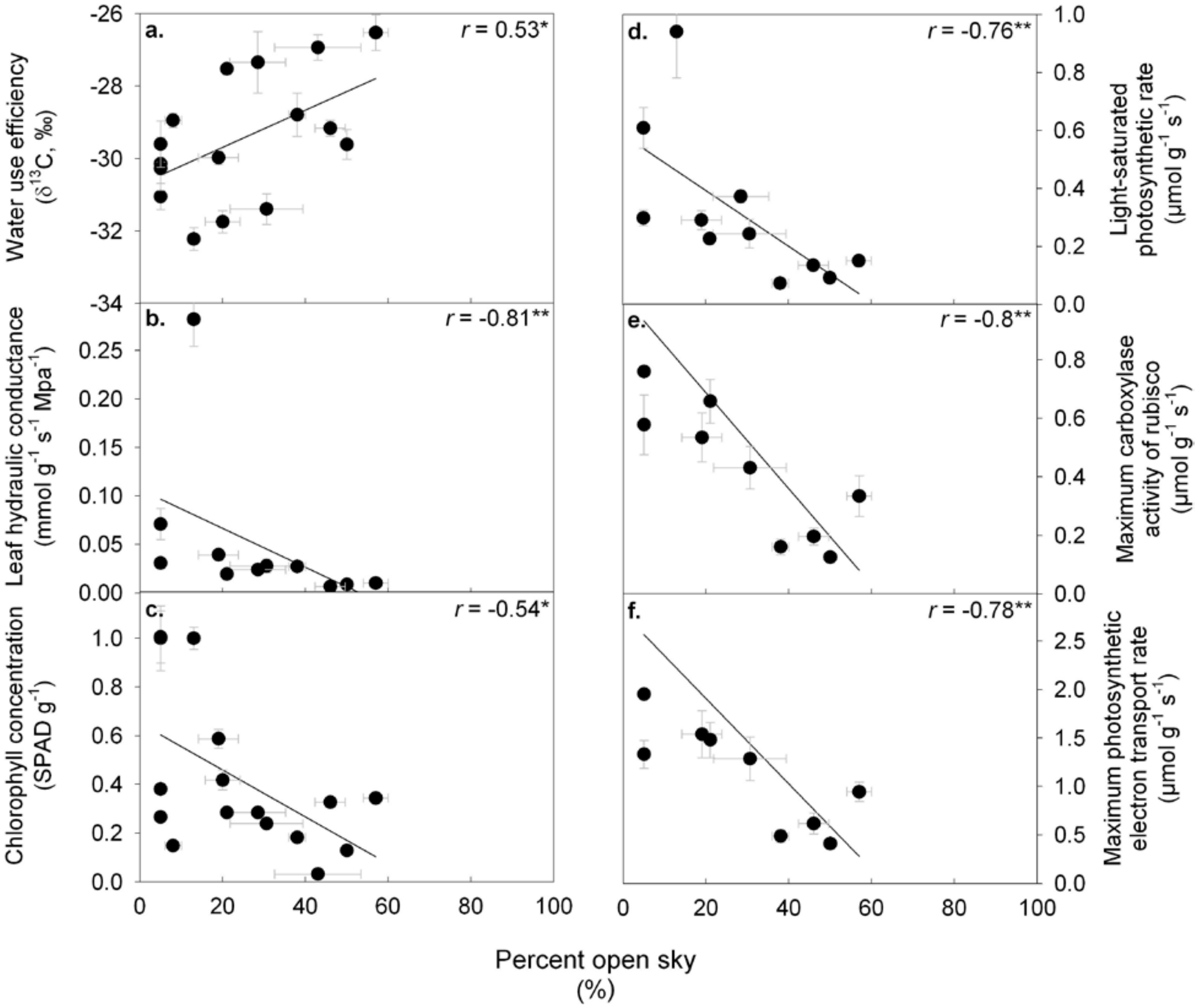
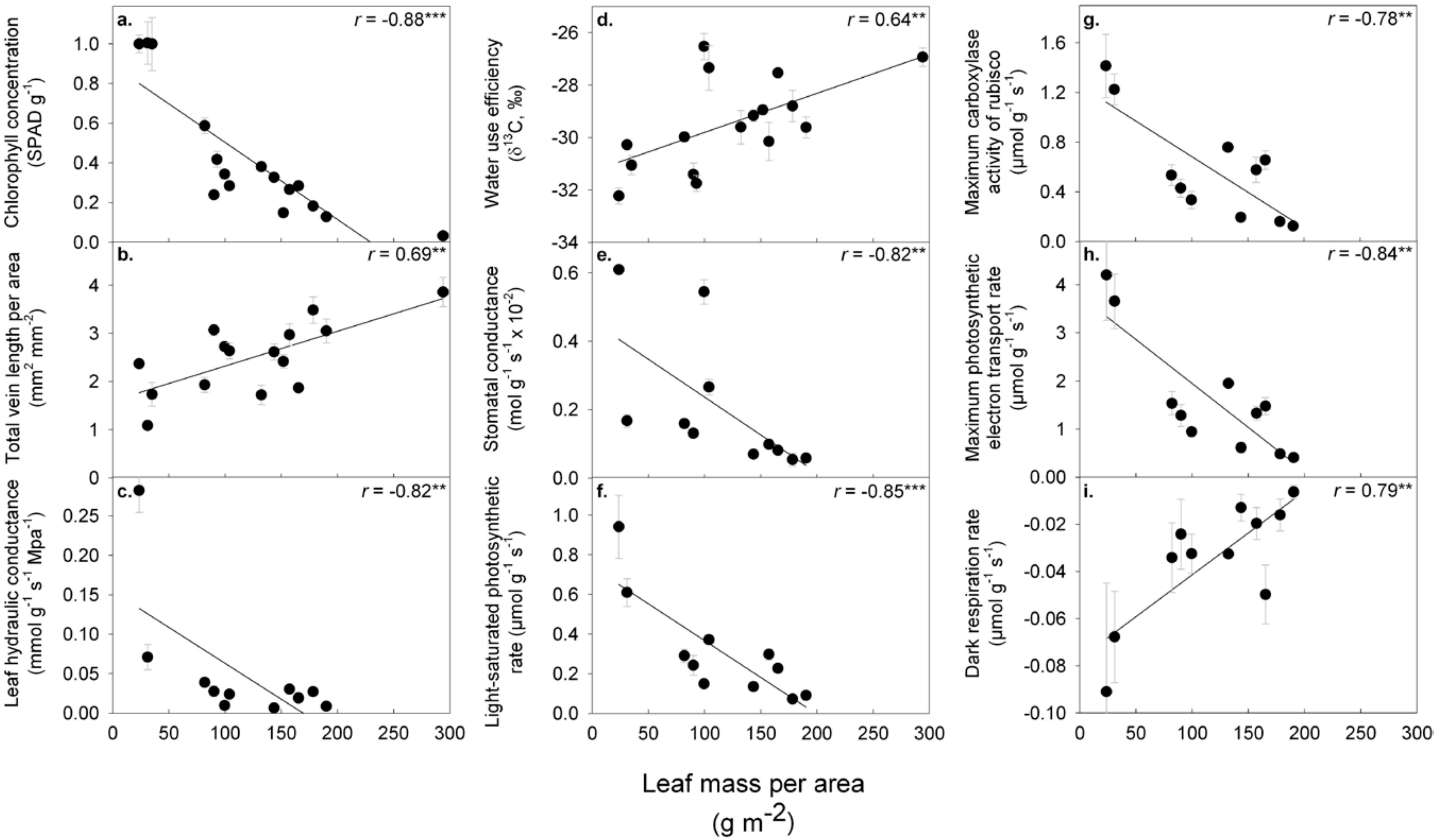

a.
r = 0.53*
Water use efficiency (δ^{13}C, ‰)
-26
-28
-30
-32
-34
b.
r = -0.81**
Leaf hydraulic conductance (mmol g^{-1} s^{-1} Mpa^{-1})
0.25
0.20
0.15
0.10
0.05
0.00
c.
r = -0.54*
Chlorophyll concentration (SPAD g^{-1})
1.0
0.8
0.6
0.4
0.2
0.0
d.
r = -0.76**
Light-saturated photosynthetic rate (µmol g^{-1} s^{-1})
1.0
0.8
0.6
0.4
0.2
0.0
e.
r = -0.8**
Maximum carboxylase activity of rubisco (µmol g^{-1} s^{-1})
0.8
0.6
0.4
0.2
0.0
f.
r = -0.78**
Maximum photosynthetic electron transport rate (µmol g^{-1} s^{-1})
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Percent open sky (%)

Fig 4.5



a.
r = -0.88***
Chlorophyll concentration (SPAD g-1)
1.0
0.8
0.6
0.4
0.2
0.0
b.
r = 0.69**
Total vein length per area (mm2 mm-2)
4
3
2
1
0
c.
r = -0.82**
Leaf hydraulic conductance (mmol g-1 s-1 Mpa-1)
0.25
0.20
0.15
0.10
0.05
0.00
d.
r = 0.64**
Water use efficiency (δ13C, ‰)
-26
-28
-30
-32
-34
e.
r = -0.82**
Stomatal conductance (mol g-1 s-1 x 10-2)
0.6
0.4
0.2
0.0
f.
r = -0.85***
Light-saturated photosynthetic rate (µmol g-1 s-1)
1.0
0.8
0.6
0.4
0.2
0.0
g.
r = -0.78**
Maximum carboxylase activity of rubisco (µmol g-1 s-1)
1.6
1.2
0.8
0.4
0.0
h.
r = -0.84**
Maximum photosynthetic electron transport rate (µmol g-1 s-1)
4
3
2
1
0.00
i.
r = 0.79**
Dark respiration rate (µmol g-1 s-1)
-0.02
-0.04
-0.06
-0.08
-0.10
0
50
100
150
200
250
300
Leaf mass per area (g m-2)

Fig 4.6

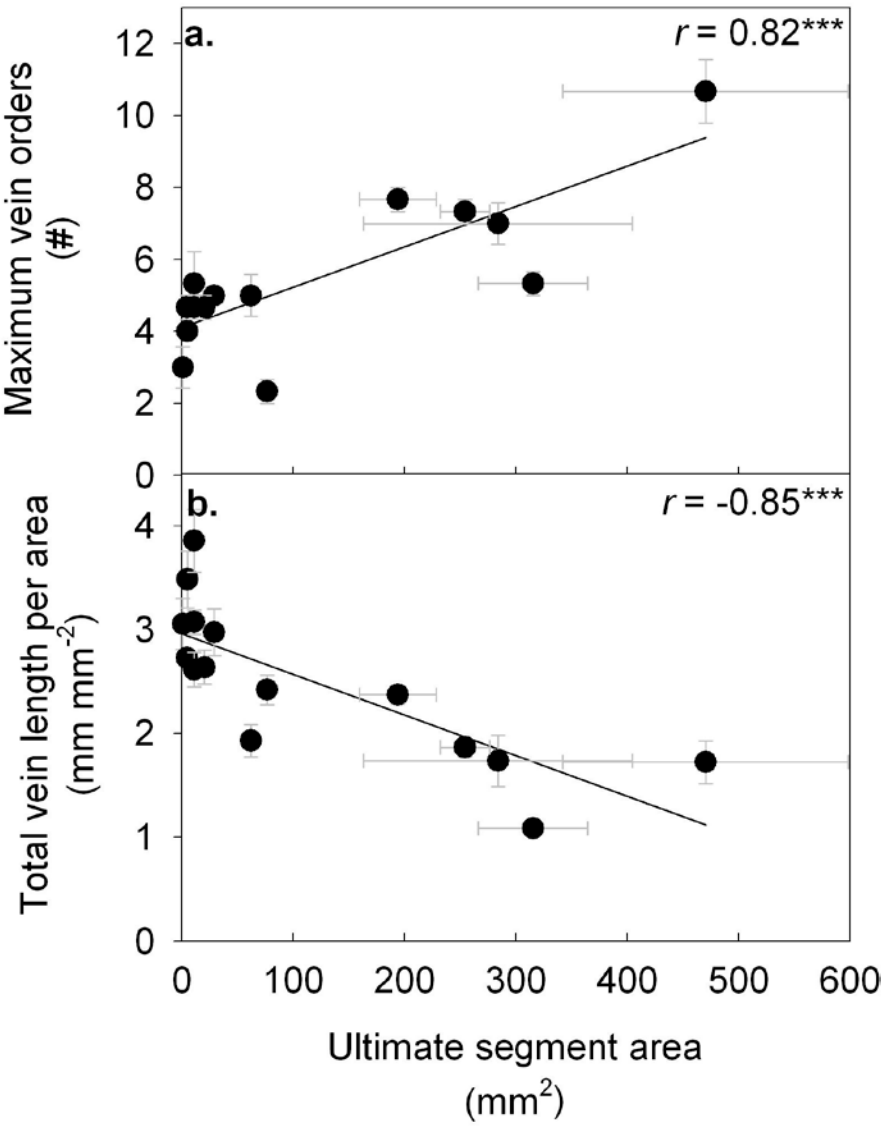

a.
r = 0.82***
Maximum vein orders (#)
0
2
4
6
8
10
12
b.
r = -0.85***
Total vein length per area (mm mm^{-2})
0
1
2
3
4
0
100
200
300
400
500
600
Ultimate segment area (mm^2)

Fig 4.7

SUPPLEMENTAL MATERIAL

SUPPLEMENTARY TABLE CAPTIONS

SuppTable 4.1 Minimum, mean and maximum trait values for all taxa, as well as trait means and *P*-values for taxa grouped by dry exposed versus moist shaded habitats for nested ANOVA significance tests; ns = not significant $^{+}P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

SuppTable 4.2 Correlation matrix with Spearman (r_s) and Pearson (r_p) coefficients and *P*-values for respective ranked, raw (untransformed), and log-transformed datasets, where highlighted cells represent relationships significant for at least the Spearman and one of the two Pearson tests; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

SuppTable 4.3 Pearson partial correlation coefficients and *P*-values for untransformed (left) and log-transformed data (right) after respectively controlling for effects of mean annual precipitation (MAP), vapor pressure deficit (VPD), and percent open sky (%OS) on relationships of leaf hydraulic conductance per area (K_{leaf}) and per mass ($K_{\mathrm{leaf,mass}}$) with vein length per area (*VLA*) for all veins; bold-type cells represent significant results for one or both tests; $^{+}P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

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CHAPTER 5

RAPID STOMATAL RESPONSES TO IRRADIANCE, CO_2 AND VAPOR PRESSURE DEFICIT IN FERNS: RESOLVING PLASTICITY AND ADAPTATION ACROSS SPECIES AND IRRADIANCES

ABSTRACT

Stomatal responses to environment are critical to balance carbon gain against water loss, but have remained enigmatic for key lineages including ferns. We tested hypotheses for the short-term sensitivity and coordination of stomatal responses of diverse tropical ferns. We measured short-term responses of stomatal conductance (g_s) to irradiance, CO_2 concentration, and vapor pressure deficit (VPD) for 13 phylogenetically and morphologically diverse tropical rainforest fern species native to open and shaded habitats, grown under controlled high and low irradiance treatments. We tested three novel hypotheses: (1) that species' responsiveness to different factors would be correlated, (2) that high irradiance grown plants would have greater responses than low irradiance grown plants, and (3) that species native to high irradiance habitats would have greater responses than those native to low irradiance habitats.

We found short-term g_s responses to all factors, with all species responding significantly to irradiance and VPD (15-48% and 7-39% opening and closing responses across the tested range, respectively) and subtle but significant opening or closing in response to CO_2 (3-16%), for 10-13 species. The magnitude of short-term g_s responses to irradiance and VPD were positively correlated, but independent of responses to CO_2. Larger absolute maximum responses were observed for species with higher light-saturated g_s. Species native to high irradiance showed greater magnitude short-term g_s responses to irradiance and CO_2 than shade species, but lower responses for VPD.

Our results support semi-coordinated and semi-independent stomatal responses to all three environmental factors. The greater sophistication of stomatal behavior than previously realized for ferns is consistent with diverse optimization of hydraulic and metabolic needs at growth and evolutionary time scales.

INTRODUCTION

Stomata respond to numerous environmental stimuli to balance whole plant hydraulic and photosynthetic needs, and these responses can influence individual fitness, species distributions, and environmental fluxes of water and carbon (Betts *et al.*, 2007; Berry *et al.*, 2010). Understanding the determinants of fern stomatal behavior will be especially valuable to inform models of plant responses to environmental change, as the habitat specificity of ferns makes them useful as 'canaries in the coalmine' indicator species (Page, 2002; Andama *et al.*, 2003; Chang *et al.*, 2009).

To better predict plant performance, we need to characterize stomatal responses to particular environmental factors, the variation in these responses within and across species and environments, and the mechanisms that coordinate these responses. Stomatal conductance (g_s) must be high enough to allow CO_2 assimilation, while avoiding excessive dehydration of the mesophyll and xylem (Cowan & Farquhar, 1977). In angiosperms generally, stomata show short-term, rapid responses (i.e., manifesting within 5-6 minutes) to open under higher irradiance and close under higher ambient CO_2 concentration (c_a) and vapor pressure deficit (VPD); however, angiosperm species show strong variation according to their metabolic versus hydraulic needs

(Fig 5.1; Franks & Farquhar, 1999; Brodribb *et al.*, 2009; Aasamaa & Sõber, 2011a). Differences in these responses across diverse fern species, including the relative magnitudes of responses to different factors, and their correlations across species, and plasticity with growth environments would be as important as in angiosperms, but have not been characterized in detail or related to ecological specialization. We investigated short-term stomatal responses to three critical environmental stimuli – irradiance, CO_2, and VPD – and associations of these responses with native habitat and growth environment for diverse tropical rainforest fern species. We focused on short-term responses of g_s (i.e., differences between minimum and maximum values measured over 5-6 min gradual step changes in irradiance and CO_2 over a duration of at least 65-100 mins, and for one step change in VPD over a duration of at least 60-210 mins) for three reasons: this allowed comparison with the bulk of previous studies of angiosperms, (e.g., Kim & Heinrich, 2003; Barbour *et al.*, 2005; Powles *et al.*, 2006; Brodribb *et al.*, 2009; Vrábl *et al.*, 2009; Woodruff *et al.*, 2009; Pasquet-Kok *et al.*, 2010; Brodribb & McAdam, 2011a; Buckley *et al.*, 2011), this approach is consistent with standard measurement recommendations (Evans & Santiago, 2012), and typically includes the majority of the responses.

Previous studies of fern stomatal behavior have shown that ferns close their stomata in response to leaf dehydration and open stomata in response to red light, as is typical for angiosperms (Brodribb & Holbrook, 2004; Doi *et al.*, 2006), but unlike angiosperms, were relatively or completely insensitive to blue light, CO_2, and VPD (Franks & Farquhar, 1999; Brodribb & Jordan, 2008; Doi & Shimazaki, 2008; Brodribb *et al.*, 2009; Brodribb & McAdam, 2011a; McAdam & Brodribb, 2013). However, these previous studies focused on 1-6 species each, 17 species in total, typically examined responses to single factors, and used measurements made heterogeneously in field, greenhouse and lab. A comprehensive approach is needed to clarify fern stomatal responses, and the coordination of responses. To quantify fern stomatal responses to multiple environmental stimuli, we cultivated 13 tropical fern species, native to open and shaded habitats, under controlled high and low irradiance treatments. We selected fern species that vary remarkably in habitat preference, morphology, and physiology within a high diversity Costa Rican rainforest (Tuomisto *et al.*, 2002; Watkins *et al.*, 2006; Moran, 2008; Watkins *et al.*, 2010). These species are also phylogenetically diverse, arising in lineages from the most basal eusporangiate Ophioglossales up to the most recently derived leptosporangiate Polypodiales, with evolutionary histories that span wide variation in climate and atmospheric CO_2 concentration (Royer, 2006; Smith *et al.*, 2006).

Additionally, we tested three overarching hypotheses. First we tested whether short-term g_s responsiveness to the three environmental factors would be correlated across species. Such coordination might be expected if their impacts were directly or indirectly transduced by a shared signaling network (Hetherington & Woodward, 2003), and/or if all ferns rely principally on changes in bulk leaf water status to generally control stomatal responses (Brodribb & McAdam, 2011a), and/or if stomatal responses for a given species are constrained by guard cell mechanics. Alternatively, if stomata respond to different environmental factors with different signals, and/or if guard cells can respond by different degrees to different factors, their responsiveness to different factors may be uncorrelated across species, allowing separate optimization. Further, because photosynthetic rate (A) is fundamentally determined by g_s and may drive feedbacks that in turn influence g_s via a hydraulic signal and/or intercellular CO_2 concentration (c_i), or via a correlated chemical signal from the leaf mesophyll (Meinzer, 2002; Mott *et al.*, 2008; Brodribb *et al.*, 2009; Nobel, 2009; Sibbernsen & Mott, 2010), we also tested for a correlation of short-term g_s responses with those of A and c_i to increasing irradiance, CO_2 and VPD, assuming predictable responses of these variables (Fig 5.1).

