

FUNCTIONAL ECOLOGY OF THE GAMETOPHYTES AND SPOROPHYTES
OF TROPICAL FERNS

By

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Botrychium lunarioides

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Ferns are an important part of both temperate and terrestrial floras, yet their ecology remains poorly understood. Although ferns are dispersed by tiny wind-blown spores, most species are limited to specific habitats; on local levels, ferns are no more widespread than angiosperms. One aspect of fern biology that poses unique ecological problems is the dependence on a free-living gametophyte. I examined the autecology and ecophysiology of the fern gametophyte to understand this structure's role in shaping fern distributions. My study showed that the gametophytes of epiphytic and terrestrial ferns respond differently to light, disturbance, and desiccation stress, and show unexpected versatility in nutrient relations. In all cases, such variation is closely linked to species ecology. Selective pressures acting on the gametophyte generation may be largely responsible for species distributions.

CHAPTER 1 INTRODUCTION

The ferns, with some 12,000 species, are the third most species group of land plants following angiosperms and bryophytes. Their intermediate evolutionary position affords the group a combination of both non-vascular and vascular plant life histories. Ferns rely on a supposedly delicate, short-lived, and usually haploid independent gametophyte (one of their connections to non-vascular plants), and in some, recruitment from the gametophyte often follows into the usually diploid sporophyte stage with lignified vascular tissue (their most obvious connection to vascular plants). It is the ecology of the gametophyte in this choreographed alternation of generations that remains largely unstudied. Such scientific deficit has been commented upon for decades (Pickett, 1914; Holttum, 1938; Cousens, Lacey, and Kelly, 1985; Greer and McCarthy, 1999), yet there has been little movement to increase our understanding of basic fern ecology.

Richard Eric Holttum (1895-1990) is arguably the founding father of fern ecology. Professor Holttum was trained at Cambridge and spent much of his time teaching and studying the ferns of Southeast Asia. By his hand one of the first great treatises of fern ecology was written (Holttum 1938). His seminal “The ecology of tropical pteridophytes” first approached the topic in a synthetic way by incorporating intimate knowledge of ferns and combining it with careful observation and questioning to get to a big-picture conclusion about the ecology of the group. He also addressed ecology of the gametophyte generation:

We are accustomed to see and to marvel at the great varied form and adaptation of the sporophytes, which are the ferns as we know them, but indeed there must be nearly as much variety of adaptation among the gametophytes. It is true that if the prothallus of *Platyserium* grew upon the forest floor, the resulting sporophyte, if produced, would find itself in uncongenial surroundings, and would not develop very far; but it is also true that the *Platyserium* prothallus must be able to develop in relatively exposed position on the tree trunk in which prothalli of many ferns would be unable to exist (Holttum 1938, pp. 421-422).

One of Holttum's key observations was the recognition of gametophyte-mediated controls on fern recruitment. *Platyserium*, commonly known as staghorn ferns, are Old World epiphytes with diverse ecology; many species grow on highly exposed emergent canopy tree trunks, but never on the forest floor. This observation combined with the copious production of wind-dispersed spores showed that dispersal is perhaps of only modest importance in the distribution of ferns: the gametophyte played the critical role in recruitment. Unfortunately, Holttum's call to arms was largely ignored and we have progressed little since the publication of his work.

Many factors have limited the study of fern gametophyte ecology. Avoidance of the gametophyte generation may have been driven by some of the comments made by Frederick Orpen Bower (Bower, 1923) in "The Ferns". Bower saw little taxonomic value in fern gametophytes and doubted their utility in advancing pteridology. Bower was such a recognized figure (and the depauperate literature of the time basically supported his ideas) that many botanists took his words to heart. Fortunately, many studies have since incorporated gametophytic characters into phylogenies, and we have learned that gametophytes have systematic value and have many taxonomic characters that allow for species or morpho-type identification (Atkinson and Stokey, 1964; Nayar and Kaur, 1971; Chiou and Farrar, 1997; Watkins Jr. and Farrar, 2005).

There has been limited application of modern ecological methodology and statistics to the study of fern gametophyte ecology. In a series of observational field and elegant lab experiments, Pickett (1913; 1914) showed that the gametophytes of *Asplenium rhizophyllum* and *A. platyneuron* could be long-lived and survive winter temperatures and extreme drought. Pickett's work helped usher in a new way of thinking about fern gametophyte ecology and later reports by Mottier (1927) and Walp (1951) showed that the gametophytes of some temperate species were essentially indeterminate and could grow *in vitro* for decades, if reproduction were prevented. This notion was further supported by Donald Farrar who developed the now classic story of tropical gametophytes growing independently of sporophytes in the temperate Appalachian Mountains (Farrar, 1967, 1971; Farrar, 1998). In many cases, these species form populations of asexually reproducing gametophytes that number in the tens of thousands and appear to survive winter freezes and summer droughts.

In a series of papers, Michael Cousens developed the concept of gametophyte safe sites and showed that multiple factors act at the level of the gametophyte to shape their distribution and recruitment (Cousens, 1979, 1981; Cousens, Lacey, and Kelly, 1985; Cousens, 1988; Cousens, Lacey, and Scheller, 1988). Cousens' work largely developed in the backdrop of earlier studies showing that fern gametophytes have a distinct ecology relative to sporophytes.

From the turn of the century with Pickett's work, to more recent work by Cousens and Farrar, we have learned that gametophytes in temperate forests have a distinct ecology relative to sporophytes, that gametophytes can be long-lived in natural and especially *in vitro* settings, and robust when dealing with abiotic stresses. Yet, the

ecology of tropical gametophytes remains essentially unstudied. The goal of this dissertation is to examine multiple aspects of fern ecology, focusing on the gametophyte generation.

CHAPTER 2
GAMETOPHYTE ECOLOGY AND DEMOGRAPHY OF TROPICAL EPIPHYTIC
AND TERRESTRIAL FERNS

Introduction

Ferns are conspicuous components of temperate and especially tropical wet forests. Yet, general fern ecology is poorly understood. Much early work was anecdotal or derived from studies and observations made from sporophytes or comments obscured in floristic inventories. A flurry of recent studies attempted to describe both the patterns of fern sporophyte diversity and the causal relations behind such patterns (Tuomisto and Ruokolainen, 1994b; Tuomisto and Dalberg, 1996; Tuomisto, Poulsen, and Moran, 1998; Tuomisto and Poulsen, 2000; Jones et al., 2006; Watkins et al., 2006). These studies were critical in developing ecological models to better understand the biology of the fern sporophyte. Yet, focusing on sporophyte ecology, only told us a small part of fern ecology. Missing are studies on the ecology of the free-living gametophyte.

Ferns alternate between two independent generations: the haploid gametophyte and the diploid sporophyte. The gametophyte is a fundamentally different organism than the sporophyte. It lacks vascular tissue, produces rhizoids instead of true roots, has poorly developed to non-existent cuticles, and is comparatively small. We know from early work that gametophytes can be more widespread and can grow in areas that are uninhabitable to sporophytes. Yet, recruitment happens in the gametophyte generation, and the resulting sporophyte distributions depend on gametophyte ecology.

Gametophyte biology is complex, and ontogeny and morphology vary tremendously among species (Atkinson and Stokey, 1964; Nayar and Kaur, 1971). A common observation is that there are the apparent fundamental differences in morphology and potentially longevity between epiphytic and terrestrial species (Dassler and Farrar, 1997, 2001). Epiphytic species often produce gametophytes with diverse morphologies that are frequently capable of asexual reproduction and are potentially long-lived. Most terrestrial species are thought to produce the short-lived, textbook cordate thallus and exhibit little ability to reproduce asexually (but see Watkins and Farrar 2005). Little quantitative data have been generated to back up such longevity claims, and I have been unable to find a single paper that describes factors that influence the distribution and mortality of tropical gametophytes.

The goal of our study was to examine the causal mechanisms of the distribution of fern gametophytes and the demography of several tropical epiphytic, hemiepiphytic, and terrestrial species. We examined the distribution of epiphytic and terrestrial gametophytes and ask what *in situ* factors control gametophyte establishment. Then we examined the gametophyte demography of 5 species from different habitat types, to assess gametophyte survival and recruitment rates and relate these to life history.

Materials and Methods

Study Site

This study was conducted at La Selva Biological Station (Heredia Province) in the Atlantic lowlands of northeastern in, Costa Rica. La Selva is a 1400 ha tropical wet forest having a mean annual rainfall of about 4,300 mm, with peaks of precipitation in June-July and November-December, and a drier period in March. Mean monthly rainfall

nevertheless never falls below 150 mm in any month during the dry season based on long-term meteorological records.

Gametophyte Transects

In order to describe the occurrence of gametophytes in nature, 425 plots of 25 cm × 25 cm were placed along 50 randomly chosen terrestrial 50m transects, and 425 25 cm × 25 cm canopy plots were placed in 9 canopy trees. The total number of gametophytes (irrespective of identification) was counted and recorded. Each quadrat was coded for level of disturbance: 0=undisturbed (<5cm² bare substrate); 1 = low disturbance (i.e. >5cm² bare substrate); 2 = medium disturbance (>5cm² bare substrate and substrate disturbed); 3= high disturbance (100% bare substrate and substrate turned over). Additionally, each quadrat with a disturbance rating of Level 1 and above was coded for the type of damage when possible. To assess light environment, a digital hemispherical photograph was taken with a Nikon Coolpix 950 digital camera (Melville, NY, U.S.A.) with a fisheye lens attachment, then analyzed using Gap Light Analyzer software (Frazer et al., 1999) to estimate the percentage of total light transmittance. Photos were taken 25 cm above each quadrat. To determine the influence of light environment on density, both number of gametophytes and percent canopy transmittance were log transformed and analyzed by regression analysis. We used ANOVA to determine the influence of level of disturbance on gametophyte density. Unless otherwise stated, all analyses were performed with the computer program JMP version 5.01 (SAS-Institute, 2005).

Disturbance Plots

To better understand the influence of disturbance and light on terrestrial fern establishment, 20 disturbance plots were established and monitored for gametophyte

density at 5 months post establishment. All plots were established on the same soil type in primary forest, with 10 plots placed in low-light understory habitats and 10 placed in high-light canopy gaps of similar age. Each plot measured 1m^2 and was divided into four $0.5\text{ m} \times 0.5\text{ m}$ subplots of increasing disturbance that were similar in degree to those found in nature. The undisturbed treatment subplot acted as the control, and no leaf litter was removed. For low disturbance level, we removed all leaf litter with no mechanical damage to the soil. The medium disturbance level was raked with a metal sand rake to disturb the first 5 cm of soil. The high disturbance level was physically turned over with a shovel, to a depth of approximately 20 cm. Gametophyte density and diversity were recorded in the center 25 cm^2 area. Litter-fall was removed from the disturbed plots weekly, and after 5 months, plots were assessed for density (and when possible, diversity of gametophytes). Light environment was determined with digital photography as discussed above. Determination of gametophyte identity was difficult, and individuals were thus lumped into “types” that in actuality may represent multiple species. Types were assigned based on morphological characters that were identifiable by the use of a 10X or 20X hand lens and were identified and organized based on: trichome presence and type, rhizoid color, gametophyte shape, and the presence and morphology of gemmae. Identification of fern species from gametophytic characters was complicated and should be taken as a conservative estimate of actual species richness. Only those gametophytes that were mature were counted. A 2X4 full factorial ANOVA was used to determine the effects of both light and disturbance intensity on gametophyte density and diversity.

Demography

In June of 2003, gametophytes from three populations of each of 5 species (See table 2-2) were located and marked in the field. Marked individuals were checked once

each month and followed for the next 15 months with one final census made at 25 months. No data were recorded during the 5-7th month. At each census, individuals were recorded as present, dead or missing, or as recruits into the sporophyte generation. When possible, individuals were coded for their cause of mortality.

In the case of terrestrial species, gametophytes were marked with a numbered aluminum nail; whereas, the epiphytic species were either marked with a nail or with a numbered tag attached to the substrate with copper wire. It was not possible in all cases to determine precisely the initial age of marked gametophytes. Therefore, individuals were chosen according to their initial size. Initial sizes were held constant within a species but differed among the species. Longevities were calculated as the time between the initial mark (treated as birth) and death of each gametophyte. Species were chosen to represent different functional types as discussed below. A major flood event took place in month 11; individuals were sampled three days before the flood (for the regularly scheduled 11 month survey) and then three days after the flood to serve as an extra survey to determine the influence of flooding. The next sample period took place on the next corresponding survey day and was recorded as the month 12 survey period. This allowed for more precise determination of mortality due to flooding rather than categorizing these individuals in the unknown category.

Gametophyte Survival Analysis

As with many demographic studies, individuals can leave the study by different avenues. Such absent samples were coded as right-censored data points (Hollander and Wolf, 1999). In this study, only those individuals that recruited into the sporophyte generation and those still alive at the end of the experiment (25 months) were recorded as censors. Censoring individuals reduces the sample size of individuals at risk after the

time of censorship. Censoring, therefore, reduces the number of individuals contributing to the curve, and each death after a censored point represents a higher proportion of the remaining population. Subsequent deaths will result in greater decreases in overall survivorship. Censorships that occur early in the study have a greater effect on survivorship curves than those removed at later periods. Thus, the data from the survivorship curve after the first censor represent an estimate and not the actual survivorship of the population. In order to clarify the survival curves, we also plotted the cumulative proportion of individuals that recruited at each time interval.

Gametophyte survival functions were estimated using non-parametric Kaplan-Meier product-limit survival functions (Collett, 2003). These analyses were also used to estimate mean life span for each species. Log-rank X^2 statistics were computed to test for homogeneity of the survival functions for all species. Weibull distributions were used to model survivorship functions and to calculate the parameters α and β . The scale parameter α is a measure of the degree of hazard for the species; whereas, the shape parameter β determines the degree of change in the hazard function over time. Large values of α correspond to low hazard levels (i.e. greater survivorship) where low values equate to rapidly decaying survivorship. Large values of β (i.e. >1) correspond to an increasing hazard rate that affects older individuals over younger individuals. With a $\beta < 1$ younger individuals are more likely to die within the period of the experiment.

Results

Transects

A combined total of 2096 gametophytes were sampled with 329 recorded from canopy, 538 from low-trunk, and 1229 from terrestrial habitats. Level of disturbance had a highly significant effect on the number of gametophytes in terrestrial habitats (Fig. 2-

2b, $r^2=0.65$, $F=53.58$, $p<0.001$) with greater percentages of gametophytes occurring in more disturbed habitats. Less than 1% of terrestrial gametophytes were found in areas without disturbance. The opposite trend was apparent in canopy habitats where level of disturbance had less influence on numbers of gametophytes (Fig. 2-2a, $r^2=0.11$, $F=2.40$, $p=0.08$) and greater percentages of gametophytes occurred in less disturbed habitats (58% of canopy gametophytes were found in areas with no disturbance).

In terrestrial transects, seven causes of disturbance were identified: leaf-litter removed, new and old root tip-ups from fallen trees, rotten logs, erosion, branch falls, and animal causes (Fig. 2-2b). A total of six causes of disturbance were identified in canopy habitat: insect, branch falls, epi-slides, physical damage, animal, and unknown causes (Fig. 2-2a). Identification of causes in the low-trunk habitat was difficult and thus was excluded from all analyses. In the case of terrestrial species, recent root tip-ups harbored the greatest number of gametophytes (>50%). The disturbance category with the greatest percentage of gametophytes in canopy habitats was animal disturbance with ~20%. In addition to disturbance, canopy openness (as a surrogate for light level) exhibited a positive effect on the number of terrestrial gametophytes (Fig. 2-3a, $r^2=0.423$, $p<0.0001$), but exhibited little influence on canopy gametophyte density (Fig. 2-3b, $r^2=0.001$, $p<0.539$).

Disturbance Plots

A total of 1247 gametophytes from 16 morpho-types were counted in the experimentally disturbed plots. There were 6 non-unique morpho-types found in the low light treatment. There were a total of 16 types with 10 unique types in the high light treatments. There was a significant and positive effect of both increasing light and disturbance and the interaction of light and disturbance on gametophyte density among

the plots (Fig. 2-4, Table. 2-1). Likewise, morpho-type richness was significantly influenced by both light and disturbance but exhibited no significant interaction (Fig. 2-4, Table.2-1).

Demography

All combined, 809 gametophytes from the five species were marked and followed throughout the demography study. A total of 263 gametophytes were marked from the understory terrestrial species *Danaea wendlandii*. The three populations of this species were all recorded in the understory of primary forests from sites that were at least 50m from trail sides. We marked 275 gametophytes of *Pityrogramma ebenea*, an abundant species often found in full to partial sun in disturbed sites such as road and trail sides. All populations of this species were recorded from disturbed sites within the forests or in open areas away from trail sides. Sixty-seven gametophytes of the understory hemiepiphyte *Lomariopsis vestita* were marked from small diameter trees in primary forests. Two canopy epiphytes were also marked: 98 from the high-light epiphyte *Vittaria lineata*, and 106 from the medium-light understory epiphyte *Campyloneurum brevifolium*.

Survival distribution functions varied significantly among species for the entire survey period (Table 2-2, log-rank $\chi^2 = 386.2$, d.f. = 4, $p < 0.0001$). *Campyloneurum brevifolium* had the highest mean longevity and was 5 times that of the lowest: *Pityrogramma ebenea* (Fig. 2-5a, 2-6a). When combined, the epiphytic species: *C. brevifolium*, *Lomariopsis vestita*, and *Vittaria lineata* had higher mean longevity than the terrestrial species (log-rank $\chi^2 = 212.3$, d.f. = 1, $P < 0.0001$). In all cases $\beta > 1$, indicating an increasing hazard rate suggesting that older individuals are more likely to die than younger individuals over the study period. Percent recruitment varied among the

species, with *C. brevifolium* < *V. lineata* < *D. wendlandii* < *L. vestita* ~ *P. ebenea*. The cumulative proportion of gametophytes recruiting varied for all species over the sampling time of the study (Fig. 2-6b). Initial recruitment was highest for both terrestrial species. More than 30% of gametophytes of *P. ebenea* had recruited between plot establishment and the first census. No additional individuals recruited beyond month eight. Initial recruitment was lower for *D. wendlandii*, but increased throughout the study period. Recruitment was lowest for *V. lineata* and *C. brevifolium* with essentially no recruitment occurring after the third census up until the 25th month (Fig. 2-6b). The percent of gametophytes still alive at the end of the study also varied from 0% in *P. ebenea* to just over 70% in *C. brevifolium* (Fig. 2-5b).

A total of 7 causes of mortality, including an unknown category, were surveyed in the field. Catastrophic habitat failure occurred when habitats were over 95% of the individuals were destroyed. This happened when entire trees fell in the case of epiphytes or when hill sides collapsed with some terrestrial species. Flooding also resulted in catastrophic failure, but was separated as unique disturbance type because we were able to directly assess its influence (Fig 2-2a). Minor erosion also resulted in the loss of some individuals as did a massive flood in month eight of the study. Fungal attack, herbivory, and physical damage (as would occur from a branch or rock fall that physically removed individuals from the population) were also identifiable causes of mortality. The unknown category likely consisted of a contribution of all of these plus unidentifiable novel disturbances. Each cause resulted in different magnitudes of mortality, with some categories completely absent from some species and/or populations (Fig. 2-2). Most %

mortality was attributable to unknown causes such as an individual simply missing from the population without any sign of disturbance, etc.

Discussion

Gametophyte Distributions

The present study clearly demonstrates the importance of disturbance for gametophyte establishment. Even minor disturbances that remove leaf litter and turn up the soil can produce sites for gametophyte establishment. Disturbance that physically turns up soil not only produces an exposed and competition free habitat, it can also expose the underlying spore bank and provide additional propagules that may further contribute to density and richness. Surprisingly, the maximum number of terrestrial gametophytes found in the lowest level of natural disturbance was three and the majority of the undisturbed sites had no established gametophytes.

