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Invasion Ecology and Response to Fire of the Nonnative Fern Lygodium microphyllum in the South Florida Everglades

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INVASION ECOLOGY AND RESPONSE TO FIRE OF THE NONNATIVE FERN *LYGODIUM MICROPHYLLUM* IN THE SOUTH FLORIDA EVERGLADES

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Nicole Sebesta

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Nicole Sebesta, and entitled Invasion Ecology and Response to Fire of the Nonnative Fern *Lygodium microphyllum* in South Florida Everglades, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Nicole Sebesta is approved.

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ABSTRACT OF THE DISSERTATION

INVASION ECOLOGOY AND RESPONSE TO FIRE OF THE NONNATIVE FERN *LYGODIUM MICROPHYLLUM* IN THE SOUTH FLORIDA EVERGLADES

by

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Professor Jennifer Richards, Major Professor

Lygodium microphyllum (Old World Climbing Fern (OWCF)) is a climbing fern native to tropical and subtropical regions of Australia, Asia, and Africa. First introduced to Florida as an ornamental in the 1960s, the fern has become a serious invasive in numerous Florida habitats, severely degrading native herbaceous and woody vegetation and altering fire behavior. One area with the greatest increase in OWCF cover is the sawgrass marsh of southern Everglades National Park (ENP), where prescribed fire is used for both maintenance of sawgrass marshes and management of OWCF infestations. However, the efficacy of OWCF control using fire in this habitat is uncertain. This dissertation investigated the response of individual OWCF plants in coastal sawgrass marshes over two years following a prescribed burn. As some OWCF occurs in brackish conditions, salinity tolerance of the fern was assessed under greenhouse conditions. Additionally, nectar production, which can influence biological control agent success, was examined in OWCF and related species.

Following the prescribed burn, surviving OWCF recovered to pre-burn sizes between 6 and 15 months after burning, depending on the burn season. Mortality was

size-dependent, with smaller plants suffering highest mortality rates. Sexual reproduction and biocontrol mite presence were both reduced for approximately 9 months post-burn. Saline conditions of 10 ppt reduced growth rates of greenhouse-grown OWCF within 6 weeks and resulted in significantly reduced biomass after 3 months as compared to plants in both 0 and 5 ppt. Spores had reduced and delayed germination in saline conditions compared to 0 ppt, and were completely prevented from germinating in 15 ppt. Foliar nectar production, previously discovered on OWCF, was documented on three additional species, including the other major Florida invasive, *L. japonicum*.

The findings of the dissertation suggest that fire is an effective management strategy for OWCF and may be improved in certain habitats by combination with biocontrol agent releases. Although OWCF is found in mildly brackish habitats, higher salinity hinders its survival, suggesting limited invasion potential in more saline areas. Finally, if foliar nectar production occurs in the field, further study of its effects on biocontrol agent success is warranted.

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ABBREVIATIONS & ACRONYMS

Abbr.	Meaning
CS	Cape Sable (prescribed burn area)
CSA	Cape Sable Site A
CSB	Cape Sable Site B
CSC	Cape Sable Site C
DMG	Damage-associated nectar (dmg)
DSB	Dry Season Burn
Dunn	Dunn's test for multiple comparisons *(z)*
ENP	Everglades National Park
EP	Test of Equal Proportions *(χ^2)*
Exp	Experimental (burned)
HB	Hell's Bay (reference area)
HBA	Hell's Bay Site A
HBB	Hell's Bay Site B
HBC	Hell's Bay Site C
JA	Jasmonic acid
KM	Kaplan-Meier survival curves
KW	Kruskal-Wallis test *(χ^2)*
L	Large plants (> ¼ m^2)
LR	Log-Rank test *(χ^2)*
M	Medium plants ($^1/_{16}$ m^2 to ¼ m^2)

ND	Non-damage nectar
Post-Rx	After (either) prescribed burn
Pre-Rx	Time period data (before Aug. prescribed burn)
Ref	Reference (unburned)
S	Small plants ($< {}^{1}/_{16}$ m^2)
SK	Scott-Knott clustering
ST	Student's T-test *(t)*
T	Timesteps (6 mo. periods, except T½ = 3 mo.) post-Rx
TF	Tanglefoot®
Trt	Treatments (Experimental and Reference together)
WSB	Wet Season Burn

CHAPTER I

I. INTRODUCTION

Introduction

Lygodium microphyllum (Cav.) R. Br., the Old World climbing fern, is a major invasive plant in central and southern Florida (Iannone et al., 2020; Pemberton and Ferriter, 1998). This nonnative fern has spread through Florida alarmingly quickly, causing ecological damage in a variety of wetland and upland habitats, both disturbed and remote, and threatening the success of Everglades restoration (Hutchinson et al., 2006; Pemberton and Ferriter, 1998). Because of this, the Florida Invasive Species Council (FISC, formerly Florida Exotic Pest Plant Council (FLEPPC)) designated *L. microphyllum* as one of Florida's most serious invasive plant species (FLEPPC, 2019; Hutchinson et al., 2006). In southern Florida, Everglades National Park, a UNESCO World Heritage Site (UNESCO, 2021) and Biosphere Reserve (UNESCO, 2015), as well as a Ramsar Convention Wetland of International Importance (Ramsar, 2014), is also threatened by this fern species. Despite the formation of a *Lygodium* task force to improve the adaptive management plan for the fern, many questions about of the biology of this species remain unanswered, hindering management progress (Enloe, 2015; Hutchinson et al., 2006). Some questions relate to the ecology of the fern, while others concern the efficacy of multiple management techniques and their interactions. Below, I review our general knowledge about invasive species, their responses to disturbance, and invasive species management. I then introduce what is known about *L. microphyllum* and outline the topics of my dissertation.

Invasive plant species

Florida's sub-tropical climate is well-suited to support a wide range of species, as evidenced by the more than 3,300 endemic species inhabiting Florida's unique variety of habitats (Wunderlin et al., 2020). Most nonnative plants in Florida, introduced by horticultural enthusiasts or arriving accidentally via disturbance, travel, or through ports, do not become invasive even if they do manage to establish. Out of the more than 1,500 exotic plants known to occur in Florida, however, nearly 200 are of major concern as they are currently invading and altering Florida native habitats (FLEPPC, 2019; Wunderlin et al., 2020). Invasive plants can outcompete native plants, reduce biodiversity, and/or alter mutualisms among native taxa or between native and exotic taxa (Holzmueller and Jose, 2009; Powell, 2016). Endangered species are especially threatened by invasive plants and animals (Hutchinson et al., 2006; Pemberton and Ferriter, 1998). The increasing presence of invasive plants in a given ecosystem can shift hydrological, biogeochemical, and even disturbance aspects of that system (Gordon, 1998). Especially damaging, for example, are invasive plants that physically alter the invaded habitats, such as vines that grow into tree canopies, making them top-heavy and subject to collapse, or invasive species that quickly fill forest gaps, shifting the proportion of seedling recruitment to understory species over tree species (Horvitz and Koop, 2001; Osunkoya et al., 2010). Aquatic invaders may grow so well in waterways that they impede water flow and deplete dissolved oxygen to levels that are lethal to invertebrates (Villamagna and Murphy, 2010), or interact in unexpected ways with other invasive plants (Wundrow et al., 2012). In addition to the ecological damage caused by botanical invasions, there are economic costs as well, including lost tourism and damage to crops

(Pimentel et al., 2005). With changes in sea level and global climate, many species may find their ranges changing, and invasive plants may benefit most (Dukes, 2010).

Plants that become invasive tend to have traits that are particularly useful for entering novel habitats. Although these vary by plant and particular environment, these traits are often similar to those of colonizer species and include possessing a short juvenile period, frequent seed or propagule production, high production of propagules, and tolerance of variable habitats (Jose et al., 2013; Rejmanek and Richardson, 1996). These traits support faster colonization and, in the case of small seed/propagule mass, better dispersal. In the extreme, ferns, with their minute, wind-dispersed spores, are exemplars of production of numerous diaspores with tiny propagule mass. High propagule production, especially in the form of minute, wind-blown spores, increases the likelihood that some spores will succeed in taking advantage of newly opened sites, both near and far.

Invasive plants and disturbance

Disturbance can affect a plant's ability to colonize and/or survive in a specific habitat, and it may play mitigative and/or facilitative roles in exotic plant invasions (Guthrie et al., 2016; Jones et al., 2019). Nonnative plants that are ill-suited to conditions associated with a particular disturbance may be prevented from successfully invading. For example, invading plants that have grown to a vulnerable stage or that do not readily resprout from protected/buried rhizomes may be unable to colonize or survive in a new habitat that is regularly disturbed by fire (DiTomaso and Johnson, 2006). Conversely, anthropocentric or naturally-occurring disturbances, such as fire or hurricanes, can create suitable conditions for invasions (Jones et al., 2019; Murphy and Metcalfe, 2016). For

example, fire disturbances exert direct effects, such as heat or smoke cues for germination, and indirect effects, such as reduced competition, increased light, or ash-derived nutrient pulses, that, depending on the native and invasive plants, may result in an increase in the proportion of invasive plants at a given site (DiTomaso and Johnson, 2006). Alternatively, hurricane-caused treefalls can be susceptible to invasion by nonnative species taking advantage of the associated, newly opened space and additional sun and water (Horvitz et al., 1998; Macdonald et al., 1991). Under certain conditions, disturbance-spurred invasion may spiral into a cascade of habitat deterioration, e.g., invading tree and vine species in La Reunion's coastal forest are less tolerant of wind than the native species, and as these replace the native canopy, the habitat becomes increasingly susceptible to damage from subsequent hurricanes, which then facilitates additional nonnative plant invasions and more damage in a positive feedback loop (Macdonald et al., 1991).

Sometimes, invasive plants may alter the behavior of the disturbance itself; fire provides an example of this effect (Brooks et al., 2004; Perry et al., 2015; Wagner and Fraterrigo, 2015). When fire-tolerant and fire-promoting plants like cheatgrass (*Bromus tectorum*) invade fire-dependent but less fire-tolerant habitats, the cheatgrass can shift the properties of the fuels in the system from a shrubland maintained by a fire return interval of approximately 100 years to a grassland that may ignite every few years (Knapp, 1996). In the Great Basin Desert of the western United States, several cycles of cheatgrass-fueled fires converted the fuel type and distribution such that fires became too frequent and intense for the native species and, in severely invaded areas, fostered a near monoculture of extremely hot- and fast-burning cheatgrass lands, where fires excluded

regeneration of native propagules (Knapp, 1996). Of course, the invasive plant-driven community shift could be in the other direction, as well, with fires becoming less frequent and/or less intense with the invasion of plants that reduce overall fuel accumulation or shift fuel types from fine (grasses) to more coarse (woody), as with Brazilian pepper (*Schinus terebinthifolius*) invasions (Doren et al., 1991; Mack and D'Antonio, 1998). The invasive, Old World climbing fern, *L. microphyllum*, has already been documented both shifting species composition and altering fire regimes (Pemberton and Ferriter, 1998).

Management methods for invasive species

A number of invasive species management methods are available, including mechanical removal, herbicides, prescribed burning, biological control, and public education efforts (DiTomaso and Johnson, 2006; Pemberton, 1998). The appropriateness of each of these methods depends on the invading plant and the conditions of the invaded area. For example, important factors are whether the invaded area is tolerant to fire, or supports endangered taxa, or is near urban developments, etc. The cost of each treatment type will vary depending on the kind of infestation, its accessibility, whether follow-up treatments are necessary, and success rates. Timing of the management application must consider the various species involved, so that maximum effect can be achieved, for example, burning when the invasive plant is more vulnerable to fire, or applying herbicides after sensitive native species have senesced (Zouhar et al., 2008).

Mean annual expenditures for management of invasive plants on Florida conservation lands (~5.2 M ha) were $44.9 ± 1.9 M (range $38.9 - 50.1 M, 2014 USD), estimated over 2009-2014 period (Hiatt et al., 2019). As travel and shipping continue,

new invasive plant introductions are likely to increase, resulting in additional plants with invasive potential. Research is turning to integrated pest management (IPM), which is the combined use of multiple management strategies, including multitrophic relationships, to suppress or prevent pest invasions. Essentially an adaptive method, management targets are regularly assessed and adjustments to the management methods made as new information is incorporated. With IPM, the use of herbicides can often be reduced, resulting in more ecologically and economically viable management of invasive plants (Ehler, 2006; Prokopy, 2003). Integrated pest management has shown promise for controlling several invasive species (Boughton and Center, 2010; Center et al., 1999; Langeland et al., 2011; Paynter and Flanagan, 2004) and can increase the effectiveness of management efforts while reducing overall costs. In fiscal year 2019, the South Florida Water Management District spent over $20 M on prevention and integrated management practices, with priority focus on four species, including *L. microphyllum* (Rodgers et al., 2020).

Within Everglades National Park, invasive plant management strategies include prescribed fire, manual removal, herbicides, biological control, education and outreach, and combinations of these where appropriate (Pemberton, 1998; Rodgers et al., 2020). When possible, prescribed fire is used to both maintain fire-dependent habitats and treat invasive plant infestations. For example, coastal sawgrass marshes and the infestations of *L. microphyllum* occurring there are treated by a fire interval of 3-10 years. Increasingly, biological control agents are also being used in the sawgrass marshes and other habitats with some success (Boughton and Center, 2010; Lake et al., 2014; Smith et al., 2014).

Lygodium microphyllum biology

Lygodium is the sole genus in the plant family *Lygodiaceae*, which is in the Schizaeales with two other families: Schizaeaceae and Anemiaceae (Schuettpelz et al., 2016). In some texts, the three groups are divided at the subfamily (Christenhusz and Chase, 2014) or genus level (Mueller 1982; Wikström, Kenrick, and Vogel 2002; Madeira, Pemberton, and Center 2008) and placed within Schizaeaceae, but later phylogenetic work supports divisions at the family level (Schuettpelz et al., 2016). Regardless, Schizaeaceae and Anemiaceae are more closely related to each other than to Lygodiaceae, the latter having vastly different morphological characters—the most striking of which is its twining habit.

In *L. microphyllum*, the creeping, protostelic rhizome grows at, or just below, the soil surface and branches dichotomously with leaves forming dorsally on the rhizome in a single rank (Mueller 1982; Tryon and Tryon 1982). From the first-produced rhizome, primary, determinate, juvenile fronds emerge, which are short—usually less than 10 cm—and simple to lobed. Secondary (adult) fronds follow, and these are indeterminate and twining, 2-3 times pinnately divided, and easily reaching over 30 m in height, given a suitable support or host tree (Mueller 1982). Fronds are foliar, but their growth is analogous to the stems of twining angiosperms in indeterminacy, circumnutation, delayed pinna (leaflet) expansion, and the presence of a resting leafbud on each pinna (Mueller 1982). Pinnae alternate along the frond (climbing leaf) rachis, but each pinna is subdivided, forming two approximately mirrored pinnules (which are once more divided into tertiary segments); the pinna ends in a central, dormant pinna bud (Mueller 1982). The pinna stalk (petiolule) terminates in the pinna bud. In the event of damage to the leaf

apex (crozier of that frond), the pinna bud of a lower pinna resumes indeterminate growth, expanding a new crozier, elongating, and developing pinnae as the previous main rachis had done (Mueller 1983). Recently, some pinna buds have been documented developing new rhizomes, as well (Figure 1.1; N. Sebesta pers. obs.; A. Carmona pers. comm., USDA). These leaf-borne rhizomes can eventually put out adventitious roots and, presumably, establish a clone, similar to reproduction by adventitious leaf-borne buds observed in some other ferns (Moran 2004). The fronds will twine around the host plant, as well as older rachises, eventually forming a mat of rachises that can be either vertical, as a skirt on a tree, or horizontal in the absence of a tall host. These mats have been documented to reach over 1 m in thickness (Lott et al., 2003).

The pinnae are sexually dimorphic, with fertile tertiary segments further lobed and developing into sorophores of enrolled leaf tissue bearing sporangia on the abaxial surface (Gandolfo et al., 2000; Page et al., 2014). Fertile pinnae tend to develop on the upper portions of the fronds, which in high-climbing individuals allows for better spore dispersal by wind (Wu et al., 2006).

Foliar nectar production was recently confirmed on *L. microphyllum* fronds, despite the lack of obvious nectaries (Sebesta et al., 2018). Foliar nectar production on ferns occurs in nearly 40 species, but it had never been reported for *Lygodium* (Weber et al., 2015). The presence of nectar can be defensive, as it often is as extrafloral nectar on angiosperms (Grasso et al., 2015), serving to attract predators (most often ants) that remove or kill herbivores from the nectar-producing plant. If foliar nectar functions similarly in *L. microphyllum*, biocontrol agents could be affected.

Lygodium microphyllum is homosporous, producing one kind of spore that can develop into either unisexual or bisexual gametophytes, facilitating a mixed mating system with three modes of sexual reproduction: intergametophytic crossing (two spores from genetically distinct plants), intergametophytic selfing (two spores from the same plant), and intragametophytic selfing (single spore) (Lott et al., 2003). The three modes are possible because of asynchronous gametophyte maturation. While asynchronous maturation promotes outcrossing and supports genetic diversity, intragametophytic selfing of hermaphroditic gametophytes facilitates successful colonization following long-range dispersal (de Groot et al., 2012; Lott et al., 2003; Soltis and Soltis, 1992). Although mixed mating systems are common in homosporous ferns, the high rates of all three modes—as found in both *L. microphyllum* and *L. japonicum*—are rare (Soltis and Soltis, 1992). Coupled with extremely high spore production (nearly 28,000 per pinnule segment) and spore viability of approximately 4 years, the potential for the fern to infest vast areas of Florida is a serious threat (Hutchinson, 2010; Stocker et al., 2008; Volin et al., 2004).

In addition to fast growth and multiple modes of sexual reproduction, *L. microphyllum* tolerates a variety of temporary stresses including cutting, freezing, inundation, and burning. In response to such stresses, it can resprout new fronds from the rhizomes within weeks following the disturbance (S. Gandiaga et al., 2009; Hutchinson and Langeland, 2014; Pemberton, 2003; Philippi and Richards, 2007; Richards et al., 2020). This tolerance for varied conditions is key for invasive species and allows *L. microphyllum* to thrive in a variety of habitats, from swamp edges and mesic sites through frequently burned freshwater marshes and more brackish areas to unburned tree

islands and forest patches (Jose et al., 2013). Even natural disturbances can aid spore dispersal and result in increased fern recruitment in, for example, hurricane-opened tree gaps (Lynch et al., 2009).

As *L. microphyllum* becomes established and forms rachis mats, native plants are shaded out and their recruitment is suppressed. Larger infestations smother and kill trees by toppling them or by altering the distribution of fuels and subsequent fire effects (Volin et al., 2013). For example, in habitats maintained by ground fires, an infestation of *L. microphyllum* builds an intermediate layer of fuel via the buildup of rachises between the ground-level and canopy vegetation, enabling fire to reach the fire-sensitive canopy, which results in greater tree mortality than would otherwise occur (Brandt and Black, 2001).

Many native plants—and the animals they support by providing forage and cover, as well as nesting materials and hunting grounds—are threatened by Old World climbing fern infestations either directly or indirectly. Some of these are already critically endangered species, like curlygrass fern (*Actinostachys pennula*) and Wright's pineland fern (*Anemia wrightii* Baker) (Call et al. 2007, Ferriter 2001, Mehltreter et al. 2010, Nelson 2000). Endangered animals whose habitats are degraded by *L. microphyllum* infestations include the Florida panther (*Puma concolor coryi*), Everglade snail kite (*Rostrhamus sociabilis*), and wood stork (*Mycteria americana*) (FMNH, 2021; Hutchinson et al., 2006). The affected plants are shaded out, exposed to abnormal fire, or toppled, while the animals lose habitat or resources or become directly trapped in the rachis mats (e.g., wading birds, tortoises, deer) (Darby and McKercher 2002, Mazzotti pers. comm. 2015).

One of the areas in southern Florida currently experiencing the greatest increase in *L. microphyllum* infestation is the southwestern coastal marshes of Everglades National Park (Hutchinson et al., 2006; Rodgers et al., 2014). Here, various habitats have become invaded by *L. microphyllum*, including coastal prairies and woody patches such as tree islands. In the coastal prairies and freshwater marshes, the dominant macrophyte is sawgrass (*Cladium jamaicense* Crantz), with shrubby patches of wax myrtle (*Morella (= Myrica) cerifera* (L.) Small) or scattered stands of cypress (*Taxodium distichum* (L.) Rich.).

My dissertation examines the ecology of *Lygodium microphyllum* in the invaded range of the coastal Everglades sawgrass marsh. Specifically, the research documents responses to prescribed burning (Chapter 2) and the species' salinity tolerance (Chapter 3). The production of foliar nectar within the *Lygodium* genus is also investigated (Chapter 4). The conclusions from this research are summarized in Chapter 5.

Figure

Figure 1.1. *Lygodium microphyllum* sporophyte, with rhizome (B) developing out of a pinna bud and producing a new climbing leaf (C). Note (A) primary rachis of the parent indeterminate leaf below the new rhizome. Photo: Andrea Carmona, USDA, October 2019.

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CHAPTER II

II. *LYGODIUM MICROPHYLLUM* REGROWTH AND SPREAD AFTER BURNING IN EVERGLADES NATIONAL PARK

Introduction

Lygodium microphyllum (Cav.) R. Br. is one of the top five invasive plants of management concern in FL, wreaking ecological havoc wherever it successfully invades (Hutchinson et al., 2006). *Lygodium microphyllum* is a vining fern that climbs hosts using indeterminate leaves that twine around supports, growing into the canopy of tall trees (Pemberton and Ferriter, 1998). It invades a variety of habitats, reducing species richness (Brandt and Black, 2001), and in the most severe cases, collapsing tree island and forest canopies over which it has formed rachis mats (Rodgers et al., 2010). Heavy infestations can carry fire into fire-sensitive habitats, resulting in tree mortality and a drastic shift in species composition (Ferriter and Pernas, 2006; Stocker et al., 2008). The species has a variety of characteristics that make it a successful invasive species, including rapid growth from underground rhizomes that produce the indeterminate climbing leaves, continuous production of spores that are dispersed short and long distances via water, wind, and animal vectors, and reproduction through multiple vegetative and sexual reproductive systems (Moran, 2004; Mueller, 1983; Mueller, 1982; Philippi and Richards, 2007; Volin et al., 2004). The species is capable of intragametophytic selfing, whereby a gametophyte becomes hermaphroditic and self-fertilizes to produce a diploid sporophyte. This reproductive mode allows a single spore to colonize a new site, enhancing successful long-range dispersal (Lott et al., 2003; Peck

et al., 1990; Volin et al., 2004). *Lygodium microphyllum* has invaded and threatens internationally important conservation sites such as Everglades National Park (ENP).

