Leaf water relations in epiphytic ferns are driven by avoidance rather than tolerance mechanisms

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Running Head: Terrestrial and epiphtic fern functional traits

# Abstract

Key Words:

# Introduction

* Paragraph: epiphytism, ferns and evoln. history
  + Following the rise of angiosperms, a major radiation event in ferns occurred in the Cretaceous period, leading to diversification and proliferation of modern fern taxa (**???**). Globally, fern species continue to adapt and thrive in shaded understory environments. Part of this recent Cretaceous radiation event in ferns (polypods), however, included oppourtunistic diversification into more complex ecological niches provided by now dominant angiosperm canopies (**???**). Consquently, one clade of ferns (Euploypoid I) has largely radiated into epiphytic niches, while a sister clade (Eupolypoid II) remained and diversified on the forest floor in tropical ecosystems (Watkins 2012).
* Paragraph: microhabitat differences
  + terrestrial vs epiphytes (cardelus)
  + Within forests, epiphytes grow at many levels. Some are restricted to the dark understory, whereas others grow on the exposed twigs of emergent tree (Watkins 2012).
  + As non-parasites, epiphyitc plants face a variety of ecological stressors, regardless of canopy position, that drive selection of functional traits key across their life history.
  + hemi-epiphyitc life form co-exists with both these groups which may represent an evolutionary transition between terrestrial and epipythic life histories.
* Paragraph: Consequences of radiation of plants into canopy, Evolution of traits
* A host of studies reveal how anatomical traits directly influence physiology in ferns in a manner that is functionally different from seed plants ((**???**), (**???**), (**???**) (**???**)).
* includes drought tolerance in gametophyte life stage, in both life stages (**???**)
* It appears likely that the canalization of stomatal traits…
* Regardless, epiphytic ferns span a vast arraw of micrhabitats that will undergo a suite of environmental stresses (high light, high ph, limited water) that should….
* Paragraph: water relations and strucutre
  + As water transport in ferns occurs exclusively through primary vascular tissue, wrapped in discrete bundles that can span the entire frond (**???**)…
  + Terrestrial ferns may have reduced water use efficiency, which would be consistent with Brodribb and McAdam’s hypothesis of inefficient stomatal control.
  + if stomata are regulated by leaf water status, what traits related to water relations allows ferns to succeed. +The evolution of xylem and that of stomata are tightly interconnected (Sperry, 2004). (read) -
* Paragraph: this study seeks…
  + looking within E1 and E2 at traits related to water relations
  + hemi as intermediaries? hemiepiphytic species begin life as epiphytes and transform into hemiepiphytes by eventually developing terrestrial root systems (**???**)

# Methods

## Study Site and Species Selection

The sites used for this study included two Costa Rican wet tropical forest locations at La Selva Biological Research Station in Heredia (84⁰00’12W, 10⁰25’52N) and Las Cruces Research Station in San Vito (8° 47′ 7” N, 82° 57′ 32” W). The La Selva site is a low elevation (ca 50 m) tropical forest, with a moderate dry season. The Las Cruces site is a premontane tropical forest located at a higher elevation (ca 1200 m). Both sites receive approximately 4m of annual rainfall (Holdridge, 1967, p. @gentry\_four\_1993).

A survey of morphological, stoichiometric, anatomical and leaf water relations parameters were conducted for six individuals from 39 fern species across three fundamentally distinct life forms (Table 1). Across both sites, 18 terrestrial, 15 epiphytic and 6 hemi-epiphytic species were collected and measured. In this study, terrestrial life forms were all collected from shaded closed canopy understories in the forest floor. Epiphyitc life forms were sampled from trunks or within tree canopies, depending on the species. Epiphytic species were collected from canopy trees using single-rope climbing techniques. Hemi-epiphytic species were all collected along lower sections of trees trunks (1-3 m). Importantly, all sampled hemi-epiphytic species are known to have root connections to forest floor soils at some point in their life history. Individuals of species were collected across multiple populations but within similar microhabitat conditions. Most sampled fern species, with the exception of hemi-epiphytes, were restricted to the eupolypod I and II clades. Vouchers for each species were deposited at the respective site of collection at either the La Selva (LSCR) or Las Cruces (LCCR) herbariums.