We also hypothesized (2) that for given species grown under high irradiance treatments, g_s would show a greater response due to plasticity. Similarly, we hypothesized that (3) at evolutionary timescales, short-term stomatal responsiveness would be correlated with species' native light habitats due to adaptation and/or ecological sorting (Givnish, 1988; Johnson *et al.*, 2000; Ackerly, 2004; Proctor, 2012). We thus predicted greater magnitude changes in g_s for open habitat and high irradiance grown plants in response to all three environmental factors for two reasons: plants from open, high irradiance conditions tend to have higher leaf hydraulic conductance (K_{leaf}) and stomatal pore area per leaf area that could facilitate greater changes in g_s and other flux traits (Brodribb & Holbrook, 2004; Sack *et al.*, 2005), and the greater extremes of irradiance, c_i, and VPD experienced by open habitat species and high irradiance grown plants may necessitate stronger responses of stomata and other flux traits to cope (Zwieniecki *et al.*, 2004; Sack *et al.*, 2005; Sack *et al.*, 2006; Buckley *et al.*, 2011).

MATERIALS AND METHODS

Common garden experimental design

Thirteen fern species from tropical lowland rainforest, selected to maximize morphological and phylogenetic diversity, were cultivated in two light environments at La Selva Biological Station, Costa Rica (84°00'12.922"W, 10°25'52.610"N). From August to September, 2010 we collected sixteen individuals of each species from the forest along with their local clay loam soil (Eutric Hapludand Andisol; Weitz *et al.*, 1997) to minimize transplant shock. We included seven species from open habitats and six species from shaded habitats (Table 5.1), and collected study individuals from at least three subpopulations per species separated by 0.5 to 2 km. Irradiance treatments were imposed with two replicate shadehouses for each irradiance treatment using layered 50% “aluminet” shade cloth (Aluminet Reforzado; CNBM, Beijing, China), a material that improves the proportion of diffuse light, thus favoring net photosynthesis and growth (Markvart *et al.*, 2010), and reflects the non-transmitted light, thus reducing cloth and air temperatures inside the shadehouses (Bailey, 1981). The % of daylight photosynthetically active radiation (PAR) transmitted into the shadehouse was determined at noon on a typical cloudy day by averaging light meter measurements (LI-250A, LI-COR, Lincoln, NE, USA) across the shadehouse benches and dividing by simultaneous measurements taken in a clearing outside the shadehouses. To reduce shock, all plants were acclimated under medium irradiance (10% daylight PAR) for six months before transferring to high irradiance (20% daylight PAR) and low irradiance (1.5 % daylight PAR) growth environments. Temperature and humidity were similar within and across the high and low irradiance growth treatments, and tracked the climate outside the shadehouses: midday measurements using the LI-COR 6400 (LI-COR, Lincoln, NE, USA) of air temperature and relative humidity (RH) averaged for five typical days were 28.0 ± 0.8 °C and 77.0 ± 6.2 % RH respectively in the high irradiance treatment and 28.5 ± 0.8 °C and 77.3 ± 6.8 % RH for the low irradiance treatment, and 29.1 ± 0.8 °C and 71.6 ± 7.2 % RH immediately outside the shadehouses. We randomized plants across benches initially and re-randomized plant locations across benches monthly to avoid any potential block effects within the treatments. Soil was kept moist by daily rainfall and additional watering on days without precipitation. Plants acclimated for three months in the high and low irradiance treatments and had established new leaves before measurements.

Measurement of gas exchange responses

From June through November, 2011, we measured light response curves, A-c_i curves and VPD responses for g_s, A, E and c_i with a LI-COR 6400 photosynthesis system (LI-COR, Lincoln, NE, USA). Because changes in E tracked changes in g_s, we focus on responses of g_s, A, and c_i.

This study focused on relatively short-term stomatal responses. Stomatal dynamics may continue to change after a shift in environmental stimulus, with dynamics continuing for several minutes, or for some species for more than 60 min, including diurnal and circadian oscillations (Meidner & Mansfield, 1968; Franks *et al.*, 1997; Franks & Farquhar, 1999; Brinker *et al.*, 2001; Hubbard *et al.*, 2007). A focus on short-term responses reduced the likelihood that g_s responses to irradiance, CO_2 and VPD were confounded by changes in other conditions. We recognize that additional studies are needed of the responses of g_s to short-term periods, as well as over much longer periods to capture long-term dynamics of gas exchange, and indeed, longer-term plastic responses, such as changes to stomatal density and aperture size concomitant with the development of new leaves (Franks *et al.*, 2012; Haworth *et al.*, 2013). Most importantly, our approach was designed to allow standard comparisons among the study species, and to the extent feasible, with species previously studied in the literature, while providing sufficient acclimation time for robust overall responses to environmental factors. A challenge with designing a study such as this one, is that typically, previous studies have reported very scantily on methodological details, e.g., equilibration times before response curves, whether curves were determined with ascending or descending steps of intensity, the number of steps, and time at each step (but see Cousins *et al.*, 2007; Tazoe *et al.*, 2011). To facilitate comparisons with future studies, we have included this information for our light, CO_2, and VPD response methods below.

Previous studies reporting a lag effect of g_s behind A, typically acclimate leaves at very low light levels before a brief higher light pulse (e.g., Kirschbaum *et al.*, 1988), but the lag time appears to depend on the particular methods, species, and functional type (e.g., Stoop *et al.*, 1991; Vico *et al.*, 2011). For example, in large leafed *Alocasia macorrhiz*a, a stable maximum g_s occurred within 20 mins time following increase in light intensity (Kirschbaum et al. 1988), but in well watered plants of *Papaya*, g_s reached the stable maximum within 3 min of the light change (Clemente & Marler 1996). Because extended acclimation time at low light may contribute to a longer g_s response time when light is increased (Gross & Chabot, 1979; Vines *et al.*, 1982; Vines *et al.*, 1983; Stoop *et al.*, 1991; Tinoco-Ojanguren & Pearcy, 1993), we acclimated leaves at high light for all response curves to standardize measurements across species by maximizing stomatal opening before changing irradiance, CO_2 or VPD, and we measured g_s light responses by moving from high to low irradiance.

Additionally, the overall stomatal responses to light, CO_2 and VPD occurred over approximately 1-3 hours as needed to ensure stability of g_s. Considering the full range of levels considered for each environmental factor (i.e., light, CO_2 and VPD), the overall response time allowed for stomatal acclimation was 85-100 min, 65-75 min, and 50-210 min respectively. To quantify total g_s responsiveness to environmental factors, we used smaller step changes of light and CO_2 given their wide ranges of stimulus intensity (0 – 1500 μmol m^{-2} s^{-1} PPFD and 0 – 40 Pa CO_2) to avoid a drastic shift in levels. For this we used step change times typical in the literature, i.e., over 5 min for the light- and CO_2 responses at each step, whereas for VPD response we used the single standard shift used in previous studies (1 to 2 kPa), i.e., over 60 min on average at each step for the VPD response. Typically, the light and CO_2 responses saturated at steps previous to the highest (or lowest) stimulus intensity applied. Though we plotted responses to the intermediate step changes in light and CO_2 (Figs 5.6, 5.7) to show the procedure, and for readers interested in the differences of slope for changes in g_s, we only examined the total responsiveness of g_s across the entire stimulus range within and across species. To examine g_s responses to each step change in stimulus intensity, we would recommend using the stabilization times we applied for the entire responses (or longer if needed for a given species) for each step change.

The times assigned for stomatal responses to step changes in irradiance, CO_2 and VPD were established based on previous studies to enable comparability (c.f. Franks & Farquhar, 1999; Brodribb & Jordan, 2008; Brodribb *et al.*, 2009; Brodribb & McAdam, 2011a) and to ensure stability (see below); for instance, Brodribb & McAdam (2011a) examined VPD responses to an approximately 1 kPa step change between 17-33 mins, Brodribb & Jordan (2008) allowed a 90 min response to three step changes in VPD, and Brodribb *et al.* (2009) examined CO_2 responses defining stability as a less than 3% change over 8 min. Notably, we used a 300 s step change in the response to irradiance and CO_2 rather than the standard protocol of 200 s step changes (Evans & Santiago, 2012). For the VPD response we allowed leaves to stabilize for 20-90 min (on average 50 min) at 1 kPa VPD before transitioning to 2 kPa and allowing leaves to stabilize for 30-120 min (on average 60 min).

We measured light response curves of g_s and photosynthetic assimilation rate (*A*) for 5-6 leaves from 4-6 different individuals in each irradiance treatment for all 13 species. To approximate ambient conditions and maximize stomatal opening, we used moderate flow rates (300-400 μmol m^{-2} s^{-1}), maintained a leaf block temperature of 29°C and VPD of 1 kPa. While we quantified minimum and maximum g_s using the extreme ends of an irradiance response curve (1500 vs. 0 μmol m^{-2} s^{-1}), we were careful to use gradual step change decreases in light intensity, resulting in a 14 step change response curve occurring over 85-100 min. We first acclimated leaves at 1000 photosynthetic photon flux density (PPFD) for 15-30 minutes until g_s and A were stable (i.e., no longer increasing or decreasing for at least five minutes given a coefficient of variation < 0.1), allowing confident resolution of maximum g_s. Next we proceeded with 300 s step decreases in irradiance intensity: 1000, 1500, 1250, 1000, 800, 600, 400, 200, 100, 75, 60, 45, 15 and 0 PPFD. Given that for our response curves, minimum g_s was achieved before the lowest environmental stimulus intensity level, we are confident that we achieved true minimum values.

We measured the responses of g_s and A to c_i for 5-6 leaves from 4-6 individuals in each irradiance treatment for ten species. We acclimated leaves as for the light responses, except that ambient chamber CO_2 concentration (c_a) was set to 5 Pa for at least 20 min until A and g_s were stable (as above) before 300 s step changes: 0, 5, 10, 20, 40, 60, 100, 150, 200 Pa, followed by a return to c_a of 40 Pa for at least 10 min until A and g_s restabilized, then leaves were held in darkness for at least 10 min before determining dark respiration (R_d). This low to high CO_2 transition approach aims to quantify the closure response that g_s would undergo over the wide range of CO_2, rather than shifting from current ambient levels (i.e., 40 Pa) to low CO_2 and from 40 Pa to 200 Pa separately. We acclimated leaves to low CO_2 to standardize measurements across species by maximizing stomatal opening, and then used stepped increases in CO_2 to obtain maximum stomatal closure. Because CO_2 levels were increased from low to high, the measurements made at high CO_2 may have been influenced by the opening response to low CO_2, a potential, so-called "low CO_2 hangover effect", and it is possible that we might have found more closure if we had adjusted CO_2 from 40 Pa straight to 200 Pa. It is unlikely, however, that there would have been less stomatal closure with our approach, given that there was very little stomatal opening when CO_2 was reduced from ambient to very low, and only in 1 species (see Results). Because such a hangover effect was not found in previous studies, Lawson *et al.* (Lawson *et al.*, 2008) used the same low-to-high CO_2 steps approach with tobacco and indeed found strong closure at high CO_2 despite the initial low CO_2 treatment. Further, Brodribb *et al.* (2009) applied multiple approaches for changing CO_2 levels, adjusting CO_2 for ferns from ambient to high CO_2, high to ambient CO_2, ambient to low CO_2, and from low to ambient CO_2, and averaged these (without testing for differences arising from these different orders of applying CO_2 levels), and reports on average lower closure responses than we found (though ours are still low relative to angiosperms). In a study of *Arabidopsis thaliana*, *Nicotiana tabacum* and *Triticum aestivum* by Tazoe et al. (2011), Fig 5.2 shows g_s responses to sequential changes in CO_2 concentration: first low (20 Pa) to high (100 Pa), then high to low, then another low to high transition. For the three angiosperm species shown, it appears that the second low to high transition, in steps like ours, led to equal or stronger stomatal closure as that observed in the initial low to high transition. This supports use of CO_2 step changes from very low to high levels to quantify the maximum g_s closure response, at least for these species.

We also measured VPD responses of g_s and A for at least for 5-6 leaves from 4-6 different individuals in each irradiance treatment for ten species, focusing on responses to a standard step change in VPD from 1 – 2 kPa as used in previous studies (e.g., Franks & Farquhar, 1999; Pasquet-Kok *et al.*, 2010; Buckley *et al.*, 2011; McAdam & Brodribb, 2013). To approximate ambient conditions and maximize stomatal opening we acclimated leaves as for light response curves, at 1 ± 0.2 kPa VPD, for 20- 90 min (on average 50 min) to ensure a stable value for *A* and g_s, defined as at least 5-10 min where the coefficient of variation was less than 0.05. We then matched the infrared gas analyzers (IRGAs) and logged for at least another 5 min to ensure continuing stability before increasing the percentage of flow through the desiccant tube (while maintaining the standard flow rate) to increase VPD to 2 kPa. Leaves were kept at 2 kPa for 30-120 min as needed until g_s and A were stable with no upward or downward trend (as above), then matched the IRGAs, and we logged at least another 5 min to correct for any drift. In angiosperms, an increase in VPD typically generates a two-phase response in g_s: the initial "Ivanoff effect", i.e., a transient spike in g_s after an increase in VPD generally explained as temporary stomatal gaping either resulting from release of xylem tension thus allowing stomatal hydration and opening, or else a reduction in back-pressure on guard cells from surrounding epidermal pavement cells as these dehydrate, followed by the expected stomatal closure, i.e., the reduction of g_s to reduce water loss (Ivanoff, 1928; Powles *et al.*, 2006; Buckley *et al.*, 2011). To characterize gas exchange responses to changes in VPD, we measured responses until stomatal closure at the high VPD level (2 kPa) was apparently complete. We also measured changes in A, c_i and water use efficiency (WUE), determined by dividing A by E at VPD 1 kPa and 2 kPa.

After each gas exchange response was measured, the area of leaf in the chamber was traced onto a transparent acetate sheet, scanned and calculated using Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA) to normalize values for chamber leaf area.

Quantification of responsiveness of flux traits to changing irradiance, CO_2, and VPD

The short-term responsivenesses of g_s, A, and c_i to each environmental factor were determined in absolute and relative terms (Table 5.2). An absolute difference in responsiveness ($Resp_{abs}$) was calculated as the difference between maximum and minimum flux trait value (e.g. max – min g_s). As this may bias responsiveness towards leaves with higher flux rates we also calculated a relative response ($Resp_{\text{rel}}$) of flux traits by dividing the absolute difference by the maximum value (e.g. (max – min g_s) / max g_s).

Notably, in the response curves, maximum and minimum g_s values did not always occur at the extreme levels of the environmental parameters. Thus, we determined absolute and relative responses in two ways. First, the maximum responsiveness (*MaxResp*) to quantify the greatest possible difference was determined using absolute minimum and maximum g_s across the range tested for the environmental variable (e.g., g_s *MaxResp* = max g_s – min g_s). Second, the standardized responsiveness (*StdResp*) was determined using trait values extracted from the same start and end points of response curves, i.e. at minimum and maximum environmental stimulus intensity (e.g., g_s *StdResp* = g_s at 1500 PAR – g_s at 0 PAR). For responses to irradiance and CO_2 concentration, the difference in flux traits at minimum and maximum environmental signal strength were taken respectively at 0 versus 1500 µmol m^{-2} s^{-1} PAR and at 0 versus 200 Pa. For VPD, because a lower VPD reflects higher air moisture, 1 kPa VPD was considered the maximum value, and 2 kPa VPD the minimum. Because VPD response phases are defined by changes in g_s, standard and maximum g_s VPD responses are the same. Maximum and standardized responsiveness variables tended to be intercorrelated, and in the description of results we refer to both standardized and maximum responses unless otherwise noted.

Statistical analysis

To test for the significance of within-species stomatal responsiveness to changes in irradiance, CO_2, and VPD, and for potential differences in direction of responses, we used paired t-tests to compare g_s at minimum and maximum values of the environmental factor for each species in each growth irradiance treatment.

Before testing species differences in responsiveness variables, we added the lowest species mean plus a standard constant of 0.0001 so that all values were positive, then log-transformed to improve normality. To test for significant differences in stomatal responsiveness across species, habitats and irradiance treatments, we used a three level nested general linear model (Minitab 16 Statistical Software 2010, Minitab, Inc., State College, PA).

To test whether stomatal responses to irradiance, CO_2, and VPD were significantly correlated across species, and to test a priori hypotheses of mechanistic associations between g_s and other gas exchange variables, we used Pearson and Spearman correlations on species means (R Development Core Team, 2010, R Foundation for Statistical Computing, Vienna, Austria). We considered trait relationships to be robustly significant if $P \leq 0.05$ for at least two of the three correlation tests (Pearson correlation on untransformed data, Pearson correlation on log-transformed data, Spearman correlation on ranked data; see SuppTable 5.5). We focused on testing only previously hypothesized relationships, and thus did not use correction for multiple correlation tests, as that would have reduced the power to test a priori hypotheses. Although we present a correlation matrix of all variables to show the structure of inter-relationships among measurements for interested readers, we do not endorse inference of non-hypothesized relationships, and we recommend statistical correction before “mining” of correlations given the risk of inflated type I error (Garcia, 2003; Moran, 2003; Givnish *et al.*, 2004; Edwards, 2006; Waite & Sack, 2009).

RESULTS

Variation in fern stomatal sensitivity to environmental factors

A first major finding is that under very low irradiance, high CO_2 and high VPD, there was substantial stomatal opening in ferns, rather than complete closure. Thus, for 11, 10 and 7 of 13 species, stomata were substantially open at 0 μmol m^{-2} s^{-1} PAR, 200 Pa of CO_2 and 2 kPa of VPD respectively. Stomatal opening at low irradiance and high CO_2 was found despite adequate equilibration times at these levels (see *Methods*).

We observed significant short-term sensitivity of fern stomata to irradiance, CO_2, and VPD (Figs 5.2-5.4) based on a comparison of standardized minimum and maximum stomatal conductance (g_s). Twelve of thirteen species showed significant rapid changes in g_s over the 1500 μmol m^{-2} s^{-1} increase in irradiance, nine of ten measured species showed a significant change in g_s in response to a 1 kPa increase in VPD, and five of ten measured species showed a significant change in g_s over the 200 Pa increase in CO_2 ($P \leq 0.05$; SuppTable 5.1). Notably, irradiance and CO_2 stimulus ranges were large, and while the step change in VPD was relatively smaller, responsiveness to this factor was still high, and in fact higher than overall short-term responsiveness to CO_2. Considering means for all species, maximum relative responsiveness ranged 15 – 48% under increasing irradiance, 3-16% under increasing CO_2, and 7-39% under increasing VPD.

Variation in the direction of stomatal responses with environmental stimuli

In general, when flux traits responded to light and VPD for a given species, they did so in the expected directions (Fig 5.1, SuppTables 5.2-5.3). However, for the short-term response of g_s to CO_2, species differed in direction. For three of the five species that responded significantly to

CO_2, g_s declined as expected at higher CO_2. However, *Ophioglossum nudicaule*, and *Pityrogramma calomelanos* significantly increased in g_s at high CO_2 (Figs 5.2, 5.4).

Partial coordination of fern stomatal responses to irradiance, CO_2 and VPD

Across fern species, the short-term g_s responses to environmental variables were partially inter-correlated and related to maximum g_s and photosynthetic rates.

The rankings of species by their maximum short-term stomatal responses to irradiance and VPD within sun- or shade-adapted categories were similar, with a tendency towards greater short-term responsiveness in species with higher flux rates (Figs 5.2-5.4). Averaged across growth irradiances, species with the highest light-saturated g_s did show the greatest absolute magnitude responses of g_s to irradiance and VPD (Fig 5.5a,b; SuppTable 5.5). To test whether this correlation was driven automatically because our measurement of light-saturated g_s did not allow enough response time (and thus species with higher responsiveness would also seem to have highest light-saturated rates), we compared the light-saturated g_s values from the light-response curve (attained after 15-30 min stabilization and an additional 10 min from initiating the previous step at 1000 µmol m^{-2} s^{-1} PAR) with those measured in the VPD response (which involved acclimating leaves at 1 kPa VPD and high irradiance for 50 min on average across species, until stability was observed). We found that the longer acclimation time to achieve light-saturated g_s as assessed in the VPD response did not necessarily lead to a higher g_s. The light-saturated g_s was in fact 21.5 % higher when assessed using light response curves than in the VPD response, despite the shorter overall equilibration time across the range of tested irradiance levels. We also tested whether the longer acclimation time provided for the VPD yielded different average maximum g_s across species. The maximum g_s for irradiance and VPD responses was strongly positively correlated ($r = 0.94$, $P < 0.001$), and an unpaired two tailed t-test demonstrated that maximum g_s was not significantly different for light and VPD responses ($P = 0.33$). We concluded, as hypothesized, that the assessment of light-saturated g_s was robust, and the correlation of maximum g_s responsiveness to light and to the step change in VPD do not arise artifactually due to insufficient response time to high irradiance, but reflect greater true responsiveness in the species with higher maximum flux rates.