To my knowledge, disturbance has never been reported to be an important factor influencing gametophyte density or shaping species distributions. However, studies on the gametophytes of temperate species have highlighted the importance of nutritional and edaphic safe sites for the gametophytes of *Lorinseria (Woodwardia) areolata* (Cousens, Lacey, and Scheller, 1988) and *Blechnum spicant* (Cousens, 1981). Little is known of other factors influencing gametophyte distributions and a few temperate studies have produced mixed results suggesting that gametophyte gender expression may influence gametophyte distribution (Klekowski, 1969; Crist and Farrar, 1983) while others have found no relationship (Holbrook-Walker and Lloyd., 1973; Greer and McCarthy, 1999). Apart from these studies, little is known of the influence of these characters on tropical fern gametophyte distributions. Numerous studies have however, examined factors behind sporophyte distributions and have fingered important roles of microclimate

(Nobel, 1978) and water availability in dry sites (Marquez et al. 1997) and edaphic factors (Tuomisto and Ruokolainen, 1994a; Tuomisto and Poulsen, 1996; Tuomisto, Poulsen, and Moran, 1998) in wet tropical sites. None of these studies have directly addressed the role of disturbance.

The nature of disturbed habitats creates a positive feedback for species that prefer disturbed sites. By their very nature such sites are unstable and result in increased mortality due to continued habitat erosion. *Pityrogramma ebenea* is perhaps the most common species of disturbed habitats at La Selva. The species produces large numbers of spores with high fecundity, and the gametophytes can be found in virtually any habitat where disturbance is present, i.e. exposed road/trail cuts and the relatively dark understory (pers. obs.). The gametophytes of this species also germinate, grow, and recruit rapidly (Fig. 2-6). Rapid development is necessary in species that occupy highly disturbed habitats. Catastrophic events are common in the habitat of this species, and in two populations such disturbance resulted in the near-complete habitat destruction and in continued habitat instability exacerbated by wet season rains. Epiphytic species are also subject to catastrophic disturbances; a single population of *Vittaria lineata* experienced this sort of disturbance following a large tree fall which resulted in 100% mortality of one of the study populations. However, these events seem relatively rare and epiphytic habitats tend to be more stable when compared to terrestrial habitats in this forest.

Based on these data, terrestrial gametophytes simply do not establish in sites that are disturbance-free. However, there are varying levels of tolerance and clearly different life histories in terrestrial species. *Danaea wendlandii* is a eusporangiate fern. The eusporangiates have many unique characters, but of particular interest for this study is the

production of liverwort-like gametophytes that are several cell layers thick. Individual gametophytes are often large and more resistant to physical damage relative to the single layered leptosporangiate species (pers. obs.). Such tough gametophytes that occur in sites with minor disturbance confer longevity. Indeed, the gametophytes of *Danaea wendlandii* exhibited 3 times the mean longevity and significantly less recruitment than those of *P. ebenea*. Two populations of *Danaea wendlandii* fell in the flood zone, and while such disturbances are clearly part of the biology of this species, this event resulted in lower mean longevities in this study. The eusporangiate biology of this species places it nearly opposite of *P. ebenea* in terms of life history strategy.

There were also surprising differences between epiphytic and terrestrial species. One emergent difference between these two groups is the percent of gametophytes that survived but did not recruit. Over 70% of the gametophytes of *C. brevifolium*, and over 50% of both *V. lineata* and *L. vestita* were alive and un-recruited by the 26th month. This, compared to the less than 5% in *Danaea wendlandii* and 0% in *Pityrogramma ebenea*. This observation highlights fundamentally different gametophytic life history strategies that have evolved in the two life forms. In fact, recent phylogenetic analysis of the ferns has revealed a recent split between terrestrial and epiphytic clades in the Eu-Polypodiales. One of the defining gametophytic characters of the epiphytic clade is indeterminate and asexually reproducing gametophytes. Dassler and Farrar (1997) have argued that such longevity and asexual reproduction is a mechanism to encourage outcrossing in epiphytic species that may carry significant genetic load. Long-lived thalli and thus genotypes can produce numerous archegonia over space and time to ensure fertilization of newly

dispersed genotypes. Such differences in life history are significant and may have been critical in the radiation from terrestrial species into canopy habitats.

Density and Species Richness

Canopy gametophyte density is clearly more sensitive to disturbance, with nearly 58% occurring in undisturbed sites. Additionally, there was not a detectable relationship between gametophyte density and light environment as was shown for terrestrial density. In general, canopy light environments are significantly higher than terrestrial sites and the lack of response to light in the former is not surprising in a habitat where this light is not limiting. However, the canopy does experience temperature and humidity extremes and it is plausible that microclimate and water availability play larger roles in these habitats relative to terrestrial sites. Such an observation would be in line with reports by Hietz and Briones (1998a) who demonstrated that within canopy distribution of fern sporophytes was largely a function of species water relations.

Quantification of gametophyte species/morphotype richness in the natural transects was abandoned largely due to time constraints. However, there was a clear light-disturbance-density relationship for terrestrial species, and for this reason we examined these variables more completely in the terrestrial disturbance experiment. In one high disturbance plot, we counted six different morphotypes with 65% of the density dominated by *Pityrogramma ebenea*. There are numerous life history strategies in the ferns, and *P. ebenea* is a species with high germination and recruitment rates. Indeed, it is possible that all of the species that were encountered in the higher disturbance plots exhibited similar life histories. Clearly there are specific differences in gametophyte ecology that influence densities at a given site. However, the near complete absence of gametophytes in undisturbed habitats suggests that disturbance may be critical to the

majority, if not all terrestrial species regardless of life history. Such natural observations combined with experimental manipulations offer strong evidence that light and disturbance both act to structure terrestrial gametophyte density and richness.

Unlike comparisons of seedling-adult distributions, fern gametophytes are completely and fundamentally different organisms from sporophytes. For this reason, gametophytes would not necessarily be expected to behave like sporophytes. Firstly, gametophytes are often, if not always more widespread than sporophytes (Peck, 1980; Peck, Peck, and Farrar, 1990). Such plasticity and simplicity in function may reduce the role that nutrients or other edaphic factors play in gametophyte distribution. Secondly, gametophytes can be long lived with some individuals living decades in culture (Mottier, 1927; Walp, 1951) and in years field settings (pers. obs.). Additionally, gametophytes may be relatively sensitive to desiccation and temperature changes; however, many studies have shown that gametophytes are relatively robust to environmental stress (Sato and Sakai, 1980; Cousens, 1981; Sato and Sakai, 1981; Cousens, Lacey, and Scheller, 1988; Ong and Ng, 1998). While survival from stress and edaphic requirements may be important, for all species, emergence in litter free sites seems critical.

Sporophyte Ecology

Gametophyte and sporophyte distributions are related; however, the point of gametophyte establishment is not necessarily the point of mature sporophyte distribution. Epiphytic species are often associated with creeping rhizomatous growth. Such growth may allow a perfectly healthy gametophyte to produce sporophytes in less than optimal conditions. Such sporophytes may have the ability to then grow into more favorable habitats where they can reproduce. The resultant mature sporophyte distributions produced by such a strategy may obscure much of the species biology. Epiphytic species

may also form gametophyte banks that are long lived and stress tolerant and like many seed banks, have the ability to wait for appropriate conditions to appear before recruiting in to the sporophyte generation. This may be especially true for species whose gametophytes also have a means of vegetative reproduction. This ability is common in epiphytes (Atkinson and Stokey, 1964; Farrar, 1990; Farrar, 1998) and has been reported in some terrestrial species (Watkins and Farrar, 2005). Such abilities question the common assumption that gametophyte 'safe sites' limit the establishment of sporophytes in epiphytic species. This hypothesis may hold more merit with terrestrial species but is unlikely as important for epiphytic species.

Conclusions

Such apparent and fundamental differences in life history and the way that epiphytic and terrestrial life forms respond to disturbance and light provides evidence for adaptively meaningful variation in life histories that has evolved in the two groups. Epiphytic species have evolved in a high light, highly competitive, yet relatively stable matrix. Such environments reduce the light limitations encountered by terrestrial species, yet they incorporate closer contact with bryophytes. Dassler and Farrar (1997) have argued that differences in gametophyte morphology and asexual reproduction between epiphytic and terrestrial species have largely evolved due to pressures from bryophyte competition. Such changes may have only been possible in canopy habitats where disturbance is less intense. Radiation into canopy habitats required a suite of adaptive characters in both the gametophyte and sporophyte generation. One major advantage of the canopy habitat is reduction in litter that can cover developing gametophyte. While canopy habitats accumulate enormous amounts of organic matter (Cardelus and Chazdon, 2005), wind often removes a significant proportion of leaves that land on internal and

especially outer branches (Nadkarni and Matelson, 1991). The presence of leaf litter may be the most important limiting factor to terrestrial species establishment as there are relatively few morphological or physiological pathways that would allow a species to survive under leaf litter. The mechanisms behind sporophyte distributions remain complicated as they clearly also rely on gametophyte ecology. This is further complicated by spore dispersal which may obscure the relationship between patterns of distribution and habitat heterogeneity. Regardless of dispersal limitations or lack thereof, terrestrial sporophytes are largely elements of disturbance past. Additional work on gametophyte ecology will need to take into account edaphic and microclimatic factors to better understand variables shaping the distribution of this magnificent group of plants.

Table 2-1. Relationship of gametophyte density and richness with three levels of experimental disturbance and two light levels

Gametophyte density			
Source	DF	F	P > F
Light	1	52.643	0.000
Disturbance	3	18.567	0.000
Light*disturbance	3	6.152	0.001

Number of species			
Source	DF	F	P > F
Light	1	46.522	0.000
Disturbance	3	6.662	0.000
Light*disturbance	3	0.364	0.779

Table 2-2. Demographic and survival analyses for the gametophytes of 5 fern species using the Wilcoxon test to compare survival distribution functions for different species

Species	Number of Gametophytes			Survival Analysis			
	Dead	Censored	Total	Mean Months	Std	X2	P > X2
<i>Campyloneurum brevifolium</i> (Lodd. ex Link) Link	19	87	106	22.9	0.57	386.2	<0.0001
<i>Danaea wendlandii</i> Rchb. f.	168	95	263	12.3	0.56		
<i>Lomariopsis vestita</i> E. Fmyn.	22	45	67	20.8	0.82		
<i>Pityrogramma ebenea</i> (L.) Proctor	165	110	275	4.1	0.28		
<i>Vittaria lineata</i> (L.) Sm.	40	58	98	16.6	1.08		

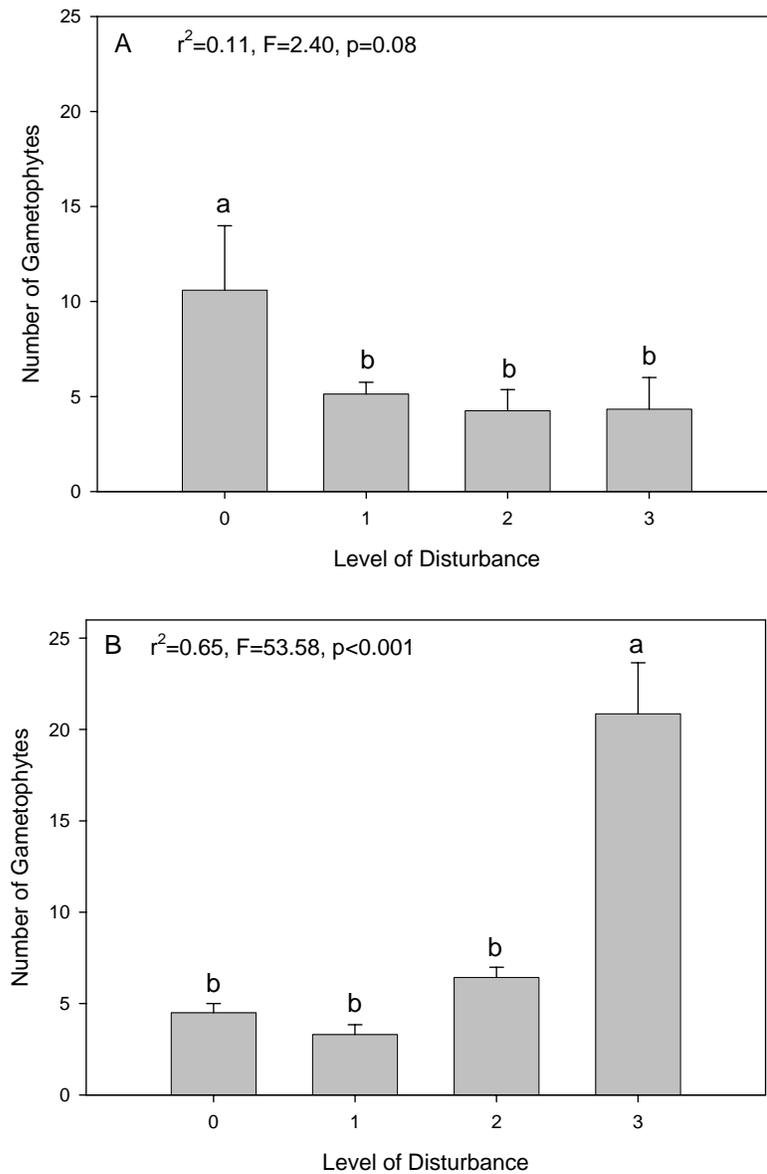


Figure 2-1. Number of gametophytes counted and their relation to disturbance from natural transects in both A) Canopy habitats and B) Terrestrial habitats. Here 0 represents no disturbance ($< 5\text{cm}^2$ bare soil), 1 = low disturbance (i.e. $> 5\text{cm}^2$ bare soil), 2 = medium disturbance ($> 5\text{cm}^2$ bare soil and soil disturbed), 3 = high disturbance (100% bare soil and soil turned over).

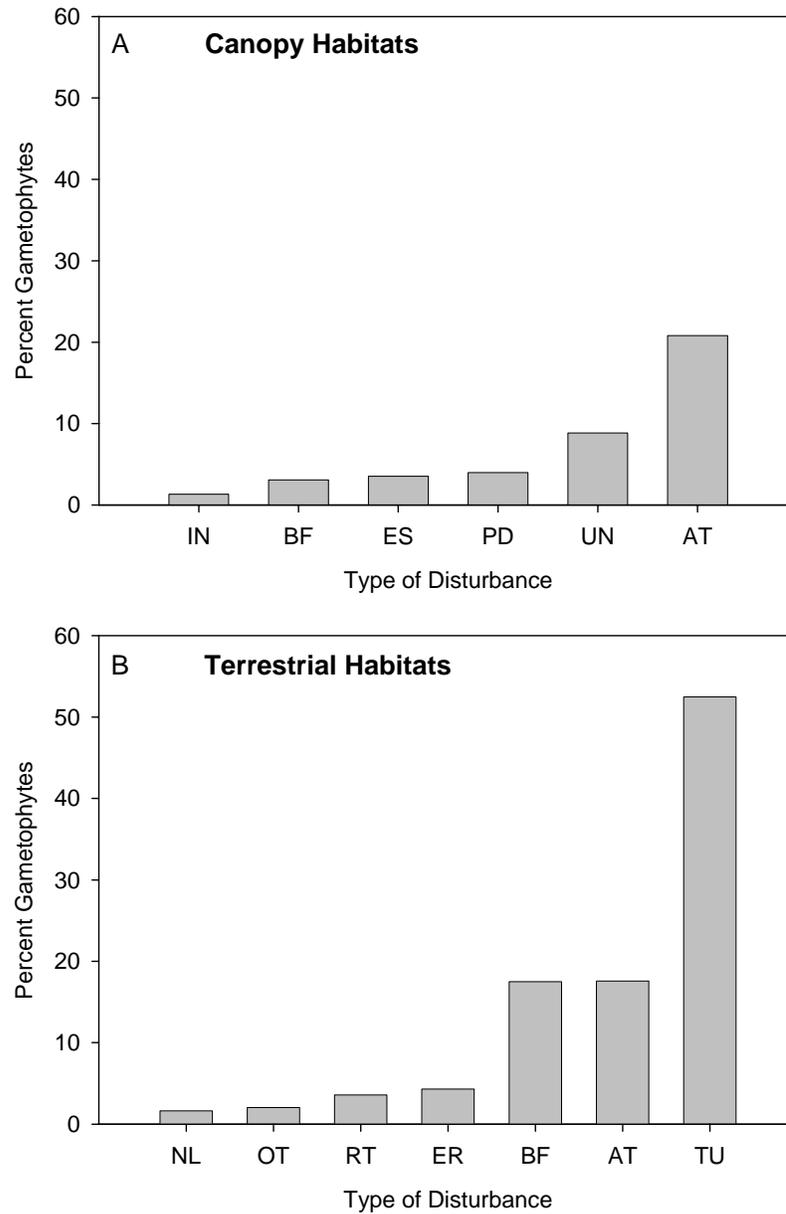


Figure 2-2. The percentage of fern gametophytes as influenced by type of disturbance identified. A) In canopy habitats (IN: insect damage, BF: branch fall, ES: epislides, PD: physical damage, UN: Unknown, AT: animal trail) and B) In terrestrial habitats (NL: no leaf litter, OT: old tip-up mound, RT: rotting tree/wood, ER: erosion, BF: branch fall, AT: animal trail, TU: recent tip-up mound)

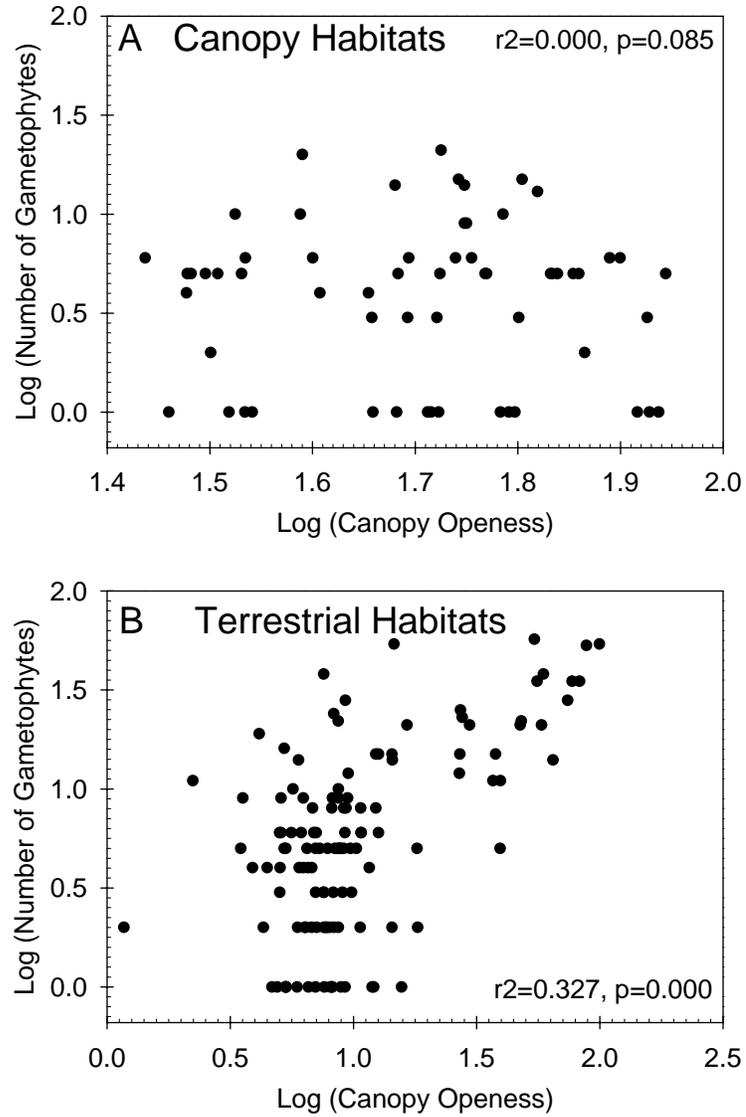


Figure 2-3. The relationship between canopy openness and gametophyte density for A. canopy and B. terrestrial species