A number of management methods are available to control invasive species populations, including mechanical removal, herbicides, prescribed burning, biological control, and public education efforts (DiTomaso and Johnson, 2006; Pemberton, 1998). Fire is a naturally occurring phenomenon in Florida habitats, historically ignited by lightning, as reported by Robertson in 1953 (Slocum et al., 2007; Smith et al., 2015), and it is used in conservation areas to manage infestations of *L. microphyllum*. The effects of burning on the fern's regrowth have been studied under greenhouse conditions (Richards et al., 2020) but have not been studied in the field (Zouhar et al., 2008). Several biocontrol agents have also been introduced for this species and two have become established within some areas of south Florida: a leaf-galling mite, *Floracarus perrepae* Knihinicki & Boczek, and a moth *Neomusotima conspurcatalis* Warren (Figure 4). Both the moth and mite have established in Jonathan Dickinson State Park, but while the moth has had mixed results in other areas (Smith et al., 2014), the mite dispersed to areas as far as Cape Sable within 5 years (Lake et al., 2014). How these biocontrols interact with fire-based invasive plant management efforts is unknown.

The goal of our research was to quantify the effects of prescribed fire on *L. microphyllum* infestations in the field. We monitored regrowth of individual *L. microphyllum* plants using ground-based surveys conducted before burning, then followed regrowth after burning to monitor patterns of re-infestation (resprouting from rhizomes or new sporeling establishment) and time to recovery. On the basis of prior greenhouse studies (Richards et al., 2020), we expected that burning would result in

greater mortality of smaller plants, and that surviving plants would remain reduced in patch size and density for 24 months post-burn. A secondary goal was to document how fire affects biocontrol agents in the field.

Materials and Methods

Study Organism

Lygodium microphyllum's characteristic and recognizable growth is composed of the twining fronds, which grow indeterminately around and up host plants. These fronds are persistent, and as new fronds twine around older fronds, they form mats of wiry rachises that remain long after the pinnule segments have senesced and abscised. The height of each individual plant is dependent on the host, as even several fronds twining together cannot support much vertical growth without additional support (Figure 2.1). In the absence of a host plant, *L. microphyllum* will gain vertical height slowly, twining upon itself, but will spread horizontally forming a mat.

Study Area

Within Everglades National Park (ENP), areas in the southwestern coastal region with the highest percent cover of *L. microphyllum* were identified in 2010-2012 Digital Aerial Sketch Maps (Rodgers et al. 2014). For our study, we chose two areas within this region that had been subjected to a 3- to 5-year fire return interval and had last burned in 2014 (Figure 2.2). The two areas were predominantly sawgrass marsh with interspersed shrubs and trees (Figure 2.3), and they were sufficiently spatially separated to reduce the chance of prescribed fire spreading from one area to the other (Figure 2.2). The treatment area, Cape Sable (CS), was experimentally burned, while the reference area,

Hell's Bay (HB), remained unburned for the duration of the study. Numerous individual *L. microphyllum* plants occurred throughout both areas, and these ranged in size from small (< $^1/_{16}$ m^2) to large (> ¼ m^2) (Figure 2.4). Plants of multiple sizes were followed to test for size-dependent variation in the effects of burning and to inform management objectives as to whether targeting smaller/newer infestations is more or less effective than targeting larger/older infestations, if targeting both is not feasible. To capture within-area variation in infestation and environmental conditions, three sites, spatially separated from each other by at least 1 km and denoted as A, B, and C, were selected in each area (Figure 2.2, Table 2.1). The sites were accessible only by helicopter; flights were provided by ENP Division of Fire and Aviation.

One pre-burn sampling event was completed for all 6 sites (CSA, CSB, CSC in CS, HBA, HBB, and HBC in HB) in January 2017, approximately 1 month before the scheduled burn in February. At each site, individual plants distributed among three size classes (small (< $^1/_{16}$ m^2), medium ($^1/_{16}$ m^2 to ¼ m^2) and large (> ¼ m^2)) were located, measured, marked with metal tags on pin flags inserted into the ground, and surveyed with a Trimble® R8 GPS (Trimble Geospatial, Sunnyvale, CA). On the basis of previous greenhouse experiments (Richards et al., 2020), smaller plants were expected to experience greater mortality than larger plants. Thus, at each experimental site, seventy plants (40 small, 20 medium and 10 large size classes) were sampled. At each reference site, thirty plants (10 small, 10 medium, and 10 large) were sampled. However, all analyses included the number of plants ultimately included (total N=275), which differed from our initial target numbers. Plants were revisited throughout the study by navigating to the location with the Trimble GPS, then identifying the plant using the tagged pin flag.

Pin flags degraded over time and were replaced as necessary. During the final survey, all pin flags, metal tags, and flagging tape were collected and removed from the sites. During the initial survey, six soil samples per site were collected for nutrient analyses. Samples were collected with a 6.5 cm diameter soil corer inserted to 10 cm depth. The soil cores were analyzed for soil carbon, nitrogen, and phosphorus concentrations, as well as pH and bulk density, at the FIU Freshwater Biogeochemistry Lab (Dr. Len Scinto, FIU, Miami, FL) using standard techniques (Serna et al., 2015). Water depth was measured near each *L. microphyllum* individual with a meter ruler. Surface water salinity was not measured directly during the initial survey, but relative soil salinity was obtained from the soil samples by suspending 1.0 g dry soil in 5.0 mL warm distilled water, mixing thoroughly, and measuring after 15 min with a salinity refractometer (VitalSine, Inc., Model SR-6, Dartmouth, Nova Scotia, Canada) (Bado et al., 2016). In subsequent surveys, when surface water was present, water was sampled near a subset of individuals and salinity was measured in the lab with the refractometer.

Experimental burn

Everglades National Park prescribed a burn to the experimental area on February 10, 2017, which successfully burned two of the three sites. The site that did not burn, CSA, was reburned August 9, 2017. The delay of 6 months for burning CSA resulted in burns during two different seasons: dry season for sites CSB and CSC, and wet season for CSA. Although all three sites experienced the same fire type (head fire, moving in the same direction as predominant wind), wetter conditions during August may have facilitated a less intense, more patchy fire, than that during drier February conditions.

The prescribed burn sites were surveyed 3 weeks after the burn to determine which plants burned, defined as aboveground biomass completely consumed, and to assess regrowth (Figure 2.5). Originally, five post-burn sampling events of both experimental (CS) and reference (HB) sites were planned to occur at 3, 6, 12, 18, and 24 months after the burn (Table 2.2). This time frame was chosen to assess whether and when *L. microphyllum* can recover to pre-burn sizes in relation to the next likely prescribed burn. In addition to the asynchronous wet and dry season burns, hurricane Irma swept through CS and HB on September 10, 2017, shortly after the CSA-reburn and just as the immediate post-burn survey for CSA and the 6-month post-burn survey for the other sites were to be conducted. As a result, we were not able to survey CSA at 3 weeks post-burn to document which plants burned, and we could not access the remaining sites for the 6-month post-burn survey. We were, however, able to visit the burned (CSA) sites 3 weeks post-Irma to determine whether the tags remained and study plants could be located after the hurricane and to sample surface water salinity. The next opportunity to survey occurred over November-December 2017, resulting in a 3-month post-burn survey for CSA, and a 9-month post-burn survey for the five other sites (Table 2.2). Subsequently, we shifted our surveys to continue at 6-month intervals, so post-burn surveys were completed at 9, 15, 21, and 27 months, except for CSA, whose final survey occurred at 21 months. Since CSA's burn was shifted 6 months from the other sites, its surveys are listed separately from the two Feb-burned sites. Below, data are first reported for Dry Season Burn (DSB), comparing the CSB and CSC experimental sites to the three reference sites (Table 2.2). Comparisons between experimental plants at CSA and the

reference sites were made using concurrent survey data. These data are referred to as the Wet Season Burn (WSB) data and are reported in full following the DSB results.

Data collected

During the initial survey, we measured water level on the top of each sawgrass/host hummock where the tagged *L. microphyllum* plant was growing, but this measurement was not useful, as the plants rarely were submerged, even as the water level noticeably changed. Thus, no water depth data is reported for the initial survey. For subsequent surveys, water depth (cm) was measured off but adjacent to the host hummock with an aluminum meter stick submerged just until resistance was met from the soil surface (Table 2.3).

Mortality and survivor regrowth were monitored post-burning (Table 2.3). For mortality, we recorded whether each plant was present or absent at each survey. If a plant was absent for two successive surveys, it was considered dead. Plant cover and leaf height were measured to document survivor regrowth. We also documented the presence and relative amount of sporulation and presence of biocontrol agents.

For small and medium plants, each plant's size class was estimated with a ¼ m^2 quadrat subdivided with string into 4 sub-quadrats, each $^1/_{16}$ m^2. A plant occupying up to 1 sub-quadrat was small, and a plant occupying between 1 and 4 sub-quadrats was medium (Figure 2.4). For large plants (occupying more than the quadrat itself), we estimated actual cover area (extent) to 0.1 m^2. Height of the tallest, living climbing leaf of each *L. microphyllum* plant was measured in centimeters using a meter stick. Sexual reproduction is reported as the % of plants within a group, such as size class, treatment, or site, that were sporulating (Table 2.3). Percent sporulation was also estimated for all

sexually reproductive plants and was documented as the percent of total above-ground leaves that had fertile leaflets, recorded as percent sporulation for each plant. Mean sporulation was the average percent sporulation for sexually reproductive plants within a group (size, treatment, or site). Biological control presence was determined by looking for either galling (rolled leaflet edges), indicative of mite (*Floracarus perrepae*) presence, or the typical "windowpanes" and frass deposits left by the moth, *Neomusotima conspurcatalis* (Figure 2.6). During the first survey, we recorded mite presence/absence per plant. In subsequent surveys we estimated mite abundance using a modified Braun-Blanquet scale documenting percent damaged leaves: 0 (absent), 1 (≤ 10%), 2 (11-25%), 3 (26-50%), 4 (51-75%), 5 (76-90%), 6 (91-100%). To document variation in plant species diversity found at the invaded sites, the species of host plant and associated species growing within a 1 m radius of each plant were recorded at each survey.

Mortality was documented during each survey and coded as 0 for present and alive, or 1 for not present, potentially dead. Under field conditions, when post-fire resprouting occurred, we used the morphology of new growth to determine whether the growth was a resprout or a newly germinated sporeling. Resprouting adult plants resume producing indeterminate climbing leaves, identifiable by the crozier and typical climbing-leaf twining habit. In contrast, primary (juvenile) leaves of newly produced sporophytes are determinate (Mueller, 1982), generally shorter than 5 cm, and more strongly lobed; several of these usually persist even after the adult indeterminate climbing leaves begin to develop. Plants that were not regrowing by the second survey following the fire and that had only dead/dried aboveground material or were apparently replaced by only new sporeling (determinate leaved) growth rather than regrowing adult leaves were

documented as dead. For missing plants, the date of the second survey was used in the mortality assessment to increase our confidence in mortality declarations. Plants that were regrowing (mature climbing leaf type) by the second post-burn survey were recoded as alive for both surveys, and their regrowth variables adjusted to 0 for the previous survey: mortality 0, height 0, extent 0, etc.; these plants were then monitored with the other surviving plants.

Regrowth variables were the same as for the initial survey and were documented at every survey through the conclusion of the study (May 2019), at 21 months for CSA and at 27 months for the remaining sites. In addition, we calculated the average weekly leaf height growth rate (cm) for each time period between surveys. Leaf growth rates were calculated by dividing the change in leaf height by the number of weeks between surveys. Change in height was the difference in height between successive surveys, except for the 3 mo. rate (immediately post-fire). For burned plants at 3 mo., the height was calculated as the leaf height at 3 mo. divided by the difference between the 3 mo. survey date and the date burned (i.e., leaf height immediately post-burn was 0). Pre-burn growth rates for DSB could not be calculated because there was only one survey preceding the burn, but they were possible for WSB because of the delay in burning, and this time period preceding the August prescribed burn is referred to as "Pre-Rx." Each post-burn period between surveys is referred to as a timestep (T), and denotes a specific 6-month period after burning: T1 = 3 months post-burn to 9 months post-burn; T2 = 9 mo. – 15 mo.; T3 = 15 mo. – 21 mo.; T4 (DSB only) = 21 mo. – 27 mo. The only exception is the first 3 months post-burn, effectively half a timestep, T½, denoting 0 mo.

– 3 mo. Because the burns were staggered, the calendar months for DSB timesteps and WSB timesteps differ (Table 2.4).

Statistical Analyses

When the CS sites were visited 3 weeks after the prescribed burn to document which plants burned, only two sites, CSB and CSC, burned nearly completely, with 94% (CSB 63/70 and CSC 69/70) of plants at these sites having burned, yielding the final number of plants included in this group as 132. Eight plants either did not burn or burned only partially, and, although data were collected on them at each survey, these were removed from analyses. Similarly, CSA plants that burned in the dry season (or failed to burn in the wet season) were removed from analyses. Data from plants that survived the entirety of the study, the survivors, were used as the core data for regrowth analyses.

Because of the offset in burn date between site CSA and the other sites, data are presented and analyzed in two groups. The five DSB sites (CSB, CSC, all HB sites) were analyzed as one group, with 132 burned plants and 91 reference plants. Data for the CSA site is presented separately. Analytically, this site is compared to samples from the HB sites that were collected contemporaneously. These four sites (CSA, HBA, HBB and HBC) are referred to as WSB, and include 52 burned plants and 87 reference plants. All statistical comparisons and visualizations were done in R v.4.0.0 (R Core Team, 2020) using additional packages as indicated.

Initial site conditions (soil nutrients) were tested for normality using Shapiro-Wilks tests in the stats package (R Core Team, 2020). Student's T-test (ST, test statistic = t) was used on normal data to test for differences between treatments, while Kruskal-

Wallis Rank Sum (KW, test statistic = χ^2) was used for non-normal data using the stats package (R Core Team, 2020).

Mortality was analyzed using Kaplan-Meier survival curves and Log-Rank tests for difference (Rich et al., 2014). Kaplan-Meier (KM) survival curves were first constructed for plant groups (overall and by initial size class) using survfit and then tested for differences using the Log-Rank test (LR, test statistic = χ^2) function, survdiff, both in the survival package (Therneau et al., 2021). Curves were visualized and presented with ggsurvplot in the survminer package (Kassambara *et al.* 2020).

Comparisons of extents (large plants only), leaf heights, and percent sporulation between treatments for size classes or pooled sizes were made with Kruskal-Wallis Rank Sum tests, followed by Dunn's test for multiple comparisons (Dunn, test statistic = *z*, *p.adjusted* given) from the stats package (R Core Team, 2020). Tests of these variables on a given group before and after (paired) an event or time period were similarly tested with Kruskal-Wallis and Dunn's tests. Changes in these variables following the fire or between timesteps were similarly tested for significance.

For comparisons of levels of mite damage across groups, the midpoint value of each class was used in analyses, such that level 1 galling = 5%, level 2 galling = 18%, level 3 galling = 38%, etc. Comparisons of proportions of plants with respect to these variables were made with the Test for Equal Proportions (EP, test statistic = χ^2) in the stats package (R Core Team, 2020).

Other R packages used include xlsx (Dragulescu and Arendt, 2020) and plyr (Wickham et al., 2011) for data importation and manipulation. Data formatting, manipulation, visualization and presentation were done using ggplot2 (Wickham et al.,

2020), reshape2 (Wickham, 2020), and lattice (Sarkar et al., 2020) packages. Summary statistics were obtained using describeBy from the psych package (Revelle, 2020).

Terminology

"Treatments" refers to the burned and reference areas together. "Experimental" or "burned" refers to one or more of the Cape Sable (CS) sites. The Hell's Bay (HB) sites are referred to as "reference" sites. "Survivors" are plants that survived to 27 mo. (or 21 mo. for CSA), having recovered from burning and/or Hurricane Irma, while "lost" plants were plants that died at any point before the final survey. Plants were lost as a result of burning or unknown causes, which included mortality after Hurricane Irma.

Results

Dry Season Burn Initial Conditions

Initial Site Conditions

Although the sites were sawgrass marsh with sawgrass, *Cladium jamaicense*, as the dominant macrophyte and primary *L. microphyllum* host, 7% of the 223 *Lygodium* plants followed were not hosted by sawgrass. In these cases, *Lygodium* was growing directly on an elevated soil mound, a stump, or a different plant; rarely was *Lygodium* growing completely by itself. More than 20 other plant species co-occurred with the surveyed *Lygodium* plants. The most common companion was *Blechnum serrulatum*, found at all sites and growing with 63% of sampled *Lygodium* plants followed. Larger *L. microphyllum* plants were found on shrubs and trees, usually *Myrica cerifera* (19%), but also *Conocarpus erectus* (5%), and even the exotic invasive *Schinus terebinthifolius* (2%). Additional notable companions were *Toxicodendron radicans* (14%, but only at

reference sites), *Mikania scandens* (4%), *Typha domingensis* (3%), *Ipomoea sagittata* (3%), and *Acrostichum danaeifolium* (3%); a complete list of associated species is provided in the Appendix. Sites CSB and CSC were dominated by sawgrass; however, neither of these approached the dense sawgrass cover present initially at the reference sites.

All the sites had peat soils with low pH and phosphorus (Table 2.5). Soils from the burned and reference areas differed in organic matter (OM) and pH, but not in total carbon (TC), total nitrogen (TN), or total phosphorus (TP) (Table 2.5). Across both treatment areas, soil TC ranged from 318.8 – 456.2 mg/g dry weight (dW), TN from 14.7 – 22.7 mg/g dW and TP from 0.35 – 0.55 mg/g dW. Soils at the experimental sites were higher in OM but lower in pH than reference sites (ST, $t_{OM} = 4.6$, $p < 0.001$; KW, $\chi^2_{pH} = 13.0$, $p < 0.001$) (Table 2.5, Figure 2.7). Nutrient ratios of N:P, C:N, and C:P did not differ between treatments (Table 2.5). The N:P ratio (wt./wt.) was well above 32 in both areas. Relative soil salinity was higher at the reference sites (KW, $\chi^2 = 5.1$, $p < 0.05$, Figure 2.7).

Initial Conditions of Plants

The experimental sites initially had 132 *Lygodium* plants that experienced the burn (74 S, 41 M, and 17 L plants), and reference sites had 91 plants (31 S, 29 M, and 31 L). Variables measured are pooled across sites within each treatment unless otherwise indicated (Table 2.3). The extents of large plants at experimental sites averaged 1.7 ± 1.4 m^2 (range 0.3 – 5 m^2), while those at reference sites were a little smaller, averaging 1.1 ±

0.9 m^2 (range 0.26 – 4 m^2); however, extents of experimental and reference large plants did not differ (KW, $\chi^2 = 2.7$, $p = 0.1$) (Table 2.6, Figure 2.8 A).

Leaf heights did not differ between treatments overall, with mean leaf heights of experimental plants 98 ± 49 cm (range 23 – 300 cm) and those of reference plants 99 ± 42 cm (range 35 – 250 cm) (Table 2.6, Figure 2.8 B). Plant leaf heights did not differ between treatments when subsetted by size class. (Dunn, $p > 0.3$ for all sizes).

At the experimental sites, 32% of plants were sexually reproductive, while at the reference sites, 53% were. The difference between treatments was significant (KW, $\chi^2 = 9.8$, $p < 0.01$) (Table 2.6). Whether a plant was reproductive was size-dependent, and the relative number of plants sporulating increased with size class. Few small plants were sporulating during the pre-treatment survey (7% at the experimental sites and 3% at the reference sites). More medium plants (54% in experimental, 69% in reference) and an even greater percent of large plants (88% in experimental, 87% in reference) were sporulating. However, percent sporulation on reproductive plants did not differ between treatments (EP, $p > 0.2$ for all size classes).

For sporulating plants (pooled sizes), mean percent sporulation averaged 8 ± 13% (experimental) and 6 ± 8% (reference) and did not differ between treatments overall (KW, $\chi^2 = 0.01$, $p = 0.9$) (Table 2.6, Figure 2.8 C) or between treatments for any size class. Percent sporulation tended to be highest on large plants, reaching as high as 50% on experimental large plants; however, mean percent sporulation remained low because many plants only had a few fertile pinnae. Only one small reference plant was sexually reproductive, and it had 40% sporulation.

When initial percent sporulation on survivors was investigated, treatments (pooled sizes) did not differ. By size class, the one small reference plant with 40% sporulation was the only small survivor that was sporulating initially. Medium and large plants were sporulating, with as much as 50% sporulation on a large experimental plant, and percent sporulation did not differ between treatments for either size class. For comparison, lost plants were initially sporulating less (max 15%) than survivor plants, although the majority were not sporulating at all. Excluding plants with 0% sporulation from analysis, the lost plants' initial percent sporulation was significantly lower than that of survivors, but only for experimental plants (KW, $Z_{exp(lost:survivor)} = 10.5$, $p < 0.01$), not reference plants (KW, $Z_{ref(lost:survivor)} = 0.7$, $p = 0.4$), but this lower sporulation may be a factor of their smaller size.

Initial Presence of Biological Controls

Only one biocontrol agent, *Floracarus perrepae*, the *Lygodium* leaf-galling mite, was found during the initial survey. This mite was found only in the experimental area, where over 80% of plants had mites present; no plants at any of the reference sites were observed to have mites (Table 2.6). Within just the DSB experimental sites (CSB, CSC), mites were found on 90% of the plants (S, 92%; M, 85%; L, 94%) (Figure 2.8 D). During this initial survey, only biocontrol presence/absence was documented.