By contrast with responsiveness of g_s to irradiance and VPD, the responsiveness of g_s to CO_2 was not significantly correlated with light-saturated g_s (SuppTable 5.5).

The absolute maximum responses of g_s to irradiance and VPD were positively correlated (Fig 5.5c). However, there were no significant correlations between the relative % responses of g_s to irradiance and VPD, between absolute and relative responses of g_s to irradiance and those to CO_2, or between absolute or relative responses of g_s to VPD and those to CO_2 (SuppTable 5.5).

Variation in fern stomatal responses with adaptation to native habitat, and the plasticity of stomatal responses with growth irradiance

The magnitude of short-term g_s responses varied significantly with native irradiance habitat and with irradiance growth environment. Species from open habitats tended to show stronger g_s responses to irradiance and CO_2, while shade habitat species tended to show stronger g_s responses to VPD (Figs 5.2-5.4, SuppTable 5.2). With respect to absolute responses, open habitat species showed greater standard absolute responses of g_s to irradiance, and greater maximum absolute responses of g_s to CO_2. With respect to relative responses, open habitat species showed greater maximum relative response to CO_2, and weaker standard and maximum relative responses to VPD compared to shade habitat species (Fig 5.4, SuppTable 5.2).

Plastic differences in the responsiveness of g_s to growth irradiance were simpler, in that plants of given species grown under the high irradiance growth treatment exhibited stronger short-term responses of g_s to all three environmental stimuli than those grown under low irradiance. High irradiance grown plants showed significantly greater absolute and relative responses of g_s to irradiance and VPD, and greater standard absolute response of g_s to CO_2 (Figs 5.6-5.8, SuppTable 5.2). Further, in response to increasing VPD, high irradiance grown plants had a greater relative magnitude response of g_s and greater relative improvement in water use efficiency as determined by instantaneous measures of A and E at VPD 1 and 2 kPa (Fig 5.4, SuppTable 5.2).

Coordination of stomatal responsiveness with magnitude changes in other flux traits

Across species, the standard relative responses of g_s and A to VPD were positively correlated (r = 0.88, P < 0.001, SuppTable 5.5). However, absolute and relative changes in the magnitude of g_s in response to irradiance and CO_2 were not significantly correlated with the responsiveness variables for A, or with R_d (SuppTable 5.5). The absolute and relative magnitude changes in g_s were significantly correlated with a depression in c_i in response to irradiance and VPD ($r > 0.65$, $P \leq 0.05$, SuppTable 5.5).

DISCUSSION

We found significant short-term stomatal responses in ferns to all three environmental stimuli – irradiance, CO_2 and VPD. Ferns showed a generally lower but wider range of responses than previously reported for angiosperms. Ferns show both plastic and apparently adaptive shifts in stomatal responsiveness, which would contribute to their habitat differentiation within the forest community. These data reveal important responsiveness, greater variability than so far reported, and more complex stomatal physiology. The striking variation in sensitivity of fern stomata to different environmental stimuli and in the magnitude of their short-term responses within and across species and growth habitats calls for a revision of the idea that all ferns lack the stomatal responses of angiosperms, or that they rely on a single simple mechanism of stomatal control to respond to all stimuli.

Fern species vary in sensitivity and direction of stomatal response to irradiance, CO_2, and VPD

As predicted, the fern species typically increased g_s under high irradiance, and low VPD to maximize photosynthesis, and decreased g_s under low irradiance, and high VPD as would be optimal to conserve water; however, not all species showed the expected closure response given increasing CO_2. The sensitivity to stimuli varied strongly across species, with more species showing significant short-term responses of g_s to irradiance and VPD than to CO_2 (12/13 versus 9/10 versus 5/10 species), and two species, including a representative of the ancient fern genus *Ophioglossum* (Smith *et al.*, 2006) showing unexpected stomatal opening in response to high CO_2. Our findings indeed confirm the relatively lower CO_2 sensitivity in ferns relative to angiosperms shown by previous studies, which found changes in fern g_s for 1-5 species ranging from 1-11 % (Brodribb *et al.*, 2009; Franks *et al.*, 2012). Our new data importantly extend the understanding of this response, showing that significant quantitative CO_2 responses do exist in ferns, ranging up to 16% stomatal closure for *Thelypteris nicaraguensis*, and can occur significantly in both directions, depending on species. This decoupling of stomatal response directions to different stimuli for given species, and differences in sensitivity to environmental stimuli across species point to more complex stimulus detection and stomatal control mechanisms in ferns than previously thought. The CO_2-induced opening of stomata in *Ophioglossum nudicaule* and *Pityrogramma calomelanos* was noteworthy as a novel observation of this pattern, and might be explained by these species having been adapted in environments with much higher levels of CO_2, thus requiring a higher CO_2 threshold to reduce stomatal apertures for water conservation. Alternatively, because these rainforest species are adapted in extremely competitive high irradiance habitats with abundant water supply, selection for optimization of carbon fixation at the expense of water conservation, all else being equal, may have effectively decoupled the stomatal closure response to high CO_2. Increasing photosynthesis in the guard cells and/or mesophyll, or signals of c_a or c_i (von Caemmerer *et al.*, 2004; Lawson *et al.*, 2011) may stimulate this opening response, and further research is needed to ascertain the source of this signal for ferns.

The significant short-term g_s responses found for ferns are aligned with the proposal by Ruszala *et al.* (2011) that stomatal responses are found generally across land plants. That study demonstrated stomatal responses to CO_2 and abscisic acid (ABA) across early vascular plants and angiosperms, and pointed to a comparable stomatal signaling mechanism, as the functionally identical OST1 gateway gene involved in *Arabidopsis thaliana* guard cell ABA signaling was also found in the early vascular plant *Selaginella moellendorffii*.

Notably, the CO_2 responses of ferns may be stronger than those observed in our study. In our study, even for those species which did show a significant CO_2 response, this did not saturate at the highest c_a (200 Pa) provided by the LI-COR 6400 using the CO_2 injector (Fig 5.7). Because the fern lineage originated in the Devonian under a CO_2 concentration that may have been four times higher than this level, i.e., ten to twenty times higher than ambient levels today (Berner, 1993; Royer, 2006), new approaches are needed to determine whether ferns would exhibit even greater magnitude depressions of g_s under substantially higher CO_2 concentrations. Fern species with an evolutionary history that includes periods with greater or longer duration exposure to high CO_2 may have a g_s response mechanism similar to angiosperms, but might respond to a wider range of CO_2 for decreasing g_s.

Stomatal responses to environmental stimuli are semi-independent

Fern stomatal responses to VPD are thought to be simpler than those to other environmental factors, as they have been suggested to be directly and passively related to the water status of leaf cells (Brodribb & McAdam, 2011a; McAdam & Brodribb, 2013). In contrast, irradiance responses would be more complicated because stomata may respond to light absorption by guard cells and mesophyll in addition to water-balance and/or c_i mediated changes in guard cell apertures, with active control depending on ion pumping. However, if guard cell control relies on the same core signaling network irrespective of environmental stimulus, short-term stomatal responses to all factors might be expected to correlate across species, with given species having more responsive stomata due to their size, structure, or biochemical sensitivity. The fern species in this study showed partially independent g_s responses to environmental stimuli. Across species, the absolute magnitude increases in g_s under high irradiance were correlated with the absolute magnitude decreases in g_s under high VPD. Thus, ferns with higher maximum flux rates are geared toward higher absolute responsiveness, allowing control of both carbon and water fluxes proportionately to their maximum transport capacity. This scaling allows high irradiance species in particular to benefit from strong responsiveness to optimize g_s (see below). However, depressions of g_s under high VPD and high CO_2 to conserve water were not significantly correlated across species. This variability suggests a reliance on different stimulus detection and response mechanisms to manage stomatal apertures. For instance, whereas VPD responses may rely on hydropassive stomatal closure (Brodribb & McAdam, 2011a), CO_2 responses may be more strongly regulated by cytosolic Ca^{2+} dynamics (Young *et al.*, 2006). This partial coordination of stomatal responses to different stimuli is consistent with a scale-free network of stomatal signaling (Hetherington & Woodward, 2003), whereby highly connected nodes (signaling molecules) may coordinate and respond to multiple stimuli, and more sparsely connected nodes allow for distinct responses. Further work is needed to investigate the independent transduction of signals to guard cells in response to different stimuli.

The magnitude of stomatal responses varies significantly with irradiance environment

We hypothesized that fern stomatal behavior would show adaptation to different habitats, as well as plasticity associated with growth environment. Specifically, we predicted that ferns adapted in high irradiance habitats would have greater magnitude short-term responses of g_s to better manage extreme and variable conditions. Likewise, we expected plants grown under more extreme conditions of high irradiance to develop leaves with a greater capacity for short-term g_s responses. Our findings broadly supported these hypotheses, though stomatal responses varied across species' light habitats according to the environmental stimulus. A major novel finding of our study was that for given species, high irradiance grown plants showed greater magnitude stomatal responses to all stimuli, though the differences in CO_2 responses across light treatments were relatively subtle.

Another major novel finding of our study was that high irradiance habitat fern species showed greater short-term responses of g_s to irradiance and CO_2 and weaker short-term responses of g_s to VPD. Adaptation to open habitats would have selected for a greater capacity to maximize photosynthesis, even under high VPD, to take advantage of high irradiance and CO_2, whereas adaptation to shade may be linked to a greater vulnerability to drying air that necessitated a stronger reduction in g_s under high VPD. All else being equal, open habitat species would lose more water under increasing VPD. Shade habitat species on the other hand showed greater stomatal closure under high VPD and improved water conservation. Future work is needed to determine whether open habitat fern species rely on other leaf traits to buffer against high VPD, such as a high leaf hydraulic conductance relative to their g_s (Brodribb & Jordan, 2008), relative to shade species, which may rely more strongly on stomatal control to reduce the impact of atmospheric drought.

High irradiance grown plants showed greater response of g_s to CO_2, irradiance and VPD, and greater relative improvement of *WUE* from VPD 1 kPa to 2 kPa compared with low irradiance grown plants. The VPD responsiveness of g_s in ferns across irradiances is thus a novel case of adaptation and acclimation tending in opposite directions. High irradiance grown plants had stronger VPD responses than shade-grown plants, whereas shade habitat species had stronger VPD responses than high irradiance habitat species. These contrary tendencies parallel those of leaf mass per area (*LMA*) in angiosperms across irradiances, where higher *LMA* is found in high-irradiance grown plants of given species, but shade-adapted evergreen plants have higher *LMA* than high light adapted species (Walters & Reich, 1999; Lusk *et al.*, 2008). Whereas a greater VPD response can help a plant to acclimate to higher irradiance by compensating for greater water loss, over long-term adaptation to high irradiance, greater tolerance of high VPD via other mechanisms would result in relative insensitivity by comparison with shade-adapted fern species. Thus we expect the specific compromise between long-term adaptation versus short-term acclimation to atmospheric drought to strongly influence the performance of fern species across their habitat distributions.

Notably, the fern species in this study exhibited short-term g_s responsivenesses to VPD similar in magnitude to those of non-woody angiosperms (Buckley *et al.*, 2011). This suggests that we extend the view from earlier studies of these responses, which focused on species that were insensitive relative to angiosperms (Franks & Farquhar, 1999; Brodribb & McAdam, 2011a). The similar range of sensitivity in ferns and angiosperms is particularly noteworthy given that previous studies of gas exchange and ABA responses have suggested that stomatal responses of ferns are entirely hydropassive (Brodribb & McAdam, 2011a), whereas those in angiosperms include active osmotic changes in guard cell potassium (Losch & Schenk, 1978) and changes in hydration due to a vapor signal (Shope *et al.*, 2008; Sibbernsen & Mott, 2010). Detailed studies on cellular-level changes in solute concentrations and turgor during fern VPD responses are needed to confirm the mechanism for such strong stomatal control in dehydrating leaves across a wide range of fern species.

Stomatal responses are variably correlated with leaf flux traits

Although A and c_i are influenced by g_s (Meinzer, 2002; Mott *et al.*, 2008; Brodribb *et al.*, 2009; Nobel, 2009; Sibbernsen & Mott, 2010), across species, the short-term responses of g_s to environmental factors were not always tightly correlated with the maximum responses of these other traits. We hypothesized that species with faster light-saturated carbon assimilation (A) and dark respiration rates (R_d) under low VPD and ambient CO_2 might achieve greater magnitude stomatal responses, due to a more rapid metabolism. However, there was no correlation across species of the magnitude changes in g_s in response to the three environmental stimuli with either greater maximum A, or with R_d, indicating that species with a slower metabolism are not constrained from adjusting g_s to optimize gas exchange. As predicted across species, differences in magnitude response of c_i, which may be an important signal for guard cell aperture modulation, were significantly correlated with changes in g_s under increasing VPD and irradiance. This tendency may have counteracted stomatal closure given that stomata would open in response to low c_i, though ferns showed low CO_2 responses. Under increasing irradiance, species that experienced a greater depression of c_i and higher A showed a greater compensatory increase in g_s to improve carbon supply rate, and thus the c_i depression may have further stimulated stomatal opening at high irradiance. The degree to which responses of g_s in ferns to irradiance and VPD are directly affected by c_i merits further investigation at the tissue level.

Conclusions

Striking variation in the sensitivity, direction, and magnitude of short-term fern stomatal responses to environmental stimuli is consistent with diverse optimization of hydraulic and metabolic needs within and across species and environments. For example, the presence of two directions of g_s responses to CO_2 in different species, the variable coordination of magnitude g_s responses to light, CO_2 and VPD, and the greater sensitivity of g_s to VPD for species acclimated to high irradiance versus greater g_s responsiveness to VPD in species adapted to shade indicate a complexity in control that would modulate adaptation and acclimation at a wide range of time scales. These different sensitivities of fern stomata to environmental stimuli emphasize the need for future studies into the independent transduction of internal signals. Whereas previous studies have emphasized that ferns can show weak or absent stomatal responses relative to angiosperm species, we found that for some responses (i.e., light and VPD), the magnitude of responsiveness can be equivalent, and consistent with complex stomatal control. Future studies of osmotic changes in fern guard cells, direct visualization of stomatal response dynamics, and investigations of coordination among stomatal and hydraulic architecture and physiology under controlled conditions will unravel the mechanics of fern stomatal behavior and its implications for species environmental tolerances. Further studies also are needed to assess combined impacts of shifts in irradiance, CO_2 and VPD on fern stomatal behavior (Aasamaa & Sõber, 2011a; Brodribb & McAdam, 2011b), and on the impacts of shifts in these environmental factors on plant water status. For instance, woody angiosperm species have shown that drought increases their sensitivity to other environmental changes (Aasamaa & Sõber, 2011b), and that stomatal responses to CO_2 are most strongly suppressed by responses to other simultaneous changes in environment (Aasamaa & Sõber, 2011b). Angiosperms have also been shown to have slower and weaker responses to VPD during drought (Brodribb & Hill, 1993; Tinoco-Ojanguren & Pearcy, 1993; Clemente & Marler, 1996; Cavender-Bares *et al.*, 2007). Tinoco-Ojanguren & Pearcy (1993) additionally demonstrated that extremes of VPD (too low, below 1 kPa, or too high, above 1 kPa) can influence the rate of stomatal induction to an increase in PPFD. This is why we maintained and used well-watered shadehouse plants and maintained a moderate VPD of 1 kPa throughout light and CO_2 response curves.

Overall, the sophistication of fern stomatal responses at growth and evolutionary timescales points to the capacity for a physiological diversification that helps to explain the wide species distributions and persistence of ferns in competitive niches within angiosperm-dominated communities (Watkins Jr. & Cardelús, 2012), and will importantly factor into fern responses to environmental change.

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TABLE 5.1 List of genus and species (family in parentheses), and their habitat type spanning understory to small clearings for shade habitat low irradiance species, and up to large clearings for open habitat high irradiance species.

Species	**Native Habitat**
Shade species	
Danaea wendlandii (Marattiaceae)	understory
Diplazium striatastrum (Woodsiaceae)	understory
Saccoloma moranii (Saccolomataceae)	understory
Cyclopeltis semicordata (Dryopteridaceae)	understory-small clearings
Blechnum occidentale (Blechnaceae)	understory-small clearings
Adiantum latifolium (Pteridaceae)	understory-small clearings
High irradiance species	
Thelypteris nicaraguensis (Thelypteridaceae)	understory-large clearings
Tectaria lizarzaburui (Tectariaceae)	small-large clearings
Campyloneurum brevifolium (Polypodiaceae)	small-large clearings
Nephrolepis biserrata (Nephrolepidaceae)	large clearings
Hemionitis palmata (Pteridaceae)	large clearings
Pityrogramma calomelanos (Pteridaceae)	large clearings
Ophioglossum nudicaule (Ophioglossaceae)	large clearings

TABLE 5.2 Variable categories, symbols, units and definitions. Short-term responsiveness traits were calculated for changes in stomatal conductance (g_s), photosynthesis (A), and intercellular CO_2 concentration (c_i) under respective increases in photosynthetically active radiation (PAR; µmol m^{-2} s^{-1}), CO_2 concentration (Pa), and vapor pressure deficit (VPD; kPa). Because VPD response phases are defined by changes in g_s, standard and maximum g_s VPD responses are the same.

Category	Variables	Units	Definition
Environmental stimulus	*Irradiance*	µmol m^{-2} s^{-1}	Light response curve irradiance from 0 – 1500 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR)
	CO_2	Pa	A - c_i curve CO_2 concentration from 0 – 200 Pa
	VPD	kPa	Stepped VPD response from 1 – 2 kPa
Flux traits	g_s	mol m^{-2} s^{-1}	stomatal conductance
	A	µmol m^{-2} s^{-1}	photosynthetic rate
	E	mol m^{-2} s^{-1}	transpiration rate
	c_i	mmol m^{-2} s^{-1}	intercellular CO_2 concentration
	R_d	µmol m^{-2} s^{-1}	dark respiration rate
	WUE		instantaneous water use efficiency determined as A divided by E for both 1 kPa and 2 kPa VPD
Standard flux trait responsiveness	$StdResp_{abs}$		Standard absolute response: difference between standardized minimum and maximum flux trait values taken from respective start and end of response curves, i.e. minimum and maximum stimulus intensity, e.g., g_s at 1500 PAR – g_s at 0 PAR
	$StdResp_{rel}$	%	Standard relative response: difference between minimum and maximum flux trait values normalized by the maximum value to avoid potential bias towards species with high flux rates, e.g., g_s at 1500 PAR – g_s at 0 PAR / g_s at 1500 PAR
Maximum flux trait responsiveness	$MaxResp_{abs}$		Maximum absolute response: difference between true maximum and minimum flux trait values from across the response curve, e.g., max g_s – min g_s
	$MaxResp_{rel}$	**%**	Maximum relative response: difference between true maximum minimum and maximum flux trait values normalized by the maximum value to avoid potential bias towards species with high flux rates, e.g., max g_s – min g_s / max g_s

FIGURE CAPTIONS

Fig 5.1 Synthesis of predicted short-term responses of physiological variables to increased irradiance, atmospheric CO_2 concentration, and vapor pressure deficit (VPD) based on studies of angiosperms: under high irradiance, photosynthetic rate (A) increases, thus decreasing intercellular CO_2 concentration (c_i) and increasing stomatal conductance (g_s) to maximize CO_2 uptake; under high CO_2, g_s should decline as c_i and A can be maintained while reducing transpiration, thus improving water use efficiency; under high VPD, g_s declines to conserve water, which would reduce c_i and A. Note that the VPD response shown is the “right way response” to close stomata in drier air.

Fig 5.2 Mean species stomatal conductance (g_s) at low versus high photosynthetically active radiation (0 vs. 1500 µmol m^{-2} s^{-1} PAR; white and light grey bars respectively) for plants grown under the low irradiance treatment versus the high irradiance treatment (dark grey and black bars respectively); open habitat species and shade habitat species are ordered from left to right by greatest to weakest maximum responsiveness of g_s, i.e., by the greatest difference between minimum and maximum g_s in response to an increase in irradiance. Greater short-term responsiveness was observed for open habitat species, for species with higher flux rates, and for plants grown under the high irradiance growth treatment. For instance, leaves of *Ophioglossum nudicaule* showed the fastest flux rates as well as the greater g_s responsiveness to irradiance than other open habitat species and shade habitat species, and exhibited greater g_s responsiveness for its high irradiance grown plants compared with the low irradiance grown plants. Significant differences between minimum and maximum g_s values were determined by paired t-test; *** $P \leq 0.001$ ** $P \leq 0.01$ * $P \leq 0.05$.