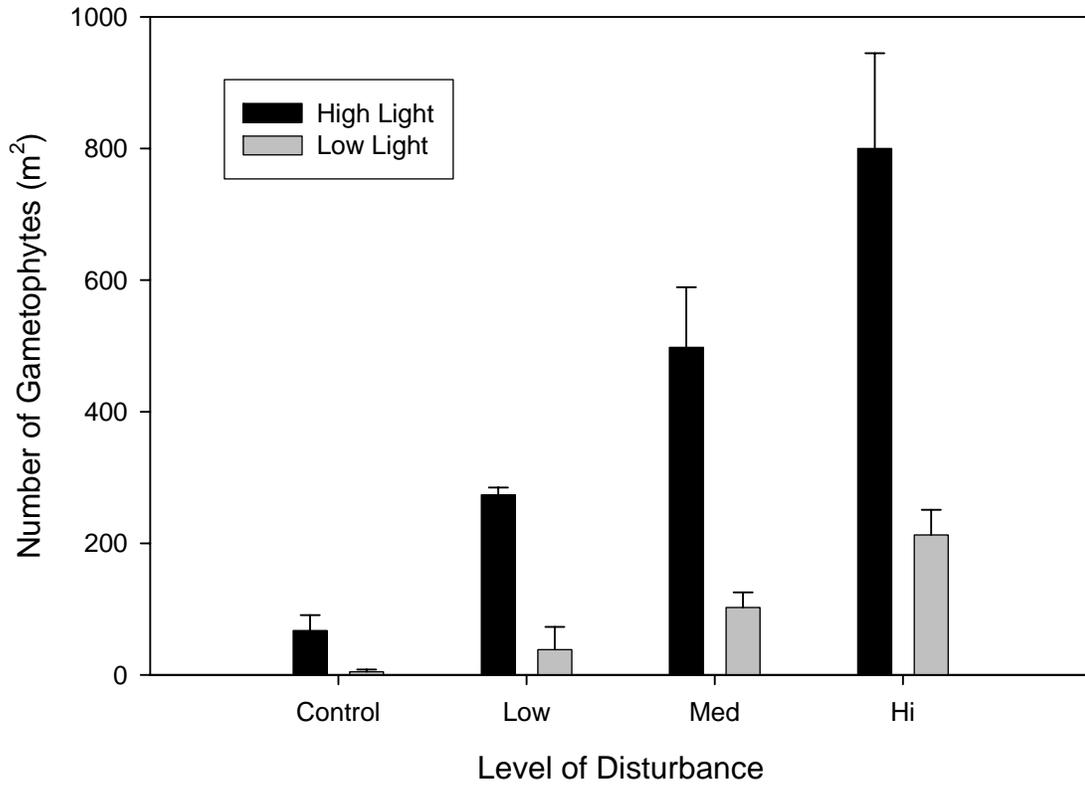


Figure 2-4. Gametophyte densities as influenced by light and disturbance in experimental plots. Control plots were those that had $<5\text{cm}^2$ of bare soil exposed. Low disturbance was created by only removing litter, medium plots were established by removing litter and raking soil to a depth of 5cm, high plots were created by removing litter and turning soil over with a spade to a depth of 20cm

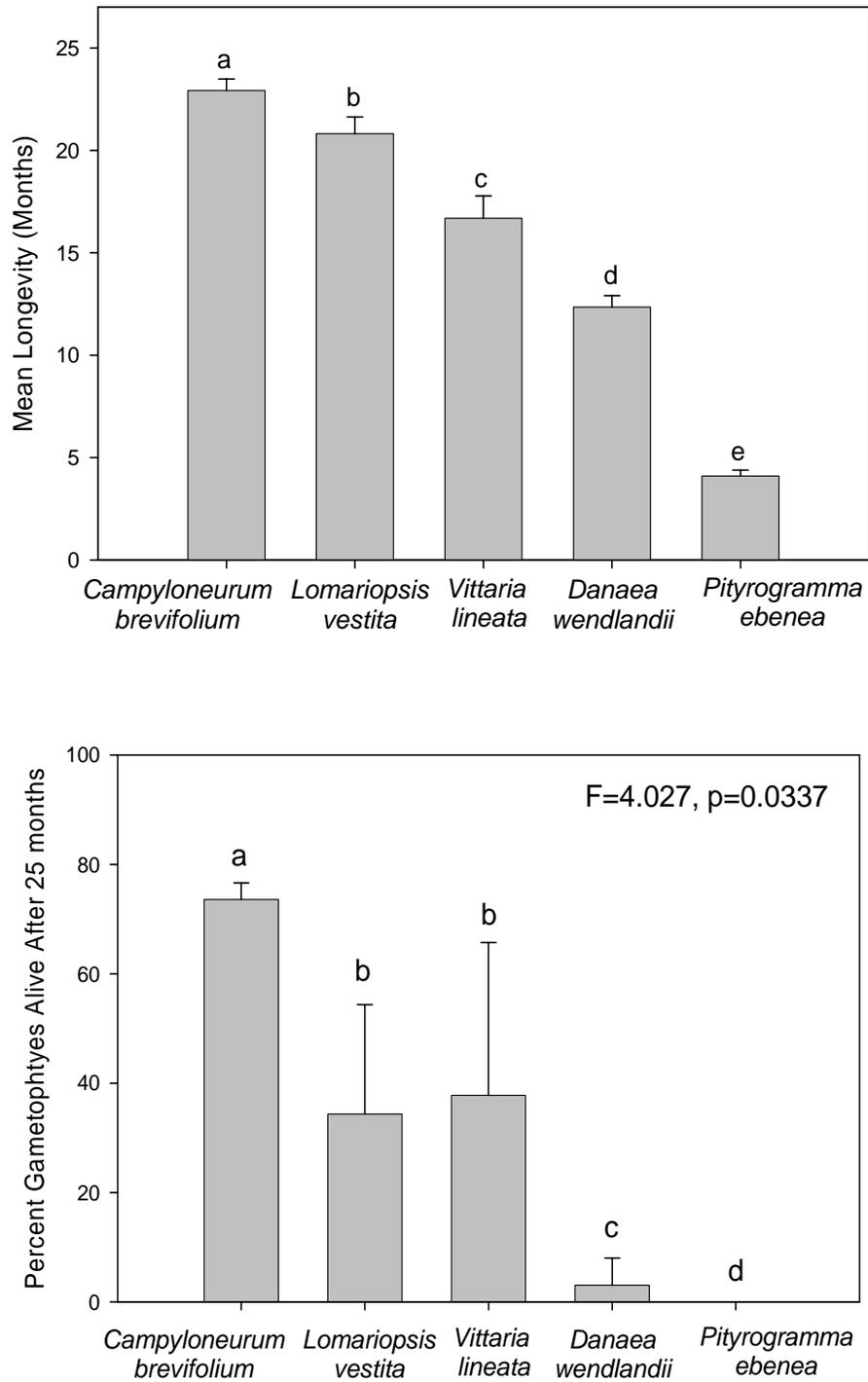


Figure 2-5. Mean longevity (months) A) and percent gametophytes still alive and un-recruited B) for the 25-month period of the study

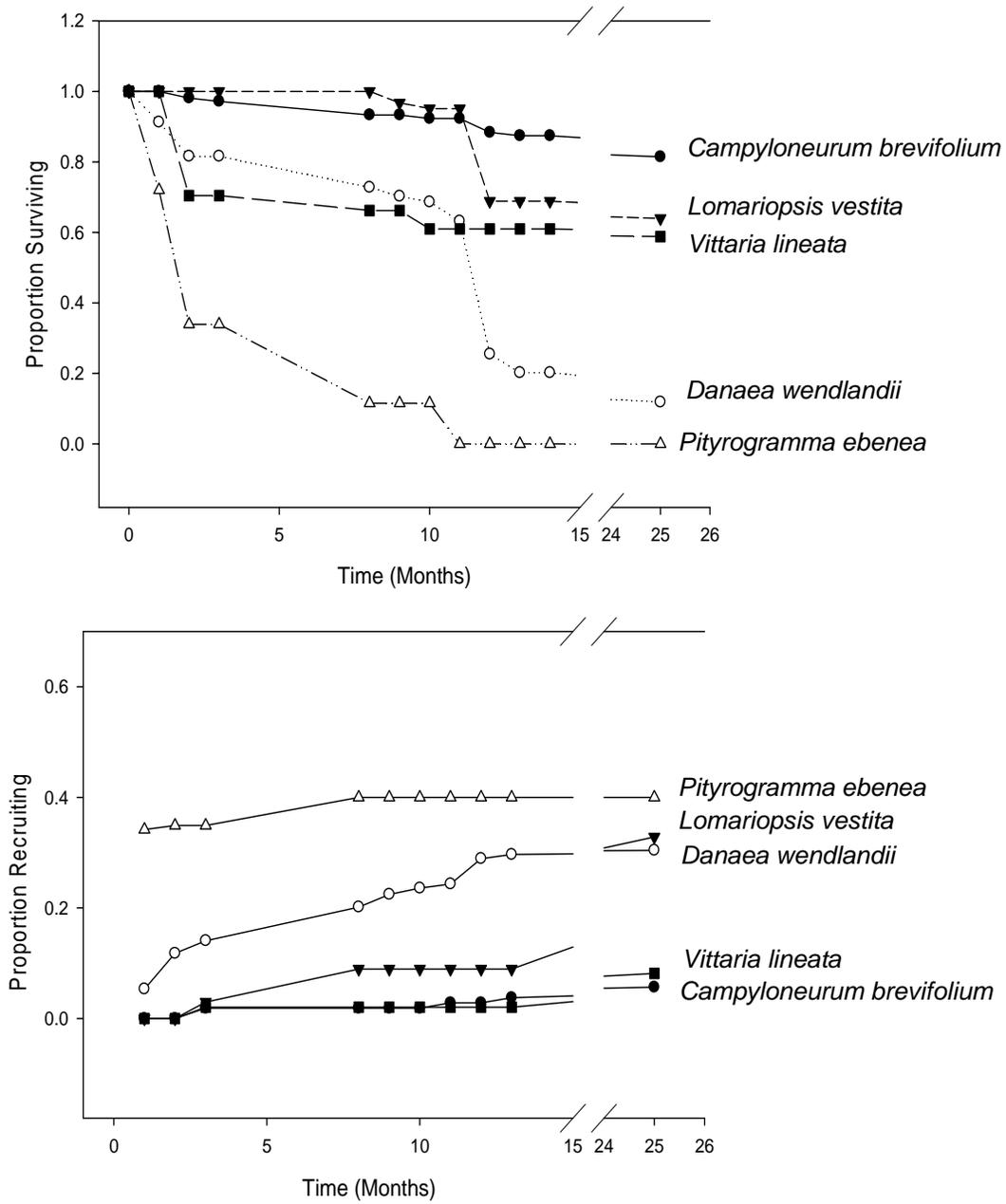


Figure 2-6. Kaplan-Meier survivorship curves A) and proportion recruiting B) of 5 species of fern gametophytes over the 25-month study period. No data were collected during months 4-7 and 15-24

CHAPTER 3
COMPARATIVE DESICCATION TOLERANCE OF TROPICAL FERN
GAMETOPHYTES: ECOLOGICAL AND EVOLUTIONARY CONSEQUENCES

Introduction

Overwhelming evidence indicates that land plants evolved from simple aquatic algal ancestors (Bold, 1957; Niklas, 1997). The radiation of once-aquatic plants onto dry land required the evolution of adaptive character suites that permitted life in what was and remains deadly dry air. To survive this environment, plants have evolved two mechanisms of surviving desiccating conditions. One mechanism is the avoidance of desiccation as demonstrated in most modern terrestrial plants and has been accomplished by the development of characters such as highly organized cuticles with effective stomatal control. Cacti of the dry deserts perhaps represent the pinnacle of avoidance with their succulent water storing stems, reduced leaves, and thick, well-developed cuticle. These characters and many other associated with desiccation avoidance are all thought to be highly derived and early land plants most certainly did not have such structures. Early plants relied on the unique mechanism of survival from desiccation, referred to as desiccation tolerance (DT). Desiccation tolerance has been differently defined; the most commonly accepted definition is survival of drying to equilibrium with surrounding air (Bewley, 1979). Such drying is more than sufficient to kill most any plant that relies on desiccation avoidance: at least 99% of the world's vascular flora (Alpert and Oliver, 2002).

Many of the characters that facilitated DT in ancestral land plants are still found in modern algae and bryophytes (Alpert, 2000; Oliver, Tuba, and Mishler, 2000; Alpert, 2005). Much as the cacti are to avoidance, the bryophytes are to tolerance. Fantastic stories exist in the literature demonstrating that some bryophytes can recover from 23 years of desiccation in herbaria (Alpert 2000 and references herein). Such remarkable abilities clearly have ecological consequences and many studies have shown that more desiccation tolerant bryophytes are often associated with more xeric habitats.

Consequences exist even within bryophyte species with one example being the overrepresentation of female gametophytes in dioecious bryophytes of xeric habitats. Such sex-based disparity is often related to greater DT in females relative to males (Stark 2005). Desiccation tolerance holds tremendous potential to influence the ecology of species (Deltoro et al., 1998; Csintalan, Proctor, and Tuba, 1999; Robinson et al., 2000; Cleavitt, 2002).

Apart from the linkage of DT to phylogeny, there have also been demonstrated differences in desiccation tolerance of different generations, such as larval-adult in some invertebrates or gametophyte-sporophyte in some bryophytes. The ability and degree of desiccation tolerance in the different stages can be radically different. In some cases a high degree of tolerance may exist in one stage but be absent in the other. For example, the gametophytes of some bryophytes exhibit a much greater degree of tolerance than sporophytes (Proctor, 2000; Proctor, 2001). Such variation also occurs invertebrates where the larvae of the fly *Polypedilum vanderplanki* exhibits greater desiccation tolerance relative to the adult stage (Watanabe et al., 2002). Within the vascular plants,

desiccation tolerance of spores and seeds is well known but much less is known about tolerance in lineages with two separate free-living stages.

The only lineage of vascular plants to exhibit two separate free-living generations is the pteridophytes. Of particular interest to desiccation tolerance are the morphological and physiological differences between these two stages in the ferns. The gametophyte is the point of gamete formation and fertilization and is small, lacks vascular tissue, and has a poorly developed to non-existent cuticle. The sporophyte produces spores and is thus the primary stage for dispersal; it has a well developed vascular system and a waxy cuticle complete with stomata. These differences alone result in unique life-cycle-mediated ecological strategies, especially as they relate to water relations. True desiccation tolerance in the sporophyte stage is known from and likely only exists in relatively few species (Gaff, 1987; Porembski and Barthlott, 2000). In a recent review on the subject, Proctor and Pence (2002) recorded that <1% (64 species) of the ferns studied exhibited DT and of those, 40 were Cheilantheid taxa that are commonly associated with desert-like habitats. Much less is known of species from tropical habitats, but DT has been recorded in genera as phylogenetically disparate as *Asplenium* and *Polypodium* (Kappen, 1964; Gaff, 1987; Proctor and Pence, 2002). The species in which it does occur are often extreme xerophytes living in deserts or other highly exposed and dry environments. As with bryophytes, the apparent degree of desiccation tolerance in the sporophyte stage has been linked with species ecological distribution (Harten and Eickmeier, 1987; Hietz and Briones, 1998b). Studies are still too sparse to determine the extent of this character in structuring populations.

Studies on desiccation tolerance of the gametophyte generation of the ferns are fewer in number. Some of the earliest comments were made by Goebel (1900) regarding the ability of the buried tubercles of *Annogramme chaerophylla* to resume growth following dry spells. A similar observation was made by Cambell (1904) on the unburied gametophytes of *Gymnogramme triangularis* that appeared to have survived a dry California summer. The first evidence was generated by Pickett (1913; 1914; 1931) who, through a series of desiccation experiments was the first to clearly show that the gametophytes of *Asplenium rhizophyllum* and *A. platyneuron* could recover growth following extreme desiccation. He also discovered that there was a greater degree of tolerance in *A. Rhizophyllum*, a species of more exposed and drier habitats, relative to *A. platyneuron*, which is often confined to more mesic sites. This was the first link of desiccation tolerance in the gametophyte generation with sporophyte distributions and species ecology. Pickett's work has largely been the last of its kind, and apart from anecdotal reports (Gilbert, 1970) and observations on the ability of the gametophytes of *Pyrossia pilosellodes* to recover from drought (Ong and Ng, 1998) nothing is known of the ability of fern gametophytes to tolerate desiccation and of their rates of recovery.

The goal of this paper is to survey a broad range of tropical fern gametophytes to determine the extent of desiccation tolerance in this phase of the fern life cycle. We first examine the ability of several species to recover from a single desiccation event. We then subject a select number of several species of varying life histories to a more extensive repeated dry down cycles and drying intensities and relate the results to the ecological distributions of the species.

Materials and Methods

Spore Material and Growth Conditions

Spore material from 12 species of varying ecology was collected from La Selva Biological Station in the Atlantic lowlands of northeastern Costa Rica at 37–100 masl. Fertile fronds were gathered in the field and put into glassine envelopes with tape-sealed seams. Envelopes with plant material inside were stored in an air-conditioned lab and allowed to dry under these conditions. Spores were brought back to the University of Florida where they were cultured. The growth of a broad sampling of species with different ecologies required extensive experimentation with culture techniques, it was discovered that species grew best on a combination of organic soil collected from canopy trees at La Selva mixed with a small amount of vermiculite. Spores were sown on this medium into 60mm x 15mm Fisherbrand Petri plates. These plates were stored in sealed clear plastic containers (Pioneer Plastics Model 395-c, Dixon, KY). Cultures were exposed to $20\mu\text{mol m}^{-2} \text{sec}^{-1}$ for 10hrs day^{-1} from GE fluorescent plant and aquarium 40watt growbulbs and watered with deionized water every 10-12 days.

Desiccation Experiments

For the initial survey experiment, 5-10 mature gametophytes (one gametophyte per Petri plate) of all 12 species (see Table 3-1) were allowed to desiccate at a vapor pressure deficit (VPD) = 1.3kPa (50% relative humidity) in a VPD controlled chamber that was constructed using one of the plastic growth boxes connected via Bevline tubing to a Licor dew point generator (Model 610, Lincoln, NE) set to a flow rate of 0.5 L min^{-1} . Samples were allowed to dry for 45min in a constant vapor pressure deficit and were removed from the box every 5min and placed in a Sartorius microbalance (Göttingen, Germany) where weight and a measurement of F_v/F_m was taken (see methods below). The

samples were then placed back into the box. The volume of the box was relatively small, and a Hobo Pro RH/Temp Data Logger (Bourne, MA) was used to verify that the container typically regained the 1.3kPa VPD within one min of the top being replaced. To evaluate recovery, upon completion of the desiccation treatment, samples were consecutively rehydrated by adding 5-10 drops of deionized water to the thallus. After 1 hr of rehydration, measurements of F_v/F_m were again made at 5min, 24hrs, and 48hrs post rehydration. Samples were then dried for 72hrs in a drying oven at 70°C and weighed to determine gametophyte dry weight. Relative water content was plotted against time and F_v/F_m .