Dry Season Burn Post-burn Conditions

Post-burn Site Conditions

Water depths, measured adjacent to, but off the hummocks hosting *Lygodium* plants, were significantly shallower at the experimental sites during the 3 month post-

burn survey, ranging from 0 to 4 cm, while at the reference sites, water depth reached 18 cm (KW, $\chi^2 = 78$, $p < 0.001$) (Figure 2.9). During the 9-month post-burn survey, depths again differed between treatment areas, but experimental sites had greater water depths (KW, $\chi^2 = 21.7$, $p < 0.001$) than the reference sites. Depths between treatment areas did not differ for the next two surveys (May 2018, and November 2018), but differed again at the final survey at 27 months, with experimental sites again having shallower water than reference sites (KW, $\chi^2 = 6.7$, $p < 0.01$).

Salinity of surface water was measured when possible, beginning with the 3-month post-burn survey (Figure 2.10). Salinity varied by site and over the surveys, with experimental sites (mean 2, range 0-5 ppt, one outlier of 10 ppt) generally having lower salinities than the reference sites (mean 5, range 2-14 ppt) (over whole study, KW, $\chi^2 = 46.4$, $p < 0.001$). Comparisons revealed statistical differences between treatments in salinity during surveys, at 9, 15, 21, and 27 months post-burn (Figure 2.10). At the 3-month post-burn survey (May 2017) only one sample (10 ppt) was obtained from the experimental area. During later surveys, significantly higher salinities were documented in the reference area, compared to the experimental sites (KW, $\chi^2 = (2.3 – 18.6)$, $p < 0.01$ for all surveys). During an immediate post-Irma (September 2017) visit, we were able to land only at the experimental sites, where all water samples were 0-1 ppt.

During the November 2017 survey at 9 months, which was the first complete survey of all sites after Hurricane Irma, measurements of surface water salinity revealed differences between burned and reference sites of up to 11 ppt. The CS sites ranged from 2-4 ppt, while HB sites ranged from 8-13 ppt in the months following hurricane Irma (Figure 2.10). In later surveys, surface salinities in both areas decreased to low single

digits, with experimental sites between 0-2 ppt, and reference sites around 3-6 ppt until the final survey, where salinity increased only in reference sites to as much as 14 ppt (Figure 2.10).

Post-burn Mortality

By May 2017, three months post-fire, 64% of burned plants and 4% of reference plants had died (LR, $\chi^2 = 80.5$, $p < 0.001$) (Table 2.7a, Figure 2.11a). Of the burned plants, the small plants were disproportionately affected: 86% of these died, while 49% of medium, and 6% of large plants died (Figure 2.11b, (Table 2.7a)). At the reference sites, 13% of small plants died (4% of all reference plants), but none of the medium or large plants did (Figure 2.11b). Mortality differed significantly between treatments for small and medium plants (LR, $\chi^2_{Small} = 51.3$, $\chi^2_{Med} = 19.5$; both $p < 0.001$), but not for large plants (LR, $\chi^2_{Lrg} = 1.8$, $p = 0.2$), during this first timestep (3 mo. post-burn). Within a treatment, mortality differed among size classes for burned plants (EP: $\chi^2_{SvM} = 15.6$, $p < 0.001$; χ^2_{MvL} 7.8, $p < 0.01$; $\chi^2_{SvL} = 37.9$, $p < 0.001$), but not for reference plants (EP, $\chi^2 = 2.2$, $p > 0.1$, all comparisons).

On September 10, 2017, hurricane Irma swept through the sites, introducing an additional disturbance. At the 9-month post-burn survey after Irma (November 2017), additional mortality was recorded at both burned (19%) and reference (31%) sites but did not differ between treatments overall (KM, $\chi^2 = 2.2$, $p = 0.1$) during this time period (Table 2.7a, Figure 2.12a). Within a class size, mortality at 9 months did not differ between treatments for small (LR, $\chi^2_{Small} = 1$, $p = 0.3$) or medium (LR, $\chi^2_{Med} = 0.6$, $p = 0.4$) plants, but differed for large plants (LR, $\chi^2_{Lrg} = 4.9$, $p < 0.05$), with 26% of large

reference plants dead, but no large burned plants dead (Figure 2.12b). Within a treatment, class sizes differed in mortality at 9 months: small (70%) burned plants had greater mortality than medium (10%) and large (0%) burned plants (EP, $\chi^2_{SvM} = 10.6$, $p < 0.01$, and $\chi^2_{SvL} = 13.2$, $p < 0.001$, respectively); reference plant mortality differed only between small and medium plants (EP, $\chi^2_{SvM} = 6$, $p < 0.05$), with 52% of small reference plants and 17% of medium reference plants dead (Figure 2.12b).

During the second year after burning, mortality slowed for both groups and did not differ between treatments overall (LR, $\chi^2 = 0.4$, $p = 0.5$), nor within size classes for the remainder of the surveys (15, 21, 27 months). Within a treatment, mortality over year 2 differed only between medium and large reference plants (EP, $\chi^2_{MvL} = 4.7$, $p < 0.05$). Over the entire 27 months, experimental plants suffered 74% mortality, differing from reference plants, which suffered 44% mortality, (LR, $\chi^2 = 34.7$, $p < 0.001$), though mortality for both treatments was size-dependent (Figure 2.13a, b).

Initial leaf heights of the subset of plants that died (lost) during the study were used to examine possible size-related factors. Overall, plants that died either after fire or after the hurricane were smaller than survivors and this was true for both treatments (KW, $\chi^2_{Exp} = 40.1$, $p < 0.001$; KW, $\chi^2_{Ref} = 7.0$, $p < 0.001$) (Figure 2.14). Initial extents of large reference plants that were lost following Irma were initially smaller than survivor large reference plants, too (Dunn, $Z_{ref,deadNov} = 2.65$, $p < 0.01$).

Post-burn Plant Regrowth

Size Classes and Large Plant Extent

Survival was related to initial size—even within the large size class—with larger plants having better survival than smaller plants (Table 2.7b). When we considered survivor large plants only, average initial extent of burned plants was 1.8 ± 1.4 m^2 (range 0.26 – 5 m^2), while initial extent of reference plants was 1.3 ± 1.0 m^2 (range = 0.26 – 4 m^2). These extents did not differ between treatments (KW, $\chi^2 = 1.5$, $p = 0.2$).

Following the burn, all experimental plants were reduced to their belowground roots and rhizomes. By 3 months post-burn, a few survivor plants in all size classes had recovered to their pre-burn size class, including all 3 small survivor plants, but the majority had not (Table 2.7b). By 9 months, survivor burned plants had increased in size, and one of three (33%) initially small plants reached medium class size. Reference survivor plants were mostly the same size class at 3 months, but by 9 months, which was after hurricane Irma, nearly half of medium and large plants were reduced by 1 – 2 size classes. Experimental plants varied less in the direction of size change, with all but one survivor plant consistently increasing in size over the surveys.

By the end of the study at 27 months, 100% of small, 88% of medium, and 100% of large survivor burned plants recovered to or surpassed their initial size class, although 13% of initially medium plants remained small plants (Table 2.7b). Although most of the survivor reference plants also increased in size, they varied more than burned plants, with several medium and large plants regressing 1 – 2 size classes over each timestep (Table 2.7b). By the last survey at 27 months, small survivor reference plants (100%) recovered

or surpassed the small class; but, while 24% of medium plants expanded to large plants, 53% of medium plants regressed to small plants (Table 2.7b). Seventy-one percent of reference plants regrew to at least as large as they were initially, but 29% had decreased by at least one size class. Mean initial extent of survivor large experimental plants, 1.8 ± 1.4 m^2, increased by 27 months post-burn to 3.3 ± 3.7 m^2, as did, to a lesser extent, that of survivor large reference plants (from 1.3 ± 1.0 m^2 to 1.7 ± 2.9 m^2) at 27 mo., but neither increase in average extent was significant (Dunn, $Z_{exp} = 0.05$, $p = 1$; $Z_{ref} = -1.82$, $p = 0.7$).

When initially large survivor plant extents were compared at 3 months (May 2017), these differed between treatments (KW, $\chi^2 = 23.4$, $p < 0.001$), because experimental plants were greatly reduced in extent by the fire (Figure 2.15). By 9 months post-burn, some burned plants that were initially large had regrown to the large class, while some large reference plant extents decreased, resulting in no difference between treatments for large plants (KW, $\chi^2 = 0.3$, $p = 0.6$) at 9 months. No difference in extents had developed between treatments by 15 or 21 months, but by 27 months, large burned plants exceeded reference plants in extents (KW, $\chi^2 = 4.2$, $p < 0.05$).

Recovery, defined as no difference between initial extents and extents at survey 'x,' occurred for experimental plants by 15 months, when differences in extent resulting from burning disappeared. Although some large burned plants further increased in extent by 27 months, these still did not differ significantly from initial extents. Furthermore, although a few reference plants expanded a lot in extent over the study, average extents did not differ from initial extents except during the November 2017 survey, when they were smaller than initial extents (Dunn, $z = -3.66$, $p < 0.01$).

Leaf Height

Overall initial mean leaf heights of survivors were 146 ± 63 cm for experimental plants and 114 ± 46 cm for reference plants, and leaf heights differed between treatments (KW, $\chi^2 = 5.7$, $p < 0.05$) (Figure 2.16). Despite the overall difference, experimental leaf heights did not differ from reference leaf heights for any size class (Dunn, $z_S = -1.25$, $p = 0.9$; $z_M = -1.31$, $p = 0.9$; $z_L = -1.23$, $p = 0.9$).

Burning reduced survivor experimental leaf heights for 15 months post-burn (Dunn, $z_{3mo,9mo,15mo} < -2.6$, $p < 0.05$ for all three surveys compared to initial heights). Reference survivor plant leaf heights were stable except for following hurricane Irma, when they decreased substantially and remained reduced compared to initial heights through 15 months (Dunn, $z_{9mo} = 4.81$, $p < 0.001$; $z_{15mo} = 4.41$, $p < 0.001$). From 15 months post-burn onward, burned survivor plants were taller than reference survivor plants (KW, $\chi^2_{15mo} = 5.1$, $p < 0.05$; $\chi^2_{21mo} = 7.2$, $p = 0.01$; $\chi^2_{27mo} = 13.4$, $p = 0.001$).

Growth Rates

Mean DSB experimental leaf growth rates were highest immediately following the burn (T½), 2.2 ± 1.3 cm/wk (max 6 cm/wk) and were higher than reference leaf growth rates during T½ through T2 (Figure 2.17, Table 2.8). By T3, reference rates peaked (1.2 ± 0.8 cm/wk), converging with decreasing experimental growth rates, and differences disappeared. During T4, all mean leaf growth rates were slightly negative, and did not differ between treatments. Mean experimental growth rates were lowest (-0.3 ± 1.5 cm/wk) during T4, while those for references were lowest (-1.0 ± 1.3 cm/wk) during T1, coincident to the occurrence of hurricane Irma.

Sporulation

Most survivor plants were initially sexually reproductive, with 71% of experimental and 63% of reference plants sporulating. Mean percent sporulation on these plants was less than 15% and did not differ between treatments (KW, $\chi^2 = 2.0$, $p = 0.2$).

At 3 months post-burn, no burned plants and only a few reference plants (22%, were sporulating, but none of these plants had more than 2% sporulation (Figure 2.18). By 9 months post-burn, only 29% of the burned plants were sexually reproductive, but percent sporulation was like initial percent sporulation (16 ± 19% sporulation (Dunn, $z_{Init:9mo} = 0.5$, $p = 1$)). Similarly, 27% of reference plants were sexually reproductive, but their percent sporulation was much lower (2 ± 3%) compared to burned plants at this 9 months post-burn survey (KW, $\chi^2 = 10.7$, $p < 0.01$).

By 15 months post burn, 56% of burned plants and 35% of reference plants were sexually reproductive, but no plant had higher than 15% sporulation and treatments did not differ in percent sporulation (Figure 2.18). Percent sporulation at 15 months did not differ from initial levels for either treatment (KW, $\chi^2_{Exp} = -1.9$, $p = 0.3$; $\chi^2_{Ref} = -1.1$, $p = 1$).

More survivor plants became sexually reproductive by the following survey (21 months) with 68% of burned and 45% of reference plants (twenty-three plants in each treatment) sexually reproductive. However, by this 21 month survey the percent sporulation on some burned plants increased substantially, with one medium burned plant reaching 60% sporulation (Figure 2.18). Burned plant percent sporulation at 21 months (17 ± 20% sporulation) was greater than percent sporulation of reference plants (4 ± 10%;

KW, $\chi^2 = 5.9$, $p < 0.05$). Percent sporulation at 21 months did not differ from initial levels for either treatment (Dunn: $z_{exp} = 0.1$, $p = 1$; $z_{ref} = -1.6$, $p = 1$).

By the final survey at 27 months post-burn, only 29% of burned and 16% of reference plants were sporulating—the lowest amount since immediately following the fire in May 2017. This survey also had the lowest % sporulation, with both experimental and reference plants showing no more than 1% sporulation, and no difference between treatments (KW, $\chi^2 = 0.4$, $p = 0.5$) (Figure 2.18).

Biological Controls

At the pre-burn survey, 90% of DSB plants and no reference plants had mites present (Figure 2.19), but when considering survivors only, 82% of the experimental plants and no reference plants were galled by mites. A single observation of low-level galling occurred on one large reference plant during the 3-month post-burn survey (May 2017), but this plant did not have galling in later surveys and was omitted from analyses.

Mites recovered at burned sites over the two years post-burn ((Figure 2.19). At 3 months, no mites were present on regrowing burned plants, but by 9 months post-burn, mites were found again on 8% of the plants. These were initially medium and large plants with galling damage of level 1 (0.01 – 10% galled). By the end of the dry season at 15 months, galling was seen only on two large burned plants, and damage was very low on both. Over the next six months, more than half (53%) of the survivor burned plants became galled, with damage on these as high as level 2 (11-25% galled), although most galled plants had level 1 damage (up to 10% galled) and were of all class sizes. Mean estimated damage during this survey (21 mo) was 5 ± 5% for all galled plants and

was the same for both medium and large plants. By 27 months, 85% of the surviving burned plants became galled, increasing in all size classes, and 100% of large plants were galled. Galling damage on one large plant attained as much as level 3 (26-50% galled), but most galled plants had only level 1 damage. During this final survey, small plants had only level 1 galling damage (median 5%), while mean estimated damage on medium plants was 7 ± 5%, and on large plants was 12 ± 7% gall damage.

Wet Season Burn Initial Conditions

Initial Site Conditions

Because the February 2017 burn failed at site CSA, some initial conditions data (soil nutrients, relative soil salinity) are from January 2017 (Table 2.5, WSB), while the number of plants sampled, water depth, and salinity are from May 2017.

At CSA, the predominant vegetation was similar to the other sites; however, more hydrophytes were also present, reflecting wetter conditions at the site (Appendix). At CSA, 90% of *L. microphyllum* plants sampled were growing on sawgrass, *C. jamaicense*. The most common associates to CSA *L. microphyllum* plants were *Blechnum serrulatum* and *Myrica cerifera*. Soils at CSA were higher in total carbon, nitrogen and organic matter (ST: $t_{TC} = 3.4$, $p < 0.01$; $t_{TN} = 4.1$, $p < 01$; $t_{OM} = 6.2$, $p < 0.001$) but lower in pH (ST, $t_{pH} = -3.1$, $p < 0.05$) than reference soils (Figure 2.20, Table 2.5, WSB). Phosphorus levels did not differ between treatment areas, but N:P ratio was greater at CSA (KW, $\chi^2 = 7.5$, $p < 0.01$) than at the other sites. Vegetation at CSA was noticeably patchier and had more standing water than the other five sites. Depths in May 2017 at CSA were greater than at reference sites (KW $\chi^2 = 88$, $p < 0.001$). Relative soil salinity (January 2017) for

CSA ranged from 5 – 15 ppt and did not differ from reference sites (ST, t_{RSal} = -1.9, p = 0.07), Figure 2.20, Table 2.5). Sampled surface water salinity in May 2017, however, was lower at CSA (2 – 3 ppt) than at the reference sites (6 – 7 ppt).

Initial Plant Conditions

Data from May 2017 were used as initial (or pre-August fire) data. At the WSB initial survey, 52 plants were followed (18 removed from analyses because of incomplete burning), with 22 S, 20 M, and 10 L. Reference plants changed some since January, and so were resurveyed, yielding 87 plants (35 S, 21 M, and 31 L) (Table 2.7a). Large plants at CSA averaged extents of 2.6 ± 2.8 m^2 (range 0.3 – 9.0 m^2) but did not differ significantly from reference large plants (1.5 ± 2.0 m^2, range 0.3 – 8.0 m^2) (Figure 2.21, Table 2.9). Leaf heights at CSA averaged 78 ± 64 cm (range 8 – 400 cm), and differed significantly from reference leaf heights (86 ± 37 cm, range 22 – 230 cm) (KW, χ^2 = 6.9, $p < 0.01$). Only 2% of CSA plants—and 17% of reference plants—were sexually reproductive in May 2017, 3 months before the August 2017 burn. Only one plant was sporulating at CSA, and those at the reference sites were sporulating at very low levels, but were higher than CSA (KW, χ^2 = 5.0, $p < 0.05$). Mites were present on 52% of experimental CSA plants in May 2017, and average galling damage on these plants was 9 ± 6% (Figure 2.21, Table 2.9).

Wet Season Burn Post-burn Conditions

Post-burn Site Conditions

Water depths were significantly greater at CSA than at references during every survey for which depths were measured in both areas, with KW $\chi^2 > 34$, $p < 0.001$ for all surveys (Figure 2.22). Salinity, meanwhile, was significantly lower at CSA than at references over all surveys (KW, $\chi^2 > 5$, $p < 0.05$ for all surveys) (Figure 2.23). The highest salinity at CSA was recorded during the March 2018 (6-month post-burn) survey, but was still no more than 5 ppt.

Post-burn Mortality

Post-fire mortality at CSA was size-dependent, with more small plants affected than other sizes (Table 2.7a). Because the hurricane occurred after the WSB but before we could survey, mortality observed could have resulted from either fire or hurricane effects or some combination of these disturbances. Overall initial mortality at CSA was 23% and did not differ from reference mortality of 31% (Figure 2.24a). Burning disproportionately affected small plants, killing 41% of these but only 15% of medium, and no large plants (Figure 2.24b, Table 2.7a). During this same period after the hurricane, the reference mortality also more severely affected small plants (49%) than medium and large plants (both 19%) (Figure 2.24b, Table 2.7a). Mortality did not differ between treatments for any size class in the early post-burn and Irma sample (T½).

Additional mortality occurred over the next 3 to 9 months in CSA, affecting only small and medium plants (Figure 2.25a, b). Reference sites were not visited at 6 months post-August burn, but similar additional mortality affected small and medium plants by 9

months. By the end of the 21 months, small burned plants had suffered 77% mortality, medium 25%, and large 0%. Over the same period, references suffered similar, size-dependent rates of mortality: small 60%, medium 38%, and large 23%. Over the full 21 months, overall mortality was near 42% for both CSA and reference sites and did not differ between treatments (LR, $\chi^2 = 0$, $p = 0.9$) (Figure 2.25b).

As for the Dry Season Burn plants, pre-fire leaf heights of CSA survivor plants were compared to pre-fire leaf heights of plants lost just after the fire and hurricane (Figure 2.26). Plants lost immediately following the disturbances were initially shorter than survivor plants for both experimental and reference groups (KW, $\chi^2_{Exp} = 12.9$, $p < 0.001$; $\chi^2_{Ref} = 11.5$, $p < 0.001$). Overall leaf heights of burned lost plants were shorter than lost reference plant leaf heights, (KW, $\chi^2_{lost(exp:ref)} = 7.8$, $p < 0.01$), but leaf heights of survivors did not differ between treatments (KW, $\chi^2_{survivor(exp:ref)} = 1.7$, $p = 0.2$). No large burned plants were lost, but initial extents of lost reference plants were smaller than those of survivor reference large plants (KW, $\chi^2_{Ref} = 8.2$, $p < 0.01$).

Post-burn Plant Regrowth

Size Classes and Large Plant Extent

All 10 large burned plants survived, and their average extent, initially 2.6 ± 2.8 m^2 in May 2017, shrank by nearly 40% by the final survey at 21 months post-burn to 1.4 ± 1.7 m^2, but this change in extent was not statistically significant (Figure 2.27). Over the 21 months following the burn, plants shifted in all sizes classes, but the direction was not consistent across sizes or seasons (Table 2.7b). Both experimental and reference extents were reduced for 3 months following the fire/hurricane (Dunn: $z_{Exp} = -4.17$, $p < 0.001$;

z_{Ref} = -3.27, $p < 0.05$) but recovered in mean extents by the following survey (experimental, 6mo.; reference, 9 mo.). Experimental extents were smaller than references only during the 3 mo. post survey (November 2017) (KW, $\chi^2_{Nov2017} = 6.9$, $p < 0.01$). By 21 months, mean extents of survivor reference large plants, initially 1.8 ± 2.1 m^2, did not surpass initial mean extents, returning to 1.6 ± 2.9 m^2.

Leaf Heights

Average initial leaf heights of CSA survivor plants were 98 ± 72 cm and did not differ from reference survivor plants, 98 ± 40 cm (KW, $\chi^2 = 1.7$, $p = 0.2$). Following fire and hurricane, plants in both treatments decreased substaintially in leaf height (Dunn, z_{Exp} = -8.0, $p < 0.001$; z_{Ref} = -3.2, $p < 0.05$) but with experimental leaf heights much shorter than reference (KW, $\chi^2_{3mo} = 39.8$, $p < 0.001$) (Figure 2.28). Burned plants remained reduced in leaf height for at least 9 months—but not 15 mo.—(Dunn, $z_{Init:9mo}$ = -3.0, $p < 0.05$), but reference plants recovered in leaf height by 9 months post-disturbance (Dunn, $z_{Init:9mo}$ = 0.8, $p = 1$).

Growth Rates

Leaf growth rates of WSB survivors varied over the timesteps, but mean burned plant growth rates were greater than those of reference plants for Pre-Rx, T½, and T1 (Tables 2.4, 2.10, Figure 2.29). Burned leaf growth rates peaked between November 2017 and May 2018 (T1), with mean rates of 1.5 ± 1.0 cm/wk, while reference growth rates peaked the following timestep (T2, 1.2 ± 0.8 cm/wk). In the final timestep (T3), leaf growth rates decreased to negative for plants in both treatments, and no longer differed.