Fig 5.3 Mean species stomatal conductance (g_s) at low versus high CO_2 concentration (0 vs. 200 Pa; white and light grey bars respectively) for plants grown under the low irradiance versus the high irradiance treatment (dark grey and black bars respectively); open habitat species and shade habitat species are ordered from left to right by greatest to weakest maximum responsiveness of g_s, i.e., by the greatest difference between minimum and maximum g_s in response to an increase in CO_2. Notably, *Adiantum latifolium*, *Hemionitis palmata*, and *Thelypteris nicaraguensis* significantly reduced g_s as expected to conserve water under high CO_2, while *Ophioglossum nudicaule* and *Pityrogramma calomelanos* showed the opposite pattern of significantly increased g_s with CO_2 (denoted by arrows). Significant differences between minimum and maximum g_s were determined by paired t-test; *** $P \leq 0.001$ ** $P \leq 0.01$ * $P \leq 0.05$.

Fig 5.4 Mean species differences in stomatal conductance (g_s) measured under dry air versus moist air (2 kPa vapor pressure deficit vs. 1 kPa VPD; white and light grey bars respectively) for plants grown under the low irradiance versus the high irradiance treatment (dark grey and black bars respectively); open habitat species and shade habitat species are ordered from left to right by greatest to weakest maximum responsiveness of g_s, i.e., by the greatest difference between minimum and maximum g_s from 1 – 2 kPa VPD. Greater short-term responsiveness was observed for open habitat species, for species with higher flux rates, and for plants grown under the high irradiance growth treatment. Significant differences between minimum and maximum g_s were determined by paired *t*-test; *** $P \leq 0.001$ ** $P \leq 0.01$ * $P \leq 0.05$.

Fig 5.5 Correlations of maximum responsiveness of g_s to irradiance and vapor pressure deficit (VPD) with light-saturated stomatal conductance (g_s).: a) the maximum absolute response of g_s to irradiance; and b) the maximum absolute response of g_s to VPD are correlated across species with light-saturated g_s at 1 kPa VPD (measured after an average 50 minute acclimation period); and c) greater maximum absolute responses of g_s to irradiance and VPD are positively correlated; *** $P \leq 0.001$ ** $P \leq 0.01$ * $P \leq 0.05$.

Fig 5.6 Short-term responses of stomatal conductance (g_s) to an increase in irradiance from 0 to 1500 μmol m^{-2} s^{-1}photosynthetically active radiation (PAR) for representative leaves of open habitat (left) and shaded habitat (right) species, from plants grown under high irradiance (open symbol) and low irradiance (closed symbol) treatments; species within each habitat type are ordered from top to bottom by the greatest difference in g_s responsiveness between their high and low irradiance treatment plants. We observed a greater magnitude increase in g_s under increasing irradiance for open habitat species, as well as for plants grown under high irradiance.

Fig 5.7 Short-term responses of stomatal conductance (g_s) to changes in atmospheric CO_2 concentration, c_a (main panels) and intercellular CO_2 concentration. c_i (inset panels) for representative leaves of open habitat (left) and shade habitat (right) species, from plants grown under high irradiance (open symbols) and low irradiance (closed symbols) treatments; species within each habitat type are ordered from top to bottom by the greatest difference in g_s responsiveness between their high and low irradiance treatment plants. We observed different direction short-term responses to high CO_2, with *Ophioglossum nudicaule*, and *Pityrogramma calomelanos* significantly increasing g_s with CO_2 concentration and *Adiantum latifolium*, *Hemionitis palmata*, and *Thelypteris nicaraguensis* decreasing g_s as expected to conserve water under high CO_2. We observed greater short-term responsiveness of g_s for species from open habitat and for plants grown under high irradiance.

Flux traits	Higher Irradiance 0 – 1500 PAR	Higher CO_2 0 – 200 Pa	Higher VPD 1 – 2 kPa
g_s	↑	↓	↓
A	↑	↑	↓
c_i	↓	↑	↓

Fig 5.1

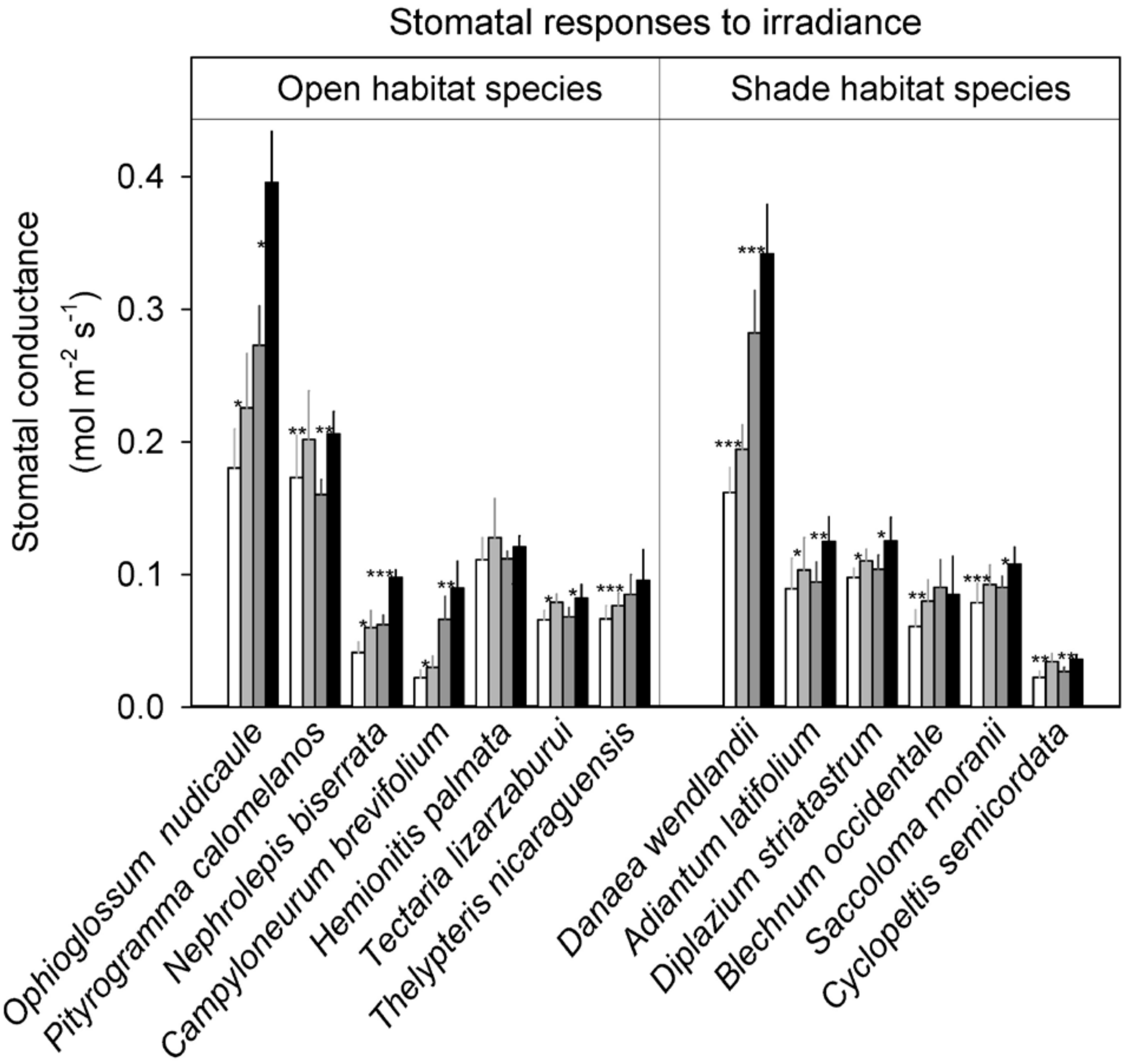
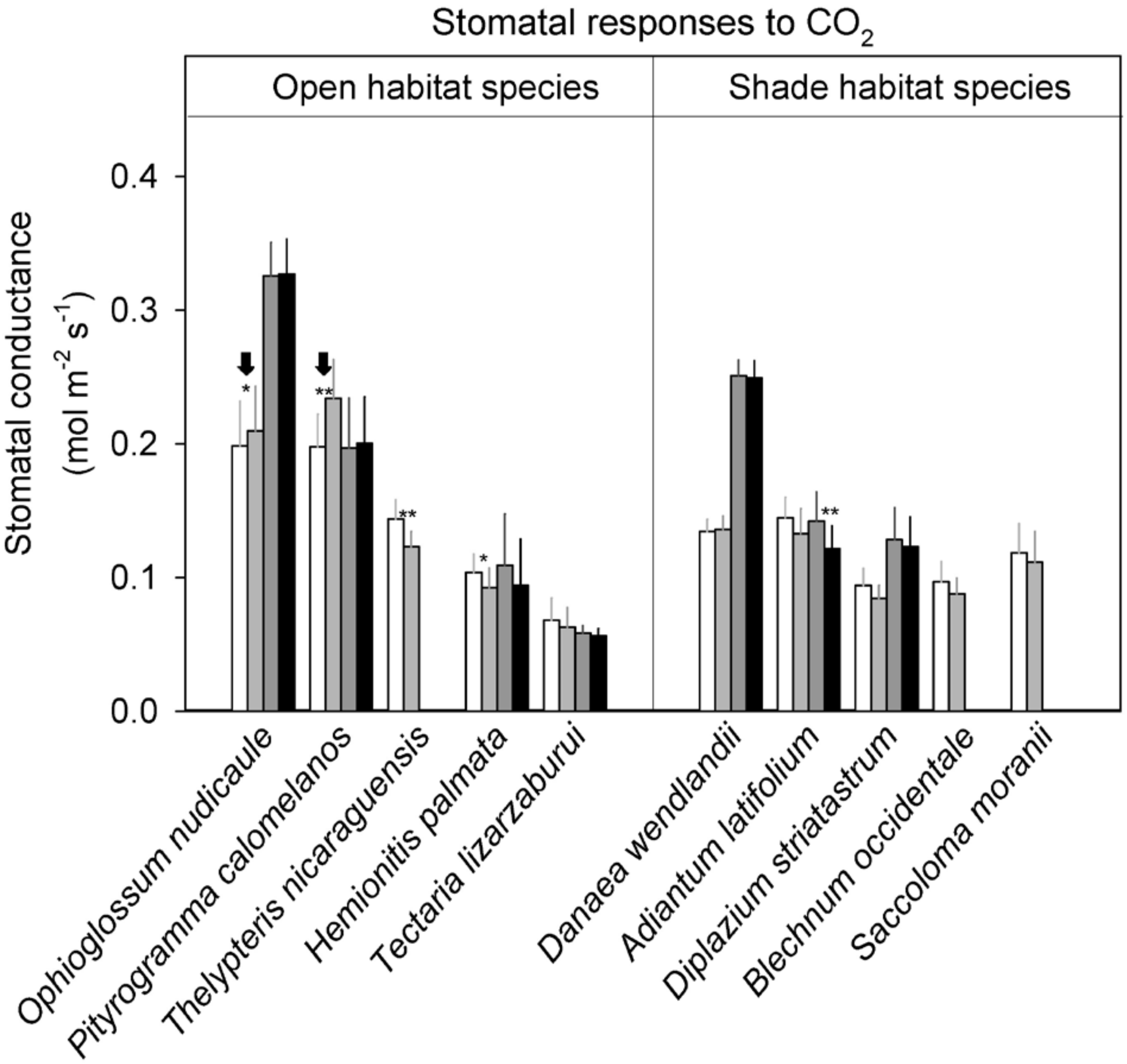

Stomatal responses to irradiance
Open habitat species
Shade habitat species
Stomatal conductance (mol m^{-2} s^{-1})
0.4
0.3
0.2
0.1
0.0
*
*
**
**
*
***
*
**
*
*
***
***
*
**
*
*
**
***
*
**
**
Ophioglossum nudicaule
Pityrogramma calomelanos
Nephrolepis biserrata
Campyloneurum brevifolium
Hemionitis palmata
Tectaria lizarzaburui
Thelypteris nicaraguensis
Danaea wendlandii
Adiantum latifolium
Diplazium striatastrum
Blechnum occidentale
Saccoloma moranii
Cyclopeltis semicordata

Fig 5.2



Stomatal responses to CO_2
Open habitat species
Shade habitat species
Stomatal conductance
(mol m^{-2} s^{-1})
0.4
0.3
0.2
0.1
0.0
*
**
**
*
**
Ophioglossum nudicaule
Pityrogramma calomelanos
Thelypteris nicaraguensis
Hemionitis palmata
Tectaria lizarzaburui
Danaea wendlandii
Adiantum latifolium
Diplazium striatastrum
Blechnum occidentale
Saccoloma moranii

Fig 5.3

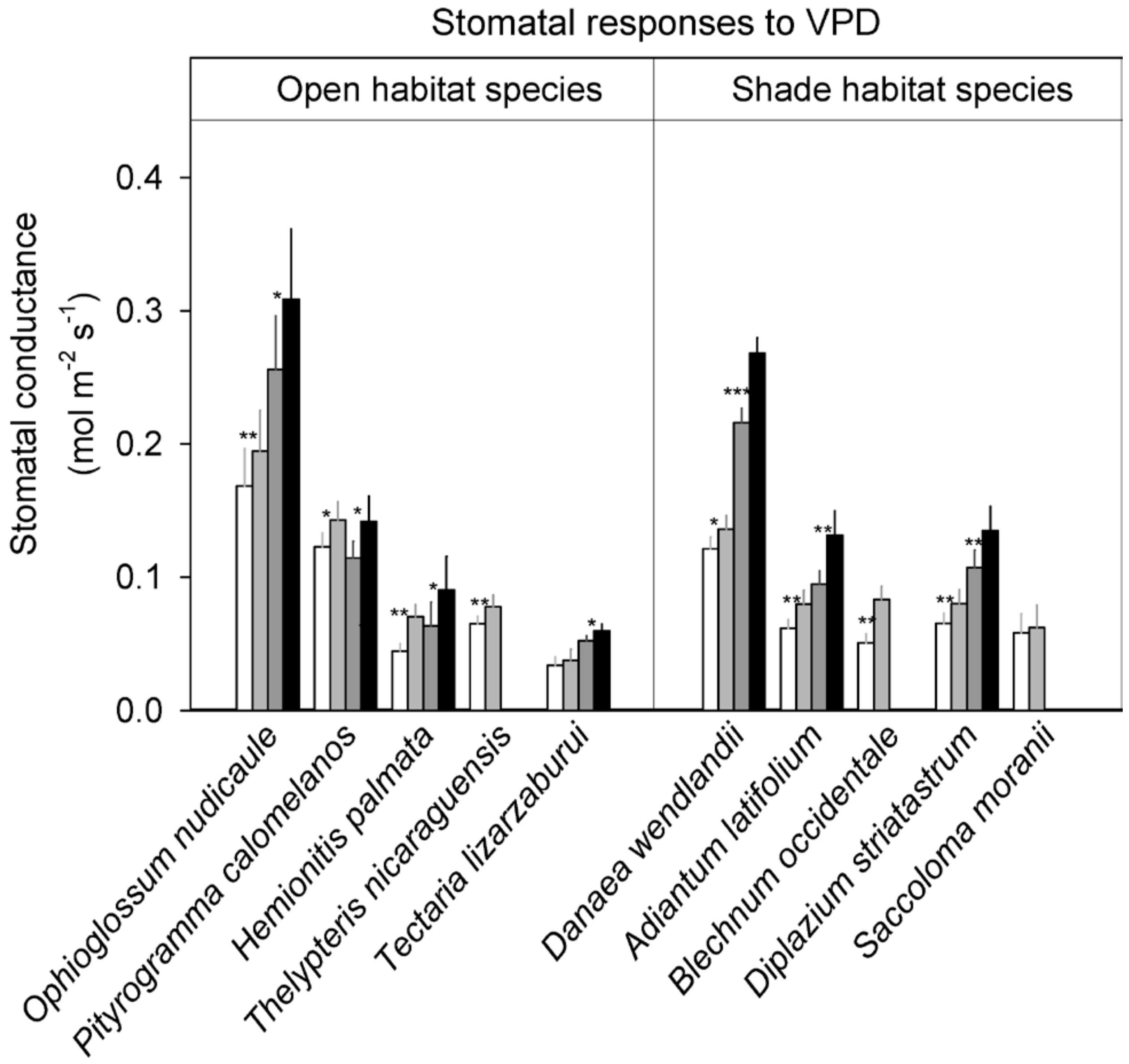

Stomatal responses to VPD
Open habitat species
Shade habitat species
Stomatal conductance
(mol m^{-2} s^{-1})
0.4
0.3
0.2
0.1
0.0
**
*
*
*
**
*
**
*
*
***
**
**
**
**
**
Ophioglossum nudicaule
Pityrogramma calomelanos
Hemionitis palmata
Thelypteris nicaraguensis
Tectaria lizarzaburui
Danaea wendlandii
Adiantum latifolium
Blechnum occidentale
Diplazium striatastrum
Saccoloma moranii

Fig 5.4

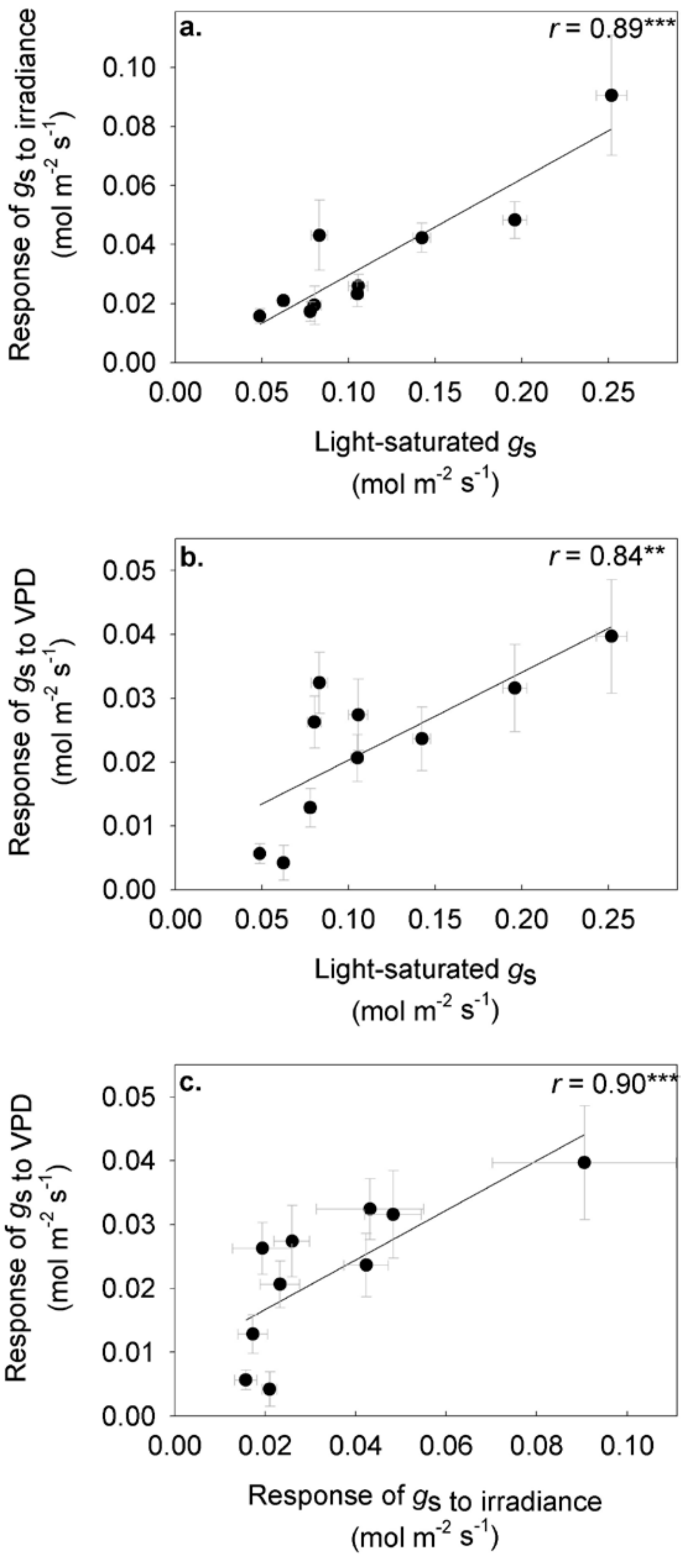

a.
r = 0.89***
Response of gs to irradiance (mol m-2 s-1)
0.00
0.02
0.04
0.06
0.08
0.10
0.00
0.05
0.10
0.15
0.20
0.25
Light-saturated gs (mol m-2 s-1)
b.
r = 0.84**
Response of gs to VPD (mol m-2 s-1)
0.00
0.01
0.02
0.03
0.04
0.05
0.00
0.05
0.10
0.15
0.20
0.25
Light-saturated gs (mol m-2 s-1)
c.
r = 0.90***
Response of gs to VPD (mol m-2 s-1)
0.00
0.01
0.02
0.03
0.04
0.05
0.00
0.02
0.04
0.06
0.08
0.10
Response of gs to irradiance (mol m-2 s-1)