A second desiccation experiment was designed to test the effect of drying intensity on recovery of F_v/F_m . The gametophytes of *Diplazium subsilvaticum* and *Phlebodium pseudoaureum*, and *Polypodium triseriale* were chosen to represent the extremes of tolerance from the initial desiccation experiment and they were dried at three different intensities VPD=0.5kPa (20% RH), VPD=1.3kPa (50% RH) and VPD=2.1kPa (80%RH) following the methods above. Gametophytes were kept at these VPD for 72hrs after which time they were rehydrated with deionized water and measurements of F_v/F_m were taken at 24, 48, and 72hr post rehydration. These values were related to the dark adapted value of F_v/F_m to determine the mean percent recovery.

A third experiment was run to examine the influence of consecutive desiccation cycles on photochemical efficiency. Plant material from six species was chosen from the survey experiment to represent the different recovery abilities. Thirty gametophytes were selected and ten were dehydrated for one, two, or three cycles at VPD=1.3kPa and kept at this level for 72 hrs using the methods described above. Material was then rehydrated

with deionized water and measurements of F_v/F_m were again made at 24hrs, 48hrs, and 72hrs post rehydration. These values were related to the dark adapted value of F_v/F_m to determine the mean percent recovery.

Chlorophyll-Fluorescence Measurements

Variation in photochemical efficiency (F_v/F_m) was measured as the desiccation dependent change in ratio of variable and maximal fluorescence F_v/F_m where F_v is the difference between the maximum (F_m) and the minimum (F_o) fluorescence emissions (Mulkey and Pearcy, 1992; Horton, Ruban, and Walters, 1996) measured using an Opti-Sciences pulse modulated fluorometer (Model OS-500, Hudson NH). Minimal fluorescence was measured under a weak pulse of modulating light over 0.8 s, and maximal fluorescence was induced by a saturating pulse of light ($8000 \mu\text{mol m}^{-2} \text{s}^{-1}$) applied over 0.8 s. The parameter F_v/F_m was first measured after 20mins dark adaptation, and this measurement was taken as the index of recovery. Dark-adapted F_v/F_m provides an estimate of the maximal quantum efficiency of Photosystem II, which in unstressed material is generally around 0.76–0.83 (Demmig-Adams and Adams, 1992).

Statistical Analysis

For the initial desiccation survey, a series of regressions was run on arcsin square root transformed relative water content (RWC) data against time for each individual within a species to determine the rate of drying of gametophytes exposed to a VPD of 1.3kPa (50% RH) over the 45min time interval. The slopes of these regression lines were calculated to generate a species mean drying rate. These rates were then analyzed by a one way ANOVA followed by a post hoc Tukey's test to determine differences among species. Linear regression analysis was used to assess the influence both final relative and absolute water content at 45min and the slope of the individual drying curves on species

recovery ability at 48h post rehydration. Percent species recovery data were also arcsin square root transformed for these analyses. The mean species drying rates, RWC, and absolute water content (AWC) at 45min were then plotted against each species' mean percent recovery at 48h. Depression in photochemical efficiency was also graphed as a function of thallus RWC and AWC.

To assess the influence of consecutive desiccation cycles (experiment 3) and VPD (experiment 2) on photochemical efficiency, a repeated measures ANOVA was performed with number of desiccation cycles or VPD and recovery time as the fixed main effects. Data were first examined for sphericity following the Mauchly criterion. Pairwise comparisons were made across recovery times with Bonferroni-adjusted multiple t-tests. Klockars and Sax (Klockars and Sax, 1986 p. 38-39) recommend using the more stringent Bonferroni-adjusted multiple t-test when the number of planned comparisons is greater than the number of degrees of freedom for between-groups. In cases where data did not meet the sphericity criterion, p-values were adjusted using both Greenhouse-Geisser and Huynh-Feldt methods based on the respective epsilons (Scheiner and Gurevitch, 2001).

Results

Desiccation Survey

For the initial desiccation survey, a series of regressions were run on each species to determine the change in relative water content (RWC) of gametophytes exposed to 1.32kPa (50% RH) over the 45min time interval. All species exhibited rapid rates of thallus relative water content loss (Fig. 3-1A) and absolute water content loss (data not shown). In all species regressions on the arcsin square root transformed data were linear (Table 3-1). In all cases, linear regressions were used to calculate the slopes of species'

RWC and AWC drying curves to determine desiccation rates. The absolute size (mass based) of individual gametophytes had little influence on the absolute rate of water loss (Fig 3-3a, $r^2=0.05$, $p=0.06$). These rates varied significantly among species with the fastest dry down in the terrestrial *Thelypteris balbisii* and slowest rates in the terrestrial *Cyclopeltis semicordata* and the epiphyte *Polypodium triseriale* (Fig. 3-3A). Depression in photochemical efficiency (F_v/F_m) as gametophytes desiccated was non-linear with respect to RWC and varied among species (Fig. 3-1B).

Species exhibited differential abilities to recover following desiccation (Fig 3-3B). This recovery ability was more closely related to the decay rate of RWC ($r^2=0.288$, $p<0.0001$) when compared to the final RWC reached ($r^2=0.193$, $p=0.0008$), the decay rate of AWC ($r^2=0.0001$, $p=0.81$), or final AWC reached ($r^2=0.097$, $p=0.008$) after 45min.

Desiccation Rates

The VPD of the different desiccation treatments significantly influenced the recovery abilities of both *Diplazium subsilvaticum* and *Phlebodium pseudoaureum*, but had little influence on *Polypodium triseriale*. For the understory terrestrial *D. subsilvaticum*, the ability to recover following the 2.12 and 1.3kPa VPD treatments was essentially non-existent. Additionally, the F_v/F_m values reached at these VPDs are suggestive of significant photoinhibition and photodamage. The 0.53kPa treatment also depressed F_v/F_m but to a lesser degree and gametophytes exposed to this treatment exhibited clear recovery following rehydration. *Phlebodium pseudoaureum* exhibited relatively less depression in F_v/F_m and exhibited greater recovery than *Diplazium subsilvaticum*. In all three VPD treatments, gametophytes exhibited recovery albeit with lower rates from the 2.12 and 1.32kPa treatments. *Polypodium triseriale* exhibited

remarkable Fv/Fm fidelity at all three rates with no significant degree of Fv/Fm depression at any desiccation intensity (Fig. 3-5, Table 3-2).

Desiccation Cycles

Six species representing different life histories and desiccation tolerance from the initial survey were chosen and exposed to multiple desiccation cycles (1, 2, or 3). In all cases excluding *Polypodium triseriale*, percent recovery was greater following one versus two or three desiccation cycles (Fig. 3-6 f, Table 3-3). Recovery ability was closely linked to species ecology with slow to no recovery following >1 desiccation cycle in the understory species that occur in more mesic habitats: *Diplazium subsilvaticum*, *Adiantum latifolium*, and *Cyclopeltis semicordata*. Within this group, *Cyclopeltis semicordata* exhibited less inhibition and the greatest recovery following a single desiccation cycle. With multiple cycles, all species were significantly inhibited and there was little evidence of recovery following 2 and 3 cycles. There was a much greater degree of recovery following 2 and 3 cycles in the species from more xeric habitats: the terrestrial *Pityrogramma ebenea*, and the epiphytes: *Phlebodium pseudoaureum*, *Polypodium triseriale*. Unlike the other species in this xeric category, *Polypodium triseriale* exhibited similar degrees of recovery from 1 and 2 cycles but experienced a much slower recovery following the third desiccation cycle.

Discussion

In all species, fern gametophytes exhibited rapid rates of water loss (Fig 1a.). With poor control of transpiration and inefficient absorptive organs, gametophytes likely rely on water derived directly from the atmosphere and/or that which flows over them. As such, gametophytes face considerable variations in water content throughout the day and must be able to withstand long periods of desiccation. This is especially true of epiphytic

gametophytes that can live for years (Watkins et al. Chapter 2). The only recourse that gametophytes have is to tolerate desiccation or perish.

The initial survey produced a surprising degree of desiccation tolerance across many species in a gametophyte that is known to require water for fertilization and is thought to require humid conditions for survival. After exposure to a VPD of 1.3kPa ($rH=50\%$) for 24 hours, all species exhibited greater than 50% recovery of the pre-treatment F_v/F_m values and the majority had recovered more than 70% of this value (Fig. 3-3). Gametophytes were dried to near constant state and thus match the definitions of desiccation tolerant by Bewley (1979) and Alpert and Oliver (2002). The species in this study are all tropical in origin and while they experience various degrees of humidity in nature, some of the more exposed canopy trees for this same forest average VPD~3.0kPa at mid-day during the dry season (Cardelus and Chazdon, 2005). The gametophytes in this study experience VPD levels of 1.3kPa, but the value remains on the extreme end of what they typically experience. There was no significant difference in the absolute water loss rates based on gametophytes mass (Fig. 3-2). In natural settings, individuals will be exposed to daily variation in RWC and their ability to recover from low relative water content is crucial. The only apparent mechanism that gametophytes have to control water loss is an increase in size and perhaps alteration of thallus morphology (see below).

Variation in VPD

To further examine the influence of desiccation intensity on recovery of pretreatment photochemical efficiency, three species were exposed to three different desiccation intensities: roughly the everyday vapor pressure deficient in nature (0.53kPa), that which is likely to reflect a typical drought event (1.32kPa) and an extreme value representing a VPD that species in this site rarely if ever experience (2.12kPa). The

results from this experiment demonstrated remarkable tolerance to desiccation intensity that is tightly linked to species ecology. *Diplazium subsilvaticum* is a low-light creek-side species and has little tolerance of desiccation intensities below 0.53kPa (80% RH) (Fig 3-5A). The biggest decrease in F_v/F_m occurred in this species between 0.53 and 1.32kPa. The two epiphytes occur in similar habitats; and, *Phebodium pseudoaureum* is typical of open and exposed habitats such as roadsides and open clearings, *Polypodium triseriale* is often found in more highly exposed areas such as fence posts and tree trunks. The level of tolerance to desiccation intensity was linked to the habitats with the most highly exposed *Polypodium triseriale* exhibiting essentially no sensitivity to desiccation intensity (Fig. 3-5C).

These patterns enforce the notion that desiccation rates can influence the survival of certain species. In some bryophytes (Gaff, 1997; Alpert and Oliver, 2002; Alpert, 2005) and at least in the sporophytes of the Hymenophyllaceae (Proctor, 2003), intermediate desiccation intensities that maintain intermediate RWC's are more likely to result in mortality compared to rapid dry downs. Alpert and Oliver (2002) have argued that one reason for this pattern is need for rapid yet organized metabolic shutdown that may not occur at slower desiccation rates. Additional studies that incorporate different drying intensities and longer time spent in these conditions are clearly needed.

The link between DT and species distributions corresponds closely with those reported for bryophyte species from xeric habitats exhibiting greater desiccation tolerance than those from mesic habitats (Oliver, Mishler, and Quisenberry, 1993; Deltoro et al., 1998; Proctor, 2001; Cleavitt, 2002; Alpert, 2005). It also suggests that there may be some connection between gametophyte and sporophyte physiologies.

Desiccation Cycles

Not only do species experience different intensities of desiccation in nature, they also experience multiple desiccation cycles throughout the day and/or growing season. The species in this study clearly exhibited different abilities to cope with consecutive desiccation cycles at a VPD of 1.3kPa with species of more mesic habitat exhibiting little ability to cope with more than one cycle of desiccation (Fig. 3-6). The more mesic creekside *Diplazium subsilvaticum* was the most desiccation-sensitive after one cycle and had the worst recovery whereas the more xeric *Adiantum latifolium* and *Cyclopeltis semicordata* had higher recoveries following one cycle (Fig. 3-6 c&b). *Polypodium triseriale*, the species of more xeric habitats also exhibited depression in Fv/Fm, but recovery was largely independent of two desiccation cycles. One aspect that was common for all terrestrial species is the relative tolerance to a single desiccation cycle and the extreme depression caused by repeated cycles. Repeated desiccation cycles likely induce radical biological damage and recovery depends more on actual DNA repair and new protein synthesis than simple recovery of PSII function related to the release of excess excitation energy. Light and desiccation combined are often a deadly combination; and that gametophytes were returned to the original culture conditions upon rehydration could have resulted in increased photodamage. This was however similar to what species experience in nature and is likely reflective of species biology.

The ability of species to recover from desiccation was more closely related to the rate of drying rather than the final RWC (Fig 3-4). While gametophytes initially seem to have relatively few options to control the rate of thallus desiccation there was variation in rates among species exposed to identical drying conditions (Fig 3-3a). For example, the terrestrial *Thelypteris nicaraguensis* a RWC decay rate that was more than twice as fast

as the canopy epiphyte *Polypodium triseriale*. The variation in desiccation rate was linked to gametophyte morphology (Fig. 3-4). Species with complex three-dimensional morphologies or those that tended to produce prothallia hairs exhibited significantly slower dry down rates than gametophytes that are one dimensional and glabrous. This observation suggests a novel mechanism that gametophytes may employ to control water loss. There is limited internal capacitance in fern gametophytes, and in a manner similar to many bryophytes, fern gametophytes with even a minor degree of morphological complexity can hold external water. Such exohydric abilities may function in a natural setting to help slow the rate of water loss. The decrease in rate was more likely a combination of water vapor becoming trapped in the folds and overlapping wings of more complex thalli and the increase of external boundary layer produced by gametophyte proliferations and hairs.

Variation in gametophyte morphology especially in complexity has long been commented upon. Dassler and Farrar (1997, 2001) have speculated that complex morphologies found in many long-lived gametophytes of epiphytic species have arisen from competition with bryophytes and as a mechanism to ensure outcrossing. One obvious trend across the taxa is that gametophytes of species from drought-prone habitats such as those in epiphytic habitats, deserts, rock outcroppings, etc., tend to produce thalli that often exhibit complex branching, overlapping wings, proliferation, and hairs. Apart from this link between gametophyte morphology and ecology there is also a link between gametophyte morphology and phylogeny. Recent phylogenetic analysis of the ferns has revealed a split between terrestrial and epiphytic clades in the Eu-Polypodiales (Pryer, Smith, and Skog, 1995). The athyrioids, thelypteroids, onocleoids, woodsoids and

blechnoids are almost entirely terrestrial; perhaps less than 1% of the known species of this clade are true epiphytes. On the other hand, the dryopteroids, lomariopsoids, elaphoglossoids, oleandroids, davallioids, and polypodioids have many epiphytic species; perhaps as many as 60-70% of the species in this group as a whole are epiphytic (Fig 3-6) (R. Moran pers. comm.). Most epiphytic species exhibit complex morphologies whereas terrestrial species often less complex ones. Increases in thallus size and complex three dimensional morphologies may provide the only water conservation mechanisms available to fern gametophytes. Complex water conserving morphology may have been critical in the radiation of ferns into canopy and more exposed habitats.

Conclusions

The data presented in this paper show remarkable desiccation tolerance in fern gametophytes. While all species exhibited recovery following an extreme desiccation event, the extent of recovery differed among species and was closely linked to the ecology of the species. The role of the fern gametophyte in controlling recruitment remains unclear, but these data suggest that gametophytes are relatively robust in dealing with desiccation especially in limited cycles. While desiccation intensity clearly influenced recovery, repeated cycles of desiccation were more likely to limit recovery and in the case of the most mesic species likely resulted in significant photodamage. The degree of recovery following desiccation and its relation to species ecology suggest that fern gametophytes exhibit adaptively meaningful variation in this character. It is likely that selective pressures acting on the gametophyte are largely responsible for the distribution of ferns and have played a major role in the evolution of ecological diversity within the group.

Table 3-1. Species and life form from the initial desiccation survey, a series of regressions were run on each species to determine the change in relative water content (slope) of gametophytes exposed to 1.32kPa (50% rH) over the 45min time interval. All species exhibited rapid and uncontrolled rates of thallus water loss. In all species regressions on the arcsin square root transformed data were linear (STE are standard errors, RWC is relative water content expressed as ((g fresh weight- g dry weight)/g saturated weight – g dry weight))*100, AWC is absolute water content expressed as mg water / mg dry mass, % rec corresponds to percent recovery of initial pre-treatment dark adapted Fv/Fm values

Species	Life Form	r2	p	Slope	STE	RWC	STE	AWC	STE	%rec	STE
<i>Adiantum latifolium</i> Lam.	Terrestrial	0.755	<0.0001	-1.891	0.101	7.375	2.138	1.340	0.164	0.579	0.116
<i>Cyclopeltis semicordata</i> (Sw.) J. Sm.	Terrestrial	0.811	<0.0001	-1.359	0.074	34.255	3.836	5.660	0.820	0.897	0.019
<i>Dennstaedtia bipinnata</i> (Cav.) Maxon	Terrestrial	0.939	<0.0001	-1.670	0.045	19.858	2.777	2.320	0.290	0.739	0.072
<i>Diplazium subsilvaticum</i> H. Christ	Terrestrial	0.862	<0.0001	-1.512	0.084	31.880	5.680	3.640	0.980	0.705	0.041
<i>Nephrolepis biserrata</i> (Sw.) Schott	Terrestrial	0.882	<0.0001	-1.419	0.094	34.858	4.298	5.540	1.170	0.823	0.056
<i>Phlebodium pseudoaureum</i> (Cav.) Lellinger	Epiphyte	0.913	<0.0001	-2.033	0.059	9.646	1.449	2.530	0.500	0.678	0.055
<i>Pityrogramma ebenea</i> (L.) Proctor	Terrestrial	0.878	<0.0001	-2.017	0.081	13.737	3.048	5.370	0.670	0.499	0.100
<i>Polypodium triseriale</i> Sw.	Epiphyte	0.973	<0.0001	-1.279	0.203	49.021	8.097	8.640	1.471	0.807	0.065
<i>Pteris altissima</i> Poir.	Terrestrial	0.834	<0.0001	-1.611	0.090	23.856	9.015	9.490	0.981	0.771	0.065
<i>Thelypteris balbisii</i> (Spreng.) Ching	Terrestrial	0.816	<0.0001	-1.490	0.120	32.866	7.795	3.130	0.211	0.828	0.070
<i>Thelypteris curta</i> (H. Christ) C. F. Reed	Terrestrial	0.711	<0.0001	-1.463	0.116	18.875	5.829	3.380	0.794	0.600	0.127
<i>Thelypteris nicaraguensis</i> (E. Fourn.) C. V. Morton	Terrestrial	0.762	<0.0001	-2.199	0.061	19.254	3.512	3.690	0.370	0.555	0.087

Table 3-2. Fv/Fm recovery results from the repeated measures ANOVA for gametophytes exposed to three different desiccation intensities: 20%RH (~0.53kPa), 50%RH (~1.32kPa) and 80%RH (~2.12kPa). The gametophytes of *Diplazium subsilvaticum* are often found in the understory, whereas those of *Phlebodium pseudoaureum*, and *Polypodium triseriale* were collected in the mid and exposed canopy respectively. Gametophytes were kept at the VPD levels for 48hrs after which time they were rehydrated with deionized water and measurements of Fv/Fm were taken at 24, 48, and 72hr post rehydration. These values were related to the dark adapted value of Fv/Fm to determine the mean percent recovery

<i>Diplazium striatastrum</i> Lellinger	df	F	p	Mauchly	χ^2	p
VDP	2	38.41	<0.0001	0.739	3.24	0.662
ID(VPD)	12	1.86	0.0741			
Recovery Time	2	7.32	0.0033			
Recovery Time*VPD	4	6.39	0.0012			
<i>Phlebodium pseudoaureum</i> (Cav.) Lellinger	df	F	p	Mauchly	χ^2	p
VDP	2	42.19	<0.0001	0.716	3.59	0.61
ID(VPD)	12	2.2	0.0482			
Recovery Time	2	24.31	<0.0001			
Recovery Time*VPD	4	7.81	0.0003			
<i>Polypodium triseriale</i> Sw.	df	F	p	Mauchly	χ^2	p
VDP	2	1.46	0.2716	0.779	2.67	0.75
ID(VPD)	12	4.53	0.3531			
Recovery Time	2	0.6	0.6352			
Recovery Time*VPD	4	5.09	0.0041			