Sporulation

At 3 months post-burn, 3% of survivor CSA plants and 22% of survivor reference plants were sexually reproductive, but none had higher than 10% sporulation (Figure 2.30). Fewer plants at CSA were reproductive than at references but this difference was not significant, and treatments did not differ in % sporulation. Through 9 months post-burn (May 2018), percent sporulation was similar between treatments, except for one burned plant with 70% sporulation; otherwise, maximum sporulation was 15% for both treatments. By 15 months (November 2018), 67% of burned plants and 45% of reference plants were sporulating, with percent sporulation greater at CSA (14 ± 21%) than at reference sites (4 ± 10%) (KW, $\chi^2 = 5.2$, $p < 0.05$). Both number of sexually reproductive plants and percent sporulation decreased substantially by the final survey in May 2019: 17% at CSA, 16% at references, and no plant in either treatment sporulating above 1%.

Biological Controls

No mites were present on any plants at 3 months post-burn (Figure 2.31, November 2017), but by 6 months post-burn, mites were beginning to return. By the survey at 9 months, mites had returned to 70% of survivor plants in CSA and mean damage estimated was 8 ± 6%, across sizes. By 15 months post-burn, 97% of plants were galled, but none above level 1 damage (median = 5% galled). These plants remained galled through the final survey at 21 months, during which 93% of medium and 100% of small and large survivor plants were galled. Level of galling damage during the last survey was the highest of any survey with mean 16 ± 14% galled. Galling damage estimates were

made for the pre-burn survey, and fewer plants were galled in May 2017 than in January 2017, suggesting a natural drop in populations during that time (data not shown).

Dry Season Burn vs Wet Season Burn Comparisons

Soil nutrients and water depth

A few soil nutrients differed between DSB (sites CSB, CSC) and WSB (site CSA) experimenal sites: TC and TN were higher, but TP was lower at CSA. Additionally, CSA had higher ratios of N:P and C:P than DSB (Table 2.5, Experimental).

Water depths were greater at CSA than at CSB and CSC during all surveys (Dunn, $z < 5$, $p < 0.001$). Surface water salinity did not differ between experimental sites of DSB and WSB in part because of the small number of samples each survey. With the exception of the single high ppt sample at DSB in the May 2017 survey, all experimental site water samples ranged between 0 and 5 ppt.

Mortality

Overall, when survival curves were compared by months-since-burn, mortality was greater for dry season burned plants than for wet season burned plants (LR, $\chi^2 = 14.8$, $p < 0.001$) (Figure 2.32a). When analyzed by size class, small and medium plants differed in mortality between burn seasons (LR: $\chi^2_S = 8$, $p < 0.01$, and $\chi^2_M = 5.7$, $p < 0.05$), but not large plants (LR, $\chi^2_L = 0.6$, $p = 0.4$) (Figure 2.32b). Mortality was size-dependent, with small plants in both dry and wet season burns more affected than medium and large plants, as were small reference plants following hurricane disturbance (Figure 2.32b).

Plant Regrowth

Leaf Height

Plants burned in either the dry or the wet season recovered in leaf heights by November 2018, and remained similar to their pre-fire heights through the final survey in May 2019 (Figures 2.16, 2.28). Reference plants, whether compared to January 2017 or May 2017 heights, similarly recovered from post-Irma decreases by November 2018 and remained similar to pre-fire survey heights through the final survey.

Growth Rates

When burned DSB and WSB leaf growth rates were compared, plants differed in every timestep except T1 (3 mo. – 9 mo. post-burn) (Figure 2.33a). DSB growth rates peaked soon after the dry season burn, in T½, but WSB growth rates did not peak until the following timestep, T1. Over the timesteps, growth rates did not change in the same directions for DSB and WSB burns. Since the burns were staggered, growth rates were also compared over true calendar months (Figure 2.33b, Table 2.11, Experimental). Mean growth rates compared by calendar month differed between dry and wet burns only twice during the entire study. First, DSB growth rates were higher than both WSB and reference growth rates immediately following the dry burn (Dunn, $z_{Dry:Wet} = -4.7$, $p < 0.001$; $z_{Dry:Ref} = -7.3$, $p < 0.001$). Second, during the period between May 2018 and November 2018 surveys, dry burned plants increased in growth rates but wet burned plants decreased in growth rate, resulting in differing rates between these groups (Dunn, $z_{Dry:Wet} = -3.2$, $p < 0.01$). By the final survey in May 2019, mean growth rates decreased

to negative for all groups, and no longer differed beween any groups (Figure 2.33b, Table 2.9).

Biological Controls

Mite galling returned sooner (between 6 and 9 mo.) following the wet season fire than following the dry season fire (over 15 mo.) (Figures 2.19, 2.31). However, both DSB and WSB survivor plants became heavily galled by the final survey, with over 80% of DSB survivors and 91% WSB survivors galled.

Discussion

L. microphyllum mortality post-disturbance

In this study, two major mortality events occurred: one following burning, and one following the hurricane. Overall mortality for burned plants following the dry season fire was much higher than mortality of either burned plants following the wet season fire or reference plants following the hurricane. Small plants were consistently disproportionately affected following both fire and hurricane disturbances, as well as in previous greenhouse experiments immediately following burning and throughout the subsequent 18 months (Richards et al., 2020). Generally, field mortality was higher for medium plants than for large plants, except following the hurricane, during which reference plant mortality did not differ between medium and large plants. Post-hurricane mortality in the reference plants coincided with a period of increased surface water salinity, and greenhouse experiments inspired by this observation showed that with exposure to salinity of 10 ppt for 5 weeks, *Lygodium* plants begin showing signs of stress (Chapter 3). Lower post-hurricane mortality observed in the experimental sites may

indicate a smaller increase in salinity at these sites than was experienced at the reference sites, although previous burning may have also already removed the plants most vulnerable to hurricane disturbance. Surface water salinity sampled 3 weeks post-Irma was < 2 ppt at the experimental sites. Though elevated two months later in November 2017, the salinity of water sampled at the experimental sites did not exceed 4 ppt, but water samples from the reference sites ranged from 8 to 13 ppt.

Overall, experimental sites were lower in both pH and salinity than were reference sites. Water depths were greater at experimental sites than at reference sites during most surveys than at reference sites. The two conditions for which data were collected over the whole study—water depth and salinity—differed substantially between treatment areas; however, the change that coincided with the mortality event post hurricane Irma was the increase in salinity at reference sites. As with post-fire mortality, small plants were most affected, followed by medium and large plants. However, after the hurricane, 19% of the large reference plants died, while after either fire, no more than 6% of large burned plants died (and no large burned plants at DSB died shortly post-hurricane). While larger plants may be more fire-resistant, salinity may affect them differently, resulting in similar (or elevated) vulnerability compared to smaller plants. Because transpiration increases with plant size (Le Maitre, 2004), larger plants may experience increased salt uptake. In the salinity experiment (Chapter 3), salt deposits were observed on all *L. microphyllum* leaf surfaces, suggesting that any salt exclusion by the roots is incomplete.

When experimental plant mortality was compared between DSB and WSB by months-since-burn (timesteps, T), the majority of mortality occurred within the first 9

months following the fire (Figure 2.32a), regardless of whether the hurricane occurred 1 or 7 months after fire. The DSB resulted in more than double WSB mortality, unless the time period including the hurricane was included, in which case DSB mortality increased to triple that of WSB. With or without the hurricane disturbance, the DSB resulted in higher mortality than the WSB. Reference plant mortality observed after the hurricane disturbance similarly tapered within one year.

An important factor in prescribed burning is fuel. Both the kind of plants and their density can greatly affect the type of fire produced, which in turn will affect chances of survival (Bond and van Wilgen, 1996). The main fire host (and fuel source) at our sites was sawgrass, which produces long, well-aerated dry fuels, in part because of persistent dead leaves. This type of fuel, especially when continuous, tends to produce a hotter, faster-moving fire than the more patchy but dense hardwood fuels (Bond and van Wilgen, 1996; Robert J. Whelan, 1995). Burning of *L. microphyllum* was most successful in the drier, predominantly sawgrass areas. And despite observations of germinated gametophytes on some hummocks post-burn, very few sporelings were documented at any of the sites, suggesting that post-fire recruitment by spores is not a major concern. Especially with a dry season burn, most spores and gametophytes on the soil surface will be destroyed by burning (Vermeire and Rinella, 2009), as *L. microphyllum* spores do not survive even short exposures to temperatures of 300°C (Sebesta et al., 2016).

L. microphyllum regrowth post-disturbance

Although a few plants were already regrowing by 3 weeks after burning, most were not. Under greenhouse conditions following a controlled burn of *L. microphyllum*

plants, resprouting was observed within as little as two weeks after burning, and all plants that survived resprouted within 3 weeks (Richards et al., 2020). How quickly plants resprout relates in part to the severity of the fire, but in the field study, resprouting of burned plants took longer than 3 weeks for most plants. This delay may relate to dry conditions persisting in the field after the dry season burn, whereas greenhouse plants were watered regularly after burning.

An unexpected observation post-fire was the resprouting of *Lygodium* from rhizomes located lower (previously underwater) and between sawgrass culms, indicating more patch connectivity than originally anticipated. This observation presents a question about actual plant size: if some of the plants we observed were connected to other clumps but were perceived as separate entities, then these plants were bigger than estimated, and perhaps better established. Variation in microhabitats could account for the survival of several initially small burned plants (3 at DSB, 5 at WSB) through the entire study; but it could also be that these were connected to larger patches and therefore better able to recover post-fire. To know for certain, each patch would need to be excavated to gauge the actual rhizome extent, or, alternatively, tested using molecular techniques to determine relatedness. Greater underground connectivity among plants in the field may increase their resistance to disturbance or management efforts. In contrast, plants in the greenhouse experiment were isolated from each other, and may have responded to burning differently had they been connected.

During the two years following the burn, leaf heights did not vary much after returning to between 100 and 200 cm, and this is likely related to growth form. Twining climbers such as *L. microphyllum* can reach extraordinary heights when provided with

adequate structural support. As such, their heights are limited by the hosts upon which they grow; sawgrass that reaches 100 – 200 cm will support *L. microphyllum* to that height. Because some *L. microphyllum* patches were initially growing on shrubs and trees, they were able to reach heights of 300-400 cm; however, when tree and shrub hosts burned, only stumps remained. *Lygodium microphyllum* regrowth in these patches then had only bare ground, small herbaceous hosts, or nearby regrowing sawgrass, and their subsequent heights—consistently below 200 cm—reflected this new limitation imposed by host type. In more sawgrass-dominated sites (or areas within sites), sawgrass flowering stalks emerge in May in southern Florida (Snyder and Richards, 2005), towering over the sawgrass leaves through the summer and often persisting into the following dry season. Increased sawgrass flowering was observed after fire in the burned sites, in addition to denser and taller sawgrass plant growth. While the increased relative density of sawgrass leaves seemed to challenge *L. microphyllum* survival (especially small plants), the numerous sawgrass flowering stalks offered ideal structural support beyond 200 cm, and *L. microphyllum* used these supports (Figure 2.34), leading eventually to increased leaf height after burning. Furthermore, increased leaf height could affect spore dispersal, and even spore production, if increased light levels encourage reproductive output (Sharpe and Shiels, 2014).

After the dry season burn (February – May 2017), experimental plants increased in leaf height at their fastest rate among all surveys, while during the period from Jan. – May 2017, reference plants had decreased leaf heights, reflecting slightly negative growth rates. The low growth rates of reference plants—as well as the unburned CSA plants—could be the result of dry season effects. As the growth rate of *Cladium*

jamaicense slows in the dry season, so too might that of *L. microphyllum* (Bellis and Gaither, 1985; Richards and Olivas, 2020). Limited host growth will further limit opportunities for *L. microphyllum* to gain height. In contrast, fire stimulated growth of the burned plants, even under slow-growth dry season conditions.

Growth rates varied somewhat over all intervals between surveys but peaked for reference plants during the May – November 2018 interval. During that summer, experimental plant growth rates were almost as high as they were immediately post-fire, and rates no longer differed between treatments. From this summer and through the next interval (November 2018 – May 2019), growth rates did not differ between treatments, but both decreased significantly compared to their previous rates. Burned plant growth rates during this final interval were the lowest during the study and were slightly negative. Differences in rates attributable to the burn disappeared between 15 and 21 months post-burn; seasonal variation in growth rates was more strongly suggested thereafter.

Stimulatory effects of burning have been well documented in other species like bracken (*Pteridium esculentum*) and cattail (*Typha domingensis*) (Miller et al., 1998; Tolhurst, 1990). Although increases in growth rates were observed for all burned plants, differences between groups disappeared over subsequent surveys, suggesting that after an initial stimulatory effect of burning on growth rates, seasonal influences become more important. Furthermore, differences in growth rates observed 3 to 9 months post-burning were on the order of a few cm/week, and although significantly statistically different, were probably not ecologically significant. Again, host height plays a sizeable role in *L. microphyllum* leaf heights and may also be seasonally influenced.

Growth rates did not account for extent, but this may be an important factor for more accurately estimating *L. microphyllum* growth and recovery post-burning. As the host changes physical position, or height, *L. microphyllum* may also change its growth direction. For example, when sawgrass grew very large (approaching 2 m) in some of the reference sites, the leaves often would fall over into a more horizontal position, taking any *L. microphyllum* fronds present with them. This reduced *L. microphyllum*'s leaf height but sometimes extended the area of cover horizontally. Following this, the *L. microphyllum* plants often shifted from apical growth to outgrowth of the pinna buds, or resprouted from the rhizome, further entangling the host leaves into a shorter mat of sawgrass leaves and *L. microphyllum* rachises. In the absence of a host, *L. microphyllum* will twine on its own climbing leaves, forming a sprawling, semi-horizontal mat, although many patches were a combination of these growth directions—making quantification challenging.

Spore production by *L. microphyllum* occurs throughout the year, but tends to peak during the late wet summer months (Philippi and Richards, 2007; Volin et al., 2004). Seasonality in sporulation was observed in this field study as well. Following the dry season burn, plants in both treatments were reduced in sporulation: the experimental plants were just beginning to resprout, but the reference plants had reduced sporulation as well, both in how many plants were sexually reproductive and in % sporulation. As burned plants regrew, reproduction resumed, with higher percent sporulation during the wet season surveys (November 2017, and November 2018). During the dry season surveys (May 2017, May 2018, and May 2019), percent sporulation dropped for both treatments to below 20%, and in the final survey (May 2019), neither treatment had any

plants with more than 2% sporulation. Both surveys with higher maximum percent sporulation occurred after peaks in water levels recorded by an Everglades Depth Estimation Network (EDEN) stage, NMP, just northeast of our reference sites (Figure 2.35). Drier season surveys found lower percent sporulation, and these similarly coincided with dry-down periods. The difference in water levels between treatments in November 2017 may also partially explain the difference observed in % sporulation, as both water depth and percent sporulation were greater at experimental sites during this survey (Figures 2.9, 2.18).

While fire is associated with stimulating some plants to set seed, it can also, with proper timing, be crucial in preventing seed set, e.g., by burning when fruits are immature and still on the plant, rather than after they have matured and are released into the potentially safer soil (Zouhar et al., 2008). Although *L. microphyllum* sporulates at low levels all year, burning before the late summer peak in sporulation could reduce both the number of spores being produced as well as the viability of spores in the spore bank.

Biological control responses to burning

Experimental sites had high presence of mite galling during the first survey in January 2017, but mites were absent three months after the fire. Mites began returning to a few dry season burned plants by 9 months post-burn but did not spread across the sites appreciably until nearly 2 years post-burn. This could be a result of the particular weather of 2017/2018, or part of the mites' seasonal vulnerability in Florida, as they have better survival in sites with slightly lower precipitation and temperatures compared to the introduced range in southern Florida (Ozman and Goolsby, 2005). Alternatively, their slow return could be the result of the particular severity/homogeneity of the dry season

burn, resulting in prolonged drier conditions that can be fatal to dispersing mites, as mites are vulnerable to desiccation during initiation of the protective leaf galls (Ozman and Goolsby, 2005). In contrast, mites at CSA had re-galled 50% of surviving plants between 6 and 9 months after the wet season burn. Recolonization, which is wind-dependent, may vary depending on the proximity and wind direction of source populations of mites, as well as ambient humidity levels (David et al., 2019; Lake et al., 2014). Regardless, once the mites re-established at the burned sites, the damage levels were higher than at any previous survey, with some plants suffering as much as 50% galling.

Mite galling can influence *L. microphyllum* growth rates under experimental shade house conditions (David & Lake 2020), but our data may be too coarse to determine whether this applied in our field sites. Although the reduction in growth rates by the final survey coincided with higher galling damage observed on the surviving plants, the growth rates on reference plants similarly decreased, despite having no mites, suggesting that mites may not be an important factor in the reduction observed in the field. Host height may also be a confounding factor. Sporulation may be affected by severe galling damage; however, at our experimental sites, mite galling and sporulation were found to co-occur on many plants in the November 2018 survey. There may be a minimum amount of galling damage needed before detriment to reproduction is observed, and perhaps this threshold is above that observed in our sites.

Seasonal effects of burning on *L. microphyllum* regrowth

The delayed reburn of CSA allowed a comparison of seasonal burning effects. To fit in with management goals, ENP often prescribes fires in the dry season. Such fires tend to burn an area more thoroughly because fuels are dry and water levels are lower.

Fires in the Everglades were historically lightning-ignited, and those occurring early in the rainy season (May-June), while it was still relatively dry, may have resulted in different outcomes compared to lighting-ignited fires later in the wet season (August-September) (Taylor, 1980). Wet season fires often result in patchier fires, which leave behind unburned patches that function as refugia for plants and animals. These patches serve as sources for propagules (vegetative or diaspore, or biocontrol agent) to recolonize the burned stretches of habitat. Wet season burning, during potentially faster growth, higher sporulation, and more water present, could have produced different outcomes than did dry season burning, and may have facilitated the observed faster return of mites to CSA.

Summary

What is the mortality from a burn and is it size-dependent?

Both dry season and wet season burning resulted in *L. microphyllum* mortality that was size-dependent, with smaller plants consistently experiencing the greatest mortality. Overall mortality was higher for plants burned in the dry season than for plants burned in the wet season (64% vs 23%), although a caveat was that WSB had fewer plants being followed, so the sample was smaller. Additionally, hurricane disturbance resulted in mortality—nearly 30% in reference plants—as well as some additional mortality for DSB plants. Finally, the co-occurrence of fire and hurricane to the WSB site may have confounded the mortality attributable to each disturbance type.

What is the rate of post-burn recovery?

Recovery to pre-burn extent of large plants occurred for DSB plants between 15 and 21 months after burning, while recovery of WSB large plant extent occurred between 3 and 6 months after burning. Following the hurricane disturbance, the large reference plants recovered to pre-disturbance extents in between 3 and 6 months. Leaf heights were linked to host type and growth, and these were subject to seasonal influences. Furthermore, if the host plant was a tall tree that burned, the new host would likely be an herbaceous plant, and this would at least reduce/delay *L. microphyllum*'s vertical growth opportunities. An important point, however, is that even though the surviving plants technically recover to mean pre-burn sizes in under two years, a large proportion of plants are killed by burning, resulting in a reduction of total *L. microphyllum* cover.

Does burning affect reproductive output?

Reproduction was reduced immediately by killing reproductive plants and by burning aboveground portions. Burned DSB plants were regrowing by 3 months but not sporulating until 9 months post-burn. Burned WSB plants were reduced in percent sporulation for at least 6 months. Reference plant percent sporulation was reduced, as well, following the hurricane, but this was true during all surveys except November 2018. Sporulation decreased for all treatments (dry season burn, wet season burn, reference) during the dry season surveys but more markedly during the final survey in May 2019. This decrease supports some seasonality to sexual reproduction in *L. microphyllum* and emphasizes the need for this to be considered when scheduling treatment.

Does burning affect biocontrol populations?

Mite populations were eliminated by burns of both seasons but began returning unaided within 9 months. However, at the sites burned in the dry season, the mites were only present in very low levels for 15 months, while at the sites burned in the wet season mite presence steadily increased from 6 months on and had produced galls on more than 50% of the survivor plants by 9 months post burn. The patchy nature of the wet season burn may have created refugia within the burned area from which mites could redisperse onto regrowing patches in the burned areas more quickly than from the edges of the more thoroughly burned DSB areas. Though the patchiness might have benefitted the mites, it may also benefit the fern, which, if spared, will continue sporulating. Research is underway by the USDA-ARS (Davie, FL) to examine combined effects of burning and biocontrol presence. Preliminary work showing reduced growth rates associated with high galling damage (David and Lake, 2020) is promising, and the preference of mites for new growth may be an opportunity for integrative management practices, as these have been successful elsewhere (Paynter and Flanagan, 2004). If mite biocontrols and prescribed fire are to be combined for the management of *L. microphyllum*, dispersal dynamics of the mite must be considered. In addition to wind-speed, there are seasonal patterns to mite density, with increases in spring and fall, and decreases in summer and winter (David et al., 2019), that may also affect post-burn mite recovery. These seasonal dynamics may help explain the difference observed in the mites' return between dry season and wet season burned areas.

Management Recommendations

Dry season burning is efficient in eliminating most small *L. microphyllum* plants in sawgrass marsh areas. In addition to consuming above ground vegetation, including reproductive fronds and rachis mats, dry season burns likely kill or damage more rhizomes, as they are more exposed to heat at the drier soil surface than when the soil is saturated or submerged. Plants that survive the burn remain reduced in leaf height and extent for approximately one year, and reproduction is halted completely for at least 3 months. Furthermore, spores on the soil surface are likely killed by the fire, reducing the spore bank.

Because surviving *L. microphyllum* plants recover in under two years, burning infested marsh habitats more frequently than the current 3- to 5-year interval is not feasible, as sawgrass leaf-derived fuel requires several years to build to levels sufficient to carry fire. However, the fact that mites eventually returned to the burned sites on their own—and in greater densities—suggests that prescribed burning and biocontrol management methods need not be mutually exclusive. Additionally, mite preference for new *L. microphyllum* growth (i.e., post-burning regrowth) and the severe reduction of fern growth by mite galling suggest that soon post-fire may be the ideal time for releases (David and Lake, 2020), possibly suppressing fern spread while the sawgrass recovers (and fuels accumulate). If this is the case, integrating burning and biocontrol methods may improve management outcomes over those of either method alone.