## Plant Material

Two complete fronds from sampled individuals were field collected in the early morning (6-7:00 am). One frond from each individual was utilized for pressure volume curves, while the other was sampled for structural morphology, lamina stoichmetry and anatomical traits. Stipes were cut at the base of the rhizome and cut ends were wrapped in wet paper towels and transported to the lab in black plastic bags. Stipes were re-cut under water and re-hydrated until time of hydraulic measurement (1-6 hours). Due to the difficulty in sampling some high canopy species; whole epiphytic individuals were carefully removed, maintained overnight in well-watered conditions in an ambient air laboratory and sampled the following day.

## Leaf Morphometric traits

Stipe length (cm) and lamina length (cm) were calculated from one sampled frond per individual. Total frond length was calculated as the sum of stipe and lamina lengths. Total lamina area for each frond was measured with a Li-3100 leaf area meter (LiCor Biosciences, Lincoln, NE, USA). Leaf mass per unit area (LMA, g cm-2) was calculated using the tissue punch method. For each individual, ten lamina punches (5 mm2) were dried to a constant mass and LMA was calculated as the total dry mass divided by the total area of all leaf punches.

## Anatomical traits

Stomatal density (SD) was measured by directly counting stomata on the abaxial leaf surface under 40x magnification. Three leaf punches (4 mm2 diameter) were sampled across random locations on different pinnae from each individual. The number of stomata in each field of view were counted in three random regions on each of three leaf punches. The stomatal density (# mm2) for each individual is presented as the mean SD across all 9 sampled regions. Individual images of stomata were directly photographed under 40x magnification (AmScope FMA050) across all three leaf punches per individual. Stomatal length (mm) and width (mm) of both guard cells were calculated for 9 stomata for each individual using Image J. Stomatal size (SS, mm2) was calculated as guard cell length multiplied by the combined width of each guard cell pair, as in Franks & Beerling (2009).

Stipes measured for vulnerability to cavitation were transversely sectioned by hand using razorblade. Stipes were sectioned at the distal end, where conduit resistivity impacts hydraulic conductivity the most. Sections were stained in toluidine blue and then mounted in glycerine on microscope slides. Each section was photographed using a light microscope mounted with a digital camera (Amscope FMA050). Section photographs were anatomically analyzed using ImageJ (National Institutes of Health, Bethesda, ML, USA). Total xylem vascular area (mm2) was considered the conduit lumen area and the wall area for all xylem in each cross section.

## Foliar chemistry

Sub-samples of foliage tissue used for lamina area calculation were collected across multiple pinnae for each individual. These sub-samples were dried to a constant mass and ground using a Wig-L-Bug (Sigma-Aldrich Co. St. Louis, USA). Nitrogen content and deltaC13 were measured using a Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer (Thermo Scientific) and corrected by comparison with certified standards.

Lamina chlorophyll content was determined on three different pinnae for each individual. Single point measurements of chlorophyll content (mg m-2) were measured within a 3 mm diameter circle with the CCM-300 chlorophyll content meter (Opti-Sciences, Hudson, NH, USA). Chlorophyll content per individual is expressed as the mean of point measurements across the entire frond.

## Pressure-volume relations

Tissue-water relations were determined with pressure–volume analysis (Tyree & Hammel, 1972) on fully expanded fronds with a Scholander pressure bomb (PMS Instruments Co., Corvallis, OR, USA). For each pressure-volume (PV) curve we sampled top most intact pinnae after full re-hydration. We generated pressure–volume curves by taking sequential water potential measurements (leaf) as fronds air dried, first in closed plastic bags (0-2 hrs), and then in open bags. The fresh mass was recorded immediately before and after each determination. Following each PV curve, foliar samples were dried to a constant mass to calculate relative water content (RWC).

For each PV curve, we graphed the relationship between 1/leaf and leaf mass to estimate parameters related to leaf turgor and bulk tissue water relations. We then calculated leaf water potential at turgor loss (tlp), the osmotic potential at full tissue hydration (o), the bulk modulus of tissue elasticity () and tissue capacitance (C) according to Sack *et al.* (2011).