Table 3-3. F_v/F_m recovery results from the repeated measures ANOVA for gametophytes exposed to 1, 2, or 3 desiccation cycles at 50% RH (VPD~ 1.32kPa). Gametophytes were kept at this level for 48 hrs. Material was then rehydrated with deionized water and measurements of F_v/F_m were again made at 24hrs, 48hrs, and 72hrs post rehydration. These values were related to the dark adapted value of F_v/F_m to determine the mean percent recovery. Adjusted p-values are G-G Greenhouse-Geisser and H-F Huynh-Feldt adjusted probabilities

							Adjusted p		
<i>Diplazium striatastrum</i>									
Lellinger	df	F	p	Mauchly	χ^2	Adjusted p	G-G	H-F	
Rate	2	3.07	0.0649	0.6301	5.081	0.0788	0.0043	0.0016	
ID(Trt)	12	3.58	0.3931						
Recovery Time	2	98.7	<0.0001			GG ϵ =	0.73		
Recovery Time*Trt	4	4.06	0.0118			HF ϵ =	0.944		
<i>Adiantum latifolium Lam.</i>		df	F	p	Mauchly	χ^2	p	G-G	H-F
Rate	2	1.53	0.2378	0.691	4.06	0.1312	0.0073	0.0029	
ID(Trt)	12	2.09	0.4982						
Recovery Time	2	324.53	<0.0001			GG ϵ =	0.764		
Recovery Time*Trt	4	5.44	0.0029			HF ϵ =	0.999		
<i>Cyclopeltis semicordata (Sw.)</i>		df	F	p	Mauchly	χ^2	p	G-G	H-F
<i>J. Sm.</i>									
Rate	2	600.01	<0.0001	0.706	3.827	0.1475	0.018	0.0094	
ID(Trt)	12	1.98	0.5498						
Recovery Time	2	7.33	0.0083			GG ϵ =	0.773		
Recovery Time*Trt	4	2.43	0.753			HF ϵ =	1		

Table 3-3. Continued

<i>Pityrogramma ebenea</i> (L.)								
<i>Proctor</i>	df	F	p	Mauchly	χ^2	p	G-G	H-F
Rate	2	57.65	<0.0001	0.857	1.688	0.4298	<0.0001	<0.0001
ID(Trt)	12	0.56	0.792					
Recovery Time	2	80.09	<0.0001			GG ϵ =	0.875	
Recovery Time*Trt	4	11.67	<0.0001			HF ϵ =	1	
<i>Phlebodium pseudoaureum</i> (Cav.) Lellinger								
	df	F	p	Mauchly	χ^2	p	G-G	H-F
Rate	2	98.81	<0.0001	0.462	8.48	0.0143	<0.0001	<0.0001
ID(Trt)	12	0.38	0.8699					
Recovery Time	2	169.52	<0.0001			GG ϵ =	0.65	
Recovery Time*Trt	4	20.49	<0.0001			HF ϵ =	0.818	
<i>Polypodium triseriale</i> Sw.								
	df	F	p	Mauchly	χ^2	p	G-G	H-F
Rate	2	24.82	<0.0001	0.492	7.78	0.02	0.0046	0.0019
ID(Trt)	12	1.65	0.548					
Recovery Time	2	87.4	<0.0001			GG ϵ =	0.664	
Recovery Time*Trt	4	6.78	0.0008			HF ϵ =	0.839	

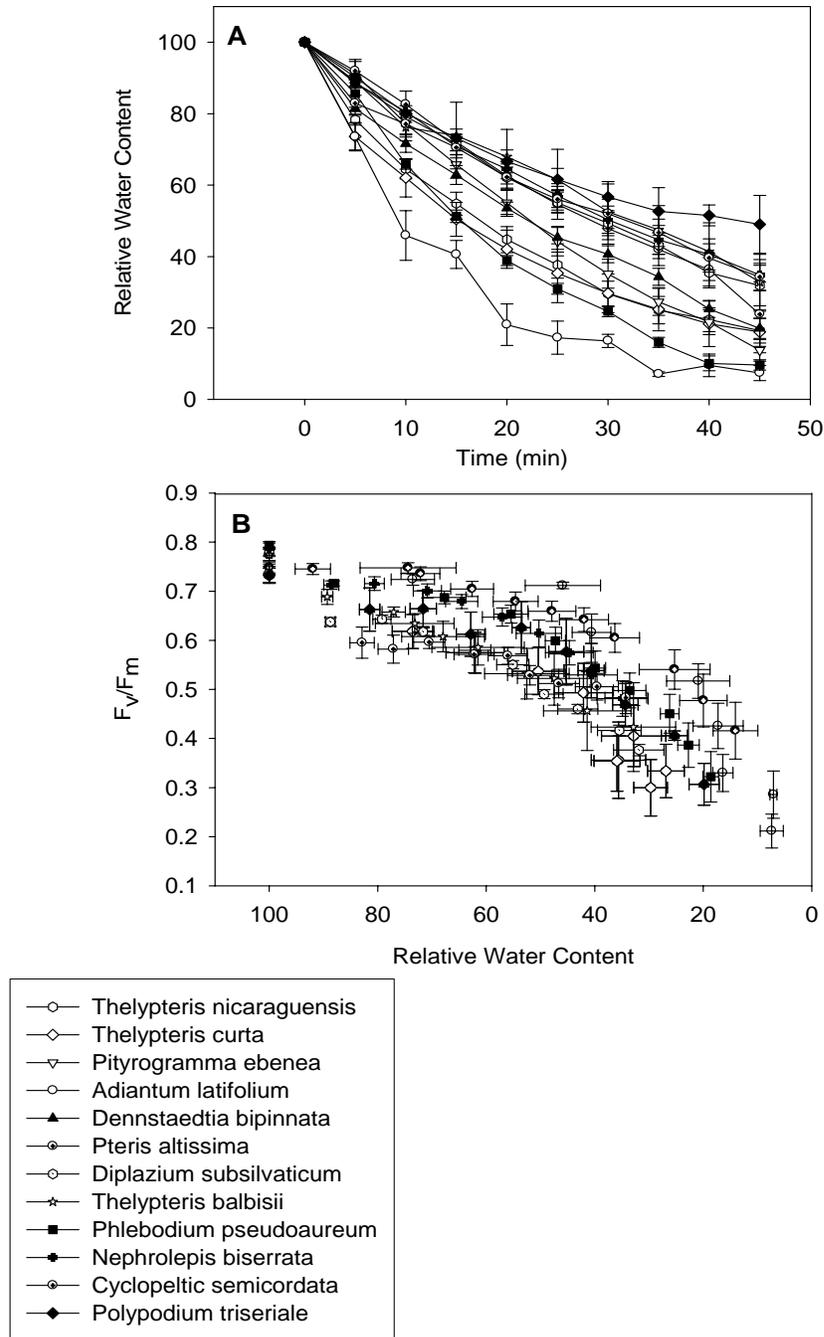


Figure 3-1. (a) Gametophyte drying curves from 12 tropical fern species of different habitats. Species were exposed to a VPD to 1.32KPa (~50%RH) for 45 min. (b) Depression of photochemical efficiency in the same gametophytes over a series of decreasing thallus water contents

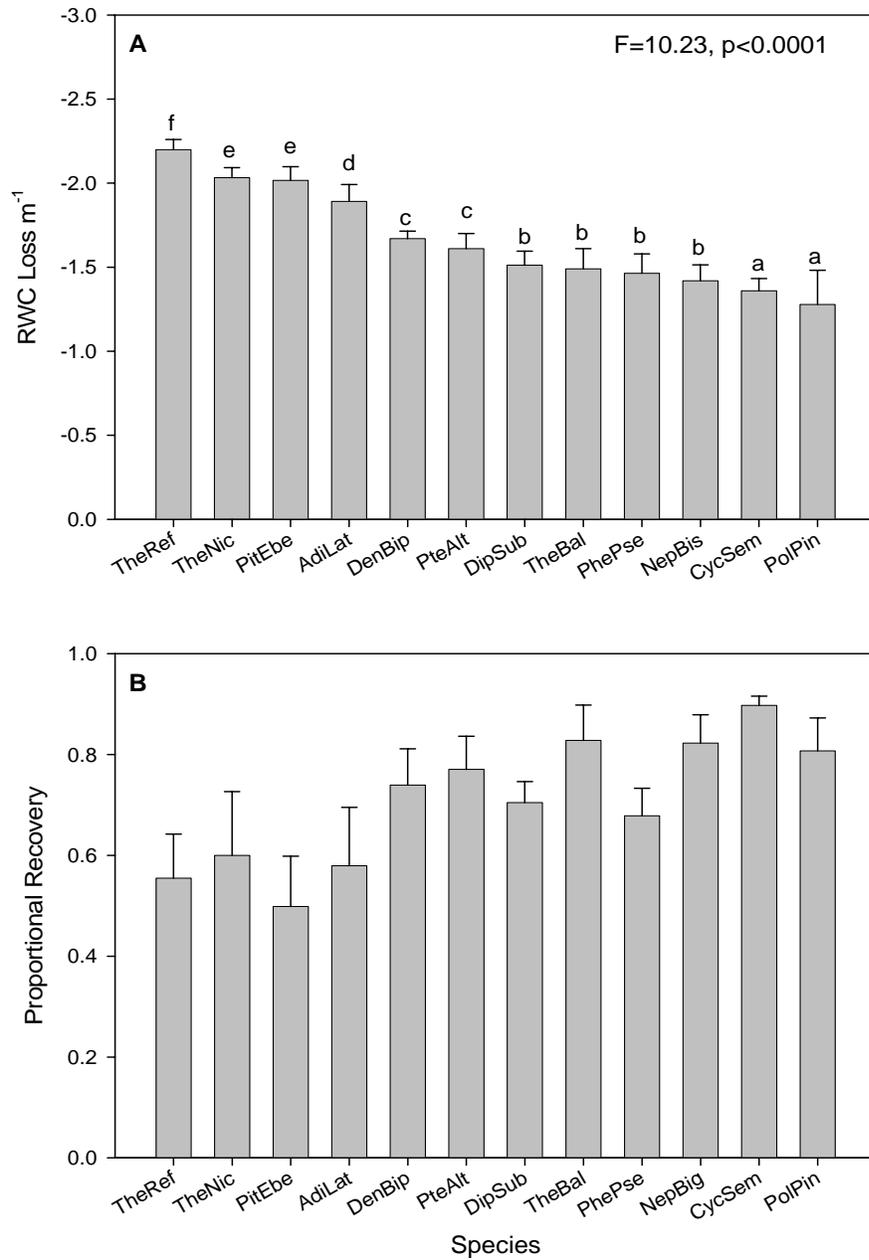


Figure 3-3. (a) Rate of thallus drying as calculated from Figure 3-1 for 12 tropical fern species of different habitats. (b) Proportional recovery of the pre-treatment dark adapted value of F_v/F_m in these same species

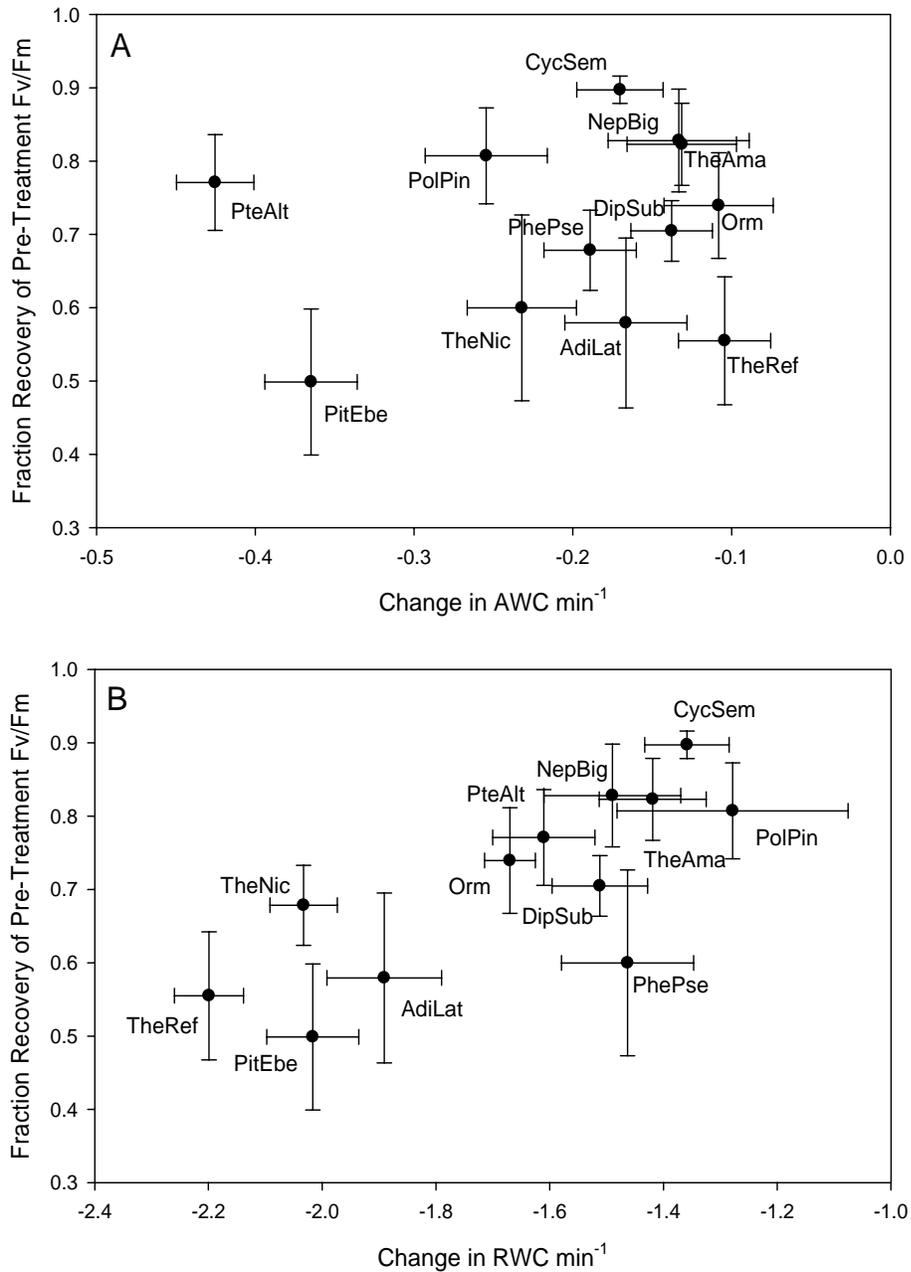


Figure 3-4. Proportional recovery of the pre-treatment dark adapted value of F_v/F_m and rate of thallus water loss expressed as (a) relative water content ((g fresh weight - g dry weight)/g saturated weight - g dry weight)*100 and (b) absolute water content (g wet weight/g dry weight)

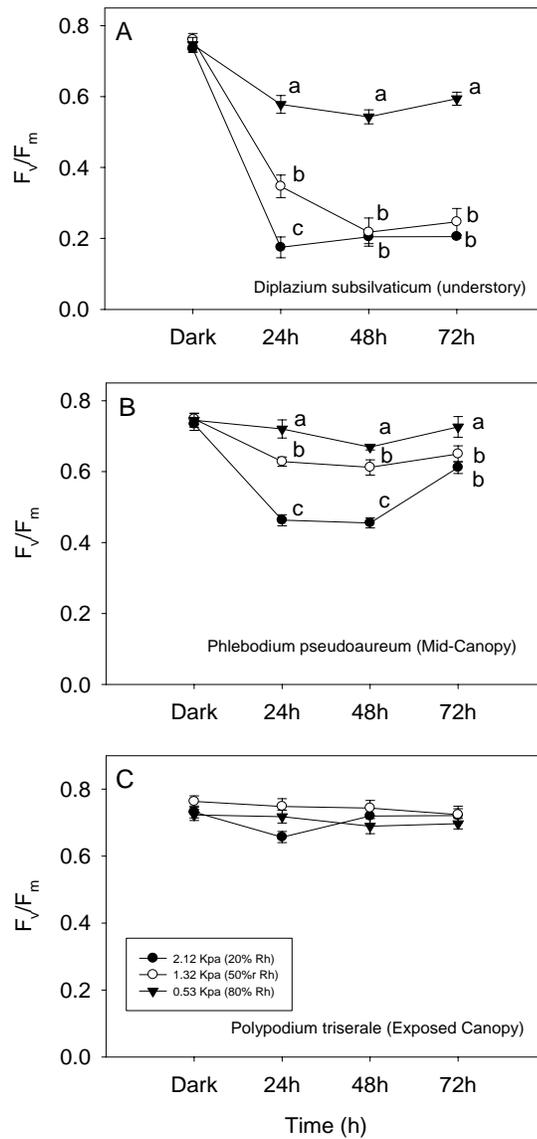


Figure 3-5. F_v/F_m recovery graphs for gametophytes of (a) *Diplazium subsilvaticum*, (b) *Phlebodium pseudoaureum*, (c) *Polypodium triserale* exposed to three different desiccation intensities: VPD~0.53kPa (20%RH), VPD~1.32kPa (50%RH), and VPD ~2.12kPa (80%RH). The gametophytes of *Diplazium subsilvaticum* are often found in the understory, whereas those of *Phlebodium pseudoaureum*, and *Polypodium triserale* were collected in the mid and exposed canopy respectively. Gametophytes were kept at the VPD levels for 48hrs after which time they were rehydrated with deionized water and measurements of F_v/F_m were taken at 24, 48, and 72hr post rehydration. Pairwise comparisons were made across recovery times with Bonferroni-adjusted multiple t-tests