Although the mites eventually returned unaided, deliberate and carefully-timed releases could be employed to facilitate faster colonization following a dry season burn

(Fellows and Newton, 1999), and to increase mite populations to a more self-sustaining presence throughout the *L. microphyllum*-infested areas. The releases could be incorporated into existing post-burn monitoring flights, consisting of aerial releases from a helicopter based on aerial biocontrol release techniques like those for a wasp, *Tetramesa romana* Walker, in Texas (Racelis et al., 2010) and a weevil, *Rhinoncomimus latipes* (Coleoptera: Curculionidae) in Pennsylvania (Park et al., 2018). Mite survival during the dry season may be higher than in the wet season in southern Florida, given mite susceptibility to high temperatures (Ozman and Goolsby, 2005), so conducting releases following a dry season burn may be preferable.

Tables

Cape Sable (CS)	Hell's Bay (HB)
CSA: 25.227468, -81.0576	HBA: 25.211183, -80.882417
CSB: 25.218865, -81.072232	HBB: 25.20578, -80.894059
CSC: 25.218783, -81.04424	HBC: 25.20558, -80.888928

Table 2.1. Coordinates of the 6 study sites in southwestern Everglades National Park.

Label Used	Actual Dates	CSB, CSC	CSA	References HBA, HBB, HBC
Jan'17	Jan.6 – Feb.16, 2017	Initial survey Col. soil cores (6/site)	Initial survey Col. soil cores (6/site)	Initial survey Col. soil cores (6/site)
FebRx	*Feb. 10, 2017*	***Rx burn (headfire)***		
Mar'17	Mar. 13, 17, 2017	3 week post-fire visit 94% burned	3 week post-fire visit CSA mostly unburned	*Not visited*
May'17	May 23 – Jun. 22, 2017	3 mo. post-Feb-burn survey	3 mo. pre-Aug-burn survey June 20, 2017	DSB 3 mo. post WSB 3 mo. pre-
AugRx	*Aug. 9, 2017*		***CSA reburn (headfire)***	
Irma	Sept. 10, 2017	*Hurricane Irma*	*Hurricane Irma*	*Hurricane Irma*
Sept'17	Sept. 25, 2017	Post-Irma CS visits	Post-Irma CS visits	*Not visited*
Nov'17	Nov. 13 – Dec. 18, 2017	9 mo. post-burn survey Nov. 13 – Dec. 18, 2017	3 mo. post-burn survey Nov. 27, 2017	DSB 9 mo. WSB 3 mo.
Mar'18	Mar. 15, 2018	*Not visited*	6 mo. post-burn survey	*Not visited*
May'18	May 6 – Jun. 26, 2018	15 mo. post-burn survey May 6 – June 26, 2018	9 mo. post-burn survey June 26, 2018	DSB 15 mo. WSB 9 mo.
Nov'18	Nov. 14 – 28, 2018	21 mo. post-burn survey Nov. 14 – 26, 2018	15 mo. post-burn survey Nov. 28, 2018	DSB 21 mo. WSB 15 mo.
May'19	Apr. 22 – May 6, 2019	27 mo. post-burn survey April 22 – May 6, 2019	21 mo. post-burn survey April 29, 2019	DSB 27 mo. WSB 21 mo.

Table 2.2. Schedule of fires and surveys. 'Label Used' refers to survey labels on graphs. DSB (Dry Season Burn dataset) includes concurrent data on references (HB) and experimental CS sites, CSB and CSC; WSB (Wet Season Burn dataset) includes concurrent data on references (HB) and experimental CS site CSA.

Variable	Definition
Water Depth (cm)	Depth of standing water just off the sawgrass hummock host
Salinity (ppt)	Salinity of surface water near a subset of plants per site
Mortality	% of plants dead (absent) at a given survey
Size class	Small (< $^1/_{16}$ m^2) Medium ($^1/_{16}$ to ¼ m^2) Large (> ¼ m^2)
Extent, Cover (m^2)	Horizontal area of a plant/patch (m^2), estimated for large plants to 0.1 m^2
Leaf Height (cm)	Height from rhizome or ground surface to top of tallest green rachis (in field)
Sexually Reproductive	Percent of plants within a group (size/treatment/site) that were sporulating
% Sporulation	Percent of above-ground leaves with sporangia/reproductive tissues on *each plant*; mean sporulation is across plants within a group
Mite Galling	present/absent, or damage as percent of leaflets galled mod. Braun-Blanquet: level 1 galling = 0.1 – 10%, median = 5%, level 2 galling = 11 – 25%, median = 18%, level 3 galling = 26 – 50%, median = 38%, level 4 galling = 51 – 75%, median = 63% level 4 galling = 76 – 90%, median = 83% level 4 galling = 91 – 100%, median = 96%

Table 2.3. Variables measured each survey.

	Months since burn	**CSA – Cal. months**	**DSB – Cal. months**
T½	0 – 3 mo.	Aug'17 – Nov'17	Feb'17 – May'17
T1	3 mo. – 9 mo.	Nov'17 – May'18	May'17 – Nov'17
T2	9 mo. – 15 mo.	May'18 – Nov'18	Nov'17 – May'18
T3	15 mo. – 21 mo.	Nov'18 – May'19	May'18 – Nov'18
T4	21 mo. – 27 mo.	*n/a*	Nov'18 – May'19

Table 2.4. Timesteps (T) defined as time-since-burn with corresponding calendar months for WSB and DSB. Timesteps are 6-month intervals between surveys; T½ is a shorter time-period—the first 3 months post-burn—but relevant to the variables in question.

		TC (mg/g)	TN (mg/g)	TP (mg/g)	OM (%)	pH	N:P	C:N	C:P	R.S. (ppt)
DSB	CSB+ CSC	409 ± 44	19 ± 2	0.48 ± .04	89 ± 3	5.6 ± 0.5	41 ± 4	21 ± 2	863 ± 108	9 ± 3
	HB	377 ± 35	18 ± 2	0.45 ± 0.07	84 ± 3	6.3 ± 0.3	41 ± 8	21 ± 2	860 ± 149	13 ± 5
	T-test, t (df)	2.1 (20) *p*=0.05	1.6 (22) *p*=0.1	1.4 (27) *p*=0.2	**4.6 (23) *p*<0.001**	**$^{KW}\chi^2$ = 13.0 *p*<0.001**	$^{KW}\chi^2$ = 0.00 *p*=1.0	$^{KW}\chi^2$ = 0.01 *p*=0.9	0.1 (28) *p*=1.0	**-2.7 (28) *p*<0.05**
WSB	CSA	435 ± 36	22 ± 3	0.43 ± 0.03	89 ± 1	5.7 ± 0.4	53 ± 8	19 ± 2	1021 ± 123	10 ± 3
	HB	377 ± 35	18 ± 2	0.45 ± 0.07	84 ± 3	6.3 ± 0.3	41 ± 8	21 ± 2	860 ± 149	13 ± 5
	T-test, t (df)	**3.4 (8) *p*<0.01**	**4.1 (7) *p*<0.01**	-0.9 (19) *p*=0.4	**6.2 (18) *p*<0.001**	**-3.1 (7) *p*<0.05**	**$^{KW}\chi^2$ = 7.5 p<0.01**	$^{KW}\chi^2$ = 2.8 p=0.1	**2.6 (10) *p*<0.05**	-1.9 (13) *p*=0.07
Experimental	CSB+ CSC	409 ± 44	19 ± 2	0.48 ± .04	89 ± 3	5.6 ± 0.5	41 ± 4	21 ± 2	863 ± 108	9 + 3
	CSA	435 ± 36	22 ± 3	0.43 ± 0.03	89 ± 1	5.7 ± 0.4	53 ± 8	19 ± 2	1021 ± 123	10 ± 3
	T-test, t (df)	-1.4 (12) *p*=0.2	**-2.9 (8) *p*<0.05**	**2.8 (11) *p*<0.05**	-0.5 (16) *p*=0.6	-0.5 (12) *p*=0.7	**-4.5 (7) *p*<0.01**	1.8 (8) *p*=0.1	**-2.7 (9) *p*<0.05**	-0.5 (11) *p*=0.6

Table 2.5. Soil Nutrients at experimental and reference sites, means ± SD. Total carbon (TC), nitrogen (TN), phosphorous (TP), organic matter (OM), acidity (pH), ratios of nitrogen:phosphorous (N:P), carbon:nitrogen (C:N), and carbon:phosphorous (C:P), and relative soil salinity (RS). Most nutrients were normally distributed and tested for difference between burned (CS) and reference (HB) areas (pooled sites) with Student's t-tests. Certain nutrient subsets were not normally distributed, so were tested with Kruskal-Wallis, and annotated as $^{KW}\chi^2$. R.S. is relative soil salinity. Statistically significant differences are **bold**. Four datasets: DSB (top group, orange), includes data for combined sites of CSB and CSC, compared to concurrent reference data; WSB (middle group, yellow), includes data for CSA compared to concurrent reference data; Experimental (bottom group, no fill) includes data for CSA compared to the combined CSB and CSC sites.

DSB	**Exp (n=132)**	**Ref (n=91)**	**Test of Difference**
Large Plant Extent (m^2)	1.7 ± 1.4 m^2	1.1 ± 0.9 m^2	KW, χ^2 =2.7, p = 0.1
Heights (cm)	98 ± 49 cm	99 ± 42 cm	KW, χ^2 = 0.6, p = 0.4
Sexually Reproductive Plants	32%	53%	**EP, χ^2 = 8.9, p < 0.01**
Mean % Sporulation of Sexually Reproductive Plants	8 ± 13 %	6 ± 8 %	KW, χ^2 = 0.01, p = 0.9
Mites Galling	+	-	n/a

Table 2.6. DSB initial plant size and status by treatment. Jan. 2017. Mean ± sd and mite presence (+) or absence (-). Kruskal-Wallis (KW, test stat χ^2), and Equal Proportions (EP, test stat χ^2) tests between treatments reported, significant differences in **bold**.

		Initial Size				3 mo. post-Rx				9 mo. post-Rx				15 mo. post-Rx				21 mo. post-Rx				27 mo. post-Rx			
		T(132)	**S**	**M**	**L**	**T(47)**	**S**	**M**	**L**	**T(38)**	**S**	**M**	**L**	**T(36)**	**S**	**M**	**L**	**T(36)**	**S**	**M**	**L**	**T(34)**	**S**	**M**	**L**
Dry Season Burn	**Burned**	S (74)	74			S (10)	10			S (3)	2	1		S (3)	3			S (3)	3			S (3)	1	2	
		M (41)		41		M (21)	18	3		M (19)	15	4		M (17)	11	4	2	M (17)	6	9	2	M (16)	2	12	2
		L (17)			17	L (16)	10	4	2	L (16)	3	3	10	L (16)		4	12	L (16)		4	12	L (15)			15
	Reference	**T(91)**	**S**	**M**	**L**	**T(87)**	**S**	**M**	**L**	**T(60)**	**S**	**M**	**L**	**T(53)**	**S**	**M**	**L**	**T(52)**	**S**	**M**	**L**	**T(51)**	**S**	**M**	**L**
		S (31)	31			S (27)	27			S (13)	12	1		S (11)	9	2		S (11)	7	4		S (11)	8	3	
		M (29)		29		M (29)	6	20	3	M (24)	20	1	3	M (19)	12	4	3	M (18)	10	4	4	M (17)	9	4	4
		L (31)			31	L (31)	2	1	28	L (23)	6	5	12	L (23)	3	3	17	L (23)	1	5	17	L (23)	2	4	17

		T(52)	**S**	**M**	**L**	**T(40)**	**S**	**M**	**L**	**T(30)**	**S**	**M**	**L**	**T(30)**	**S**	**M**	**L**	**T(30)**	**S**	**M**	**L**
Wet Season Burn	**Burned**	S (22)	22			S (13)	13			S (5)	4	1		S (5)	4	1		S (5)	4	1	
		M (20)		20		M (17)	16	1		M (15)	11	4		M (15)	7	8		M (15)	9	6	
		L (10)			10	L (10)	7	2	1	L (10)		3	7	L (10)	1	2	7	L (10)	1	2	7
	Reference	**T(87)**	**S**	**M**	**L**	**T(60)**	**S**	**M**	**L**	**T(53)**	**S**	**M**	**L**	**T(52)**	**S**	**M**	**L**	**T(51)**	**S**	**M**	**L**
		S (35)	35			S (18)	17	1		S (14)	11	2	1	S (14)	9	4	1	S (14)	10	3	1
		M (21)		21		M (17)	15	1	1	M (14)	9	4	1	M (14)	8	4	2	M (13)	7	4	2
		L (31)			31	L (25)	6	5	14	L (25)	4	3	18	L (24)	1	5	18	L (24)	2	4	18

Table 2.7a. Size class change matrices for all plants. "T(#)" is the total number of plants alive for each treatment group per survey. Initial size class (number of plants alive) in left column of each survey. Small (S), Medium (M), and Large (L) across the top indicate sizes at each survey. Decreasing numbers within an initial size class over subsequent surveys reflect plant deaths. Dry Season Burn "Burned" (132) are plants combined from sites CSB and CSC. Wet Season Burn "Burned" (52) are plants from CSA. "Reference" counts are combined across all three reference sites.

		Initial Size				3 mo.			9 mo.			15 mo.			21 mo.			27 mo.		
		T(34)	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**
Dry Season Burn	**Burned**	S (3)	3			3			2	1		3			3			1	2	
		M (16)		16		14	2		13	3		10	4	2	5	9	2	2	12	2
		L (15)			15	9	4	2	2	3	10		3	12		3	12			15
		T(51)	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**
	Ref	S (11)	11			11			10	1		9	2		7	4		8	3	
		M (17)		17		2	13	2	13	1	3	10	4	3	9	4	4	9	4	4
		L (23)			23	1		22	6	5	12	3	3	17	1	5	17	2	4	17

		T(30)	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**
Wet Season Burn	**Burned**	S (5)	5			5			4	1		4	1		4	1	
		M (15)		15		15			11	4		7	8		9	6	
		L (10)			10	7	2	1		3	7	1	2	7	1	2	7
		T(51)	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**	**S**	**M**	**L**
	Ref	S (14)	14			13	1		11	2	1	9	4	1	10	3	1
		M (13)		13		11	1	1	8	4	1	7	4	2	7	4	2
		L (25)			24	5	5	14	3	3	18	1	5	18	2	4	18

Table 2.7b. Size class change matrices for survivor plants. "T(#)" is the total number of survivor plants in each treatment group. Small (S), Medium (M), and Large (L) across the top indicate sizes at each survey. Dry Season Burn "Burned" (34) are plants combined from sites CSB and CSC. Wet Season Burn "Burned" (30) are plants from CSA. Reference "Ref" (51) are combined across all three reference sites.

DSB	T ½	T1 + Irma	T2	T3	T4	Overall
Burned	2.2 ± 1.3	1.0 ± 1.3	1.3 ± 1.0	1.6 ± 1.3	-0.3 ± 1.5	1.2 ± 1.5
Reference	-0.8 ± 1.5	-1.0 ± 1.3	0.2 ± 0.9	1.2 ± 0.8	-0.5 ± 0.9	-0.2 ± 1.4
KW, χ^2	**46.4, p<0.001**	**36.0, p<0.001**	**23.1, p<0.001**	2.2, p=0.1	1.8, p=0.2	**83.9, p<0.001**

Table 2.8 DSB mean ± sd (cm/week) growth rates per timestep by treatment. T ½ is the first 3 months following the dry season burn. T1 (May'17-Nov'17) is the first 6-mo. timestep and includes hurricane Irma, while T2, T3, and T4 are the 6-mo. timesteps of Nov'17-May'18, May'18-Nov'18, and Nov'18-May'19, respectively. Overall refers to the entire 27 months post-February burn. Maximum growth rate highlighted in orange, minimum in blue. Significant statistics are **bold**.

WSB	Exp (n=52)	Ref (n=87)	Test of Difference
Large Plant Extent (m^2)	2.6 ± 2.8 m^2	1.5 ± 2.0 m^2	KW, χ^2 = 1.8, p = 0.2
Heights (cm)	78 ± 64 cm	86 ± 37 cm	**KW, χ^2 = 6.9, p < 0.01**
Sexually Reproductive Plants	2%	17%	**EP, χ^2 = 6.1, p < 0.05**
Mean % Sporulation of Sexually Reproductive Plants	0.1 ± n/a %	1.0 ± 0.4 %	**KW, χ^2 = 5.0, p < 0.05**
Mites (mean galling damage)	9 ± 6 %	-	n/a

Table 2.9 WSB initial plant size and condition by treatment, May 2017. Mean ± sd, mite presence (+) or absence (-). Kruskal-Wallis (KW, test statistic χ^2), and Equal Proportions (EP, test stat χ^2) tests between treatments reported, differences in **bold**.

WSB	Pre-Rx	T½ + Irma	T1	T2	T3	Overall
Burned	-0.3 ± 2.0	0.9 ± 1.1	1.5 ± 1.0	0.6 ± 1.3	-0.7 ± 1.1	0.6 ± 1.6
Reference	-0.8 ± 1.5	-1.0 ± 1.3	0.2 ± 0.9	1.2 ± 0.8	-0.5 ± 0.9	-0.1 ± 1.4
KW, χ^2	**5.8, p<0.05**	**37.5, p<0.001**	**26.8, p<0.001**	**5.3, p<0.05**	0.007, p=0.9	**20.1, p<0.001**

Table 2.10 WSB Mean growth rates per timestep by treatment. Pre-Rx is the time between Jan. and May 2017 surveys. T ½ is the first 3 months following the burn and includes hurricane Irma. T1-T3 are the 6-mo. timesteps of Nov'17-May'18, May'18-Nov'18, and Nov'18-May'19, respectively. Overall refers to the entire 21 months post-August burn (excludes 'Pre-Rx'). Maximum growth rate highlighted in orange, minimum in blue. Significant statistics are **bold**.

	May 2017	Nov. 2017	May 2018	Nov. 2018	May 2019	Overall
DSB						
Burned	**Feb-Rx** 2.2 ± 1.3	1.0 ± 1.3	1.3 ± 1.0	1.6 ± 1.3	-0.3 ± 1.5	1.2 ± 1.5
Reference	-0.8 ± 1.5	-1.0 ± 1.3	0.2 ± 0.9	1.2 ± 0.8	-0.5 ± 0.9	-0.1 ± 1.4
KW, $\chi^2_{CSB.CSCvHB}$	**46.4, *p*<0.001**	**36.0, *p*<0.001**	**23.1, *p*<0.001**	2.2, *p*=0.1	1.8, *p*=0.2	**83.9 *p*<0.001**
WSB						
Burned	-0.3 ± 2.0	**Aug-Rx** 0.9 ± 1.1	1.5 ± 1.0	0.6 ± 1.3	-0.7 ± 1.1	0.6 ± 1.6
Reference	-0.8 ± 1.5	-1.0 ± 1.3	0.2 ± 0.9	1.2 ± 0.8	-0.5 ± 0.9	-0.1 ± 1.4
KW, χ^2_{CSAvHB}	**5.8, *p*<0.05**	**37.5, *p*<0.001**	**26.8, *p*<0.001**	**5.3, *p*<0.05**	0.007, *p*=0.9	**20.1, *p*<0.001**
EXPERIMENTAL						
DSB	**Feb-Rx** 2.2 ± 1.3	1.0 ± 1.3	1.3 ± 1.0	1.6 ± 1.3	-0.3 ± 1.5	1.2 ± 1.5
WSB	-0.3 ± 2.0	**Aug-Rx** 0.9 ± 1.1	1.5 ± 1.0	0.6 ± 1.3	-0.7 ± 1.1	0.6 ± 1.6
Dunn, *z*	**4.7, *p*<0.001**	0.1, *p*=0.9	-0.7, *p*=0.5	**3.2, *p*<0.01**	1.3, *p*=0.7	**4.1, *p*<0.001**

Table 2.11. All growth rates (Mean ± SD) by calendar months. Maximum growth rate highlighted in orange, minimum in blue. Significant statistics are **bold**.

Figures

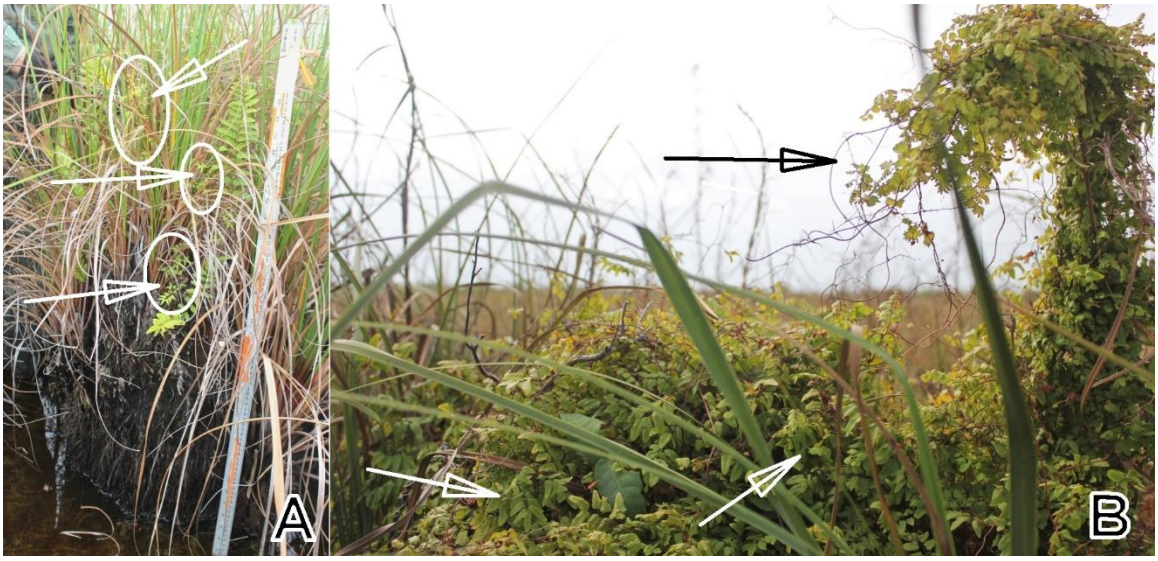

Figure 2.1. Study organism growth habits. Small *L. microphyllum* plant with pinnae circled growing on a sawgrass hummock with *Blechnum serrulatum* (uncircled fronds) (A) and large *L. microphyllum* plant overtopping sawgrass flowering stalk (B). White arrows indicate *L. microphyllum* fronds; black arrow indicates *L. microphyllum* fronds overtopping sawgrass inflorescence and cascading out/down.