Fig 5.5

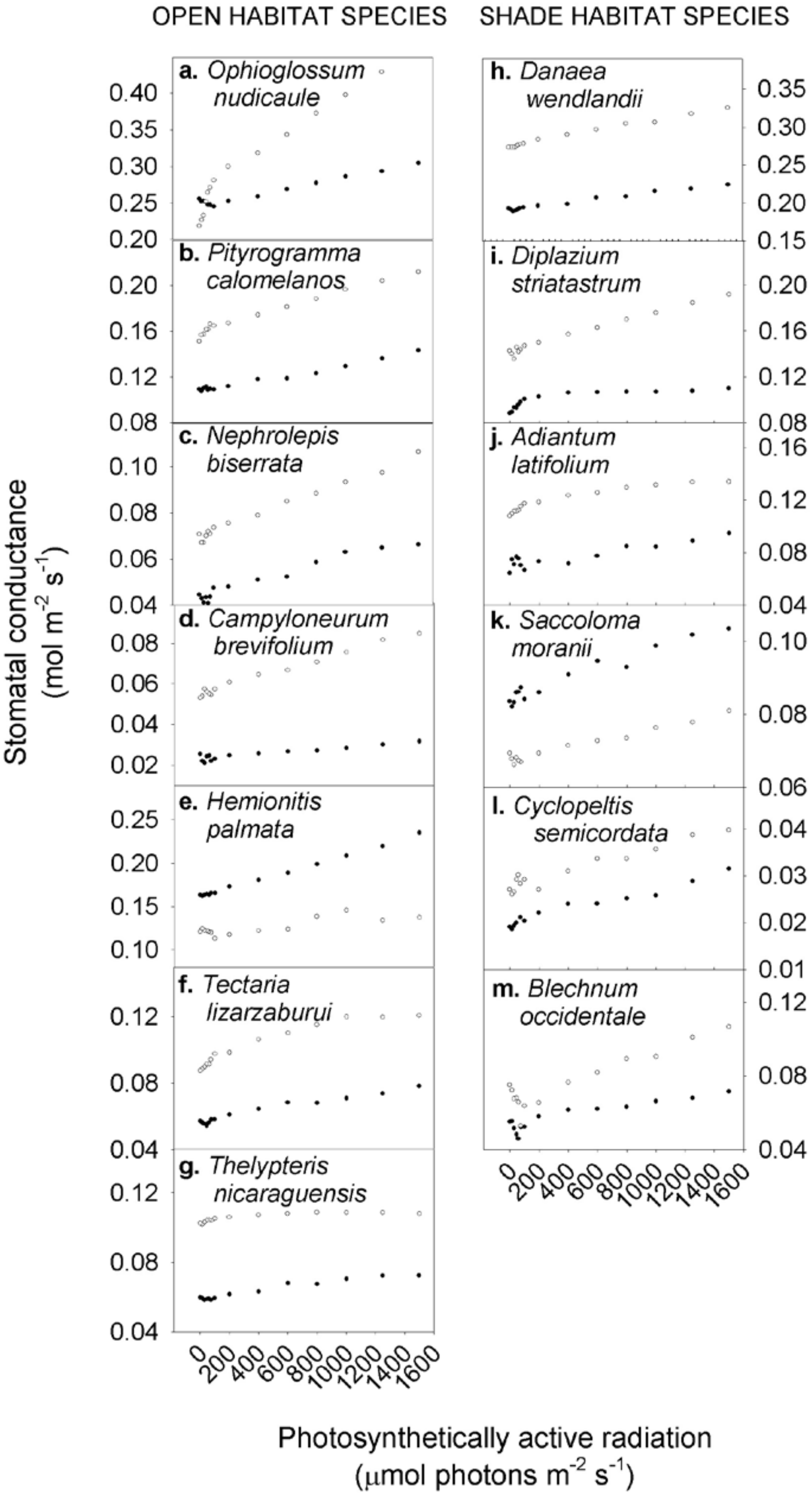

OPEN HABITAT SPECIES
SHADE HABITAT SPECIES
a. Ophioglossum nudicaule
b. Pityrogramma calomelanos
c. Nephrolepis biserrata
d. Campyloneurum brevifolium
e. Hemionitis palmata
f. Tectaria lizarzaburui
g. Thelypteris nicaraguensis
h. Danaea wendlandii
i. Diplazium striatastrum
j. Adiantum latifolium
k. Saccoloma moranii
l. Cyclopeltis semicordata
m. Blechnum occidentale
Stomatal conductance (mol m-2 s-1)
Photosynthetically active radiation (μmol photons m-2 s-1)
0
200
400
600
800
1000
1200
1400
1600

Fig 5.6

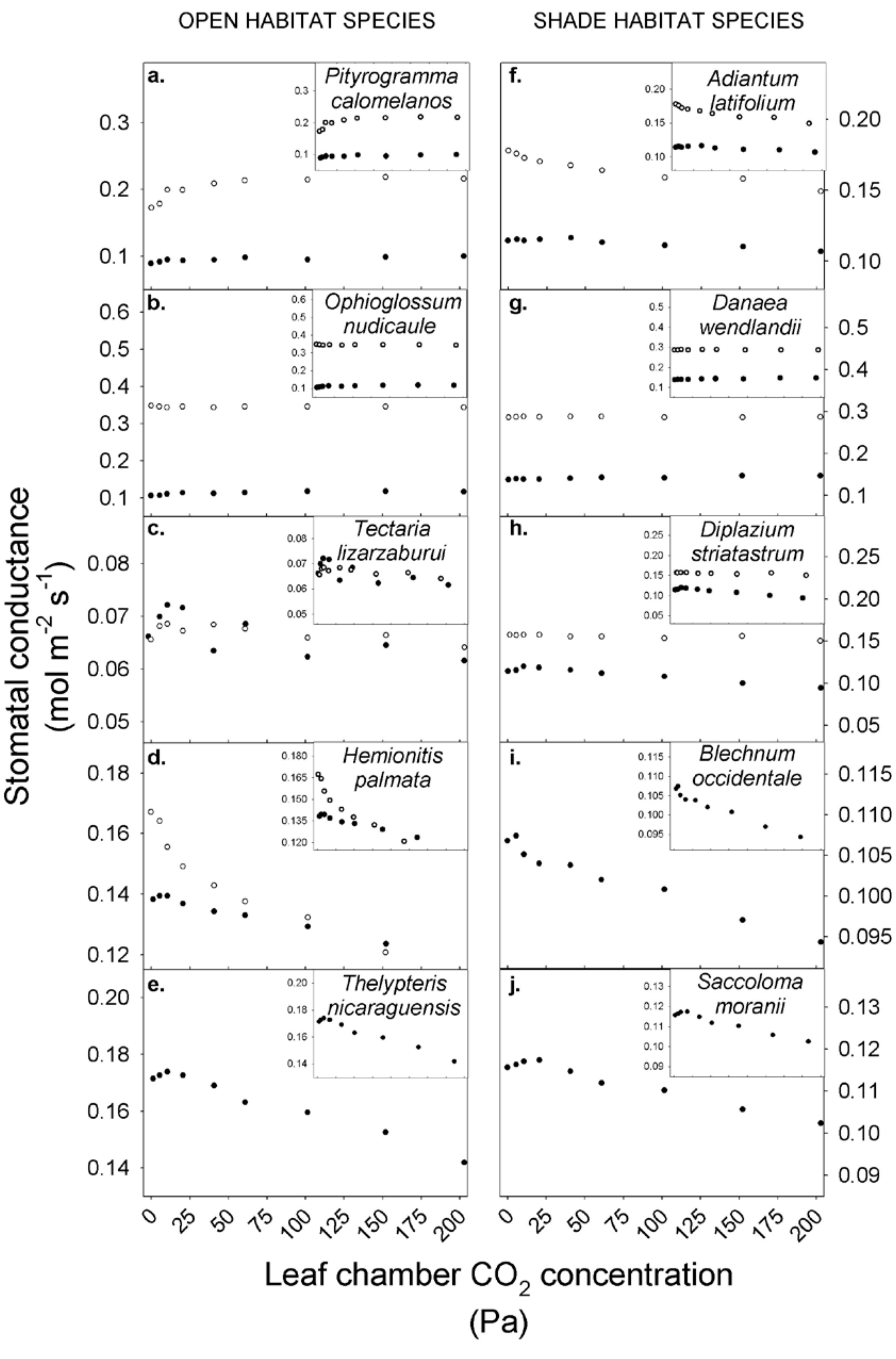

OPEN HABITAT SPECIES
SHADE HABITAT SPECIES
a.
Pityrogramma calomelanos
b.
Ophioglossum nudicaule
c.
Tectaria lizarzaburui
d.
Hemionitis palmata
e.
Thelypteris nicaraguensis
f.
Adiantum latifolium
g.
Danaea wendlandii
h.
Diplazium striatastrum
i.
Blechnum occidentale
j.
Saccoloma moranii
0.1
0.2
0.3
0.1
0.2
0.3
0.4
0.5
0.6
0.05
0.06
0.07
0.08
0.12
0.14
0.16
0.18
0.14
0.16
0.18
0.20
0.10
0.15
0.20
0.1
0.2
0.3
0.4
0.5
0.05
0.10
0.15
0.20
0.25
0.095
0.100
0.105
0.110
0.115
0.09
0.10
0.11
0.12
0.13
Stomatal conductance (mol m^{-2} s^{-1})
0
25
50
75
100
125
150
175
200
Leaf chamber CO_2 concentration (Pa)

Fig 5.7

SUPPLEMENTAL MATERIAL

SUPPLEMENTARY TABLE CAPTIONS

SuppTable 5.1 Families, species, habitat type and irradiance treatment ranked in phylogenetic order (PHYLO RANK) based on Smith et al. (2006) from most recently derived (top) to most ancient (bottom), with paired t-test results for significant differences in stomatal conductance (g_s) at minimum and maximum environmental signal strength in response to increasing irradiance (0 – 1500 PAR), increasing CO_2 concentration (0 – 200 Pa), and increasing vapor pressure deficit (VPD; 1 – 2 kPa). Symbol "--" represents insufficient data for statistical comparison; *** $P \leq 0.001$ ** $P \leq 0.01$ * $P \leq 0.05$ † $P < 0.09$.

SuppTable 5.2 Minimum, mean and standard deviation, maximum trait values from vapor pressure deficit (VPD) short-term responses for plants grouped by habitat origin (open vs. shade), and for plants grouped by irradiance treatment (high vs. low) with significance values from three level nested ANOVA of habitat<species<treatment; *** $P \leq 0.001$ ** $P \leq 0.01$ * $P \leq 0.05$. Trait legend included at right.

SuppTable 5.3 Minimum, mean and standard deviation, maximum trait values from irradiance short-term responses for plants grouped by habitat origin (open vs. shade), and for plants grouped by irradiance treatment (high vs. low) with *P*-values from three level nested ANOVA of habitat<species<treatment; *** $P \leq 0.001$ ** $P \leq 0.01$ * $P \leq 0.05$. Trait legend included at right.

SuppTable 5.4 Minimum, mean and standard deviation, maximum trait values from CO_2 short-term responses for plants grouped by habitat origin (open vs. shade), and for plants grouped by irradiance treatment (high vs. low) with *P*-values from three level nested ANOVA of habitat<species<treatment; *** P ≤ 0.001 ** P ≤ 0.01 * P ≤ 0.05. Trait legend included at right.

SuppTable 5.5 Correlation matrix with correlation coefficients from left to right for Pearson test on untransformed data, Pearson test on log-transformed data, and Spearman rank test, on variables from irradiance short-term responses in yellow, variables from vapor pressure deficit (VPD) short-term responses in orange, and variables from CO_2 short-term responses in blue; green highlighted cells indicate trait relationships significant for at least two of the three correlation tests; *** P ≤ 0.001 ** P ≤ 0.01 * P ≤ 0.05. Trait legend included at right.

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CHAPTER 6

THE PLASTICITY AND ADAPTATION OF LEAF FORM AND PHOTOSYNTHESIS IN TROPICAL FERNS

ABSTRACT

Shifts in leaf form and photosynthesis physiology in response to light have been well quantified in angiosperms, but scarcely in ferns, a distinct major lineage with independent leaf evolution. We examined 13 phylogenetically diverse tropical rainforest fern species native to sun and shade habitats in high and low irradiance treatments in a common garden to test hypotheses of adaptive and plastic differences in leaf functional traits associated with habitat versus growth irradiance, and collected data on leaf traits and light distributions for the 30 most common understory fern species at La Selva Biologial Station, Costa Rica. We tested for plastic differences in leaf form, composition, nutrients, and photosynthetic physiology with growth irradiance, and for potentially adaptive differences between sun and shade species. We also tested for correlations of leaf mass per area (*LMA*) with nutrient and flux traits. These tropical ferns showed strong adaptive and plastic differences with irradiance parallel to those observed in angiosperms, as well as key differences in optimization of carbon assimilation. Considered on a leaf area basis, high irradiance grown ferns showed faster rates of photosynthesis, respiration, higher irradiance compensation (I_c) and saturation (I_s) points, and faster maximum carboxylation (V_{cmax}), and electron transport (J_{max}) rates. Shifts between ferns native to low versus high irradiance habitats were parallel to plastic changes with growth irradiance, but for a smaller set of traits; with no difference in 11 traits. Differences in *LMA*, N per mass (N_{mass}), chlorophyll (*Chl*) concentration, *Chl/N*, *C/N*, quantum efficiency (*QE*), and ratios of V_{cmax} and J_{max} to leaf N were only associated

with acclimation to irradiance, and with comparable photosynthetic rates per mass across the growth irradiance treatments. By contrast, J_{max}/V_{cmax} only differed by habitat, with shade species exhibiting a higher J_{max}/V_{cmax}, potentially due to inherited differences in species photosynthetic sensitivity to CO_2. Leaf area, *LMA*, and lifetime integrated water-use-efficiency (*WUE*) tended in different directions for shade habitat species versus low irradiance grown plants, further demonstrating key differences in the adaptation versus acclimation responses of ferns to irradiance environment. Investment in high *LMA* was strongly correlated with lower N and photosynthesis per mass in accordance with global trends for the angiosperm leaf economics spectrum, as well as greater *WUE*. These results indicate convergence among angiosperms and ferns in their overall plasticity and adaptation to irradiance, as well as the ways in which their leaf traits shift in different directions for plastic versus adaptive responses to light.

INTRODUCTION

Ferns are a highly speciose radiation of land plants that dominated terrestrial biomes 350 mya, and still exhibit the greatest diversity in leaf form today. Previous studies of variation in leaf form and function focused mainly on the currently dominant angiosperms (e.g., Ackerly and Reich 1999; Wright et al. 2004; Peppe et al. 2011; Scoffoni et al. 2011; Sack et al. 2012). However, determining how ferns vary in leaf composition and physiology across environmental resource gradients is important to better inform models of ecosystem responses to environmental change, as the habitat specificity of ferns makes them excellent indicator species (e.g., Andama et al. 2003; Chang et al. 2009). Also, determining patterns for ferns will indicate recurring evolutionary trajectories or contrasts in optimization (e.g., Brodribb and McAdam 2011; Pittermann et al. 2011; Franks et al. 2012; Haworth et al. 2013; McAdam and Brodribb 2013).

Further, it has been unclear how much of the variation in fern leaf traits is related to adaptation to particular habitats and/or to acclimation to growth conditions. Angiosperms have shown strong variation in leaf traits in response to irradiance at growth and evolutionary timescales (e.g., Walters and Reich 1999; Evans and Poorter 2001; Poorter et al. 2009; Marino et al. 2010; Scoffoni et al. 2011). To test for hypothesized adaptive differences in fern leaf traits associated with habitat openness, and for variation in the capacity of fern species to plastically adjust their leaf traits as an acclimation response to growth irradiance, we designed a common garden experiment using seven open habitat species and six shade habitat species (Table 6.1) grown under high and low irradiance treatments. We tested a priori hypotheses of leaf trait associations with irradiance environment based on trends reported for angiosperms. A recent study found these for mosses (Waite and Sack 2011a).

We hypothesized that variation in fern leaf form, composition, nutrients and physiological traits related to photosynthetic performance (Table 6.2) would be strongly related to both irradiance habitat and growth irradiance to maximize carbon assimilation. Consistent with exposure to low irradiance in angiosperms (e.g., Givnish 1988; Reich et al. 1992; Kull and Niinemets 1998; Walters and Reich 1999; Evans and Poorter 2001; Hogewoning et al. 2010), we expected compensation for light limitation in shade habitat species and low irradiance grown ferns via greater investment to benefit net leaf photosynthesis at low light levels, including lower respiration rates, lower photosynthetic irradiance compensation (I_c) and saturation (I_s) points, larger leaves with greater relative surface area relative to support mass (i.e., lower leaf mass per area, *LMA*), higher concentrations of chlorophyll and N, a higher *Chl/N* ratio, and lower C and *C/N*. For ferns from the high irradiance habitat and growth treatment, to reduce vulnerability to desiccation we expected higher *LMA* and a less negative carbon isotope ratio (∂^{13}C), which may indicate lifetime integrated water-use-efficiency (*WUE*, see Methods).

For physiological traits related to photosynthetic performance, we expected faster light-saturated rates of photosynthesis ($A_{\max}$) and dark respiration (R_d) rates per area and per mass for high irradiance grown plants. Associated with the faster photosynthetic rates for high irradiance grown plants, we expected higher I_c, I_s, quantum efficiency (*QE*), maximum carboxylation rate (V_{cmax}), photosynthetic electron transport rate ($J_{\max}$), and greater ratios of RuBP carboxylation and electron transport relative to leaf N (V_{cmax}/N, $J_{\max}/N$) (e.g., Evans et al. 2000). We also tested whether ferns, similar to angiosperms and gymnosperms, would maintain a proportional investment into RuBP regeneration and carboxylation ($J_{\max}/V_{\mathrm{cmax}}$) across growth irradiances (e.g., Tinoco-Ojanguren and Pearcy 1995; Wohlfahrt et al. 1999). We expected similar shifts in traits between species native to deeply shaded versus open habitats.

Finally, given global leaf economic trends of increased investment in leaf durability and longevity at the expense of carbon assimilation rates (Wright et al. 2004; Shipley et al. 2006; Karst and Lechowicz 2007; Waite and Sack 2011a; Lloyd et al. 2013; Osnas et al. 2013; Westoby et al. 2013) we predicted that species with high *LMA* would show a correlated high N per area (N_{area}), but low N per mass (N_{mass}) and light-saturated photosynthesis per mass (A_{maxmass}), as well as higher *WUE* to support longer leaf lifespan by helping to avoid desiccation (Ni and Pallardy 1991; Edwards et al. 2012; Creese and Sack *in prep-a*). These tests would establish convergent evolution of leaf form and photosynthetic light responses in ferns as previously shown for angiosperms and mosses.

MATERIALS AND METHODS

Common garden experimental design

Thirteen fern species from tropical lowland rainforest were cultivated in shadehouses providing two light environments at La Selva Biological Station, Costa Rica (84°00'12.922"W, 10°25'52.610"N). From August to September, 2010 we collected sixteen individuals of each species from the forest along with their local clay loam soil (Eutric Hapludand Andisol; Weitz et al. 1997) to minimize transplant shock. We included seven species from open habitats and six species from shaded habitats (Table 6.1), and collected study individuals from at least three subpopulations per species separated by 0.5 to 2 km. To avoid pseudoreplication, irradiance treatments were imposed with two replicate shadehouses for each irradiance treatment using layered 50% "aluminet" shade cloth (Aluminet Reforzado; CNBM, Beijing, China), a material that improves the proportion of diffuse light, thus favoring net photosynthesis and growth (Markvart et al. 2010), and reflects the non-transmitted light, thus reducing cloth and air temperatures inside the shadehouses (Bailey 1981). The % of daylight photosynthetically active radiation (PAR) transmitted into the shadehouse was determined at noon on a typical cloudy day by averaging light meter measurements (LI-250A, LI-COR, Lincoln, NE, USA) across the shadehouse benches and dividing by simultaneous measurements taken in a clearing outside the shadehouses. To reduce shock, all plants were acclimated under medium irradiance (10% daylight PAR) for six months before transferring to high irradiance (20% daylight PAR) and low irradiance (1.5 % daylight PAR) growth environments. Temperature and humidity were similar within and across the high and low irradiance growth treatments, and tracked the climate outside the shadehouses: midday measurements using the LI-COR 6400 (LI-COR, Lincoln, NE, USA) of air temperature and relative humidity (RH) averaged for five typical days were 28.0 ± 0.8 °C and

77.0 ± 6.2 % RH respectively in the high irradiance treatment and 28.5 ± 0.8 °C and 77.3 ± 6.8 % RH for the low irradiance treatment, and 29.1 ± 0.8 °C and 71.6 ± 7.2 % RH immediately outside the shadehouses. We randomized plants across benches initially and re-randomized their locations across benches monthly to avoid any potential block effects within the treatments. Soil was kept moist by daily rainfall and additional watering on days without precipitation. Plants acclimated for three months in the experimental irradiance treatments and had established new leaves before measurements.