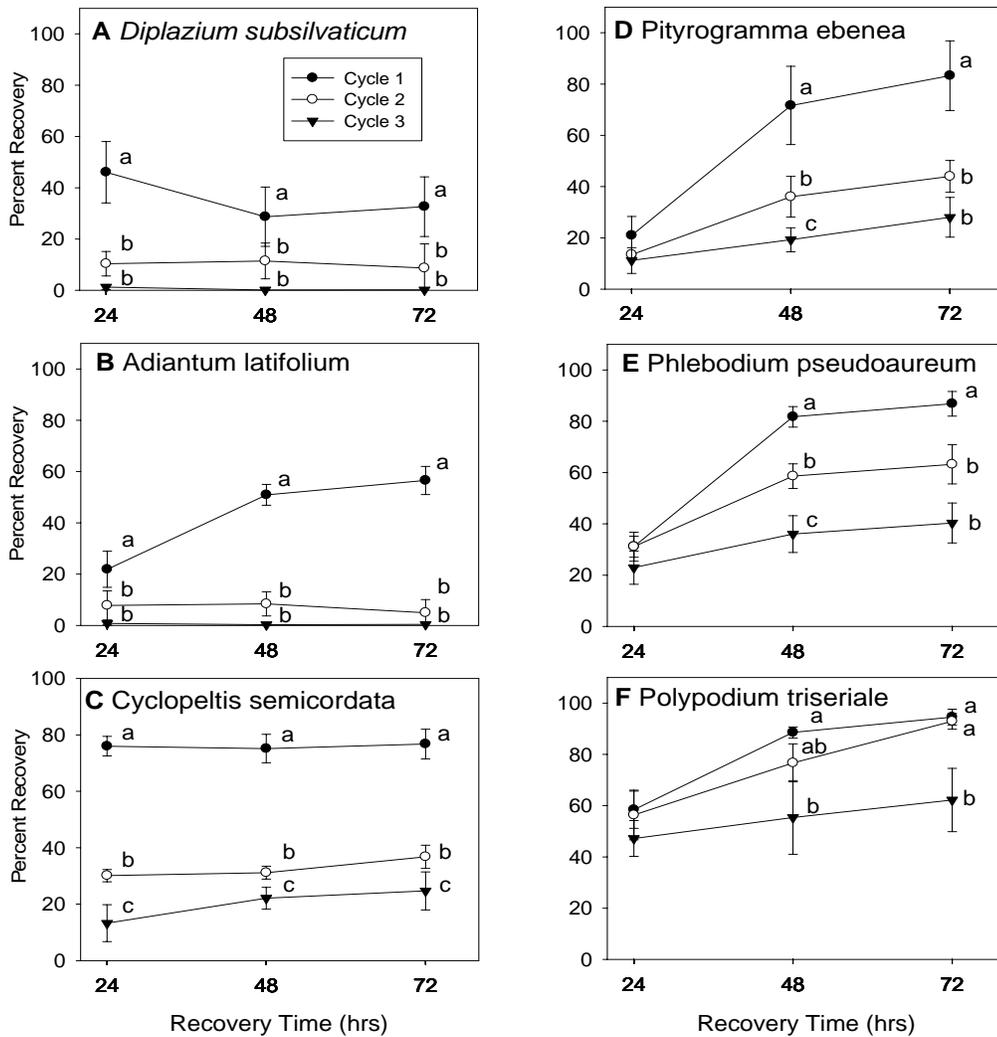


Figure 3-6. Proportional F_v/F_m recovery results for gametophytes exposed to 1, 2, or 3 desiccation cycles at $VPD \sim 1.32 \text{ kPa}$ (50%RH). Gametophytes were kept at this level for 48 hrs. Material was then rehydrated with deionized water and measurements of F_v/F_m were again made at 24hrs, 48hrs, and 72hrs post rehydration. These values were related to the dark adapted value of F_v/F_m to determine the mean percent recovery. Pairwise comparisons were made within recovery times with Bonferroni-adjusted multiple t-tests. (a) *Diplazium subsilvaticum*, (b) *Adiantum latifolium*, (c) *Cyclopeltis semicordata*, (d) *Pityrogramma ebenea*, (e) *Phlebodium pseudoaureum*, (f) *Polypodium triseriale* (see Table 3-1 for species habitat information)

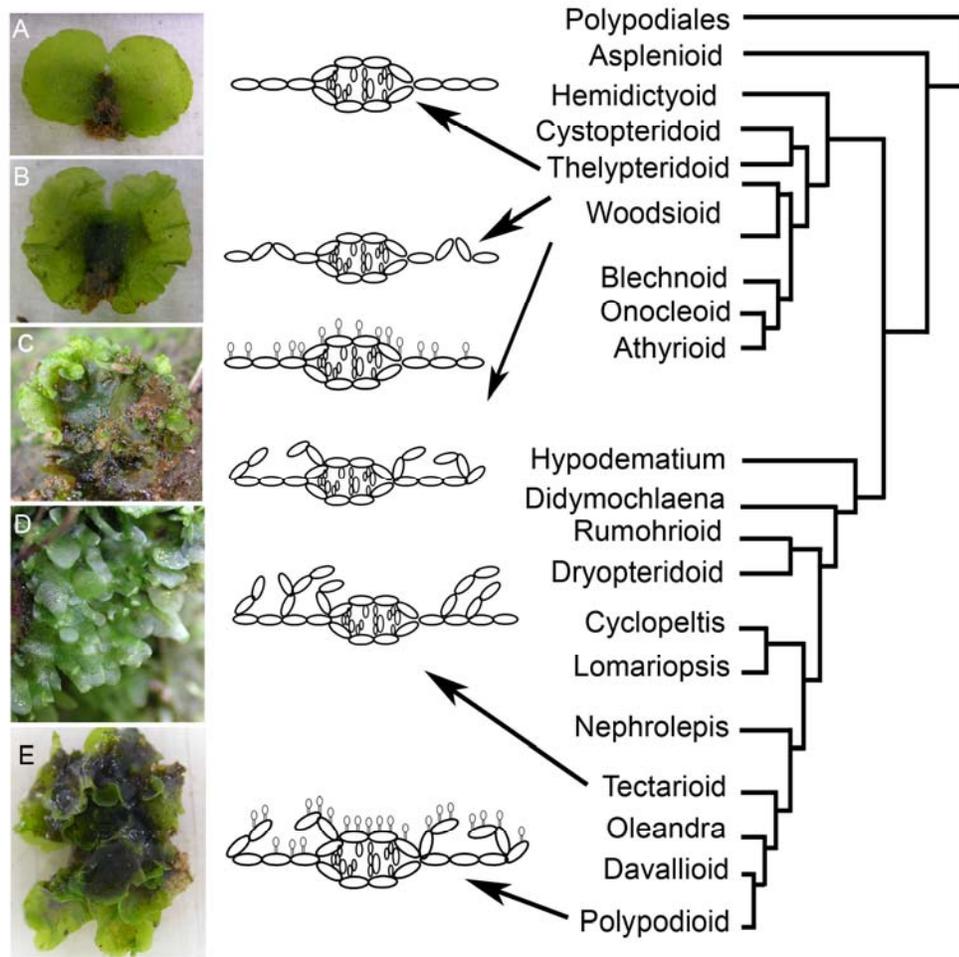


Figure 3-7. Morphology in fern gametophytes is diverse and is closely related to species ecology and phylogeny. Gametophytes of species from drought prone habitats such as those in epiphytic habitats, deserts, rock outcroppings etc. tend to produce thalli that often exhibit complex branching, overlapping wings, proliferation and hairs; whereas, species from more buffered habitats have less ornamented and simple morphologies. The athyrioids, thelypteroids, onocleoids, woodsoids and blechnoids are almost entirely terrestrial, perhaps less than 1% of the known species are true epiphytes. On the other hand, the dryopteroids, lomariopsoids, elaphoglossoids, oleandroid, davallioid and polypodioids have many epiphytic species, perhaps as many as 60-70% of the species in this group as a whole are epiphytic (R. Moran pers. comm.). Most epiphytic species exhibit complex morphologies whereas terrestrial species often less complex ones. Such differences in morphology are significant and may have been critical in the radiation from terrestrial species into canopy habitats. A) *Adiantum latifolium*, B) *Thelypteris sp.1*, C) *Thelypteris sp.2*, D) *Vittaria*, E) *Campyloneurum*

CHAPTER 4
NITROGEN-15 NATURAL ABUNDANCE AND NITROGEN USE STRATEGIES OF
THE GAMETOPHYTES AND SPOROPHYTES OF TROPICAL EPIPHYTIC AND
TERRESTRIAL FERNS

Introduction

A central goal of plant ecology is to develop a mechanistic understanding of species distributions in both space and time. One important abiotic factor that is known to influence both plant performance and distribution is nitrogen. In N-limited systems or in systems where N availability is heterogeneous, plants can compete for N in many ways, one of which is by partitioning this resource in both space and time or by uptake of different chemical forms of N. Such partitioning may result in species coexistence through sorting along resource gradients which ultimately structures species distributions.

The partitioning and uptake of different chemical forms of N has been receiving increased attention largely due to the revelation that plants can circumvent the N cycle and directly uptake organic N, in the form of amino acids from the soil solution (Chapin, Moilanen, and Kielland, 1993a; Kielland, 1994, 1997; Lipson and Nasholm, 2001; Finzi and Berthrong, 2005). While it has been known for some time that plants can acquire organic N, early work in tundra and boreal ecosystems demonstrated that a large component of an individual's N budget could be supported by direct uptake of organic relative to inorganic N (Chapin, Moilanen, and Kielland, 1993b; Kielland, 1994, 1997). Subsequent studies have shown that plants from a wide range of ecosystems can directly access organic N as an important part of their N nutrition (Lipson and Nasholm, 2001). Few studies, however, have examined the ability of plants from tropical wet forests to

take up organic nitrogen and to our knowledge no studies have examined this ability in the ferns.

The ferns pose a unique set of ecological constraints due to both the dispersal of tiny wind-blown spores and the occurrence of an independent and free-living haploid gametophyte. The mineral nutrition of fern sporophytes is not well studied; however, evidence indicates that fern sporophytes behave as seed plant sporophytes when confronted with increased inorganic N (Prange and Ormrod, 1982; Walker and Aplet, 1994; Pillai and Ong, 1999). Less is known of the mineral nutrition of gametophytes and unlike sporophytes (which rely on well developed root systems), fern gametophytes are thought to rely primarily on rhizoid uptake of nutrients with possible uptake of water and nutrients across the thallus (Racusen, 2002). The ability of fern gametophytes to grow on different N forms was reviewed by Miller (1968), and there is limited evidence to show that the gametophytes of at least one species can grow well on specific mixtures of amino acids in the absence of inorganic forms.

For plants other than ferns, changes in $\delta^{15}\text{N}$ values have been shown to occur through ontogeny and with increases in plant size (Zotz, 1997; Schmidt, Stuntz, and Zotz, 2001; Hietz and Wanek, 2003; Reich et al., 2003; Zotz et al., 2004; Casper, Forseth, and Wait, 2005). In the case of hemiepiphytic plants, changes occur as a direct result of the connection of once aerial roots to terrestrial water and nutrient pools (Putz and Holbrook, 1989; Field, Lawton, and Dawson, 1996; Wanek et al., 2002). As ferns continue their life-cycle from gametophyte to sporophyte, radical changes in their ecophysiology, especially nutrient relations are likely to occur.

The goal of this paper is to determine if species differ in their ability to take up different forms of nitrogen in both the gametophyte and sporophyte generations and to tie this to the natural abundance of $\delta^{15}\text{N}$ to determine if species access different nitrogen source pools. We then compare these data across epiphytic and terrestrial species and a developmental series of a hemiepiphytic species to better understand the dynamics of nitrogen nutrition of different life forms.

Material and Methods

Study Site

This study was conducted at La Selva Biological Station of the Organization for Tropical Studies in Heredia Province, Costa Rica (10°26' N, 84°00' W). La Selva is a 1400 ha tropical wet-forest positioned in the Caribbean lowlands with an average monthly temperature of 25.8 °C and annual rainfall of 4000 mm per year (Sanford et al., 1994). The site boasts a diversity of ferns with multiple species from epiphytic, hemiepiphytic and epiphytic life forms (Grayum and Churchill, 1987).

Study Species

The gametophytes and sporophytes of 10 species were field collected from 100X100m grids to control for differences in soil type in the terrestrial species and from the trees in the case of epiphytic species (Table 4-1). The following species were sampled:

Adiantum latifolium Lam. is a terrestrial species that is common in disturbed areas in both primary and secondary forests. The species typically grows along trail sides and can be encountered under a wide range of light and soil regimes.

Danaea nodosa (L.) Sm. and *Danaea wendlandii* Rehb. F. are both eusporangiate ferns and as such have gametophytes that are several cell layers thick. *Danaea*

wendlandii is perhaps the most common fern at La Selva and often grows on upland well drained sites and in disturbed understory habitats. *Danaea nodosa* has similar habitat, but is more often associated with wetter sites becoming most abundant along creek sides.

Diplazium subsilvaticum H. Christ is a terrestrial arborescent species that is common along creek banks and wetter areas in both primary and secondary forests.

Lomariopsis japurensis (Mart.) J. Sm. and *Lomariopsis vestita* E. Fourn are both understory hemiepiphytes. In both species, the gametophytes develop on the trunks of small trees and remain epiphytic throughout their life. Young sporophytes produce roots that grow down and contact the soil and rely on the host tree for support. Adult plants never lose contact with the forest floor.

Olfersia cervina (L.) Junze has been classified as both a terrestrial and hemiepiphyte species. It is restricted to grow on soils with high organic content and is most commonly found growing on rotting logs, but can also grow on large tree trunks where sufficient detritus has accumulated.

Elaphoglossum latifolium (Sw.) J. Sm. and *Campyloneurum brevifolium* (Lodd. Ex Link) Link are both canopy epiphytes with the former more often found in highly exposed portions of the canopy and often on bare bark. The latter species also occurs in exposed sites, but is most common in the inner canopy rooted in canopy soil organic matter.

Antrophyum lineatum (Sw.) Kaulf: An understory epiphyte that grows on the trunks of living trees. Both gametophytes and sporophytes are common in primary and secondary forests at La Selva.

Isotopic Natural Abundance and $\delta^{15}\text{N}$ Labeled Uptake

Foliar and gametophytic samples for natural abundance of all nine species were field collected within a 3-wk period during June 2005. Collections were brought back to the lab where they were washed in deionized water to remove all soil and then dried at 60C for 48h. Samples were analyzed for nitrogen concentration and isotope ratio using a Costech elemental analyzer coupled with a Finnigan Delta XL PlusTM continuous flow mass spectrometer at the University of Florida, Gainesville. Based on repeat analyses of NIST peach leaves standard (SRM 1547; $\delta^{15}\text{N}$ 1.91‰), average 1s precision was 0.07‰ for $\delta^{15}\text{N}$.

In order to compare uptake of both organic and inorganic N forms we used excised root techniques (Treseder and Vitousek, 2001) in sporophytes and whole plant uptake in gametophytes of *Danaea wendlandii*, *Lomariopsis vestita*, and *Campyloneurum brevifolium*. Immediately prior to the uptake trials, plants were field collected and brought back to the lab where roots or gametophytes were rinsed with deionized water to remove soil and other debris. For sporophytes, fine roots were selected, excised, and placed into a series of solutions containing increasing concentrations of $\delta^{15}\text{N}$ labeled organic and inorganic N forms (see below). For gametophyte trials, 2-5 individual gametophytes of similar size and maturity were placed directly into a separate set of solutions. All samples were allowed to incubate for 60min in solutions containing 0 (deionized water only), 10, 50, 100, 300, and 500 μmol concentrations containing only labeled NH_4^+ , NO_3^- , (99 atom%), a cocktail of equal proportions of the amino acids: Aspartic and Glutamic Acids, and Glycine (98 atom%), and a cocktail of all solutions ($\text{NH}_4^+ + \text{NO}_3^- +$ the 3 amino acids). All solutions were amended with 0.01 mol/L sucrose as an energy source and 0.5 mmol/L CaCl_2 to maintain membrane integrity (Kielland

1997). Immediately following the incubation trial, roots and gametophytes were removed and rinsed for 2min in a solution containing 1mmol/L KCl to remove any excess $\delta^{15}\text{N}$ from the external surfaces. The material was then dried at 60C for 73h, weighed, and ground for analysis of $\delta^{15}\text{N}$ using the same Finnigan Delta XL Plus™ continuous flow mass spectrometer.

Nutrient Uptake Calculations

$\delta^{15}\text{N}$ enrichment was calculated as $F = [T(A_S - A_B)] / A_F$, where F is the weight of N derived from the $\delta^{15}\text{N}$ tracer, T is the total weight of N in the sample, A_S is atom% excess $\delta^{15}\text{N}$ in the labeled sample, A_B is atom% excess $\delta^{15}\text{N}$ in the natural abundance sample, and A_F is atom% excess in the $\delta^{15}\text{N}$ tracer (Knowles and Blackburn, 1993). To calculate the kinetic uptake parameters of maximum uptake (V_{max}) and the saturation constant (K_m), we fitted the data to a Michaelis-Menten function. We also quantified the μmol s of N per g root dry mass against the solution concentration using $V = V_{\text{max}} S / K_m + S$, where V is the velocity of uptake, and S is the concentration. The parameter V_{max} is an estimate of the maximum uptake rate for a given ion and is controlled by the activity of membrane bound proteins specific to that ion. The value of experimental calculations of V_{max} is that it gives an estimate of the root's/gametophyte's total capacity of ion uptake. The value K_m is estimated to describe the affinity of specific membrane-bound proteins to a given ion and is related to the capacity to utilize low concentrations of this ion. Roots with lower K_m values have higher affinities at low concentrations for the ion in question.

Results

$\delta^{15}\text{N}$ Natural Abundance and N concentration (mg g^{-1})

Species differed significantly in $\delta^{15}\text{N}$ and N concentration (mg g^{-1}) values ($F=3.06$, $p=0.0053$ and $F=3.69$, $p=0.0009$ respectively). There were also significant differences

within and between life forms ($\delta^{15}\text{N}$ $F=3.74$, $p=0.029$; N concentration (mg g^{-1}) $F=10.66$, $p<0.0001$), generations ($\delta^{15}\text{N}$ $F=15.52$, $p=0.0002$; N concentration (mg g^{-1}) $F=57.65$, $p<0.0001$), and a significant life form by generation interaction ($\delta^{15}\text{N}$ $F=16.95$, $p<0.0001$; N concentration (mg g^{-1}) $F=8.85$, $p=0.0004$) (Fig. 4-1, 4-2a). Within both hemiepiphytic and terrestrial species, the $\delta^{15}\text{N}$ values of gametophytes were more enriched $F=64.93$, $p<0.0001$ and $F=3.84$, $p=0.0061$) than that of sporophytes; whereas, the opposite was the case for the epiphytes ($F=3.99$, $p=0.059$). Across life forms, the gametophytes of terrestrial species were slightly depleted yet not significantly so when compared to epiphytic and hemiepiphytic species ($F=2.63$, $p=0.086$). Greatest differentials were observed among the sporophytes with hemiepiphytes significantly more depleted than terrestrial species than epiphytic species ($F=17.12$, $p<0.0001$). Gametophytes exhibited significantly higher N concentration (mg g^{-1}) relative to sporophytes within each life form and varied between life forms with epiphytes and hemiepiphytes exhibiting higher N concentration (mg g^{-1}) in gametophytes and sporophytes than terrestrial species (Fig. 4-2b). To better understand ontogenetic shifts that occur in hemiepiphytic ferns, we measured variation in ^{15}N natural abundance of different stages of *Lomariopsis vestita*. There was a clear series of increasing $\delta^{15}\text{N}$ enrichment from epiphytic gametophytes and sporophytes to terrestrially-rooted adult sporophytes (Fig. 4-3).

$\delta^{15}\text{N}$ Labeled Uptake

The gametophytes and sporophytes of both *Danaea wendlandii* and *Campyloneurum brevifolium* exhibited uptake capacity of both inorganic and organic forms of N. For both species uptake of NO_3^- in both gametophytes and sporophytes was limited. The gametophytes of both species had higher V_{max} for all N forms than sporophytes. The gametophytes and sporophytes of *C. brevifolium* had higher V_{max}

values for the amino acid mix followed by NH_4^+ and the all solution cocktail (Fig. 4-4a&b and Fig. 4-5a&b) and all solution cocktails. The gametophytes of *D. wendlandii* had highest V_{max} values for the all solution cocktail where the V_{max} for NH_4^+ and amino acid was similar (Fig. 4-5c). K_m values exhibited considerable variation both within and between species and generations. In all cases, K_m was lowest and therefore affinity highest for NO_3^- relative to all other N forms (Fig. 4-6a-d).

Discussion

The results from the uptake studies indicate that ferns show preference for specific N forms and that they do so differently in sporophyte and gametophyte generations. In the case of the epiphytic *C. brevifolium*, both gametophytes and sporophytes exhibited high potential for uptake of amino acid N followed by inorganic NH_4^+ (Fig. 4-5a-b). Uptake potentials shifted slightly in the gametophytes of the terrestrial *D. wendlandii* with uptake of amino acids and NH_4^+ essentially equal. The gametophytes and sporophytes of the epiphytic species exhibited higher uptake capacities for N-derived from amino acids relative to the terrestrial *Danaea wendlandii*. *Campyloneurum brevifolium* is a mid- to low-canopy species that is frequently rooted in canopy soil; whereas, *Danaea wendlandii* is a species that is always rooted on mineral soil. The occurrence of species on such different soil types is likely to produce radically different nitrogen nutrition and produce different nutrient use strategies between such species.