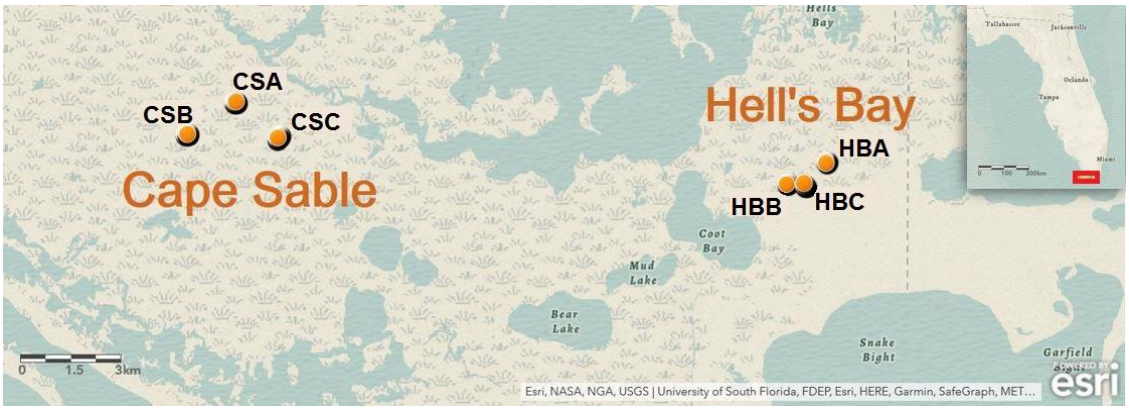

Figure 2.2. Location of *L. microphyllum* Cape Sable and Hell's Bay sampling areas and sampling sites (CSA, CSB, CSC; HBA, HBB, HBC). Cape Sable sites were burned (CSA in the wet season, CSB and CSC in the dry season), while Hell's Bay sites were reference sites.

Figure 2.3 Typical sawgrass marsh vegetation at the sites, with occasional shrubs, trees, and sawgrass flowering stalks. Flagging tape marks a couple of *Lygodium* patches at CSC; numbered metal tags are in among the sawgrass culms.

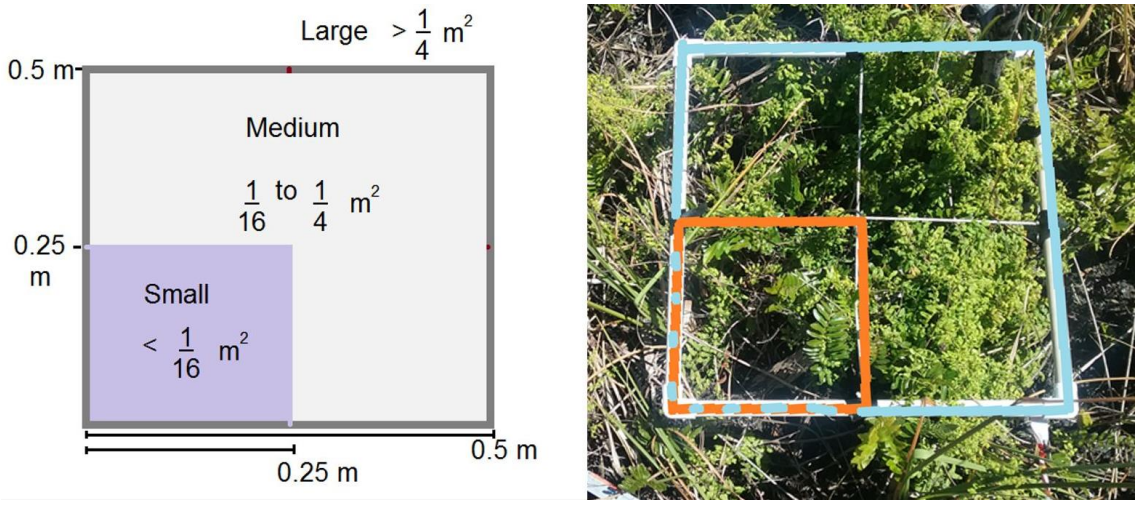

Figure 2.4. Size classes defined using subdivided quarter-meter quadrat (left). A plant confined within the orange-outlined subdivision of the quadrat was considered small, a plant larger than this but smaller than the blue outline of the ¼ m^2 quadrat was a medium, and a plant larger than the quadrat was large and had its extent estimated.

Figure 2.5. Three weeks post-burn, sawgrass regrowth at CSB (March 17, 2017) (left), and *Lygodium* remnant rhizomes (R) and leaf bases (LB) on a burnt hummock (right).

Figure 2.6. Normal pinna (top left), severe damage by the *Lygodium* leaf-galling mite, *Floracarus perrepae* (bottom left),"window paning" and frass by the *Lygodium* moth *Neomusotima conspurcatalis* larvae (right).

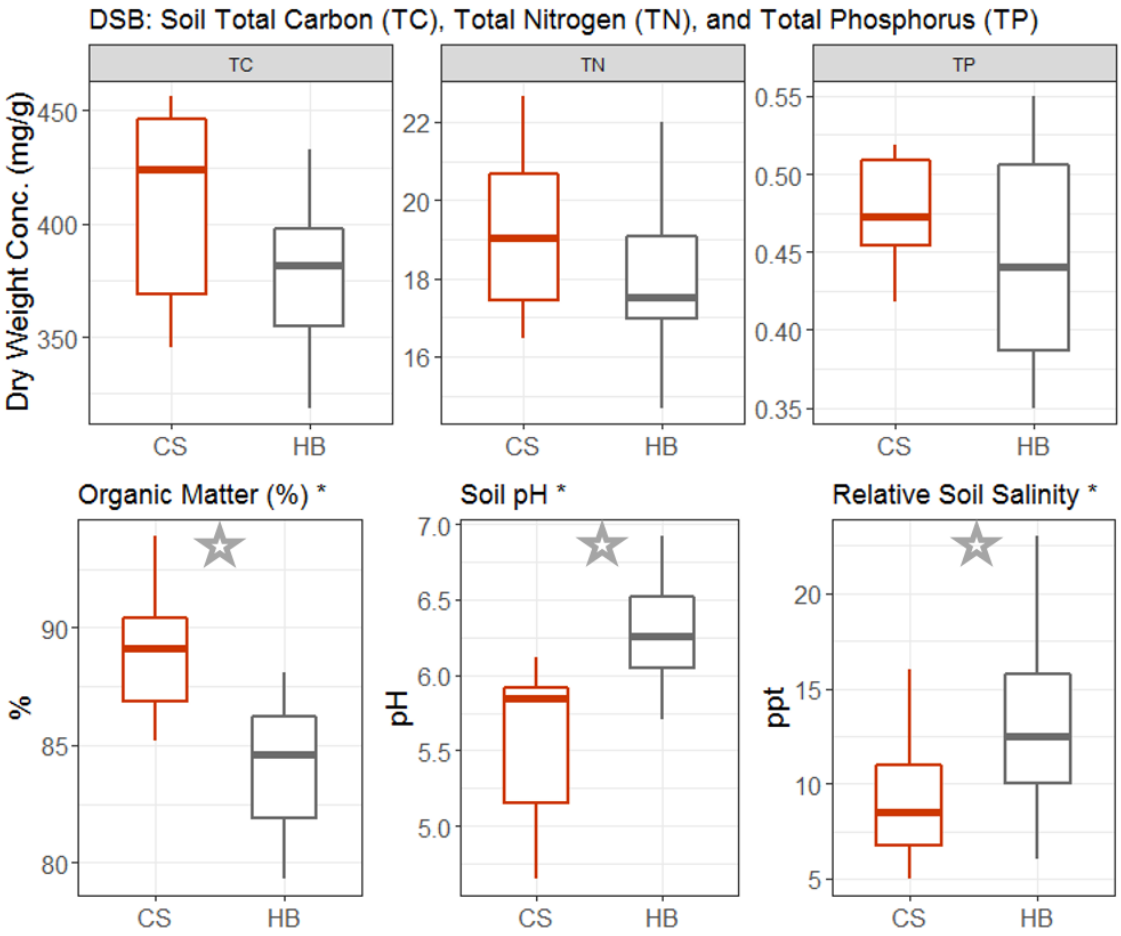

Figure 2.7. DSB soil nutrient concentrations (mg/g) for total carbon (TC), total nitrogen (TN), total phosphorus (TP) (top row); organic matter (OM), pH, and relative soil salinity in experimental (CS – red)) and reference (HB - gray) areas. Sampled January 2017. Note: scales differ for each nutrient, but all in units of mg/g, OM is %, pH is unitless, and relative soil salinity is in parts per thousand (ppt); gray star indicates significant difference between treatments for marked nutrients.

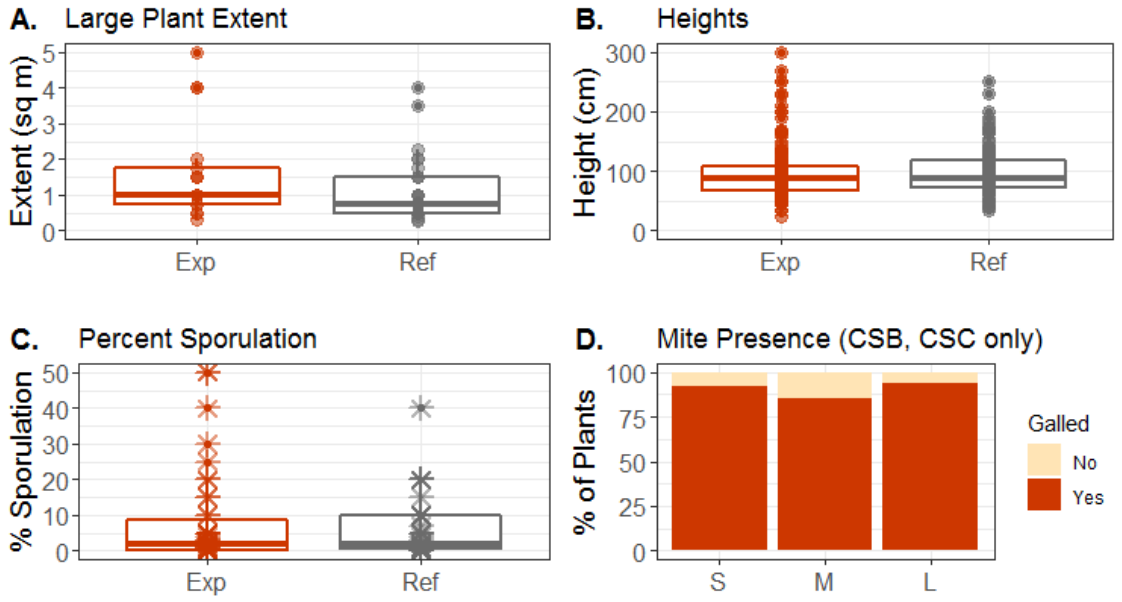

Figure 2.8. DSB initial (January 2017) experimental ("Exp," red) and reference ("Ref," gray) extents of large plants (A), leaf heights (B), level of sporulation on reproductive plants (C), and mite presence (red) or absence (tan) on experimental plants by size class (D; size classes are small (S), medium (M), and large (L), as described in Figure 2.4). No plants had mite galling at the reference sites.

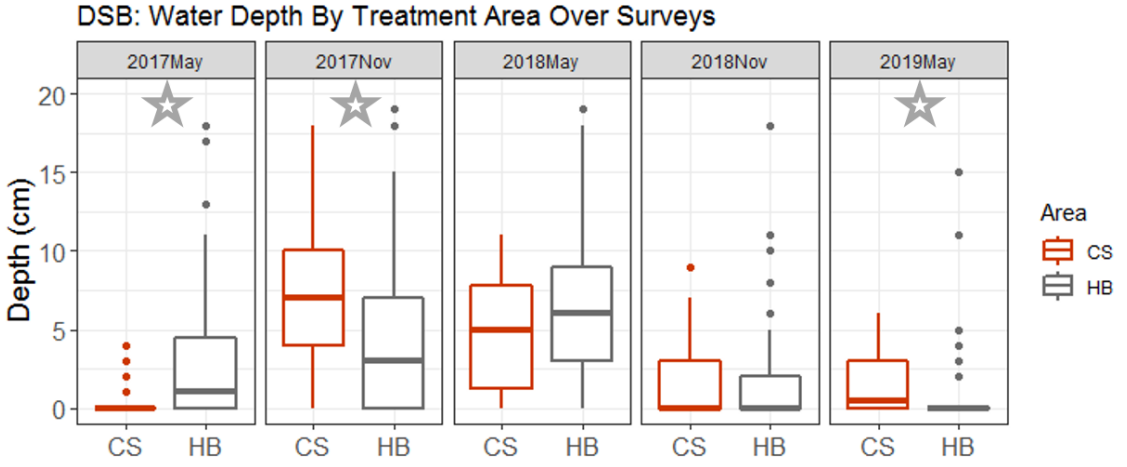

Figure 2.9. DSB Average water depth by treatment area (over all surveys). Water depths differed between treatment areas during the May 2017 (3 mo. post-burn), Nov. 2017 (9 mo. post-burn), and May 2019 (27 mo. post-burn) surveys. Gray star indicates significant difference. CS = Cape Sable; HB = Hell's Bay.

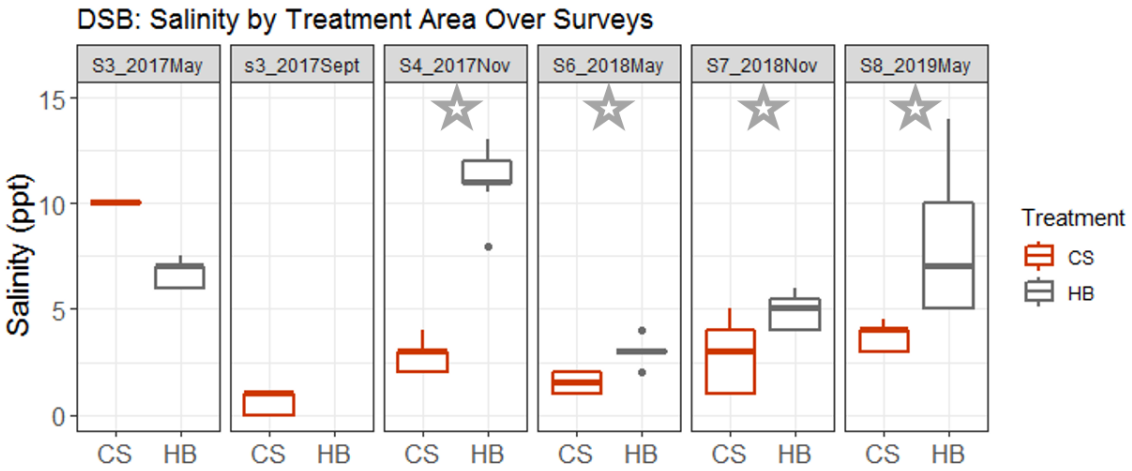

Figure 2.10. DSB surface water salinity over surveys for treatment areas. Only a single sample was obtained from the experimental sites in 2017May. The reference area was not visited immediately post-Irma (2017Sept). Gray star indicates statistical difference ($p < 0.01$). CS = Cape Sable; HB = Hell's Bay.

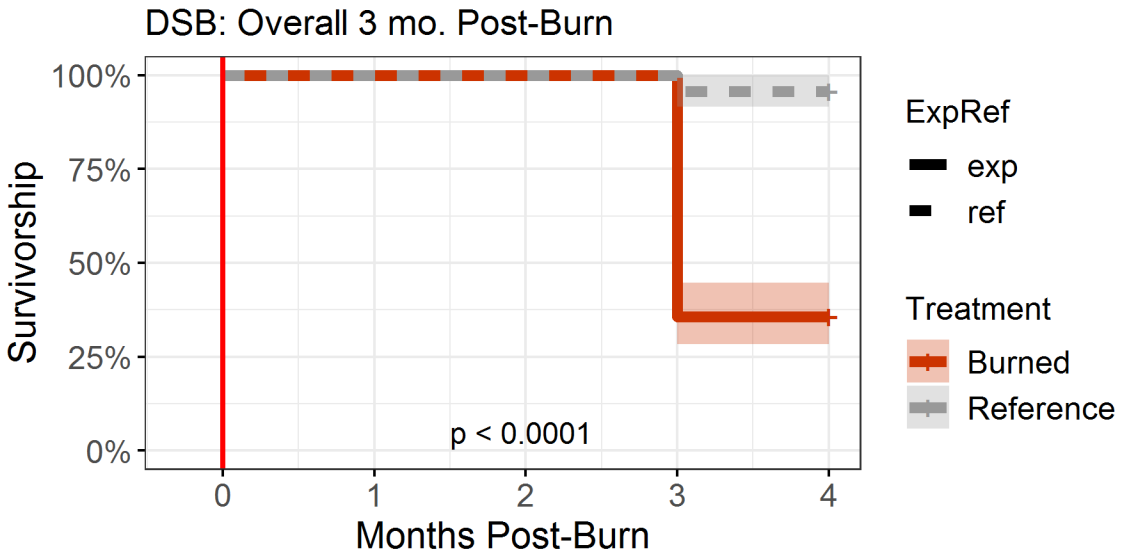

Figure 2.11a. Overall early post-fire mortality by treatment. Experimental (solid red), reference (dashed gray). Vertical red line at 0 indicates February fire.

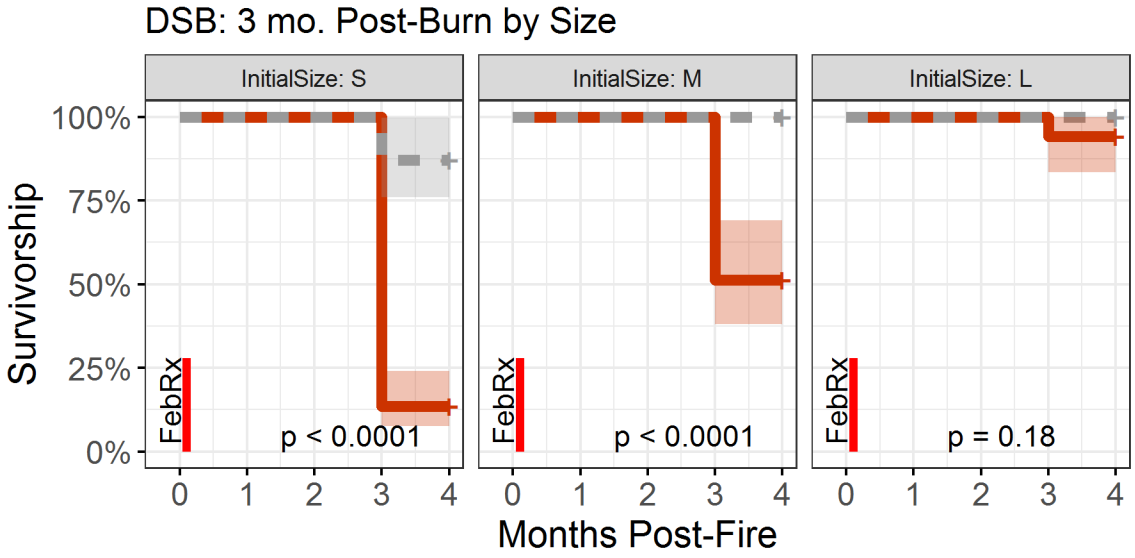

Figure 2.11b. Early post-fire mortality by initial size class. S, M, and L = small, medium and large plants, respectively. Experimental (solid red), reference (dashed gray); red vertical line segment (FebRx) indicates February fire.

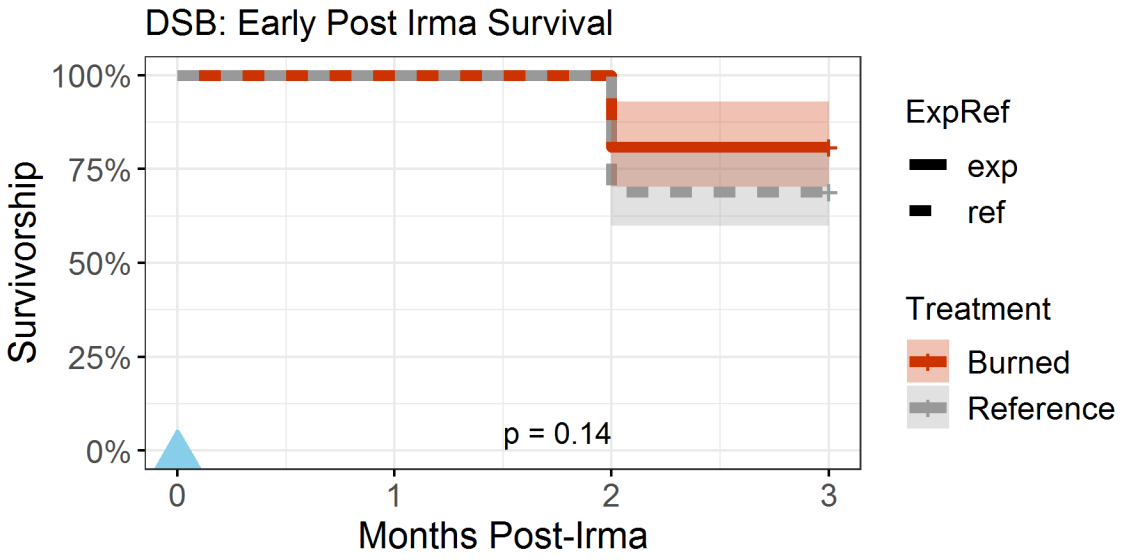

Figure 2.12a. Overall post-Irma mortality. Experimental (solid red), reference (dashed gray); blue triangle indicates hurricane Irma.