Field sampling of the most common understory fern species

We additionally sampled 30 common fern species from the understory at La Selva Biological Station, which included an overlap of seven species from the common garden (SuppTable 6.4). For at least five individuals per species, we obtained leaf form and nutrient data (see below), and quantified site irradiance by averaging at least five PAR measurements using a light meter (LI-250A, LI-COR, Lincoln, NE, USA) at midday under diffuse light conditions. Species were grouped into three habitat light categories: "low light" (≤ 2 % PAR), "medium light" (2 – 5 % PAR), and "high light" (> 5 % PAR; SuppTable 6.4).

Measurement of gas exchange responses

From June through November 2011, we measured photosynthetic responses to irradiance and CO_2 by constructing light and CO_2 response curves with a LI-COR 6400 photosynthesis system (LI-COR, Lincoln, NE, USA).

We measured light response curves of net photosynthetic assimilation rate (*A*) for 5-6 leaves from 4-6 different individuals in each irradiance treatment for all 13 species. To approximate ambient conditions and maximize stomatal opening, we used moderate flow rates (300-400 µmol m^{-2} s^{-1}), maintained a leaf block temperature of 29°C and VPD of 1 kPa, and to fully induce maximum photosynthetic rates, we acclimated leaves at 1000 µmol m^{-2} s^{-1} PAR for 15-30 minutes until stomatal conductance (g_s) and A were stable (i.e., no longer increasing or decreasing for at least five minutes, and the coefficient of variation < 0.1 before commencing 300 s step changes in photosynthetic photon flux density (PPFD): 1000, 1500, 1250, 1000, 800, 600, 400, 200, 100, 75, 60, 45, 15 and 0 PPFD (Givnish et al. 2004; Funk and Vitousek 2007; Evans and Santiago 2012). Leaves were returned to ambient conditions for at least 10 min until A and g_s restabilized, then were held in darkness for at least 10 min for measurement of dark respiration (R_d).

We measured the responses of A to changes in ambient CO_2 for 5-6 leaves from 4-6 individuals in each irradiance treatment. We acclimated leaves as for the light responses, except that chamber CO_2 concentration was set to 5 Pa for at least 20 min until g_s and A were stable (as above) before 300 s step changes: 0, 5, 10, 20, 40, 60, 100, 150, 200 Pa (Bernacchi et al. 2003; Long and Bernacchi 2003; Evans and Santiago 2012). Protocols were developed to allow measurements to be made rapidly to avoid potentially confounding effects of time of day and to ensure sufficient time for full induction to high and low light to and high and low CO_2. While other measurements would be feasible, such as a single step transition from high to low stimulus intensity, we do not expect this to change our findings.

For each CO_2 response curve we determined maximum CO_2 saturated photosynthetic rate (A_{max}), maximum carboyxlation ($V_{c,max}$), electron transport (J_{max}) and dark respiration (R_d) rates. We determined $V_{c,max}$ and J_{max} at 25°C (using PS-FIT_7.3; Farquhar et al. 1980; Bernacchi et al. 2003; Long and Bernacchi 2003).

We fitted light response curves to plots of A versus PAR (Sigmaplot 10.0; Systat Software, Inc., San Jose, CA, USA) as an exponential rise to maximum (Iqbal et al. 1996):

$$A = A\max - ae^{-bx} \qquad \text{eqn 1}$$

Where $A_{\max}$, a and b are fitted parameters. Given the fitted equation for each species, we estimated the PAR leading to 90% saturation of A as saturation irradiance (I_s). To determine further light-response parameters associated with low irradiance we fitted a linear regression to the relationship between A and PAR at low irradiance (30-75 µmol m^{-2} s^{-1}) and determined the irradiance compensation point (I_c) as the x-intercept, the quantum efficiency of CO_2 assimilation per area (QE) as the slope, and the dark respiration rate per area (R_d) as the y-intercept, i.e., the negative A at zero PAR.

To normalize values for chamber leaf area, the leaf area inside the chamber was traced on a transparent acetate sheet, scanned and measured using Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA). Leaf mass-based assimilation rate ($A_{\text{max,mass}}$), quantum efficiency (QE_{mass}), carboxylation ($V_{\text{cmax,mass}}$), electron transport ($J_{\text{max,mass}}$), and dark respiration ($R_{\text{d,mass}}$) were determined by dividing their values per area by the leaflet mass per area (see following section). We then divided $J_{\text{max,mass}}$ by $V_{\text{c,max,mass}}$ to obtain the ratio of electron transport rate to carboxylation efficiency ($J_{\max}/V_{\text{c,max}}$), and divided $V_{\text{c,max,mass}}$ and $J_{\text{max,mass}}$ by lamina nitrogen concentration per mass (N_{mass}; see following section) to obtain V_{cmax}/N and $J_{\max}/N$.

Leaf form, composition and nutrient composition

Plants were harvested December 2011 following completion of gas exchange measurements to quantify differences in leaf and leaflet form, composition, and nutrient composition. Hereafter fern leaflets are referred to as ultimate segments (USEGs), the highest order division of photosynthetic lamina tissue for a leaf (Creese and Sack *in prep-b*). In our study the USEG represented the lamina of an entire (non-pinnatifid) leaf, or the highest order division of lamina for a 1- or 2-pinnately compound leaf. Determining traits at the USEG-level removes any potential confounding effects of rachis tissues when considering traits such as nutrient concentrations (Creese and Sack *in prep-b*) and mass-based photosynthetic traits. To measure leaf and USEG areas we used a leaf area meter (LI-COR 3100; LI-COR Biosciences, Lincoln, NE, USA). Chlorophyll concentration (*Chl*) was averaged from at least three measurements taken from the central lamina (excluding the primary vein) of each leaf using a chlorophyll meter (model SPAD-502, Konica Minolta, New Jersey, USA), and divided by USEG mass per area (*UMA*) to obtain concentration per mass (Chl_{mass}); we then divided Chl_{mass} by lamina nitrogen concentration per mass (N_{mass}) to obtain *Chl*/*N*. For a given species, SPAD measurements are linearly related to total chlorophyll (a + b) per unit area (Marquard and Tipton 1987; Singha and Townsend 1989; Fanizza et al. 1991; Manetas et al. 1998). To determine leaf mass per area (*LMA*), which included stipe and rachis tissues, and USEG mass per area (*UMA*), which included only the photosynthetic lamina tissue, leaves were oven dried at 70 ºC for at least one week before mass measurements with an analytical balance (Mettler Toledo, Columbus, OH, USA). Dried lamina tissue was then analysed by accelerator mass spectrometry (UC Davis Stable Isotope Facility, California, USA) to determine nutrient concentrations and isotope ratios including C, N, C/N, and ∂^{13}C, a measure of lifetime integrated *WUE* (see above). These nutrient analyses were performed using a Thermo GC/C-IRMS system composed of a Trace GC Ultra gas chromatograph (Thermo Electron Corp., Milan, Italy) coupled to a Delta V Advantage isotope ratio mass spectrometer through a GC/C-III interface (Thermo Electron Corp., Bremen, Germany). Although we measured instantaneous water-use-efficiency (*WUE* = *A* / transpiration), we analyzed $\partial^{13}C$ to reflect a longer-term integrated *WUE* as smaller stomatal apertures and persistently lower stomatal conductance discriminate against the influx of heavier ^{13}C isotopes (Farquhar and Richards 1984) to provide a more reliable indicator of species' tendencies for stomatal behavior. All area-based flux traits and nutrient concentrations were additionally divided by *UMA* for expression per unit mass. Because leaf identity for gas exchange measurements and functional traits from the subsequent harvest were not always identical due to leaf turnover, flux traits per mass, V_{cmax}/N, and J_{max}/N, were calculated at the species × treatment level (Table 6.2).

Statistical analysis

We tested for significant differences in leaf traits using untransformed and log-transformed data to improve normality. To test for significant differences across species, habitat, and irradiance treatments (SuppTable 6.1), we used a three level nested general linear model with irradiance treatment nested within species identity nested within habitat irradiance and reported P values from the untransformed or transformed data that provided the most robust significance (Minitab 16 Statistical Software 2010, Minitab, Inc., State College, PA).

For composite traits calculated from two traits where the leaf identity was not always identical (e.g., $A_{max,mass} = A_{max} / UMA$), we calculated mean values at the species × treatment level, and to account for potential differences in variance, we calculated standard error using propagation of error (Beers 1957):

$$\frac{\partial z}{z} = \left(\frac{\partial x}{x}\right) + \left(\frac{\partial y}{y}\right) \qquad \text{eqn 2}$$

Where ∂z and z respectively are the standard error and mean for trait z, which is calculated by dividing x by y, determined by the standard errors and means of the input variables x and y, when. To test for significant differences in leaf traits determined at the species × treatment level (see above), we used a one-way ANOVA to test for differences due to species identity, and paired *t*-tests to assess differences due to growth irradiance treatment (SuppTable 6.1).

To test for correlations among leaf traits associated with the worldwide leaf economics spectrum (e.g., Wright et al. 2004), we used Pearson and Spearman correlations on species means for each irradiance treatment (R Development Core Team, 2010, R Foundation for Statistical Computing, Vienna, Austria). We considered trait relationships to be robustly significant if they had been hypothesized a priori (see Introduction), and if P was ≤ 0.05 for at least two of the three correlation tests (Pearson correlation on untransformed data, Pearson correlation on log-transformed data, Spearman correlation on ranked data). We present correlation matrices of all variables for the high and low irradiance grown plants (SuppTable 6.2) so that readers may examine the intercorrelative structure of our variables; however, we do not endorse significant trait relationships that were not previously hypothesized, and would recommend statistical correction for any such "mining" of relationships given the greater risk of type I error (Garcia 2003; Moran 2003; Givnish et al. 2004; Edwards 2006; Waite and Sack 2009).

RESULTS

Variation in fern leaf traits

The fern species in our study exhibited extraordinary variation in leaf form, composition, nutrients, and photosynthetic physiology, with species × treatment values showing 3-fold to over 1000-fold variation in form and composition traits, 1 to 4-fold variation in nutrient compositional traits, and 1 to 20-fold variation in physiological traits related to photosynthetic performance

(Figs 6.1-6.6, SuppTables 6.1-6.3). Notably, physiological traits were more variable when expressed on a leaf mass basis than on a leaf area basis (SuppTable 6.1).

Shifts in fern leaf form and physiology with growth irradiance

Ferns showed strong plasticity in photosynthetic physiology traits. Species from high irradiance grown plants showed faster photosynthetic and respiration rates per area ($P < 0.05$, Figs 6.4, 6.5 SuppTable 6.1). The same tendencies applied for photosynthetic and respiration rates per mass ($P < 0.05$, SuppTable 6.1), and hereafter, significant differences between traits associated with irradiance environment, and significant trait-trait correlations refer to variables on both a leaf area- and leaf mass-basis unless otherwise noted. As expected, I_c, I_s, QE, V_{cmax}, and J_{max} were also significantly higher for high irradiance grown plants ($P < 0.001$, Figs 6.4, 6.5, SuppTable 6.1). Notably, $V_{cmax,mass}$ did not vary significantly with irradiance, though $J_{max,mass}$, V_{cmax}/N, and J_{max}/N were significantly greater for high irradiance grown plants ($P < 0.05$, SuppTable 6.1).

Fern leaf form and composition also showed strong plasticity in response to growth irradiance (SuppTable 6.1). Plants grown in the low irradiance treatment showed higher leaf and USEG areas ($P < 0.001$, Fig 6.3, SuppTable 6.1). For low irradiance grown plants, *LMA* (and *UMA*) was significantly lower, and chlorophyll concentration was significantly higher ($P < 0.001$, Fig 6.3, SuppTable 6.1). Additionally, low irradiance grown plants showed significantly lower C_{area}, N_{area} C/N, ∂^{13}C, a higher N_{mass} and *Chl/N* ($P < 0.05$, Fig 6.3, SuppTable 6.1).

Adaptive shifts in fern leaf form and physiology for species native to low and high irradiance

Ferns showed strong associations of photosynthetic physiology traits with native habitat irradiance. Species from open habitats showed faster photosynthetic and respiration rates per area ($P < 0.05$, SuppTable 6.1). As expected, I_c, I_s, V_{cmax}, and J_{max} were also significantly higher for open habitat species ($P < 0.001$, Figs 6.4, 6.5, SuppTable 6.1), while J_{max}/V_{cmax} was significantly higher for shade habitat species ($P < 0.001$, SuppTable 6.1); differences in *QE* were not significant ($P > 0.05$, Fig 6.4, SuppTable 6.1).

Leaf form varied in opposite directions for habitat type versus growth irradiance, with species from shade habitats exhibiting smaller leaf and USEG areas, whereas plants grown in the low irradiance treatment showed higher leaf and USEG areas (Fig 6.3, SuppTable 6.1). With respect to leaf composition, there was no significant difference in *LMA* or chlorophyll concentration between open and shade habitat species, whereas for low irradiance grown plants, *LMA* (and *UMA*) was significantly lower, and chlorophyll concentration was significantly higher (SuppTable 6.1). Leaf nutrient traits were not significantly associated with habitat type, except for a higher $\partial^{13}C$ in shade habitat species ($P < 0.001$, SuppTable 6.1).

Correlations of leaf traits across species within irradiance treatment groups

As expected, *LMA* and *UMA* were significantly negatively correlated with N_{mass}, and $A_{max,mass}$ ($r = -0.80$ to -0.84, $P < 0.01$) across high and low irradiance grown ferns, though only *UMA* was positively correlated with N_{area} ($r = 0.58$, $P < 0.001$, SuppTable 6.2). Leaf mass per area was also positively correlated with *WUE* for the low irradiance grown species ($r = 0.59$-0.62, $P < 0.01$, SuppTable 6.2).

Differences in leaf traits between habitat light categories for the 30 common fern species

Trends in leaf form, composition, and nutrient traits for the 30 common fern species paralleled those found in the common garden study, with greater leaf area in the higher light species (though USEG area was greater in low and medium light groups), greater *LMA* for the low and medium light groups, greatest chlorophyll concentration for medium light species, and higher ∂^{13}C for the highest and lowest irradiance species.

DISCUSSION

Shifts in fern leaf traits with habitat openness and growth irradiance: convergence with angiosperms

Differences in fern leaf traits across habitats and growth irradiances revealed strong commonalities in trait shifts between adaptation and acclimation in response to irradiance. The variation related to irradiance explained a large proportion of an exceptional overall variation in form, physiology and composition, as the fern species in our study exhibited 1 to 1080-fold variation in leaf form, composition, nutrients, and photosynthetic physiology traits, with most variation in leaf form and physiology. Notably, physiological traits per mass were more variable than these traits on a per area basis, indicating an important role for leaf (and USEG) mass per area in driving fern physiological responses to irradiance. Variation in each group of leaf traits was strongly associated with habitat type and growth irradiance. Overall, more traits showed plastic shifts than significant differences on average between species adapted to low and high irradiance. In certain cases traits showed shifts in different directions between adaptation and plasticity in response to irradiance, or a shift in only one case, indicating that traits are differently constrained at growth and evolutionary timescales. Strikingly, some of these opposite shifts for plasticity vs. adaptation are analogous to those previously found for angiosperms, indicating a very high level of convergence in these two major terrestrial plant lineages, in their independent leaf evolution across light gradients.

Leaf photosynthetic physiology varied distinctly with habitat openness versus growth irradiance

As hypothesized, open habitat species and high irradiance grown plants showed faster rates of carbon assimilation and respiration per leaf area under high irradiance. However, physiological traits were variably associated with habitat and growth irradiance, suggesting key differences in the adaptability and plasticity of tropical fern leaf traits. In tandem with faster respiration and photosynthetic rates per area, open habitat species and high irradiance grown plants showed higher I_c, I_s, V_{cmax}, and J_{max} consistent with optimization of carbon assimilation per area at the leaf-level. Additionally, QE, $J_{max,mass}$, V_{cmax}/N, J_{max}/N were higher for high irradiance grown plants, but $A_{max,mass}$ was similar across growth treatments. This suggests that modifications to *LMA* are especially important to help compensate for reduced photosynthesis per area under low growth irradiance to optimize carbon assimilation on a per mass basis.

We hypothesized that the proportional investment in RuBP regeneration to carboxylation (J_{max}/V_{cmax}) would be similar across growth irradiances as seen for angiosperms (Kull and Niinemets 1998; Hikosaka 2005), and indeed, J_{max}/V_{cmax} was maintained for plants of species grown across high and low irradiance treatments. The J_{max}/V_{cmax} was significantly higher for shade habitat species compared with open habitat species. Shifts in the balance between J_{max} and V_{cmax} influence the rate of photosynthesis at low versus high CO_2 because photosynthetic rate is more limited by CO_2 given greater reliance on RuBP carboxylation than regeneration. Thus, higher J_{max}/V_{cmax} increases the relative rate of photosynthesis at high CO_2 compared to that at low CO_2 (Sage 1994; Medlyn 1996), and is believed to enhance the CO_2 stimulation of photosynthesis (c.f. Lewis et al. 1996; Medlyn et al. 1999; Onoda et al. 2005). Differences in photosynthetic sensitivity to CO_2 in these fern species may be related to their evolutionary history, as our species range from the most ancient to recently derived clades, and the fern lineage originated in the Devonian under a CO_2 concentration that may have been ten to twenty times higher than ambient levels today (Berner 1993; Royer 2006). Further, stomatal sensitivity to CO_2 was shown to be highly variable among the tropical fern species in our study, with a representative of the ancient fern genus *Ophioglossum* (Smith et al. 2006) showing unexpected stomatal opening in response to high CO_2 (Creese et al. *in prep*). Such differences in the sensitivity of fern species to CO_2 across habitats could lead to strongly divergent responses of open versus shade adapted fern species to rising levels of CO_2. Greater species-level resolution of the fern phylogeny is needed to facilitate comparative phylogenetic studies of fern photosynthetic biochemistry to explore mechanisms that may be driving differences in stomatal and photosynthetic responses to CO_2, and species differences in the balance of RuBP regeneration and carboxylation processes across high and low irradiance adapted ferns.

Leaf form, composition, and nutrients vary distinctly with habitat openness versus growth irradiance

We hypothesized that to maximize carbon gain under low irradiance, shade habitat species and plants grown under low irradiance would exhibit larger leaves and leaflets (USEGs), higher chlorophyll concentrations, and lower *LMA* (and *UMA*, hereafter indicated by mention of *LMA*). As expected, low irradiance grown plants increased leaf area and chlorophyll concentrations and decreased *LMA* and *WUE* (as measured by $\partial^{13}C$); however, shade habitat species showed smaller leaf areas, greater *WUE*, and no significant differences in *LMA* or chlorophyll concentration compared with open habitat species. These contrasting trends are similar to those in shade adapted evergreen angiosperm species to optimize carbon balance at the whole-plant level through slower growth and greater investment in leaf durability and lifespan associated with higher *LMA* (Walters and Reich 1999, Lusk et al. 2008): these shade tolerant species have smaller leaves of higher *LMA* than light demanding species – the opposite shift that occurs during a plasticity response to low vs. high irradiance, in which shade-grown plants have larger leaves with lower *LMA*. Given that our tropical fern species relied on adjustments to *LMA* only to cope with differences in growth irradiance, it appears that compensatory changes in other leaf traits precluded adjustments to *LMA* that would reduce growth potential over evolutionary timescales associated with habitat irradiance. With respect to *WUE*, which may be important to help reduce likelihood of desiccation under high light (Ni and Pallardy 1991; Edwards et al. 2012; Creese and Sack *in prep-a*), it may be too limiting of carbon gain and growth in species adapted in open habitats, and instead would provide a valuable safety net for shade adapted species more vulnerable to desiccation, and which lack other compensatory mechanisms of drought tolerance. Thus we expect these specific compromises between long-term adaptation versus short-term acclimation to irradiance to strongly influence the performance of fern species across their habitat distributions.

Notably, leaf nutrient concentrations did not differ significantly between open and shade habitat species suggesting that ferns may have compensatory nutrient assimilation and allocation processes that maintain nutrient concentrations within a tight margin across diverse species, habitats, and substrates compared with other plant groups (Hou 1950; Wegner et al. 2003). Such a nutrient leveling mechanism may help to explain why ferns have not shown the same changes in nutrient concentrations and ratios as angiosperm and moss species across habitats and elevation gradients (Wegner et al. 2003; Waite and Sack 2011a; Creese and Sack *in prep-b*).

Although chlorophyll and nutrient concentrations were similar on average for high and low light adapted species, these traits did show plastic variation with growth irradiance. In addition to higher chlorophyll concentration, low irradiance grown plants invested in greater N_{mass}, *Chl/N*, and consequently had a lower C/N than high irradiance grown plants, to help maximize light capture through greater investment in the photosynthetic apparatus (Givnish 1988; Poorter and Evans 1998; Rozendaal et al. 2006).