Canopy soil is fundamentally different from terrestrial soil in that the former is almost entirely organic. In a study on soil nutrients at La Selva, Cardelus and Mack (pers. com.) have shown that canopy soil organic matter had significantly greater bulk nitrogen, NH_4^+ , and dissolved organic nitrogen than terrestrial forest floor soils and that nitrogen

mineralization rates were significantly lower in the canopy. They were also able to show that within canopy soils, the concentration of NO_3^- was low and NH_4^+ and dissolved organic nitrogen dominate this matrix. For this reason, it is not surprising that epiphytic species would exhibit preferential uptake of NH_4^+ and organic nitrogen.

Both the uptake rate (V_{max}) and half saturation constants (K_m) were higher for NH_4^+ than either amino acids or NO_3^- for the sporophytes of *Danaea wendlandii* (Fig 4-5, 4-6). In the gametophytes of this species, the values of K_m for amino acids were several fold higher than those from the sporophytes and from the gametophytes and sporophytes of *Campyloneurum brevifolium*. Uptake rates of amino acids and NH_4^+ from these same gametophytes were not significantly different. These patterns indicate that NH_4^+ is an important N source for both, but especially, terrestrial sporophytes in this study. NO_3^- concentration within terrestrial soils at this site is high (Cardelus and Mack pers. com.). Nitrate is highly mobile and ferns must compete with microbes and other plants for this resource and may have partitioned uptake to NH_4 and amino acids to avoid or lessen this competition. Such plants would not be highly invested in NO_3^- carriers and would be expected to exhibit higher affinities for this ion at lower concentrations; a result demonstrated by this study (Fig. 4-6).

The importance of amino acids as components of species' N budgets is receiving increased attention and has been shown in species from tundra (Kielland, 1994), to temperate (Finzi and Berthrong, 2005) and subtropical (Schmidt and Stewart, 1999) ecosystems. We believe that our data are some of the first to demonstrate this ability in species from tropical lowland forests and clearly the first to do so in the ferns. The significance of amino acids varies but clearly makes up a critical component (>50% by

some estimates) of species' N budgets from tundra ecosystems (Kielland 1994). In many ways, canopy soil is functionally equivalent to tundra and boreal soils as it is organic in origin and potentially has high concentrations of free amino acids. As such, there may be convergence to greater investment in amino acid uptake in organic soils.

The gametophytes of both species had higher uptake rates of all N forms excluding NO_3^- relative to sporophytes. There are fundamental differences in anatomy and morphology of these two stages of the life cycle and comparisons across stage must be made with care. Gametophytes produce primitive yet functional rhizoids that likely aid in nutrient uptake (Smith, 1972a, 1972b), yet they may also take in nutrients via diffusion across cells of the thallus (Racusen, 2002). This could result in much greater gametophyte surface area and transporter density compared to root surface area and result in greater uptake per unit mass in gametophytes. Expression of uptake rates on an area basis is made difficult as the gametophytes of both species develop complex three dimensional morphologies that make accurate determination of area difficult.

There are a number of mechanisms that control the natural abundance $\delta^{15}\text{N}$ signatures in plant tissues and for this reason, $\delta^{15}\text{N}$ signatures reflect a series of integrated fractionation events (Evans, 2001; Robinson, 2001; Dawson et al., 2002). In spite of the complexities of interpreting natural abundance $\delta^{15}\text{N}$ signatures, such data provide evidence of a process when confounding variables that influence fractionation can be identified or eliminated (Robinson, 2001). Differences in plant $\delta^{15}\text{N}$ signatures have been shown to be primarily related to 1) uptake of different N sources with distinct signatures (Robinson, 2001), 2) N availability and plant demand (Kolb and Evans, 2003), 3)

mycorrhizal associations (Hobbie and Hobbie *in press*), 4) rooting depth (Nadelhoffer and Fry, 1994) or a combination of these events (Dawson et al., 2002).

Both N availability and plant demand can have impacts on tissue $\delta^{15}\text{N}$ values through kinetic fractionations Kolb and Evans (2003) have shown that when N supply is greater than a plant's assimilatory ability, plant tissues can become highly depleted in ^{15}N relative to the source. This, however, only occurs in ecosystems where external N concentrations are high and such kinetic fraction seems unlikely to be responsible for the differences observed in our study. It is possible that mechanisms other than supply and demand drive kinetic fractionations differently in gametophytes and sporophytes. Unfortunately, little is known of how root vs. rhizoid/thallus uptake differentially fractionate N.

Ectomycorrhizal associations can result in large fractionation events (8-10 ‰) whereas fractionation caused by arbuscular mycorrhizal symbioses seems to have little effect on $\delta^{15}\text{N}$ values of host plants (Schmidt and Stewart, 2003). Both terrestrial and epiphytic leptosporangiate fern species have arbuscular mycorrhizal symbioses in the sporophytes, but never in the gametophytes (Gemma, Koske, and Flynn, 1992). The differences in natural abundance $\delta^{15}\text{N}$ signatures between gametophytes and sporophytes are unlikely due to such associations.

Plant uptake from source pools with different $\delta^{15}\text{N}$ signatures is a major contributor influencing tissue values. Plants can take up both organic and inorganic nitrogen and each of these sources has different signatures: NO_3^- is highly depleted relative to NH_4^+ which can be more depleted than amino acids. In our data, gametophyte natural abundance $\delta^{15}\text{N}$ signatures are either significantly depleted or equal to sporophyte

values. The data from the uptake experiment indicate that NO_3^- is not a major source of either gametophyte or sporophytes N nutrition and that source alone is not responsible for the differences between gametophyte and sporophytes. Sources can however be derived from N species from enriched soil or highly depleted atmospheric sources. Evidence indicates that epiphytes rely heavily on depleted atmospheric N sources (Hietz et al., 2002) and the result is major offset of $\delta^{15}\text{N}$ values when compared to terrestrially rooted plants (Watkins, unpublished data). This may also help explain the differences between gametophytes and sporophytes. As throughfall leaches through the canopy to the forest floor, it may contain significant concentrations of depleted N (Cardelus and Mack pers. com.) which may then be taken up more directly by gametophytes. Canopy epiphytic gametophytes also rely on depleted N species and may exhibit greater direct atmospheric uptake than sporophytes. Our uptake data indicate that overall uptake rate in gametophytes is much greater than that in sporophytes. This may provide gametophytes greater opportunity for uptake of depleted pools that may be rapidly diluted by rainfall.

Several studies have now shown that tissue $\delta^{15}\text{N}$ values from deep rooted individuals are more enriched relative to shallow rooted individuals (Nadelhoffer and Fry, 1988; Nadelhoffer and Fry, 1994; Handley and Scrimgeour, 1997). Rooting depth is a potentially major contributor to the observed differences in $\delta^{15}\text{N}$ values between the surface rooted gametophytes and deeper sporophyte roots.

One way to observe shifts in N sources is to follow ontogenetic series and track changes in $\delta^{15}\text{N}$ values. The gametophytes of *Lomariopsis vestita* are epiphytes on understory trees and produce epiphytic sporophytes with roots that grow down the trunk into the soil. The difference between gametophytes and young sporophytes that were not

attached to the soil were large with the latter being more enriched (Fig. 4-3). This is possibly due to a combination of atmospheric uptake in gametophytes and quickly shifts to direct root uptake in sporophytes that may be more efficient in tapping nutrients from more enriched soil pools. Terrestrially rooted young and adult individuals would be predicted to exhibit enriched $\delta^{15}\text{N}$ signatures relative to epiphytic individuals. Such plastic abilities are critical in the life of hemiepiphytes that face frequent water stress and a temporally heterogeneous nutrient environment.

Conclusions

The observed differences in foliar $\delta^{15}\text{N}$ values and evidence of differential uptake of N forms indicate that ferns can partition N by form. The preference of N form varied with greater preference on organic N in the epiphytic vs. terrestrial species and between gametophytes and sporophytes. These data indicate that there are different nutrient use strategies between the two life forms and between generations. Individuals that can circumvent mineralization in the N cycle by direct amino acid uptake may have a tremendous competitive advantage relative to those relying on inorganic forms. What is critically needed are studies that incorporate dual labeled C and N isotopes to precisely determine if amino acids are hydrolyzed at the cell membrane or if they are taken directly into plants.

Species	Life Form	Distribution
<i>Adiantum latifolium</i> Lam.	Terrestrial	Disturbed areas, high - medium light
<i>Danaea nodosa</i> (L.) Sm.	Terrestrial	Understory, low light
<i>Danaea wendlandii</i> Rchb. F.	Terrestrial	Understory, disturbed low light areas
<i>Diplazium subsilvaticum</i> H. Christ	Terrestrial	Understory - exposed mesic areas
<i>Lomariopsis japurensis</i> (Mart.) J. Sm.	Hemiepiphyte	Understory secondary and primary forests
<i>Lomariopsis vestita</i> E. Fourn	Hemiepiphyte	Understory secondary and primary forests
<i>Olfersia cervina</i> (L.) Junze	Hemiepiphyte-Terrestrial	Understory, on mounds of organic matter or decaying trees
<i>Elaphoglossum latifolium</i> (Sw.) J. Sm.	Epiphyte	Highly exposed, on bare bark in canopy
<i>Campyloneurum brevifolium</i> (Lodd. Ex Link)	Epiphyte	Moderate light, rooted in soil, inner canopy
<i>Antrophyum lineatum</i> (Sw.) Kaulf	Epiphyte	Understory secondary and primary forests

Table 4-1. Species, life form and ecology for the natural abundance and uptake experiments

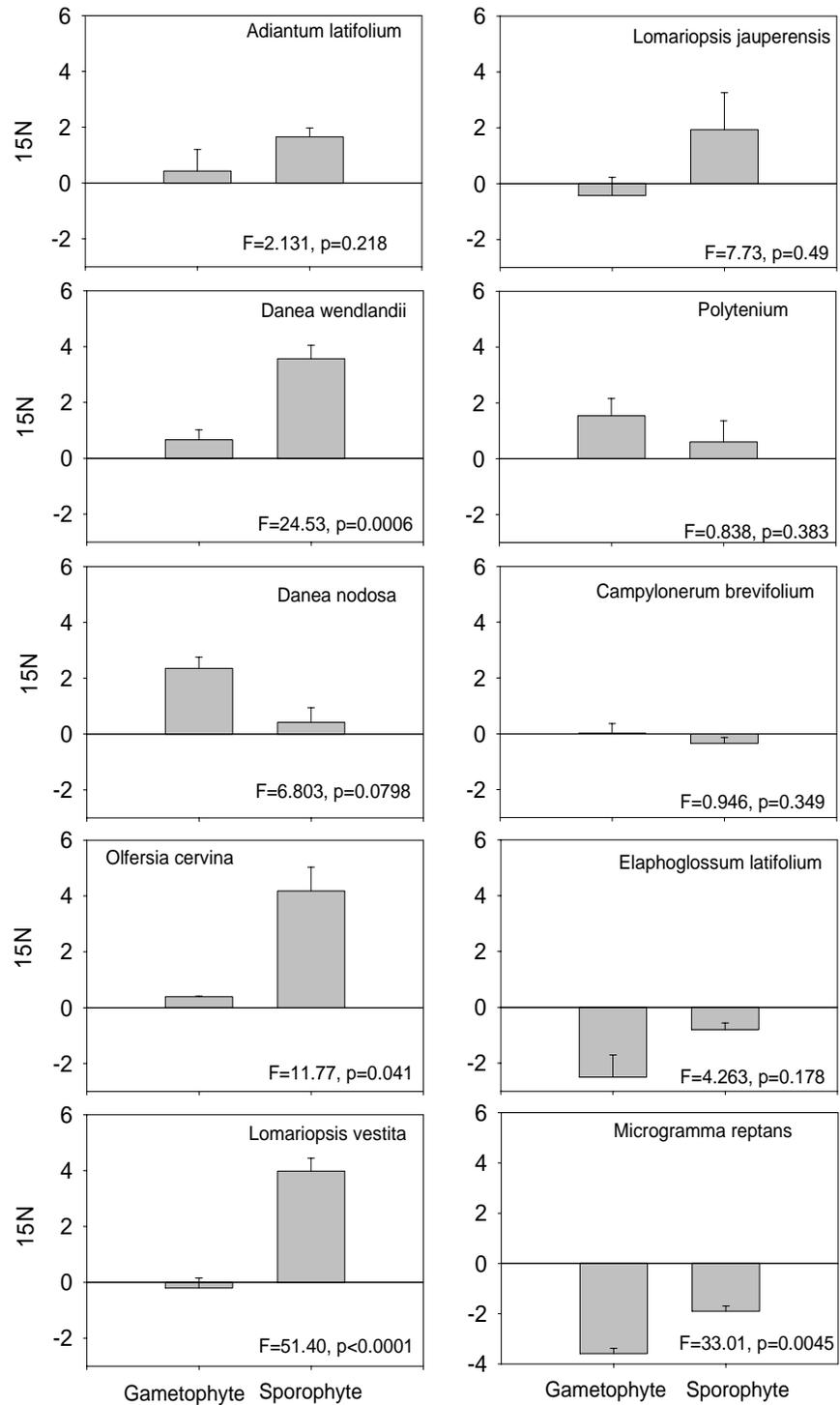


Figure 4-1. Sporophytic and Gametophytic $\delta^{15}\text{N}$ natural abundance signatures of 10 tropical fern species. Tissue was field collected from 100X100m grids to control for differences in soil type in the terrestrial species and from the same trees in the case of epiphytic species

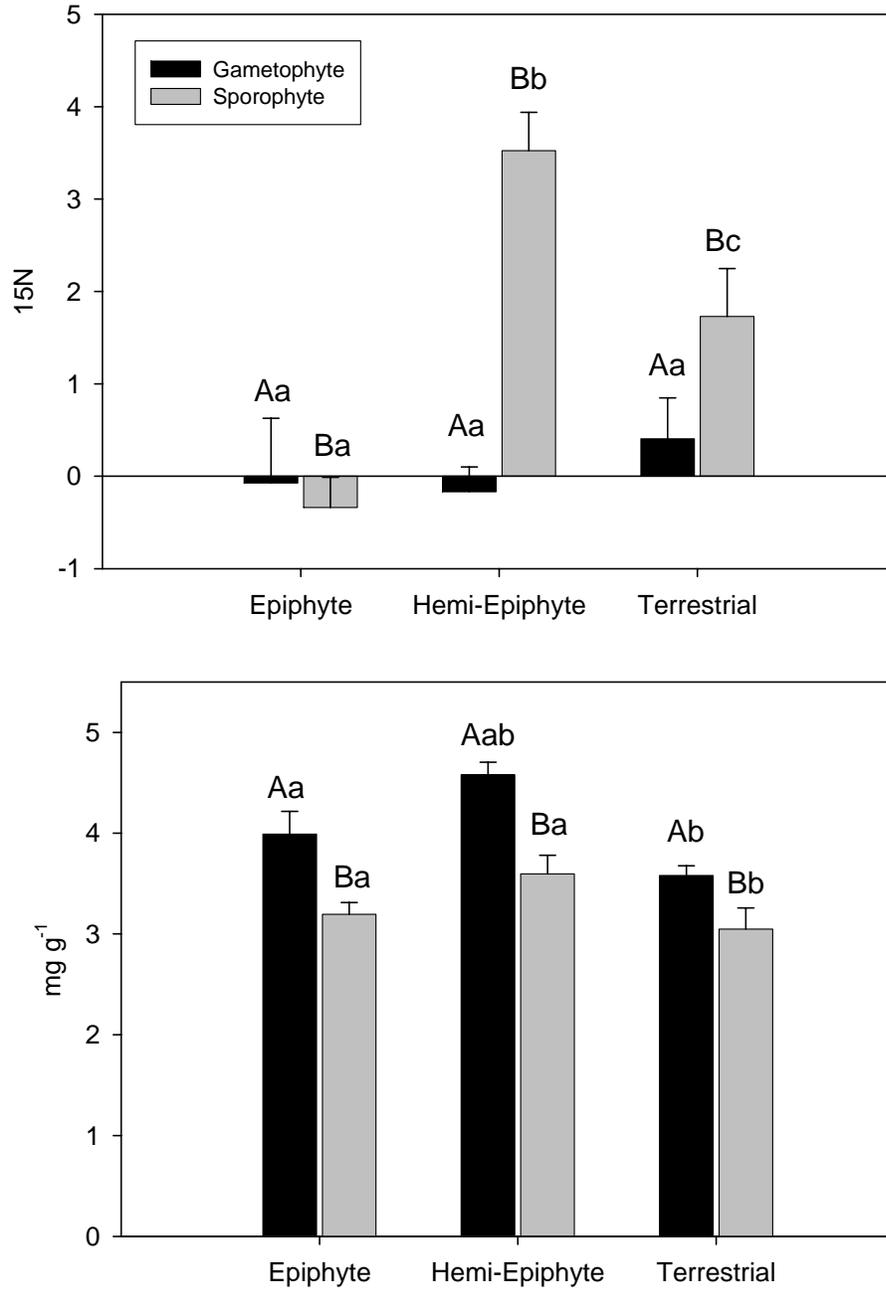


Figure 4-2. Sporophytic and Gametophytic (a) $\delta^{15}\text{N}$ natural abundance signatures and (b) N concentration (mg g^{-1}) of epiphytic, terrestrial, and hemiepiphytic tropical fern species. Post hoc test were generated using Tukey tests. Capitol letters refer to within life-form whereas lower case letter refer to across life form comparisons

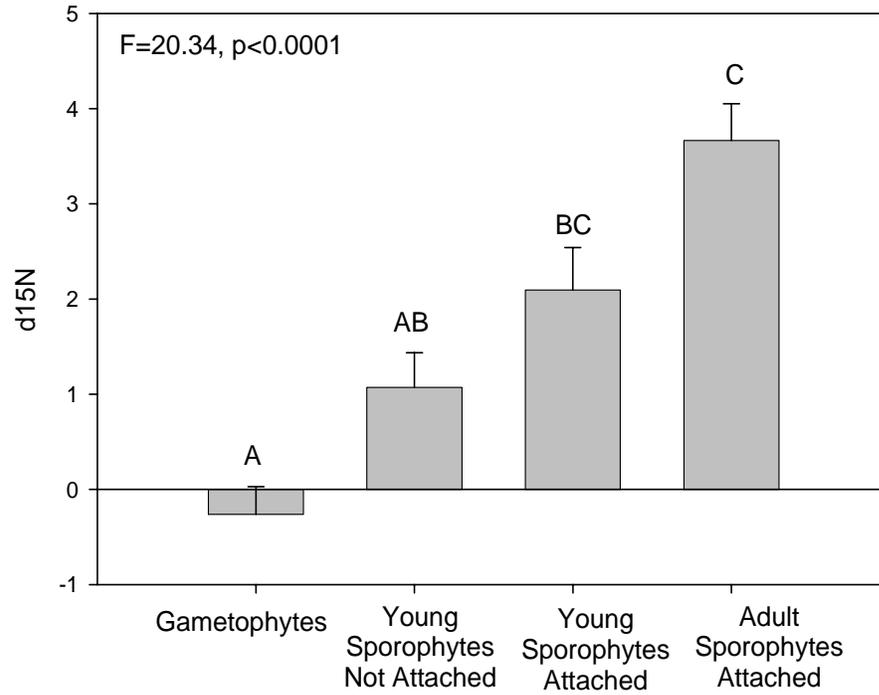


Figure 4-3. $\delta^{15}\text{N}$ natural abundance signatures from the hemiepiphytic fern *Lomariopsis vestita*. Gametophytes of this species are completely epiphytic on understory trees. Young sporophytes are initially produced that have true roots, but no connection to the forest floor at very early stages. Young sporophytes eventually attach to the soil and rooting depth increases with adult plants