## Statistical analysis

Linear mixed-effect models were used to test responses of functional traits to categorical fixed effects of life form and site. The interaction between life form and site was tested to confirm any potential environmental or climate influence on trait patterns. Generally, there were few life form x site interactions, so models with life form and site as main effects were compared to full models (AIC scores) and the most parsimonious model was selected. To test for broad differences among life forms, individual plant species were treated as random effects in each model. Tukey’s post-hoc test were performed in conjunction with ANOVA to determine which mean values of functional traits were different among fixed effect treatments with the ‘multcomp’ package (**???**). We utilized a type 3 ANOVA due to an unbalanced design with the limited number of hemi-epiphytes species available. When interactions were present, we computed pairwise comparisons with the ‘emmeans’ package (Lenth, 2018) to investigate interactions between life form and site.

The conditional and marginal r2 values were calculated as per Nakagawa & Schielzeth (2013)

**A phylogenetic tree for these 30 fern species was constructed based on chloroplast rbcL sequences obtained from the GenBank website (**[**http://www.ncbi.nlm.nih.gov/genbank/**](http://www.ncbi.nlm.nih.gov/genbank/)**). Phylogenetic analyses for each matrix were carried out using the maximum likelihood method in PAUP\* v.4.0b10 [44]. Schneider et al. (2004) has integrated Colysis and major components of Microsorum into Leptochilus by using nucleotide sequences derived from three plastid loci [45]. For simplicity, the old Latin names of species in Colysis and Microsorum were used in the present study. The phylogenetic signal (K-statistic) for each trait was calculated using ‘picante’ based on the R package. Such K-statistics can express the conservatism of traits. Cases where the K-value is ,1 indicate convergent traits while K.1 represents that traits are more conserved than would be presumed from a Brownian expectation [47]. Relationships among variables were evaluated by both pair-wise Pearson correlations in the R package and a phylogenetically independent contrast (PIC). Possible evolutionary associations were assessed via PIC analysis, utilizing the molecular phylogenetic tree. This PIC analysis was examined with the ‘‘analysis of traits’’ module in Phylocom, which calculates the internal node values for continuous traits [48].**

For bivariate trait relationships, responses of dependent variables were analysed with linear mixed-effect models, with species as a random effect and life form as a categorical fixed effect. Explained variance (R2) of mixed models were computed as in (**???**), in which the marginal R2 represents variance explained by fixed factors and the conditional R2 by both fixed and random factors. Tests of allometric relationships between log-transformed biomass components were implemented using standardized major axis regression in the ‘smatr’ package in R (**???**). Principal component analysis, utilizing the ‘vegan’ package (**???**), was used to explore how measured functional traits were distributed and co-varied among species (mean values) and life form. All tests of statistical significance were conducted at an level of 0.05. All analyses were performed with R 3.5.1 (**???**).

# Results

## Frond morphology and anatomy

Functional shifts in frond structural relationships for epiphtic species likely represent adaptation to the reduce the path length of water transport to lamina tissues. Total frond length was reduced by 26 % in epiphytic (59±2.5) compared to terrestrial (85±3.3) species, with frond length of hemi-epiphytes an intermediate between both groups (*P* = 0.021). The reduction in total frond length was driven by a large reduction (-54 %) in stipe length in epiphytic compared to terrestrial species (*P* = 0.001, Figure 1A). The majority of epiphytic and hemi-epiphytic ferns had stipes restricted to less than 20 cm (80% quantiles), while maximum stipe length of terrestrial ferns from the same probablity distribution was 42.3 cm. Allometric relationships between stipe length and lamina area were functionally different between epiphytic species compared to terrestrial and hemi-epiphytic species (Figure 1B). Log relationships of stipe length and lamina area were positively correlated for each life form, however, pair wise differences were detected in both the slopes (*P* < 0.001) and elevation (*P* < 0.001) of this structural relationship for epiphytic species.

Total xylem area was 65 % smaller in epiphytic species compared to terrestrial species (*P* = 0.003), with hemi-epiphytic species has intermediates (Figure 2A). Consequently, terrestrial species produced more xylem area per unit leaf area than epiphytic or hemi-epiphytic species, resulting in higher Huber valuec (*P* = 0.001). (**add means here if no figure**). Overall, increases in total xylem area were postively correlated with increases in stipe length (*P* < 0.0001, Figure 2B), with a marginally significant effect of fern life form. The relationship between total xylem area and stipe length in hemi-epiphytes

**data check for stipe xylem regression, pick back up here** **you cannot have a large stipe without a large xylem area**

No differences were detected in total lamina area between any of the life form groups, due to the large amount of variation in lamina area across species (R2 marginal = 0.17 & R2 conditional = 0.89). However, leaf mass per unit area was 67% higher in epiphytic compared to terrestrial species, with hemi-epiphytic species intermediates between both life forms (*P* = 0.002, Figure 1C).