The leaf economics spectrum: adherence and exception to global trends

Although ferns represent the second largest radiation of land plants after angiosperms, they have not been well represented in studies of global patterns for the leaf economics spectrum (Wright et al. 2004; Shipley et al. 2006; but see Karst and Lechowicz 2007). Compared with these broad patterns, our tropical fern species showed the same trend of decreasing N_{mass} and A_{mass} with increasing *LMA* (Wright et al. 2004; Shipley et al. 2006) – previously unsupported in temperate ferns (Karst and Lechowicz 2007) – but no significant relationship with photosynthesis per area, a pattern also observed for Southern California fern species from exposed and shaded habitats (Creese and Sack *in prep-a*). Only *UMA* was significantly correlated with higher N_{area}, whereas for Southern California fern species, *LMA* calculated for leaf blades versus only lamina tissue was significantly correlated with higher N_{area} (Creese and Sack *in prep-a*). This suggests that trait associations for the leaf economics spectrum in ferns depend on how nutrient and biomass is partitioned across their lamina, rachis, and stipe tissues, and that co-variation in these leaf economic traits may be uncoupled. Comparative phylogenetic studies of ferns with variable *LMA* are needed to assess differences in their lifespan, nutrient concentrations, venation architecture and differences in the size, number, and wall thickness of leaf cells (c.f., Shipley et al. 2006) to investigate the mechanisms supporting or decoupling relationships between *LMA*, N concentration and photosynthesis per area. Notably, our tropical fern species showed the same correlation of greater *WUE* with high *LMA* as Southern California fern species, Hawaiian mosses and other tracheophytes (Hultine and Marshall 2000; Waite and Sack 2011b; Creese and Sack *in prep-a*). Given that A_{area} was not significantly greater in our high *LMA* ferns, it is unlikely that the relationship between ∂^{13}C and *LMA* is due to greater ^{13}C discrimination from a faster photosynthetic rate (Farquhar 1989). Instead, we hypothesize that the correlation of greater ∂^{13}C and *LMA* apparent for tropical and mediterranean-type climate fern species may be due to co-selection of high *LMA* and ∂^{13}C for more conservative water use to protect more expensive, longer lived leaves – or that the greater discrimination of ^{13}C is due to differences in (g_{m}) mesophyll conductance (Niinemets et al. 2009). Therefore, comparative studies of fern ∂^{13}C and g_{m} are needed.

CONCLUSIONS

This common garden and field study provide a novel first assessment of differences in plasticity and adaptation in ferns. This study further demonstrates strong overlap in fern adaptive and acclimation responses to irradiance compared with angiosperms, as well as key differences in their optimization of carbon assimilation at growth and evolutionary timescales. As expected, and similar to angiosperms, open habitat and high irradiance grown ferns showed higher I_{c}, I_{s}, V_{cmax}, J_{max} and faster respiration and carbon assimilation rates per area. However, plastic adjustments in leaf area, *LMA*, N_{mass}, chlorophyll concentrations, *Chl/N*, *C/N*, *QE*, V_{cmax}/N, and J_{max}/N supported comparable photosynthetic rates per mass across the growth irradiance treatments (though low irradiance grown plants still exhibited a lower respiration rate per mass). We hypothesize that this capacity to optimize carbon assimilation per mass at the growth timescale would be especially beneficial to species growing in tropical rainforests with fast plant turnover rates and highly variable light gap dynamics (Chazdon 1988; Rozendaal et al. 2006). Notably, trends for leaf area and *WUE* tended in opposite directions for ferns grouped by habitat versus growth irradiance, and parallel the contrary responses of these same tropical fern species in their responses to increasing VPD (Creese et al. *in prep*). We expect that the greater VPD responsiveness of g_s and higher *WUE* for high irradiance grown plants and shade adapted species is important to reduce their vulnerability to desiccation, whereas open habitat species have inherited and/or evolved other compensatory mechanisms of desiccation tolerance. These cases of adaptation and acclimation tending in opposite directions emphasize the importance of specific compromises between long-term adaptation versus short-term acclimation to irradiance and moisture availability in determining the performance of fern species across their habitat distributions. Thus comparative studies of tropical fern desiccation tolerance-related traits, leaf phenology, and relative growth rates are needed to investigate potential differences in optimization of leaf hydraulic protection and efficiency mechanisms (e.g., Pittermann et al. 2011), leaf lifespans, and carbon balance at the leaf and whole-plant levels for species from open and shade habitats.

Also similar to angiosperms, ferns did not plastically adjust their ratio of J_{max}/V_{cmax} in response to growth irradiance. Shade species exhibited a higher J_{max}/V_{cmax}, which may be due to inherited differences in species photosynthetic sensitivity to CO_2. Despite the strong trade-off of *LMA* with $A_{max,mass}$ in accordance with global trends for the leaf economics spectrum, our tropical fern species did not follow the trend of higher *LMA* in shade habitat species as seen for evergreen angiosperms (Walters and Reich 1999). Therefore, while acclimation to low irradiance in tropical ferns relies on adjustments to *LMA* to optimize carbon assimilation per mass, adaptation to maximize shade tolerance appears to depend on smaller leaf size, increased investment into the photosynthetic apparatus, and slower respiration rates without adjustments to *LMA*. These differences in the adaptation and acclimation of tropical ferns to irradiance environment provide a new foundation for modeling their responses to environmental change at growth and evolutionary timescales.

These results point to evolutionary convergence among angiosperms, mosses, and ferns in overall plasticity and adaptation in response to irradiance, and the ways in which the trends shift in different directions for plastic versus adaptive responses to light. To better understand this convergent evolution, further studies into the genetic, developmental, and functional mechanisms constraining morphospace and physiospace of land plant lineages with independent leaf evolution are needed (McGhee 2007; Wilson and Knoll 2010; McGhee 2011). Identification of key differences in the evolution of fern leaf physiology across varying habitats will further improve our understanding of species niche partitioning and the maintenance of diversity in ferns as in angiosperms.

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TABLE 6.1 List of genus and species (family in parentheses), and their habitat type spanning understory to small clearings for shade habitat low irradiance species, and up to large clearings for open habitat high irradiance species.

Species	**Native Habitat**
Shade species	
Danaea wendlandii (Marattiaceae)	understory
Diplazium striatastrum (Woodsiaceae)	understory
Saccoloma moranii (Saccolomataceae)	understory
Cyclopeltis semicordata (Dryopteridaceae)	understory-small clearings
Blechnum occidentale (Blechnaceae)	understory-small clearings
Adiantum latifolium (Pteridaceae)	understory-small clearings
High irradiance species	
Thelypteris nicaraguensis (Thelypteridaceae)	understory-large clearings
Tectaria lizarzaburui (Tectariaceae)	small-large clearings
Campyloneurum brevifolium (Polypodiaceae)	small-large clearings
Nephrolepis biserrata (Nephrolepidaceae)	large clearings
Hemionitis palmata (Pteridaceae)	large clearings
Pityrogramma calomelanos (Pteridaceae)	large clearings
Ophioglossum nudicaule (Ophioglossaceae)	large clearings

TABLE 6.2 Variable categories, symbols, units and definitions.

Traits	Units	Trait Description
Environmental stimulus		
Irradiance	μmol photons m^{-2} s^{-1}	Light response curve irradiance from 0 – 1500 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR)
CO$_2$	Pa	CO$_2$ response curve concentration from 0 – 200 Pa
Leaf form & composition		
LA	cm^2	Leaf area
$USEGA$	cm^2	Ultimate segment (USEG) area
LMA	g cm^{-2}	Whole leaf mass per area
UMA	g cm^{-2}	Leaf USEG mass per area
Chl	SPAD units	Chlorophyll concentration per unit area
Chl_{mass}	SPAD g^{-1}	Chlorophyll concentration per unit mass
Chl/N		Ratio of chlorophyll to leaf N per mass
Nutrients		
C_{area}	mg m^{-2}	Leaf USEG Carbon concentration per area
C_{mass}	g 100 g^{-1}	Leaf USEG Carbon concentration per mass
N_{area}	mg m^{-2}	Leaf USEG Nitrogen concentration per area
N_{mass}	g 100 g^{-1}	Leaf USEG Nitrogen concentration per mass
C/N		Ratio of leaf USEG Carbon to Nitrogen per mass
WUE ($\partial^{13}C$)	‰	Leaf USEG lifetime integrated water-use-efficiency as ^{13}C to ^{12}C ratio
Light response curve flux traits		
A_{max}	μmol m^{-2} s^{-1}	Maximum photosynthetc rate per area at saturating irradiance
I_{c}	μmol photons m^{-2} s^{-1}	Light compensation point
I_{s}	μmol photons m^{-2} s^{-1}	Light saturation point
QE	mol CO$_2$ (mol photons)$^{-1}$	Quantum yield or efficiency of photosynthesis
R_{d}	μmol m^{-2} s^{-1}	Dark respiration rate per unit area
CO$_2$ response curve flux traits		
$A_{\text{max,lrc}}$	μmol CO$_2$ m^{-2} s^{-1}	Maximum photosynthetc rate per area at 40 Pa CO$_2$
V_{cmax}	μmol CO$_2$ m^{-2} s^{-1}	Maximum rate of rubisco carboxylase activity per area, 25 ˚C
J_{max}	μmol e$^-$ m^{-2} s^{-1}	Maximum photosynthetic electron transport rate per area, 25 ˚C
$J_{\text{max}}/V_{\text{cmax}}$		Ratio of electron transport rate to rubisco carboxylase activity
$R_{\text{d,lrc}}$	μmol CO$_2$ m^{-2} s^{-1}	Dark respiration rate per unit area for the CO$_2$ response curve

Traits	**Units**	**Trait Description**
Traits calculated at species × treatment level		
$A_{\text{maxmass,lrc}}$	µmol g^{-1} s^{-1}	Maximum photosynthesis per mass at saturating irradiance
QE_{mass}	mol CO$_2$ g^{-1} (mol photons)$^{-1}$	Quantum yield or efficiency of photosynthesis per mass
$R_{\text{dmass,lrc}}$	µmol g^{-1} s^{-1}	Dark respiration rate per unit mass for the light response curve
$A_{\text{max,mass}}$	µmol g^{-1} s^{-1}	Maximum photosynthesis per mass at 40 Pa CO$_2$
$V_{\text{cmax,mass}}$	µmol g^{-1} s^{-1}	Maximum rate of rubisco carboxylase activity per mass
$J_{\text{max,mass}}$	µmol g^{-1} s^{-1}	Maximum photosynthetic electron transport rate per mass
$R_{\text{d,mass}}$	µmol g^{-1} s^{-1}	Dark respiration rate per unit mass for the CO$_2$ response curve
V_{cmax}/N	mmol CO$_2$ (mol N)$^{-1}$ s^{-1}	Ratio of rubisco carboxylase activity to Nitrogen per mass
J_{max}/N	mmol e^{-} (mol N)$^{-1}$ s^{-1}	Ratio of electron transport rate to Nitrogen per mass

FIGURE CAPTIONS

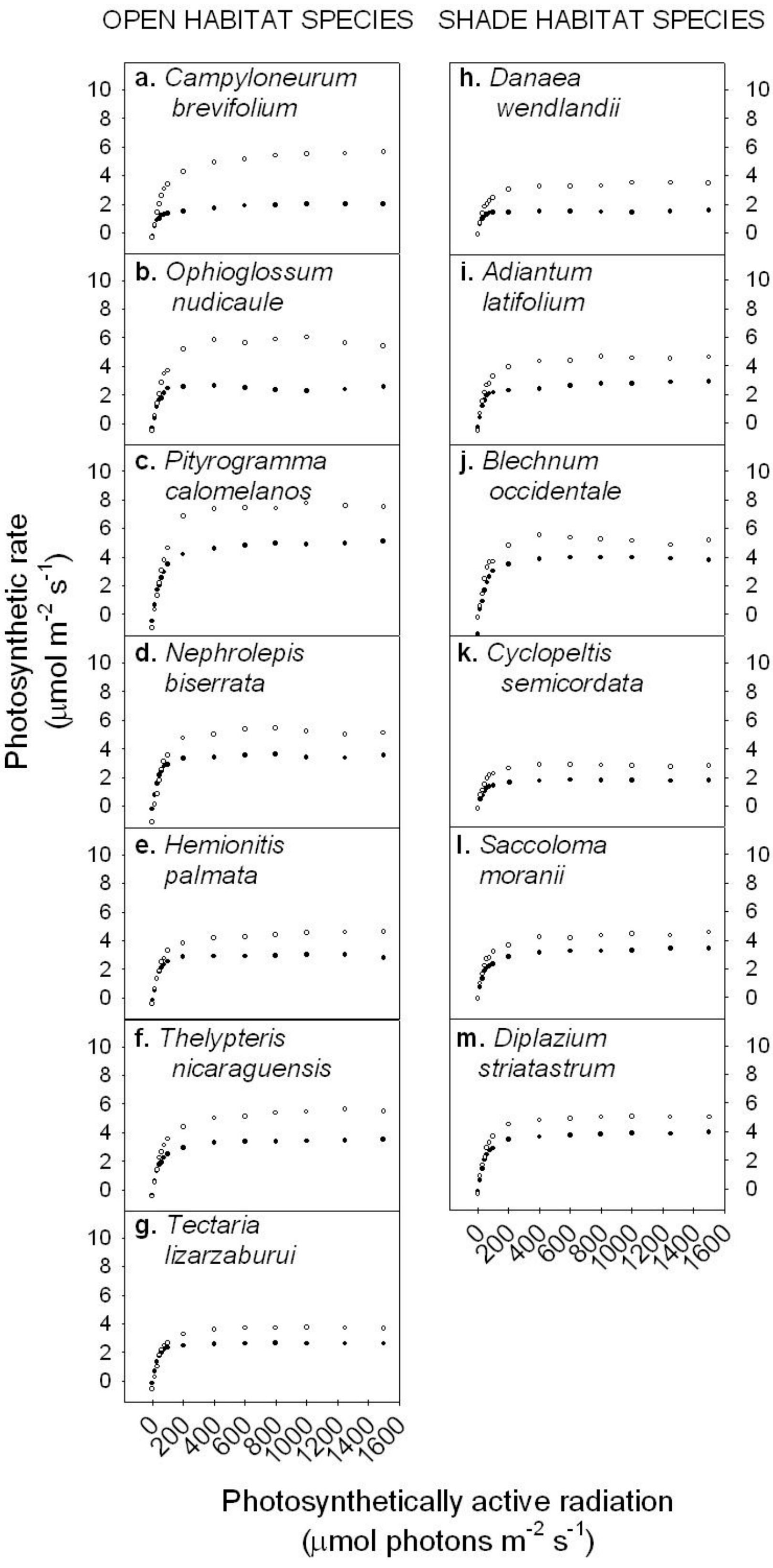
Fig 6.1 Responses of photosynthetic rate (*A*) to an increase in irradiance from 0 – 1500 µmol m^{-2} s^{-1}photosynthetic photon flux density (PPFD) for representative leaves of open habitat (a – g) and shaded habitat (h – m) species, from plants grown under high irradiance (open circles) and low irradiance (closed circles) treatments; species within each habitat type are ordered from top to bottom by greatest mean difference in maximum photosynthetic rate between high and low irradiance grown plants.

Fig 6.2 Responses of maximum photosynthetic rate (*A*) to changes in chamber atmospheric CO_2 concentration for representative leaves of open habitat (a – e) and shade habitat (f – j) species, from plants grown under high irradiance (open circles) and low irradiance (closed circles) treatments; species within each habitat type are ordered from top to bottom by greatest mean difference in maximum photosynthesis between high and low irradiance plants.

Fig 6.3 Differences in mean leaf and ultimate segment (USEG) form, composition and nutrient traits for high irradiance habitat species and grown plants (open bars) and low irradiance habitat species and grown plants (black bars), arranged by habitat (left) and growth irradiance (right); *P*-values determined via hierarchical ANOVA tests, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, * $P \leq 0.05$, †$P \leq 0.1$

Fig 6.4 Differences in mean light response curve traits for high irradiance habitat species and grown plants (open bars) and low irradiance habitat species and grown plants (black bars), arranged by habitat (left) and growth irradiance treatment (right); *P*-values determined via hierarchical ANOVA tests, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, * $P \leq 0.05$, †$P \leq 0.1$

Fig 6.5 Differences in mean CO_2 response curve traits for high irradiance habitat species and grown plants (open bars) and low irradiance habitat species and grown plants (black bars), arranged by habitat (left) and growth irradiance treatment (right); *P*-values determined via hierarchical ANOVA tests, $***P \leq 0.001$, $**P \leq 0.01$, $* P \leq 0.05$, $†P \leq 0.1$



OPEN HABITAT SPECIES
SHADE HABITAT SPECIES
a. Campyloneurum brevifolium
b. Ophioglossum nudicaule
c. Pityrogramma calomelanos
d. Nephrolepis biserrata
e. Hemionitis palmata
f. Thelypteris nicaraguensis
g. Tectaria lizarzaburui
h. Danaea wendlandii
i. Adiantum latifolium
j. Blechnum occidentale
k. Cyclopeltis semicordata
l. Saccoloma moranii
m. Diplazium striatastrum
0
2
4
6
8
10
0
200
400
600
800
1000
1200
1400
1600
Photosynthetic rate (μmol m⁻² s⁻¹)
Photosynthetically active radiation (μmol photons m⁻² s⁻¹)

Fig 6.1

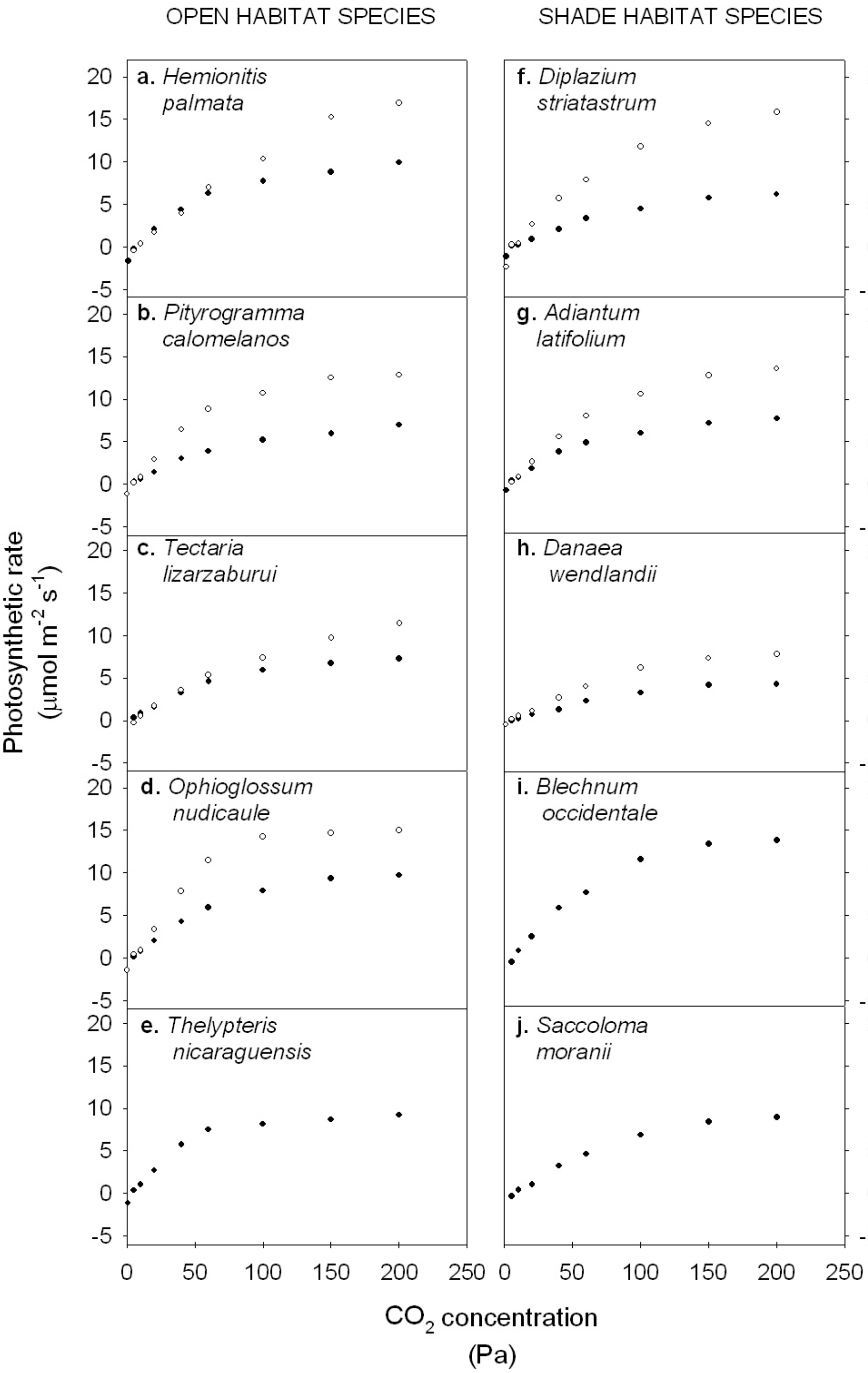

OPEN HABITAT SPECIES
SHADE HABITAT SPECIES
a. Hemionitis palmata
b. Pityrogramma calomelanos
c. Tectaria lizarzaburui
d. Ophioglossum nudicaule
e. Thelypteris nicaraguensis
f. Diplazium striatastrum
g. Adiantum latifolium
h. Danaea wendlandii
i. Blechnum occidentale
j. Saccoloma moranii
Photosynthetic rate (µmol m-2 s-1)
CO2 concentration (Pa)
20
15
10
5
0
-5
0
50
100
150
200
250