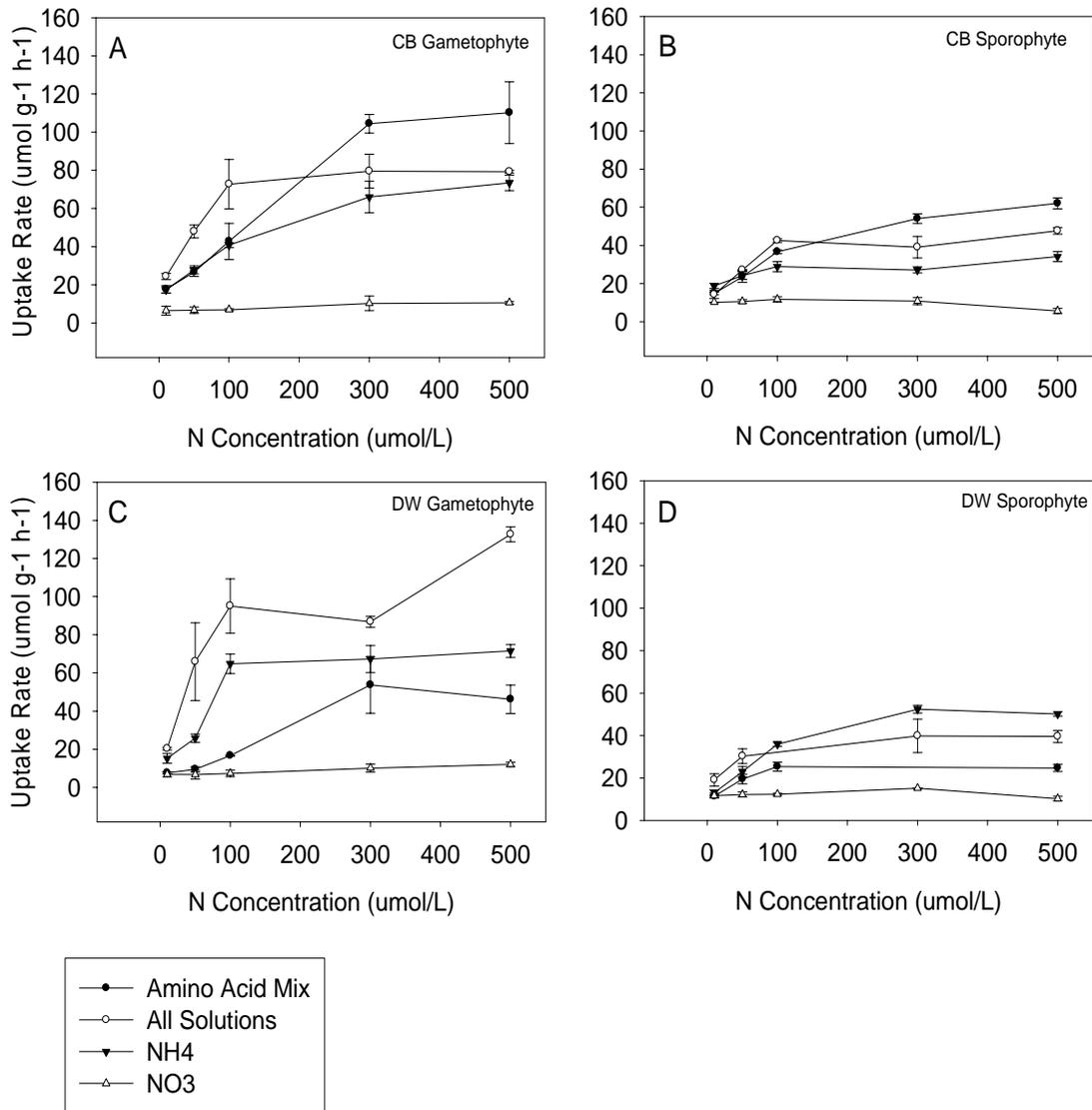


Figure 4-4. Uptake curves from $\delta^{15}\text{N}$ labeled solutions. Fine roots were selected, excised, and placed into a series of solutions containing increasing concentrations of $\delta^{15}\text{N}$ labeled organic and inorganic N forms. For the gametophyte trials individual gametophytes of similar size and maturity were placed directly into a separate set of solutions. All samples were allowed to incubate for 60min in solutions containing only ^{15}N labeled NH_4^+ , NO_3^- , (99 atom%), a cocktail of equal proportions of the amino acids: Aspartic and Glutamic Acids, and Glycine (98 atom%), and a cocktail of all solutions (NH_4^+ + NO_3^- + the 3 amino acids)

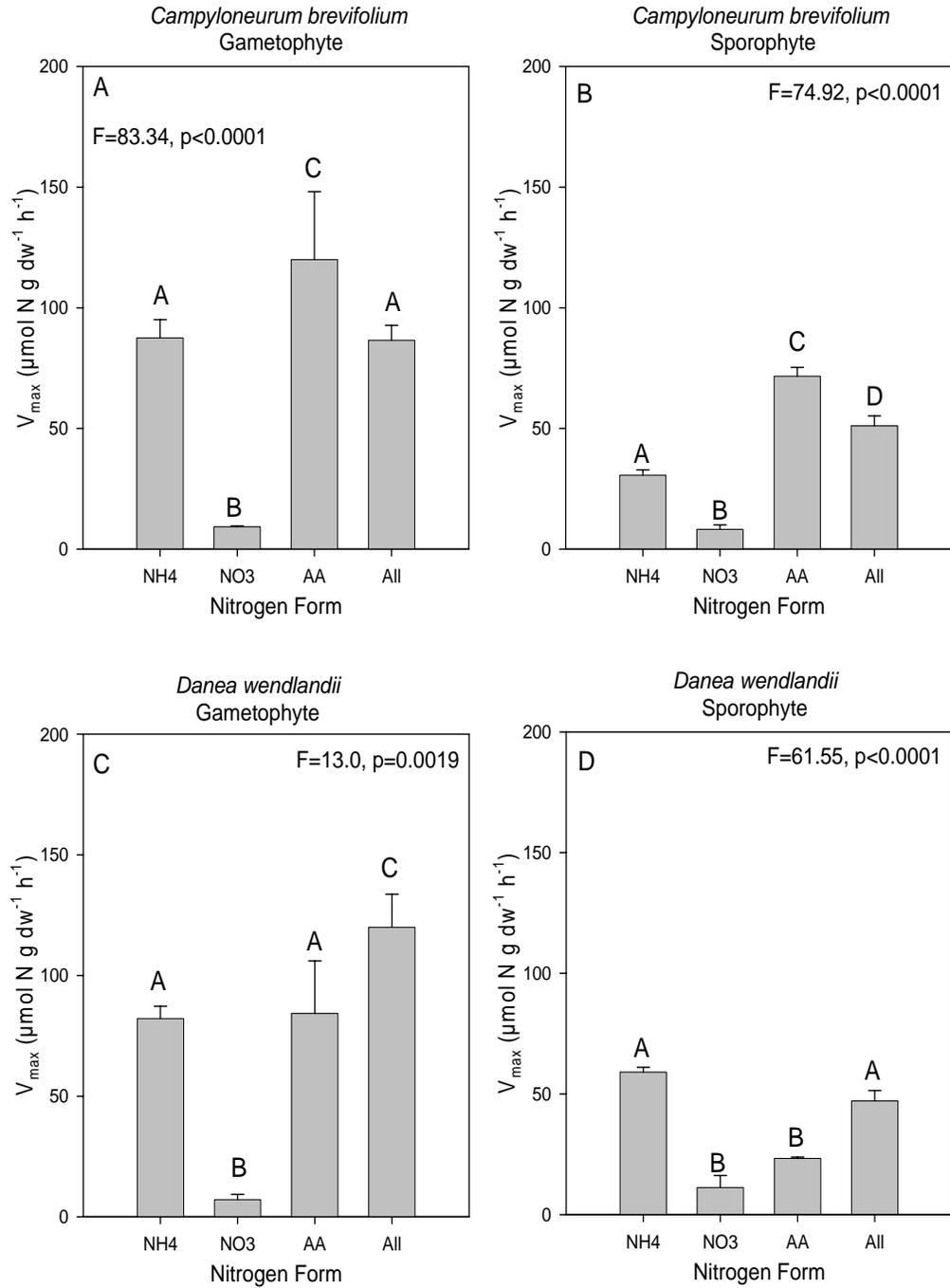


Figure 4-5. Uptake saturation values (V_{max}) of each N form derived from Michaelis-Menten functions of the data from Fig. 4-2, error bars are standard errors. *Campyloneurum brevifolium* is a mid-canopy epiphyte of exposed habitats; *Danaea wendlandii* is a low-light understory terrestrial species

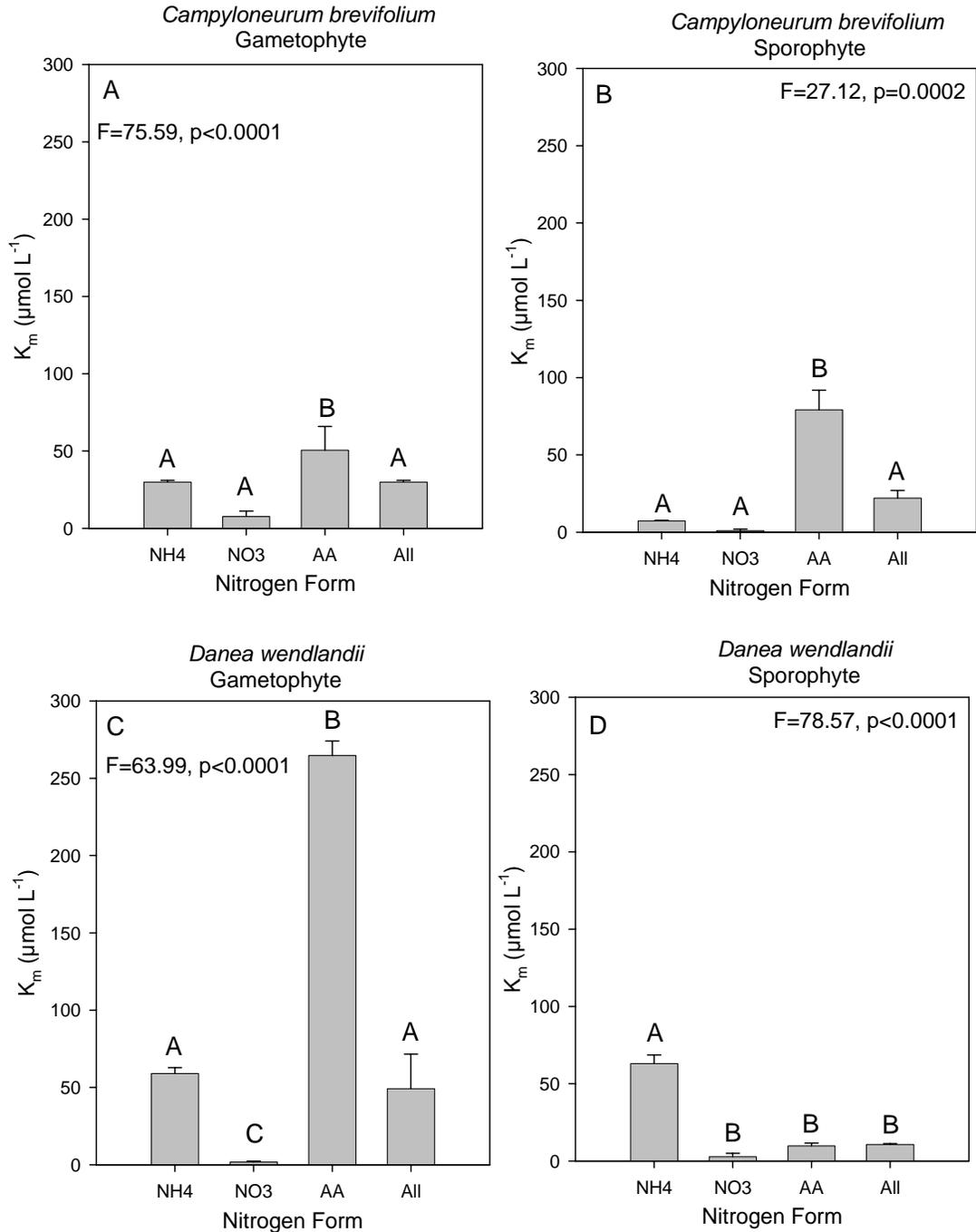


Figure 4-6. $\frac{1}{2}$ Uptake saturation values (K_m) of each N form derived from Michaelis-Menten functions of the data from Fig. 4-2, error bars are standard errors. *Campyloneurum brevifolium* is a mid-canopy epiphyte of exposed habitats; *Danaea wendlandii* is a low-light understory terrestrial species.

CHAPTER 5 CONCLUSIONS

This dissertation is one of the first attempts to examine and apply modern ecological and ecophysiological techniques to the study of fern gametophyte ecology. This work has demonstrated that fern gametophytes can be extremely long-lived *in situ* and that there are differences in factors that influence the distribution and demography of epiphytic and terrestrial ferns. Differences in life history and the way that epiphytic and terrestrial life-forms respond to disturbance and light provide evidence for adaptively meaningful variation in life histories that has evolved in the two groups. Epiphytic species have evolved in a high light, highly competitive, yet relatively stable matrix. Such environments reduce the light limitations encountered by terrestrial species, yet they incorporate closer contact with bryophytes. These habitat mediated conditions may be largely responsible for the observed variation in longevity.

Dassler and Farrar (1997) have argued that differences in gametophyte longevity between epiphytic and terrestrial species have largely evolved due to pressures from the genetic consequences of intergametophytic selfing. Asexually reproducing indeterminate gametophytes of many epiphytes can produce large and long-lived clones. Such clones greatly increase the longevity of individual genotypes which is hypothesized to increase the chance of outcrossing. The data generated from chapter one clearly show that epiphytic gametophytes are significantly longer lived than terrestrial species. One remarkable discovery is that epiphytic gametophytes can live for years where even

terrestrial species on relatively stable substrates rarely lived beyond 6 months. I have observed gametophytes of some understory epiphytes that are over 6 years old.

Are the differences in longevity between epiphytes and terrestrial species related to some adaptively meaningful variation between the life-forms as has been hypothesized, or is it simply a result of some intrinsic instability of epiphytic vs. terrestrial habitats? The answer to this question remains elusive as there is no clear understanding of the differences in disturbance between epiphytic and terrestrial habitats. The data in my study would indicate that epiphytic habitats are far more stable than terrestrial habitats. This clearly needs to be established and a much greater survey of demography needs to be undertaken to better understand the extent of the differences that I have reported.

Gametophytes that can live for months and especially those that live for years have to cope with stress associated with extreme abiotic variation. The second part of my dissertation has revealed a surprising and completely unexpected degree of extreme gametophyte stress tolerance to desiccation across several species. All species surveyed exhibited more desiccation tolerance than current pteridological dogma would suggest. In addition, such tolerance was clearly linked to species sporophyte ecology with those from drought prone habitats, such as the epiphytes in the study, exhibiting greater degrees of tolerance compared to those in mesic habitats. Epiphytic species were also robust in dealing single dry down events and exhibited significantly greater recovery following extreme desiccation intensities and multiple desiccation cycles compared to more mesic terrestrial species. Not only are epiphytic species longer-lived, they are also considerably more desiccation tolerant: two characters that are likely connected.

Stress tolerance has been shown to structure some bryophyte communities (Cleavitt, 2002) but I have been unable to find any additional work to suggest that ferns are sorting along clines of stress tolerance. Much additional work needs to be completed on gametophyte physiology to truly understand the role that gametophytic longevity and desiccation tolerance plays in sorting species. Additional studies need to include combinations of temperature and light stress with desiccation to develop a better understanding of the interaction of these characters and the relative tolerance of more species. This work also has basic science applications beyond ferns and can be applied to aspects directly related to genetic engineering of desiccation tolerance in crop plants.

One factor that seems closely tied to sorting of terrestrial fern sporophytes are edaphic factors (Tuomisto, 1998, Tuomisto, 1994, Tuomisto, 2002). The role that nutrients play in shaping fern gametophyte distributions is virtually unknown. My work on nitrogen relations of tropical fern gametophytes has revealed unexpected versatility in nitrogen acquisition between both gametophytes and sporophytes and between epiphytic and terrestrial species. Of great significance was the discovery that ferns can partition nitrogen by form and have the ability to take up amino acids and use them as an important component of their nitrogen budgets. The importance of amino acid uptake as critical components of species' N budgets is currently receiving increased attention and has been shown in species from tundra (Kielland, 1994), to temperate (Finzi and Berthrong, 2005) and subtropical (Schmidt and Stewart, 1999) ecosystems. Nitrogen associated with amino acids is clearly important in both the N cycle of tropical fern gametophytes and sporophytes and such flexibility in accessing different nitrogen forms may provide species with differential competitive abilities result in one mechanism by

which species are sorted along nutrient gradients. What is critically needed are studies that incorporate dual labeled C and N isotopes to precisely determine if amino acids are hydrolyzed at the cell membrane or if they are taken directly into plan.

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BIOGRAPHICAL SKETCH

James Edward (Eddie) Watkins, Jr. was born on 12 March, 1974 in Ozark, Dale County, Alabama. He attended the now-condemned Flowers Elementary School where his first introduction to science was a project on turtles by his first grade teacher Mrs. Hopper. He graduated to attend D.A. Smith Middle School. It was there, under direction of his eighth grade teacher, Dena Byers he first began to develop an understanding of scientific experiment. He competed in several regional science fairs, making it as far as the Alabama State Science Fair for his work on factors controlling the rate at which mice could exit a complicated maze.

During these years, he spent much of his time fishing, hunting, building forts, and observing nature from his daily hikes in to the forests surrounding his home. During these early years he developed a true connection with the natural world. He gained his early understanding of how this world was put together by his first biological mentor Ms. Linda Dees: science teacher at Carroll High School. During these formative high school years he began to put together what he would do for the rest of his life. One of the most important developments came when Ms. Dees required his freshman biology class to complete a plant collection. This Eddie did by only collecting live ferns that were then transplanted into the school's nature preserve. In the course of this collection, he discovered two of the rarest ferns in Alabama and went on to publish some of this work in a peer-reviewed journal. During his early fern forays, he made the acquaintance of Professor Warren Herb Wagner, Jr.; the world's leading fern authority at the time, and

continues to inspire Eddie's work today. After graduation, he attended Auburn University, where he worked under the direction of Dr. John D. Freeman and Dr. Robert S. Boyd. In the lab and courses of Dr. Boyd that Eddie was finally able to put nature and experimental sciences together and begin to develop an understanding of the complexities of ecology. After graduation, he attended Iowa State University under Dr. Donald Farrar where he attained an MS degree with his thesis on *Thelypteris burksiorum*. These years were paramount to his Pteridological development, as Dr. Farrar is one of the last old-school fern biologists and was as smitten with ferns as Eddie. Eddie graduated in 2000 and spent a year living with his wife Catherine in Costa Rica, studying the magnificent array of ferns at La Selva Biological Station and beyond. He then returned to the South where he began his doctoral studies with Dr. Stephen Mulkey and Dr. Michelle C. Mack. After completing of his doctoral studies, Eddie will begin a post doctoral fellowship in the lab of Michelle Holbrook at Harvard University.