Epiphytic and hemi-epiphytic species had 51% lower stomatal density (36±1.7 mm-2) compared to terrestrial species (72±3.1, *P* < 0.001). Stomatal length was similar across all species, however, guard cell width differed across life forms. Mean width of individual guard cells was 18 % smaller in terrestrial species compared to hemi-epiphytic of epiphytic species. Overall, stomatal size of terrestrial and epiphytic species were statistical similar, yet epiphytes had broadly higher stomatal size. Stomatal size in hemi-epiphtic species was 27 % larger than terrestrial or epiphytic life forms (*P* = 0.044).

## Foliar chemistry

Foliar nitrogen content was 29.8% lower in epiphytic ferns compared to terrestrial and hemi-epiphytic ferns (*P* = 0.007). Foliar 13C for terrestrial and hemi-epiphytic species were more negative that epiphytic species (*P* = 0.004, Figure 2x). Additionally, foliar13C for fern species at the higher elevation Las Cruces site were less negative (-32.6 &permil) than fern species at the low elevation La Selva site (34.0 &permil,*P* = 0.015).

Total chlorophyll content was similar between terrestrial and epiphytic species, although epiphytic species had a lower range of chlorophyll content. Hemi-epiphytic species had similar chlorophyll content to terrestrial species, but were 37 % higher than epiphytic species (\*P = 0.031).

## Frond hydraulic traits

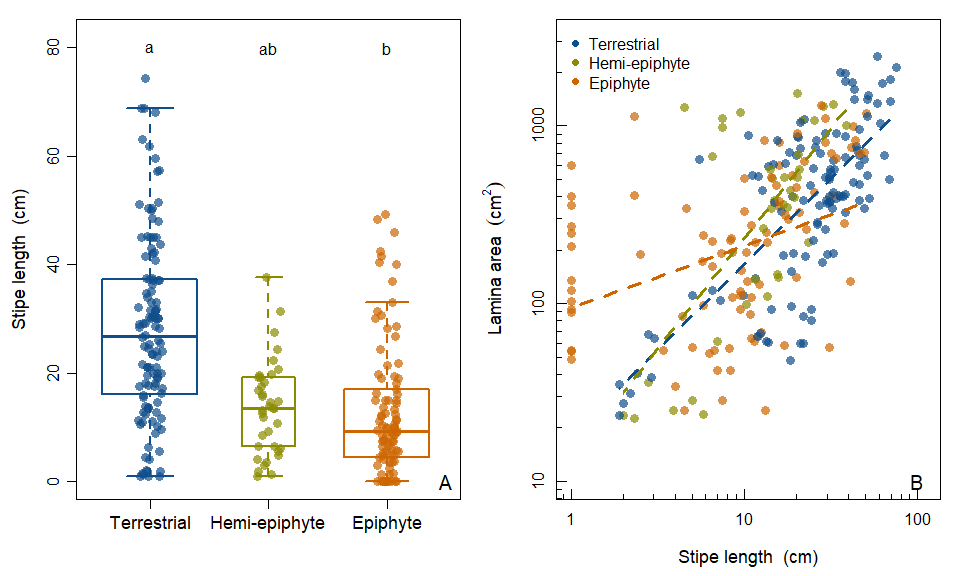
Surprisingly, minimal functional differences in drought tolerance where detected among the three life ferns from pressure volume curve parameters (Figure 2a). The turgor loss point (tlp) varied by fern life form (*P* = 0.042), however, post-hoc multiple comparisons did not detect differences in tlp across terrestrial, hemi-epiphytic or epiphytic ferns. Broadly, terrestrial and hemi-epiphytic fern species had slightly lower tlp than epiphytic species (Figure 2b). Additionally, the osmotic potential (o) decreased in terrestrial and hemi-epiphytic species (*P* = 0.009, Figure 2C), while the modulus of elasticity () was similar across all life forms. Consequently, the broad decreases in tlp among terrestrial and hemi-epiphytic fern species appeared to be driven by osmotic adjustments via solute accumulations rather than shifts in cell wall flexibility (see Bartlett *et al.* (2012)).