Fig 6.2

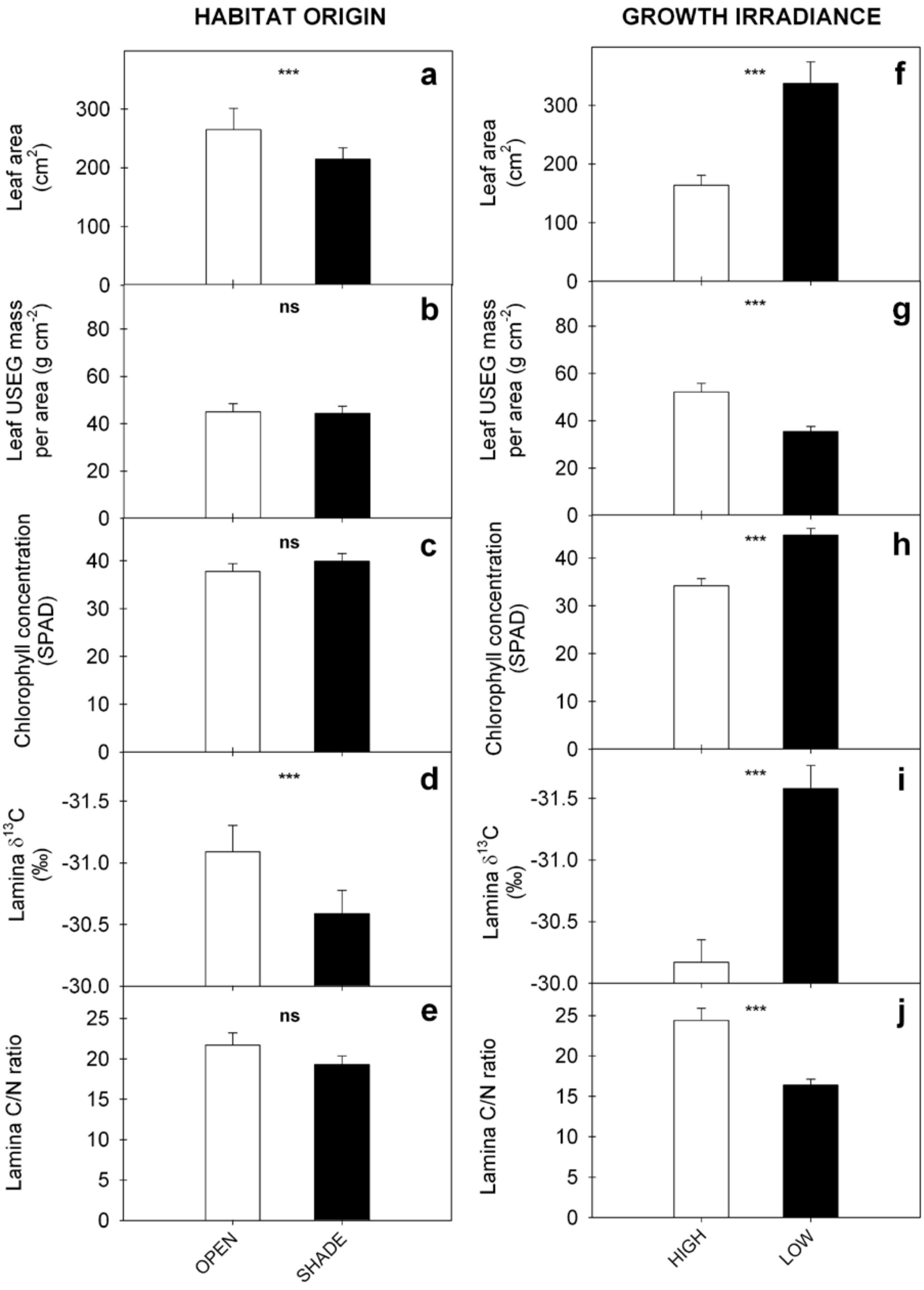

HABITAT ORIGIN
GROWTH IRRADIANCE
a
f
b
g
c
h
d
i
e
j
***
ns
Leaf area (cm^2)
Leaf USEG mass per area (g cm^{-2})
Chlorophyll concentration (SPAD)
Lamina δ^{13}C (‰)
Lamina C/N ratio
OPEN
SHADE
HIGH
LOW

Fig 6.3

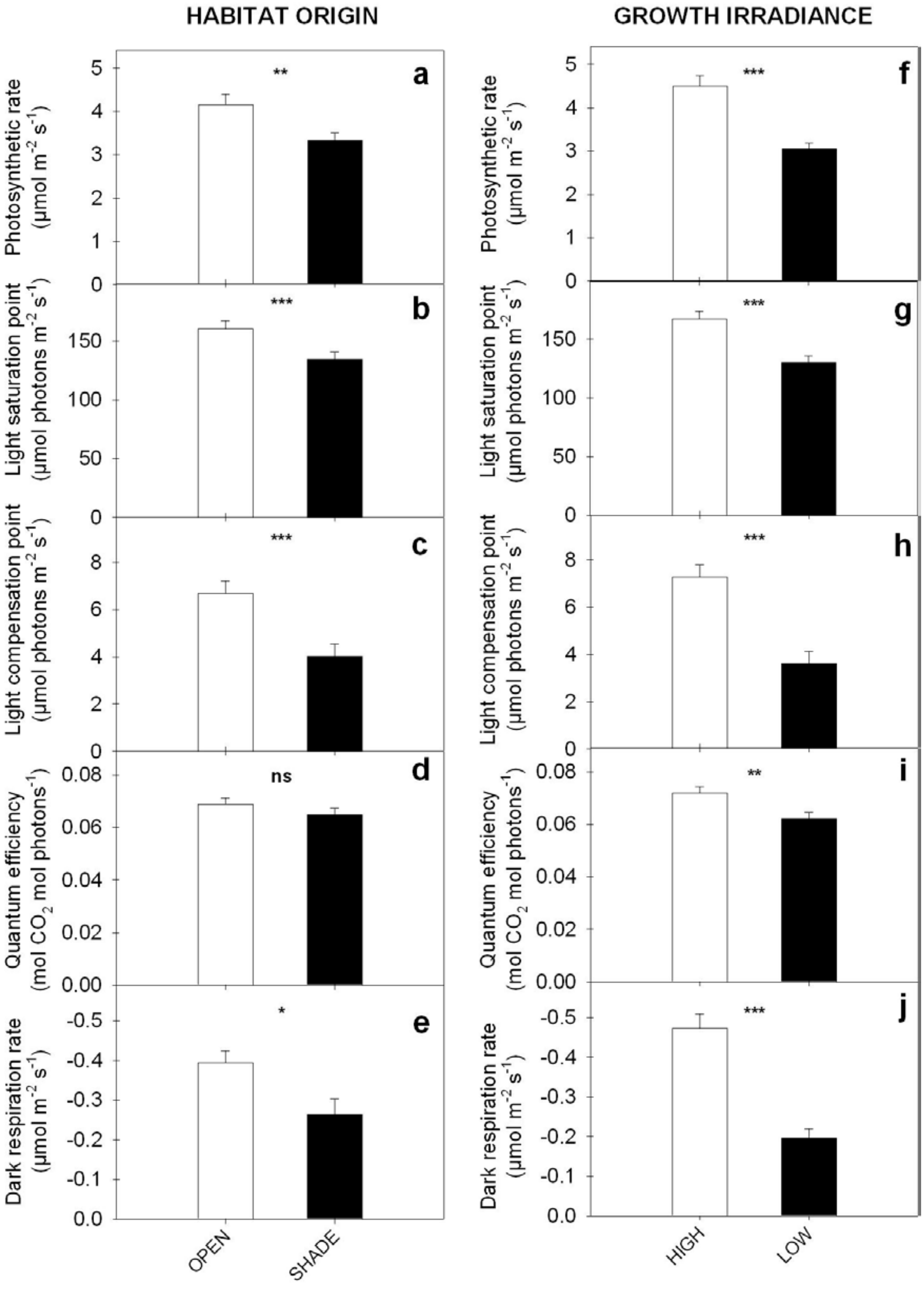

HABITAT ORIGIN
GROWTH IRRADIANCE
Photosynthetic rate (μmol m^{-2} s^{-1})
Light saturation point (μmol photons m^{-2} s^{-1})
Light compensation point (μmol photons m^{-2} s^{-1})
Quantum efficiency (mol CO$_2$ mol photons^{-1})
Dark respiration rate (μmol m^{-2} s^{-1})
a
**
b
***
c
***
d
ns
e
*
f
***
g
***
h
***
i
**
j
***
OPEN
SHADE
HIGH
LOW

Fig 6.4

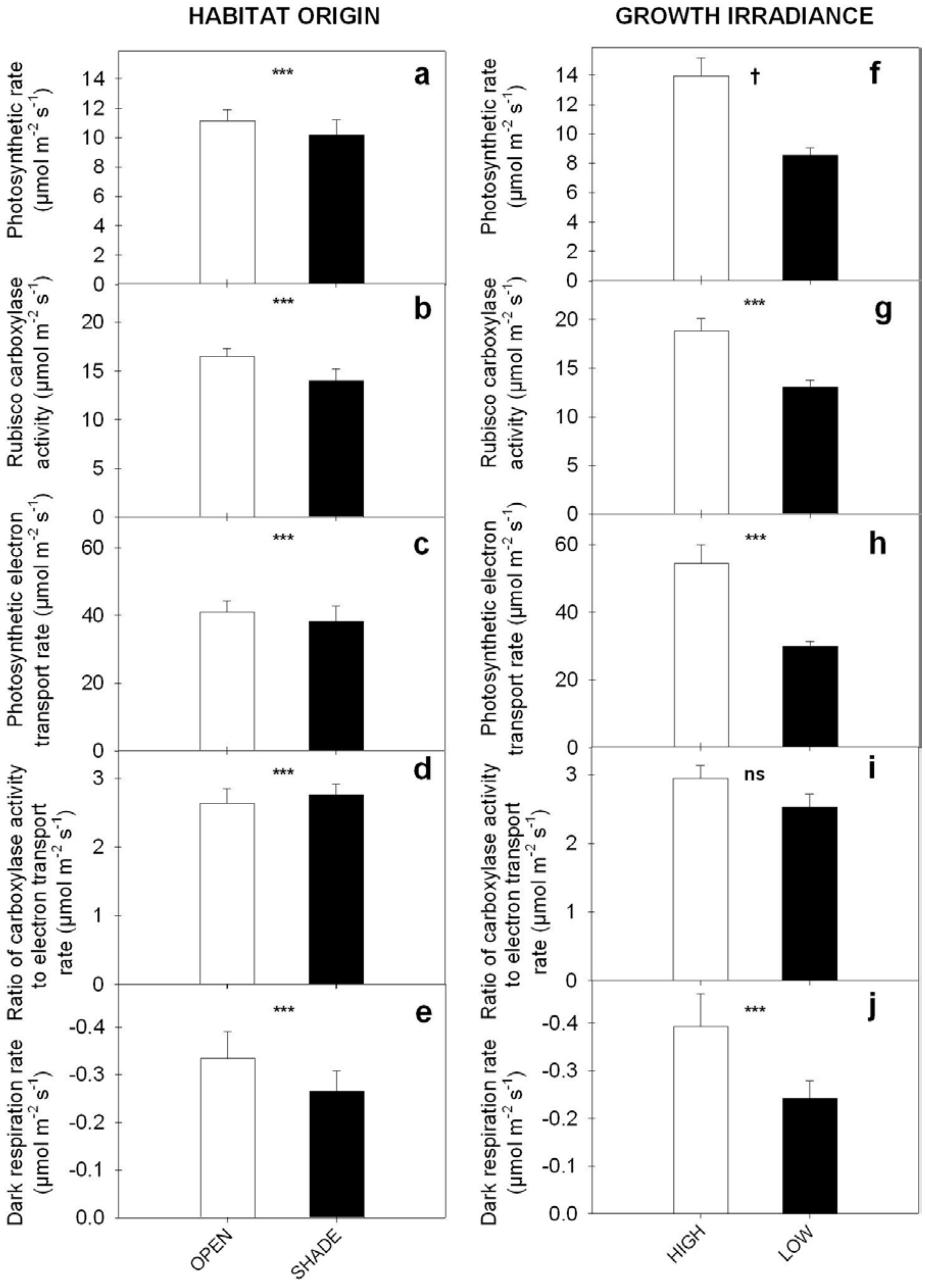

HABITAT ORIGIN
GROWTH IRRADIANCE
Photosynthetic rate (µmol m^{-2} s^{-1})
Rubisco carboxylase activity (µmol m^{-2} s^{-1})
Photosynthetic electron transport rate (µmol m^{-2} s^{-1})
Ratio of carboxylase activity to electron transport rate (µmol m^{-2} s^{-1})
Dark respiration rate (µmol m^{-2} s^{-1})
a
b
c
d
e
f
g
h
i
j
***
†
ns
OPEN
SHADE
HIGH
LOW

Fig 6.5

SUPPLEMENTAL MATERIAL

SUPPLEMENTARY TABLE CAPTIONS

SuppTable 6.1 Minimum, mean and standard error, and maximum trait values for species grouped by habitat origin (open versus shade), all species, and for species grouped by growth irradiance treatment (high versus low irradiance); *P*-values for species grouped by habitat and by growth treatment were determined by general linear model (see Methods) significance tests except for composite traits calculated at the species × treatment level where species differences were determined by one-way ANOVA and differences between irradiance growth treatments via paired t-test; ns = not significant $^{+}P \leq 0.1$ $^{*}P \leq 0.05$ $^{**}P \leq 0.01$ $^{***}P \leq 0.001$. Note that the minimum light compensation point and maximum dark respiration rate from the light response curves appear were not significantly different from zero for two leaves of *Danaea wendlandii*.

SuppTable 6.2 Correlation matrix with Spearman (r_s) and Pearson (r_p) coefficients and *P*-values for respective ranked, raw (untransformed), and log-transformed datasets of species × treatment means for the high irradiance treatment (above) and low irradiance treatment (below) where highlighted cells represent relationships significant for at least two tests; $^{*}P \leq 0.05$ $^{**}P \leq 0.01$ $^{***}P \leq 0.001$.

SuppTable 6.3 Species × treatment means (above) and standard errors (below) for all variables; standard errors for composite traits were determined using propagation of error (see Methods).

SuppTable 6.4 Means and standard errors for site irradiance, leaf functional traits and nutrients for the 30 most abundant fern species at La Selva Biological station ordered by increasing habitat irradiance; bold values are means for species grouped by habitat irradiance, and asterisks denote species that were also included in the common garden study.

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CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

Studies of variation in leaf traits have focused mainly on angiosperms. However, understanding how ferns vary their leaf form and physiology across species and environments is important to better inform models of ecosystem responses to environmental change, as the habitat specificity of ferns makes them excellent ‘canaries in the coalmine’ indicator species. Identifying the drivers of variation in fern leaf traits is also vital to building predictive models for species ecological tolerances, distributions, and evolutionary trajectories. The goal of this dissertation was to provide a foundation for such work by exploring drivers of variation and potential sources of constraint related to leaf form and performance. This allowed me to develop explanations of how ferns have acclimated and adapted to diverse environments to generate extant patterns of abundance and distribution.

The size-scaling of plant and leaf parts to maintain functional proportions was a significant factor in explaining diversity in fern anatomy and morphology across species and environments, from differences in the density of veins to the size and shape of leaves. Growth forms, or as termed in Chapter 3, the terrestrial versus epipetric ‘habitats’, were also strongly associated with variation in plant size and shifts in other leaf traits to preserve an economy of scale, and an economy of stress tolerance. Adherence to global trends for the leaf economics spectrum – investment in higher leaf mass per area (LMA) at a cost of lower photosynthesis – also explained variation in leaf form and physiology due to different resource allocation patterns in leaves. However, further study of differences in biomass and nutrient allocation patterns influencing this leaf economics spectrum across fern assemblages is recommended because these correlations varied across the fern floras of Southern California and Costa Rica. In particular, for both floras, LMA was significantly correlated with photosynthetic rate on a per mass basis, while photosynthesis per area was significantly correlated with LMA in Southern California ferns but was only significantly correlated with leaflet (or ultimate segment) mass per area in Costa Rican ferns. This suggests that proportional investment into photosynthetic and non-photosynthetic leaf tissues is optimized at different scales – leaf or leaflet – depending upon the species. To ascertain the extent to which the trait associations reported in this dissertation are mechanistic or arise due to co-selection, and to identify factors constraining fern adaptive space, genetic and developmental studies are needed.

The ferns studied also ranged widely across resource gradients, and differences in factors such as moisture and irradiance were strongly associated with variation in leaf form and performance at growth and evolutionary timescales. I found evidence that ferns can adapt both by optimizing rates of resource capture, metabolism and growth, and/or by optimizing stress tolerance and survival, with traits linked to slow growth. Nevertheless, high flux rates for hydraulic and gas exchange traits were not consistently associated with high irradiance, as seen for angiosperms. Rather, optimization of carbon gain under high irradiance appears to be strongly mediated by moisture availability. For the mediterranean-type climate ferns of Southern California where water availability was typically more limited and irradiance more saturated, leaf hydraulic conductance and rates of gas exchange were decoupled from vein density in dry open habitat species compared with moist shaded species (unless differences in irradiance were accounted for by partial correlation). Thus, it was in fact the shaded site species that exhibited faster flux rates in spite of their lower vein densities, and differences in these traits were associated with variation in leaf mass per area (LMA) across habitats. For the tropical rainforest ferns of Costa Rica where light was more limited and water more abundant, open habitat species exhibited faster flux rates than shade habitat species, and these species did not significantly differ in their LMA, despite strong differences in habitat irradiance. These species grown under high and low irradiance treatments did show the expected trend of higher LMA for high light grown plants compared with low light grown plants. This suggests that shifts in LMA are important for acclimation to irradiance habitat at growth timescales, and at evolutionary timescales only when water availability is limited. To assess whether these Costa Rican fern species show vein patterns similar to those for the Southern California species with respect to light environment, and to additionally determine to what extent ferns can plastically adjust their venation architecture to optimize hydraulics and carbon gain under different irradiance growth conditions, I collected and fixed leaves of replicates for all these species grown under the high and low irradiance growth treatments for future study.

I expect that the distribution of fern species is also strongly influenced by variation in the sensitivity, direction, and magnitude of their stomatal responses to changes in irradiance, CO_2, and vapor pressure deficit (VPD). In fact, the species studied here showed a wider range of stomatal responses than previously reported for ferns and angiosperms. To determine to what extent these differences in stomatal sensitivity are related to phylogenetic history and past CO_2 atmospheric concentrations, a fully resolved fern phylogeny at the species level is needed. Advances in technology for measuring CO_2 response curves are also needed to support CO_2 reference concentrations comparable to the higher levels experienced in fern history. Nevertheless, the data presented here demonstrate a greater responsiveness of stomata to VPD for species acclimated to high irradiance versus greater responsiveness to VPD in species adapted to shade that indicates a complexity in control that would modulate adaptation and acclimation at a wide range of time scales. This also reveals a greater sophistication of fern stomatal behavior than previously realized. Notably, the trend for acclimation and adaptation tending in opposite directions applied to leaf size and lifetime integrated water-use-efficiency (WUE), and also appeared with respect to leaf area and LMA. I expect that the greater VPD responsiveness and WUE of high irradiance grown plants and shade adapted species is important to reduce their vulnerability to desiccation, whereas open habitat species have evolved other compensatory mechanisms of desiccation tolerance. This emphasizes the importance of specific compromises between long-term adaptation versus short-term acclimation to irradiance and moisture availability, and the need for comparative studies of fern desiccation tolerance-related traits.

The next step in fern physiology research will utilize these measures of fern leaf performance to predict their responses to environmental change. These measures can also be scaled up to performance of the fern understories and forest subcanopies to examine their contribution to ecosystem fluxes of carbon, nutrients, and moisture. To do this, I have collected functional trait, nutrient, and irradiance data for the 30 most abundant fern species at La Selva Biological Station in Costa Rica to compare with local eddy flux data.

By providing the most comprehensive examination of fern leaf biology to date, this dissertation sheds new light on the evolution of fern diversity in comparison to other plant groups, and provides a new framework for exploring the universality and idiosyncrasy of mechanistic relationships among leaf form and physiology traits across all plants. This dissertation is also a call to action for deeper investigation into fern biology as the ferns hold great promise for improving our understanding and management of plant responses to environmental change.