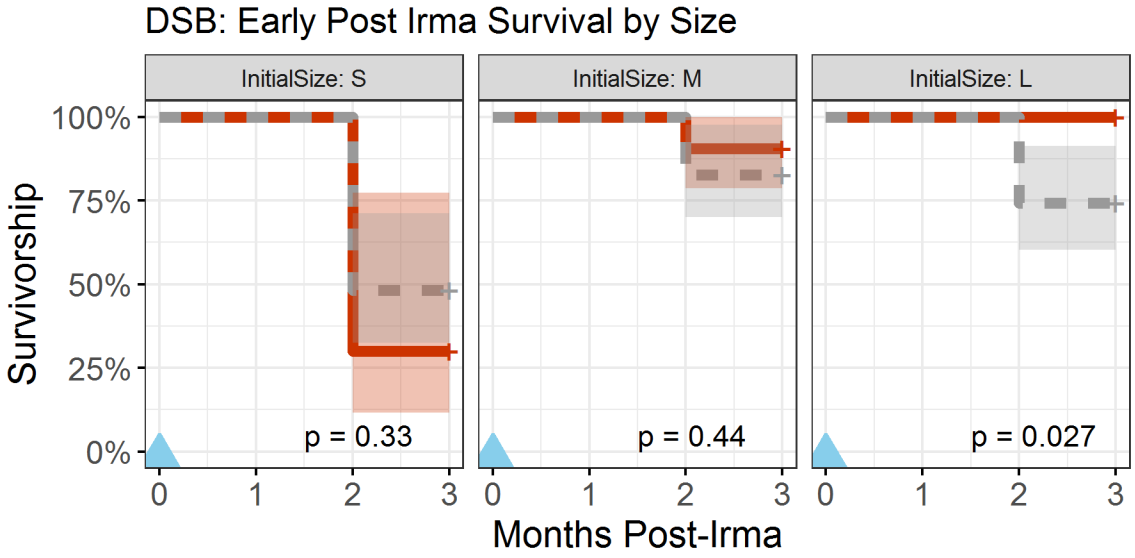

Figure 2.12b. Early post-Irma mortality by initial size class. S, M, and L = small, medium and large plants, respectively. Hurricane Irma (blue triangle), experimental (solid red), and reference (dashed gray).

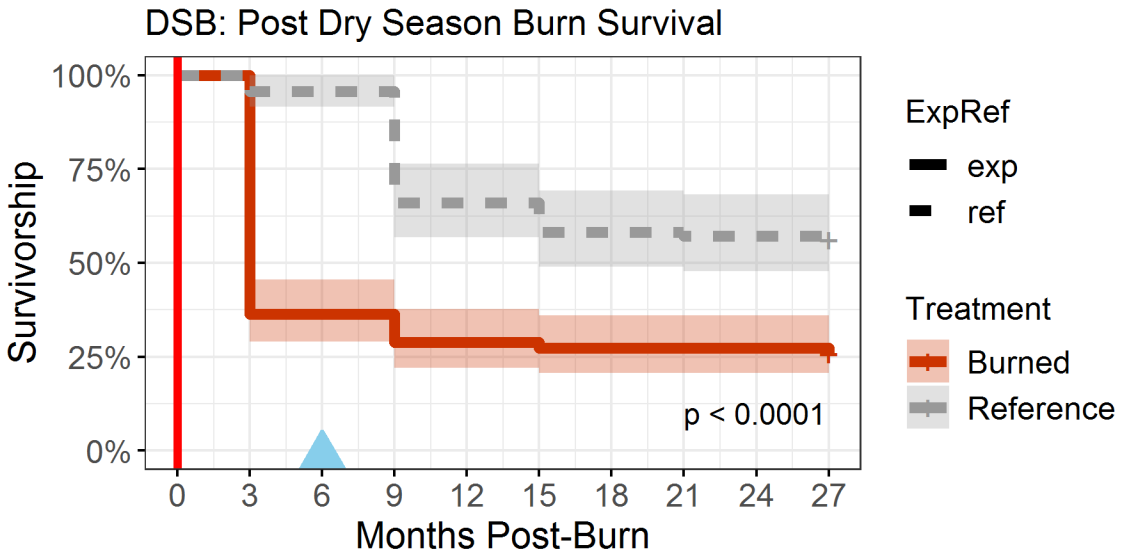

Figure 2.13a. Kaplan-Meier survivorship curves for DSB over 27 months post-burn, all plant sizes pooled. Experimental (solid red) and reference (dashed gray) survivorship. Red vertical line (at 0) indicates February burn; blue triangle (at 6) indicates hurricane Irma.

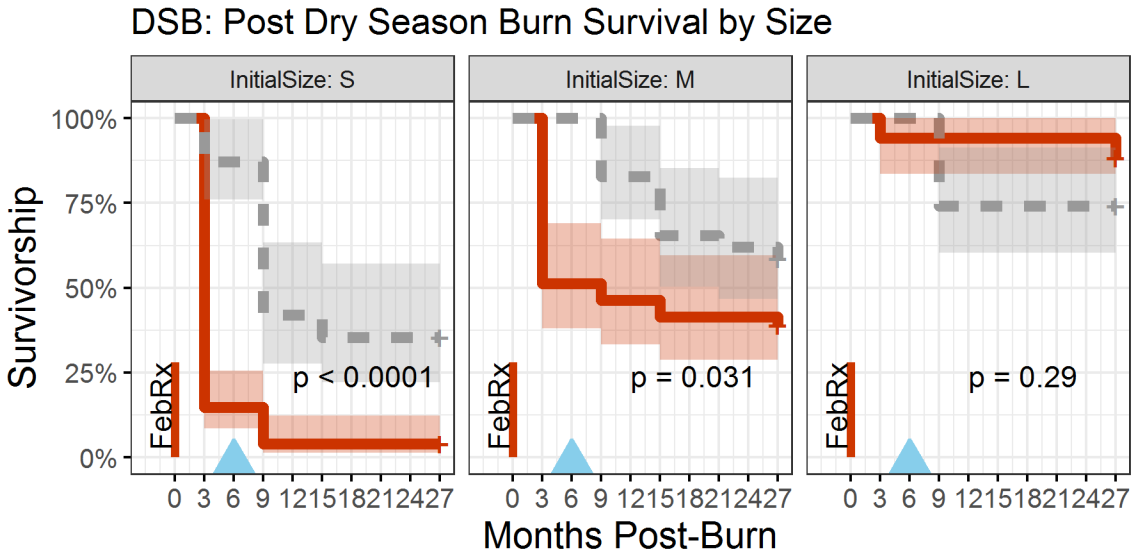

Figure 2.13b. Kaplan-Meier survival curves for *L. microphyllum* plants by initial size class. Burned (solid red) and reference (dashed gray) plants from 0 to 27 months. Red vertical line segment (FebRx) indicates February burn (red line at 0). Blue triangle indicates the occurrence of hurricane Irma at 6 months. S, M, and L = small, medium and large plants, respectively.

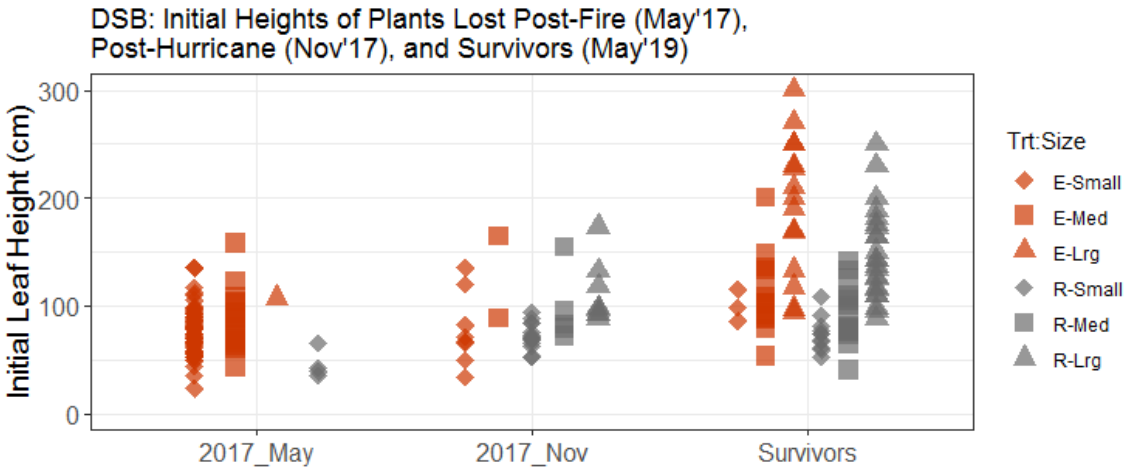

Figure 2.14. Initial heights of burned (red) and reference (gray) plants that died post fire (2017_May) or post hurricane Irma (2017_Nov), and plants that did not die by 27 months (Survivors). Shapes indicate initial size class: diamond = small, square = medium, triangle = large. E = experimental sites; R = reference sites.

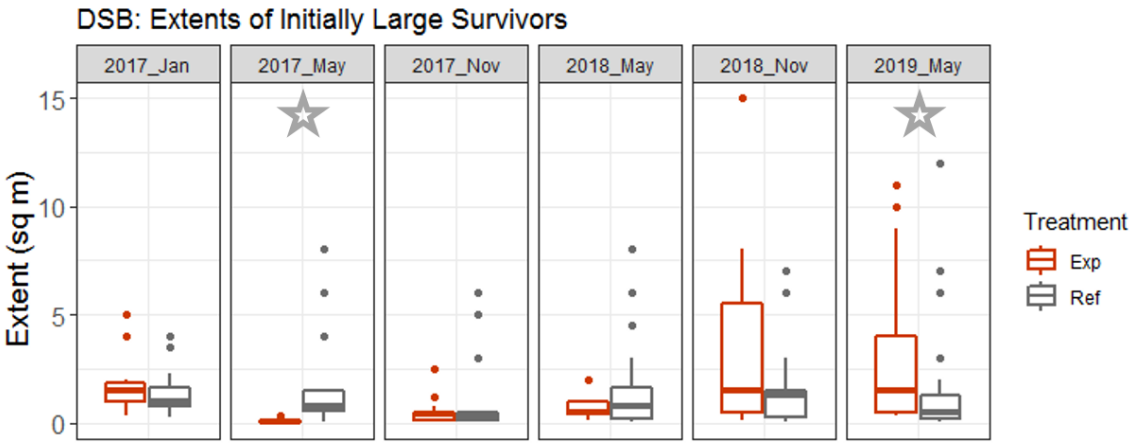

Figure 2.15. DSB extents of initially large plants over surveys. Experimental (Exp, red) and references (Ref, gray). Gray star indicates difference at $p < 0.05$.

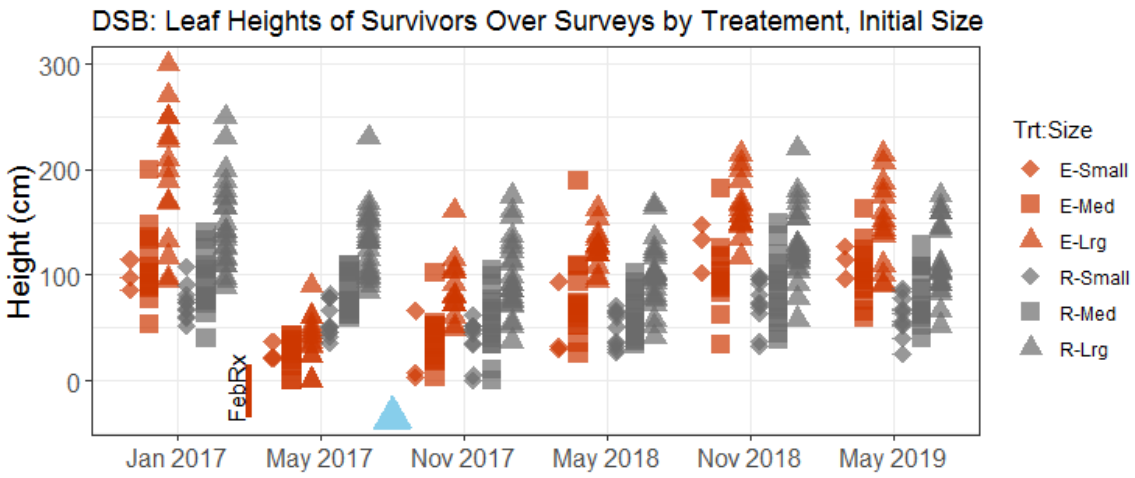

Figure 2.16. DSB leaf heights of survivors over all surveys, grouped by treatment and initial size class (Trt:Size). Experimental (E, red) and reference (R, gray); diamond = small, square = medium, triangle = large. Red vertical line (FebRx) indicates February burn; large blue triangle indicates hurricane Irma. Darker hues within a group indicate overlapping records.

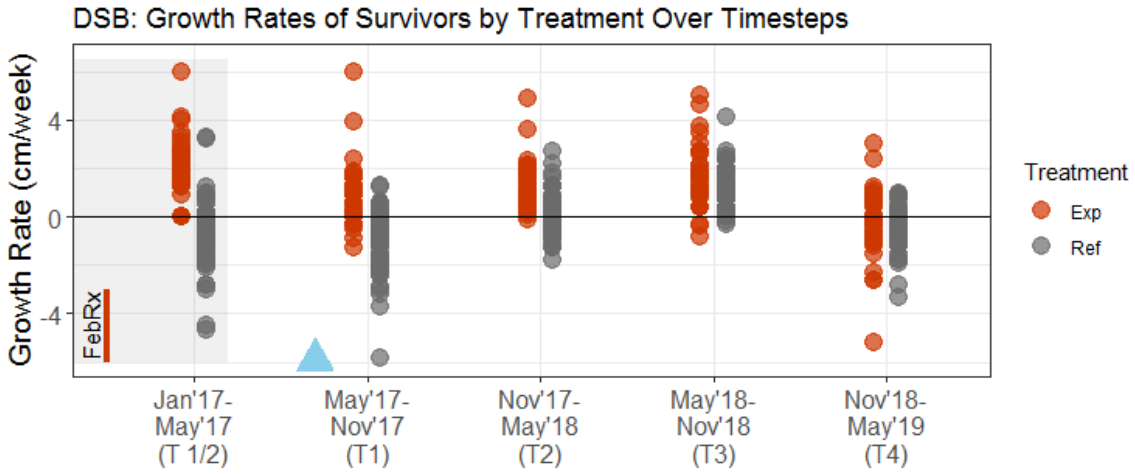

Figure 2.17. Growth rates for pooled sizes of burned (Exp, red) and reference (Ref, gray) plants over four timesteps (T). Darker hues within a group indicate overlapping sample records. Vertical red line segment (FebRx) indicates February fire, blue triangle indicates hurricane Irma (September 2017). Gray-shaded region covers initial survey (Jan 2017) to the 3-month post-burn survey (May 2017) and is shorter than the following 6-month periods between surveys (referred to as T½). Growth rates during T ½ for experimental plants were calculated with respect to months-since-burn, while growth rates for reference plants were calculated over the time between Jan. and May.

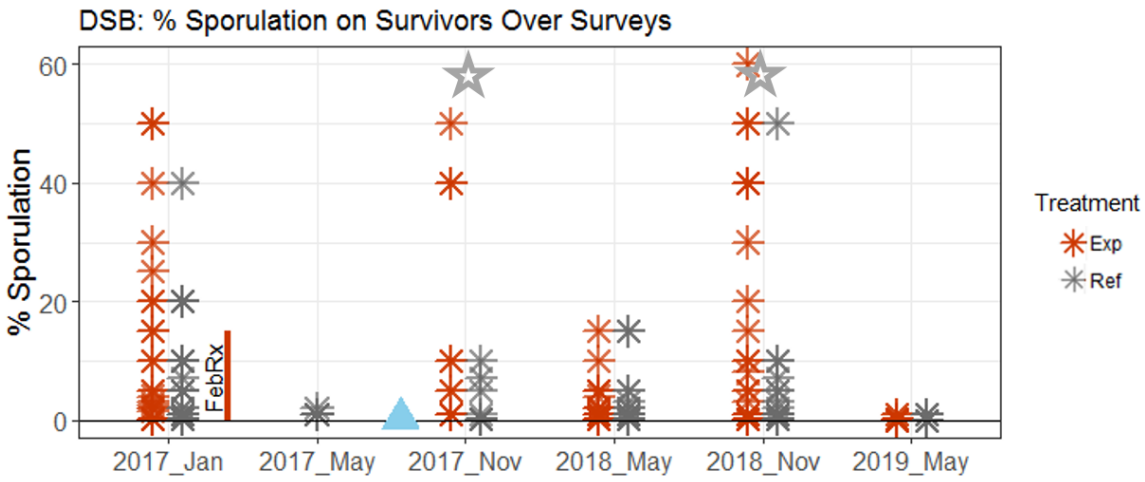

Figure 2.18. Percent sporulation per individual, grouped by treatment, over surveys. Experimental (Exp, red), reference (Ref, gray), and darker symbols indicate overlapping records. Vertical red line (FebRx) indicates dry burn, blue triangle indicates hurricane Irma. Gray star indicates significant difference in percent sporulation between Exp and Ref.

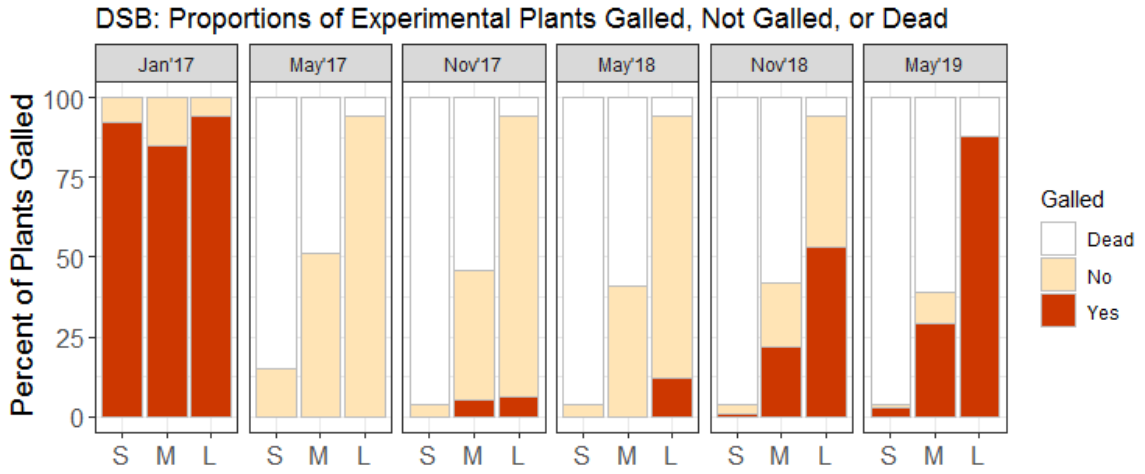

Figure 2.19. Proportion of initial experimental plants galled (red), not galled (tan), or dead (white) at each survey. Small, medium, and large plants (S, M, L, respectively). The burn occurred in February 2017, shortly after the first survey, and hurricane Irma occurred between the May and Nov. 2017 surveys.

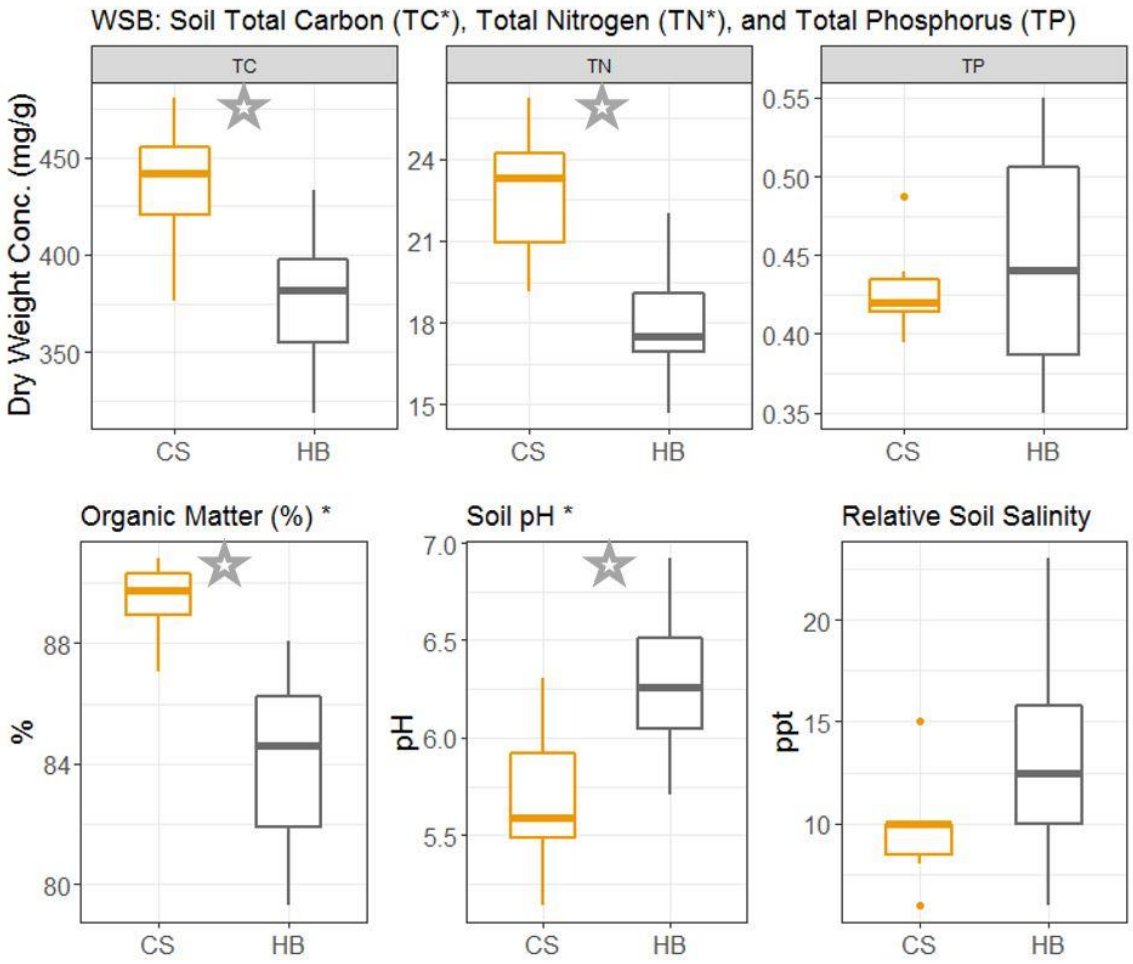

Figure 2.20. WSB soil nutrients concentrations (mg/g) for total carbon (TC), total nitrogen (TN), total phosphorus (TP) (top row) and organic matter (OM), pH, and relative salinity (ppt) (bottom row) in experimental (CS – yellow)) and reference (HB - gray) areas). Sampled January 2017. Notes: scales differ for each nutrient, but all in units of mg/g; gray star indicates significant difference between treatments.