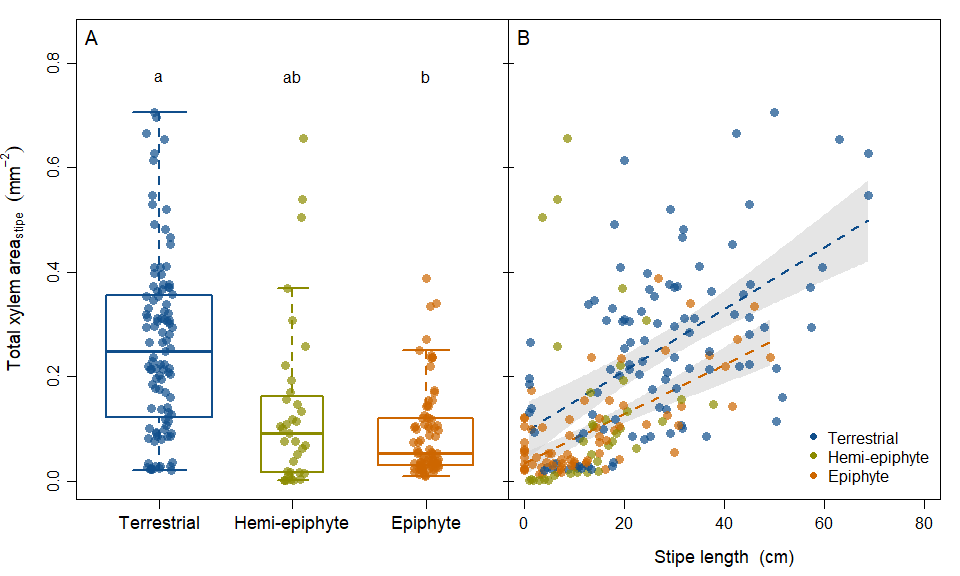
Tissue capacitance at turgor loss point (**capacitance zero?**) was 54 % lower in epiphytic compared to terrestrial species (*P* = 0.010), with hemi-epiphytic species has intermediates. **add normal capactiance instead**

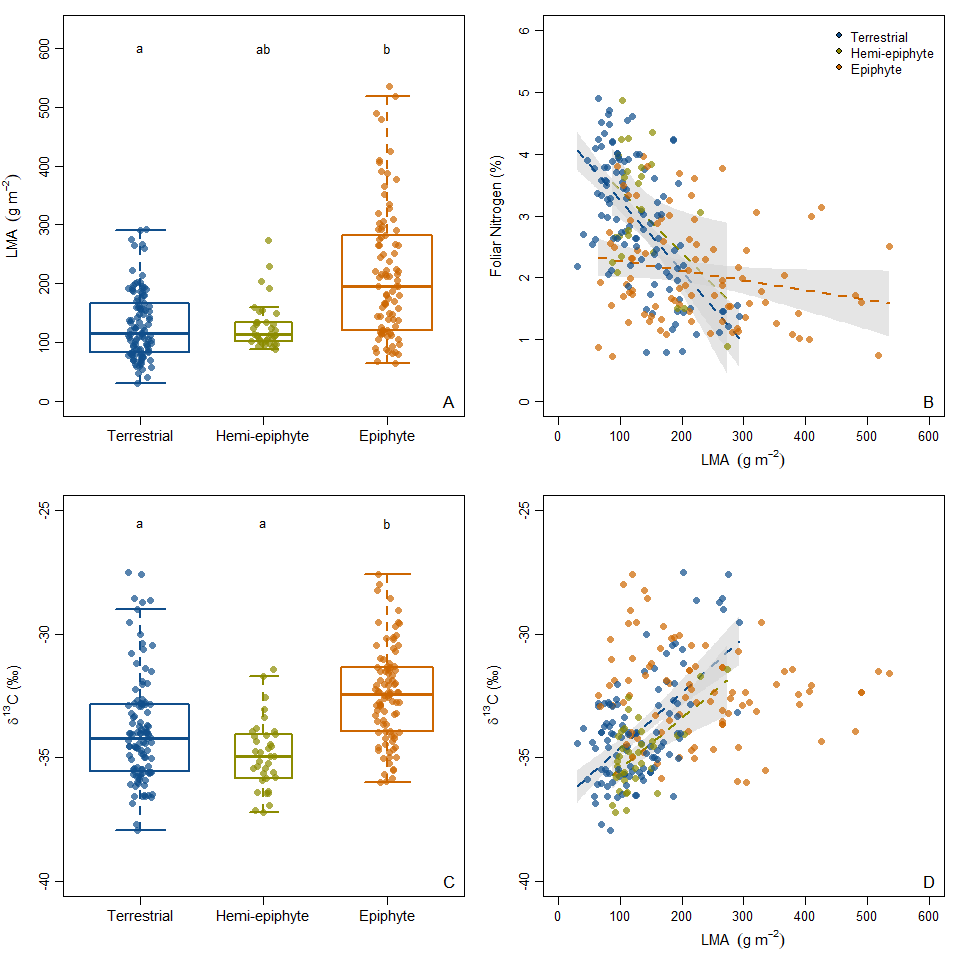
## Bivariate traits relationships:

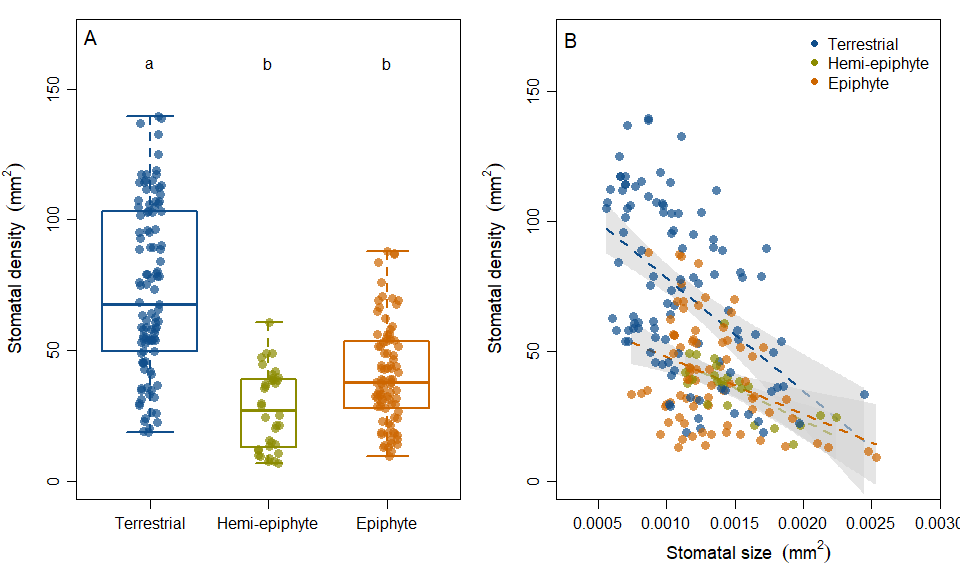
* leaf area/stipe or others with with xylem
* lma and nitrogen
* density vs size (not much with stomata and other traits)
* others?

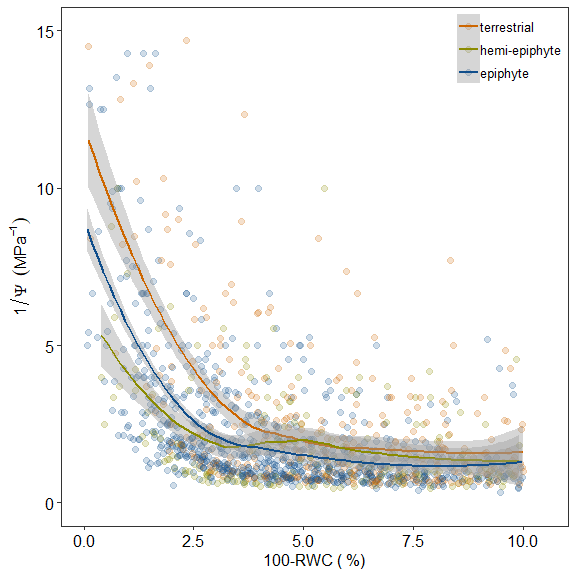
# Figures

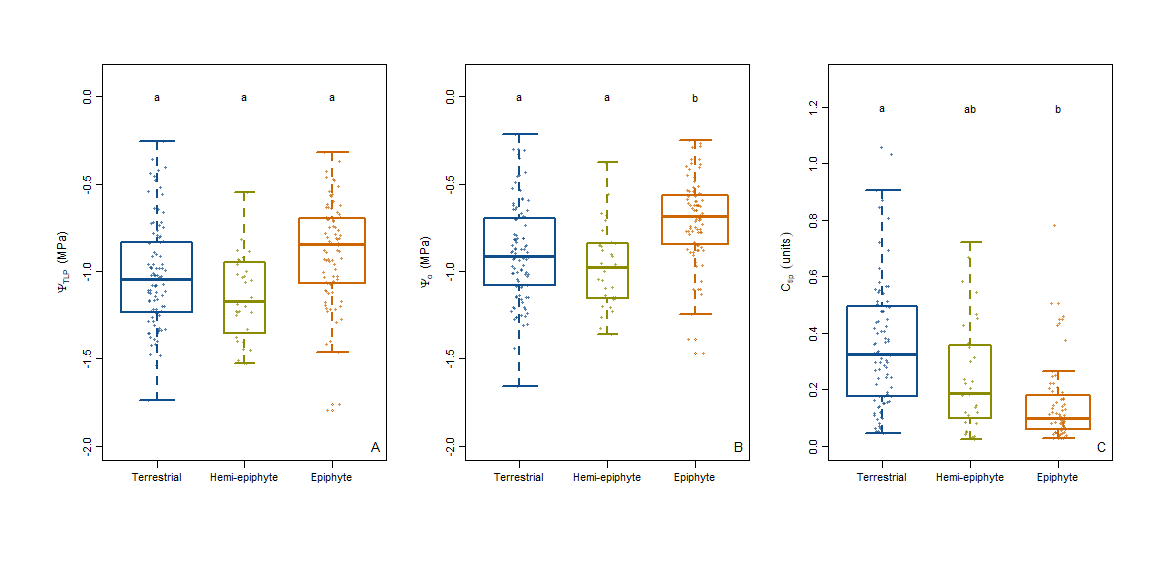
 Figure 1. Functional shift in path length to transport water, via stipe length, alters how different fern life forms build conductive structures

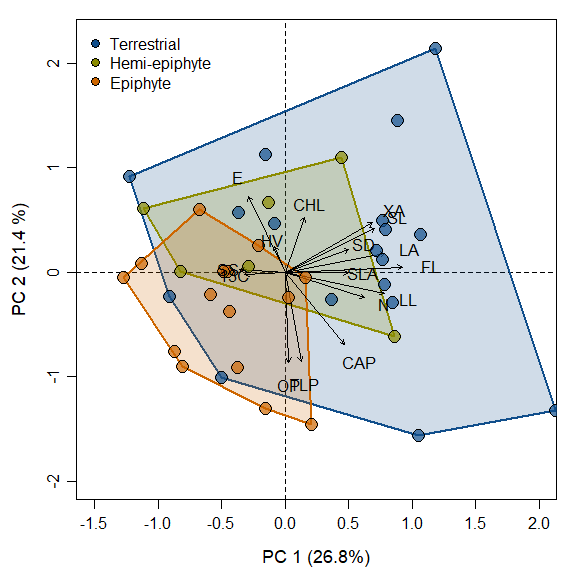
 Figure 2. Stipe length and xylem anatomy define water supply.

 Figure 3. Shifts in LMA between life forms alters relationships with nitrogen and water use efficiency

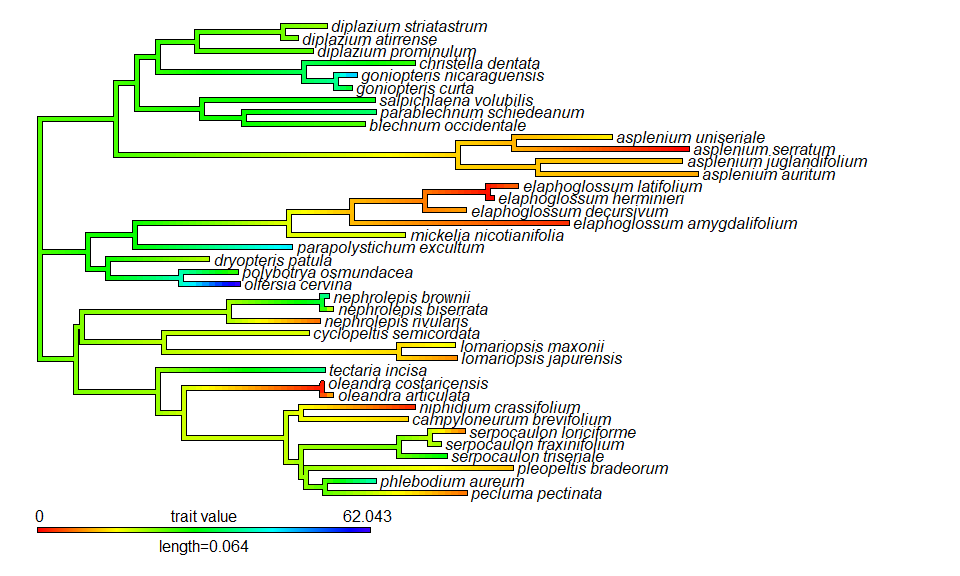
 Figure 4. Stomatal traits and xylem area are functional different between life forms.

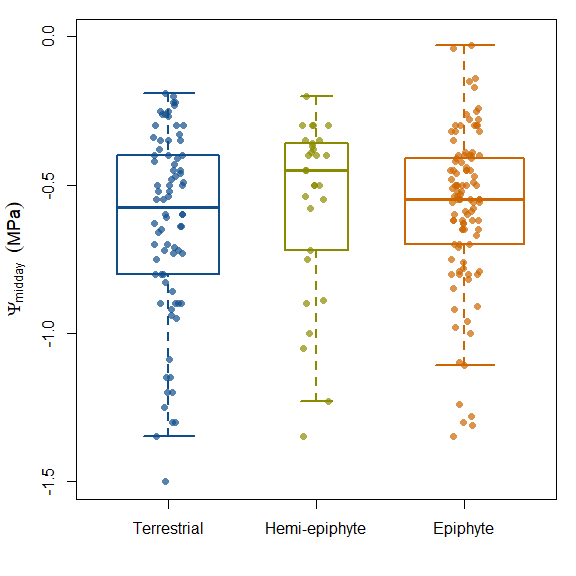
 Figure 5A. Global view of pv curves. I am currently making this with ggplots, which means it looks different and I need to figure out how to combine with pv parameter figures (below).

 Figure 5B. Epiphytic not more drought tolerant. Osmotic adjustments, not shifts in cell wall flexibility explain differences in TLP. I am not sure what capacitance at TLP means. There are possibly a few low outliers that may help TLP stats….

 Figure 6. This PCA is a mostly a placeholder, I imagine we will replace it with trait phylogeny mapping

# More Plots

 Ancestral state reconstruction for frond length

 Midday water potential similar across life forms.

# Discussion

## Do biomechanical relationships define water transport capacity?

* Differences in slopes of scaling realtionships between epiphyitc and terrestrial (more than with phyloegny) ferns have been detected with LMA and petiole width, suggesting a balance is required between optimizing hydaulic support and providing sufficient biomechanical support (**???**)
* Foliar Narea increases with LMA () in terrestrial temperate ferns understory [(**???**); \*campany

## Do canalized traits define leaf water status?

# Acknowledgements

We would like to thank Juliette Bechard, Kathleen Bynon, Luke Calderaro and Alexandra Russell for their hard work in the field and in the lab. We would also like to thank Rodolfo Quiros Flores and Bernal Matarrita Carranza for their organizational support at each OTS field station.

# Bibliography

Bartlett MK**,** Scoffoni C**,** Sack L. **2012**. The determinants of leaf turgor loss point and prediction of drought tolerance of species and biomes: A global meta-analysis. *Ecology Letters* **15**: 393–405.

Franks PJ**,** Beerling DJ. **2009**. Maximum leaf conductance driven by CO2 effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences* **106**: 10343–10347.

Gentry AH. **1993**. *Four Neotropical Rainforests*. Yale University Press.

Holdridge LR. **1967**. Life zone ecology. *Life zone ecology.*

Lenth R. **2018**. Emmeans: Estimated Marginal Means, aka Least-Squares Means.

Sack L**,** Pasquet-Kok J**,** Contributors P. **2011**. PrometheusWiki Leaf pressure-volume curve parameters.

Tyree MT**,** Hammel HT. **1972**. The Measurement of the Turgor Pressure and the Water Relations of Plants by the Pressure-bomb Technique. *Journal of Experimental Botany* **23**: 267–282.