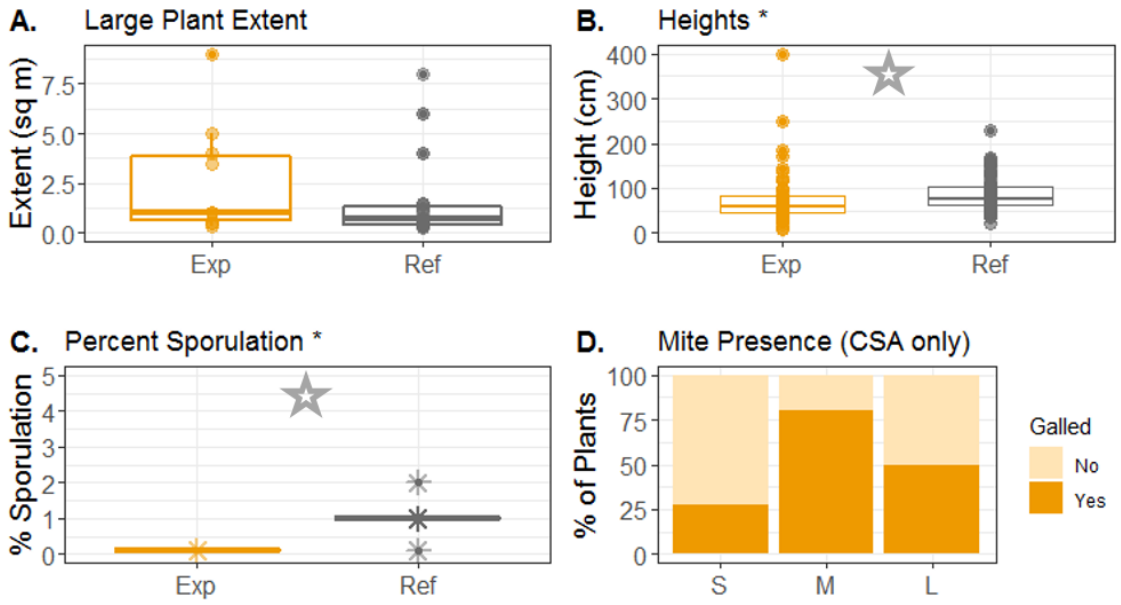

Figure 2.21. WSB initial (May 2017) experimental (Exp, yellow) and reference (Ref, gray) extents of large plants, height, percent sporulation on reproductive plants, and mite presence (yellow) or absence (tan) on plants by size class (S = small, M = medium, and L = large) at experimental sites. No plants had mite galling at the reference sites. Note very low scale for percent sporulation. Gray star indicates significant statistical difference between Exp and Ref.

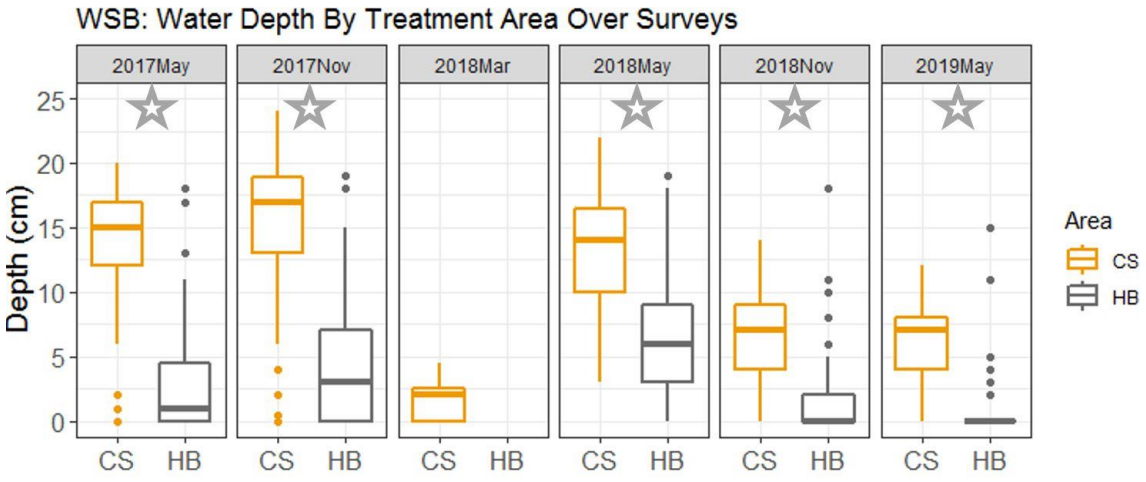

Figure 2.22. Surface water depth over surveys. Experimental (CS = CSA (burned), yellow) and reference (HB, gray). Depths differed at all surveys except for March 2018, during which references were not visited. Gray star indicates significant statistical difference.

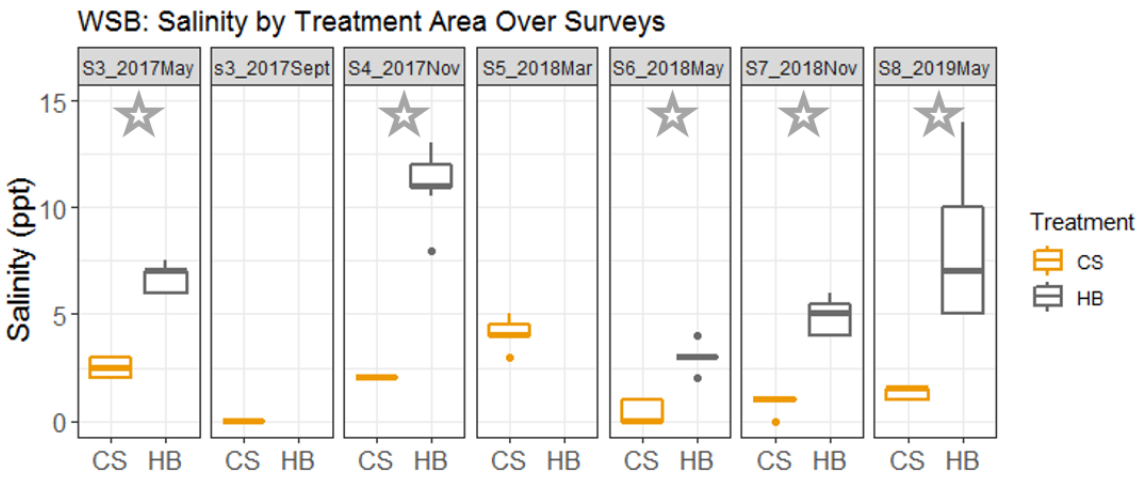

Figure 2.23. WSB salinity over surveys (S3-S8). Experimental (CS = CSA (burned), yellow) and reference (HB, gray). S3_2017Sept was the post-Irma visit made only to experimental sites in September. S6_2018Mar was a 6-month post burn visit made only to CSA. Gray star indicates significant statistical difference.

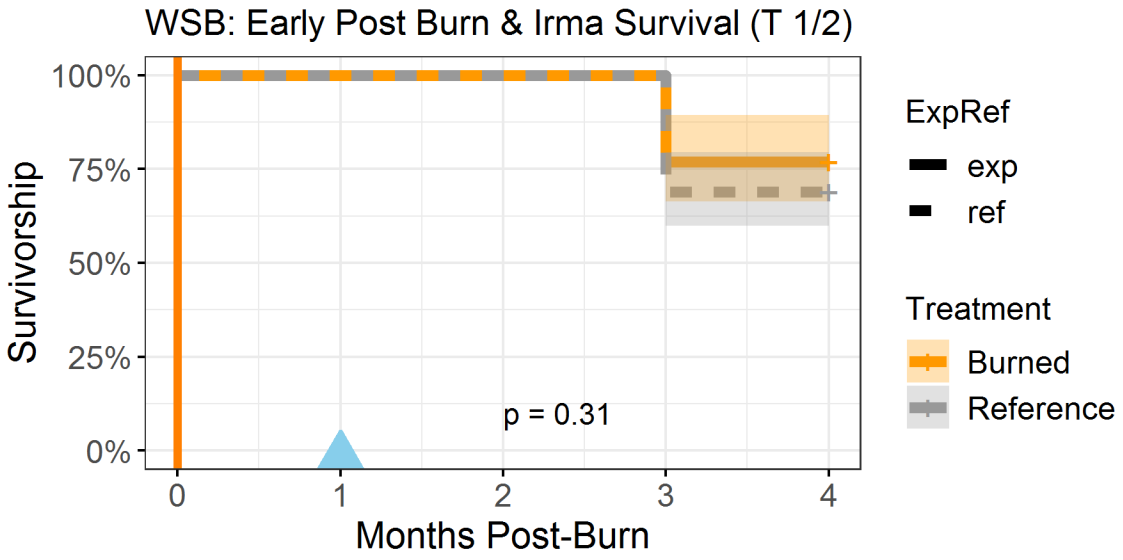

Figure 2.24a. Overall immediate post-fire/Irma mortality in experimental (CSA (burned), solid yellow) and reference (HB, dashed gray) sites. Vertical line at 0 indicates Wet Season Burn (orange, at 0 months), and blue triangle indicates hurricane Irma (at 1 month).

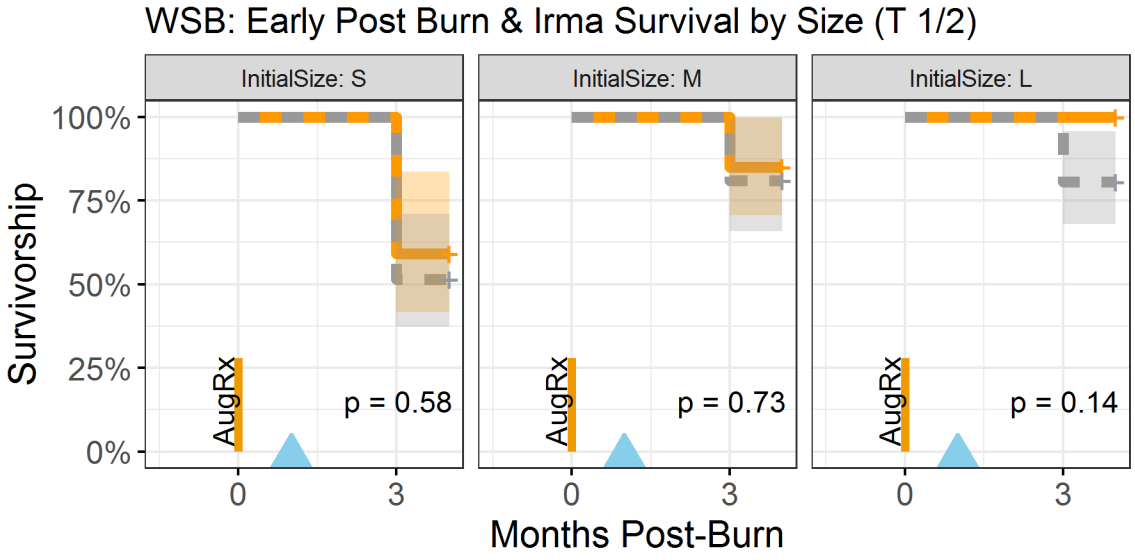

Figure 2.24b. Immediate post-fire/Irma mortality by size for experimental (CS = CSA (burned), solid yellow) and reference (dashed gray) sites. Differences in mortality between treatments were not significant for any size class. S = small, M = medium, and L = large. Verticl line (AugRx) indicated Wet Season Burn (yellow, at 0 months), and blue triangle indicates hurricane Irma (blue, at 1 month).

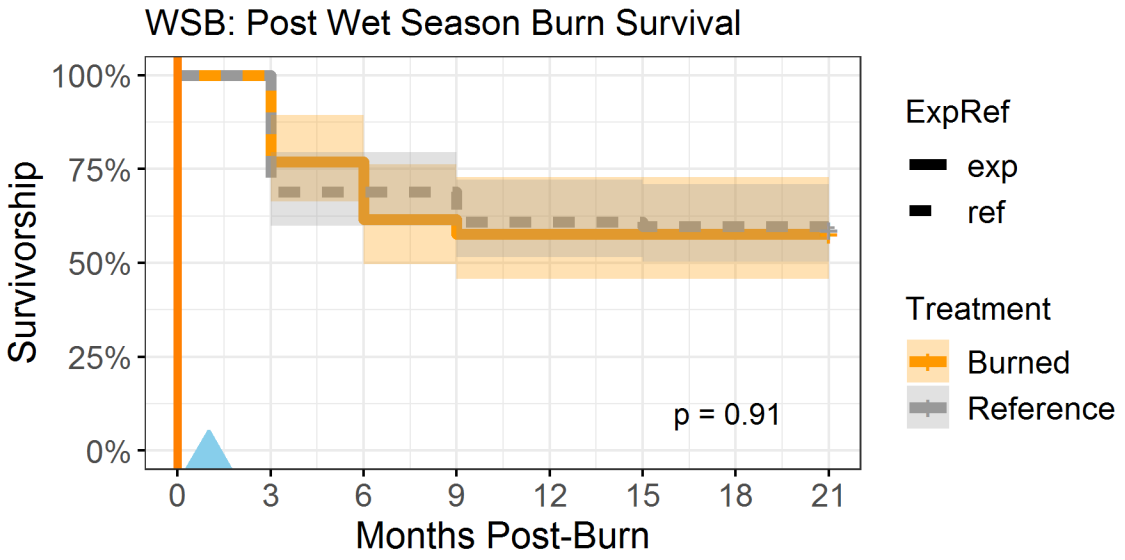

Figure 2.25a. Overall WSB (21 mo.) mortality following fire/hurricane for experimental (CS = CSA (burned), solid yellow) and reference (dashed gray) sites. Vertical line at 0 indicates Wet Season Burn (orange, at 0 months), and blue triangle indicates hurricane Irma (at 1 month).

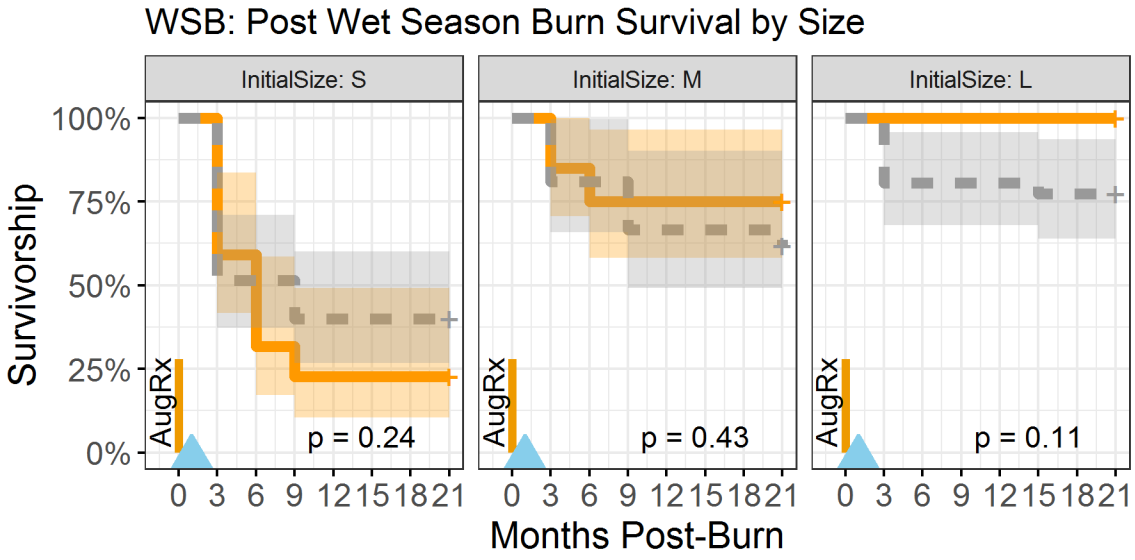

Figure 2.25b. Mortality following WSB/hurricane for experimental (CSA (burned), solid yellow) and reference (HB, broken gray) plants, by initial size class. Vertical segment indicates August burn (AugRx, yellow line at 0), blue triangle indicates hurricane Irma (blue at 1 month). No difference between treatments for any size. S = small, M = medium, and L = large.

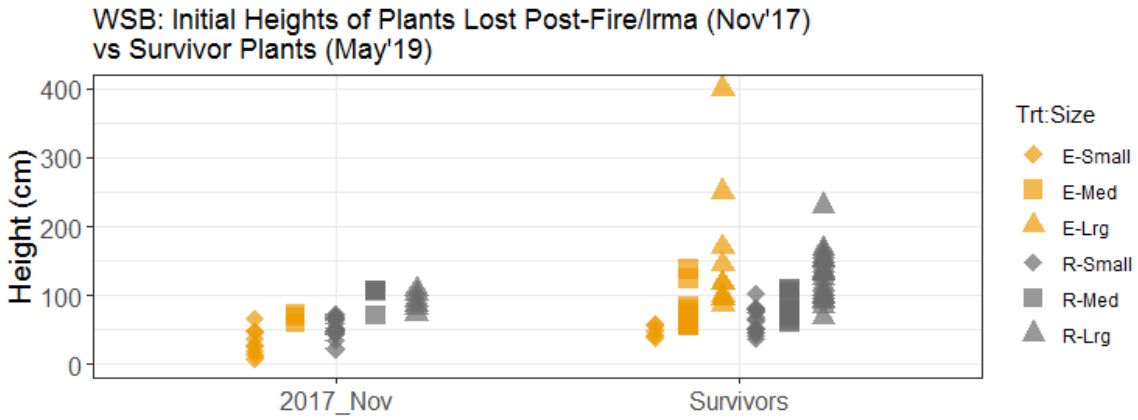

Figure 2.26. Initial heights of WSB plants that died (Lost) immediately following August fire or September hurricane Irma (2017_Nov), and Survivors, by initial size class. Shapes indicate initial size class: diamond = small, square = medium, triangle = large. E = experimental sites; R = reference sites.

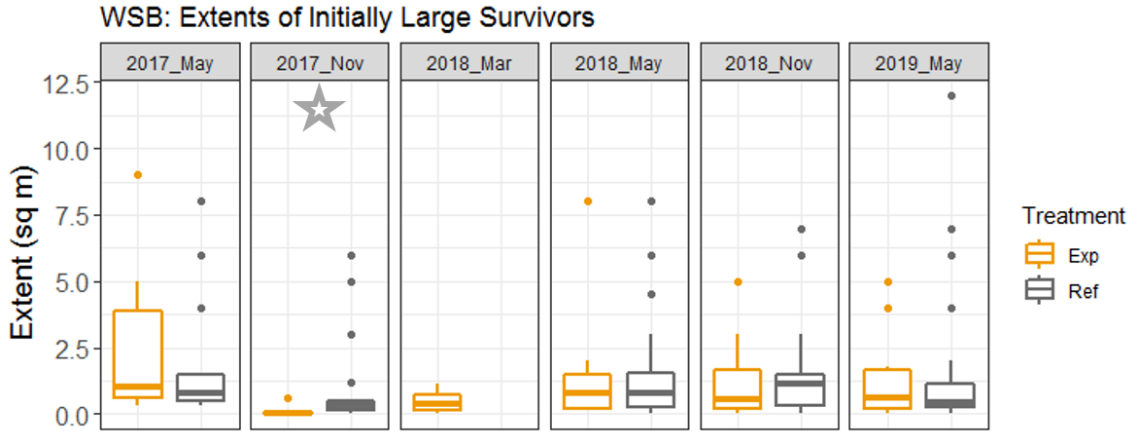

Figure 2.27. WSB extents of initially large plants over surveys 2017_May to 2019_May. Exp = experimental (CSA (burned), yellow) and Ref = reference (gray). Reference sites were not visited during the March 2018 survey. Gray star indicates statistical difference at $p < 0.01$.

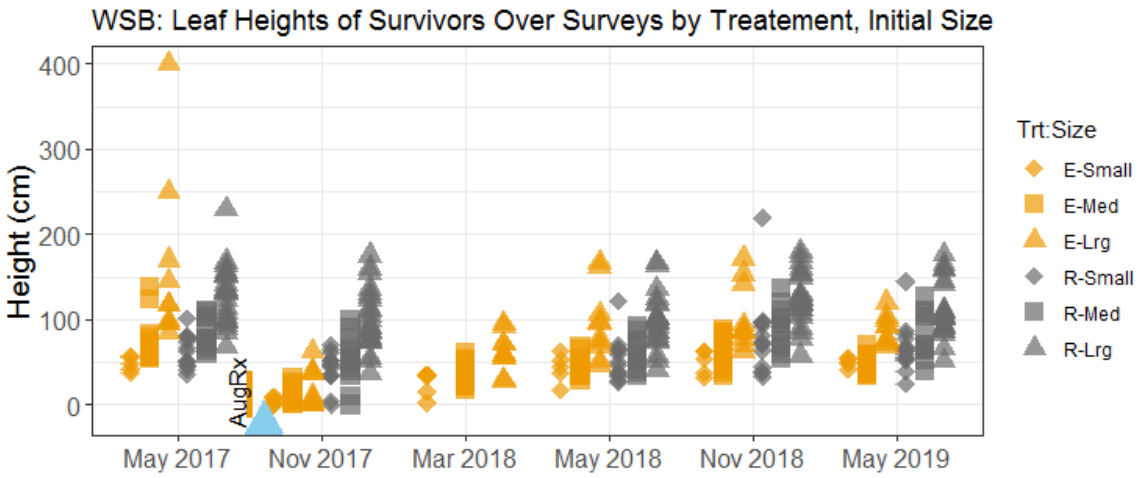

Figure 2.28. WSB leaf heights of survivors over all surveys, grouped by treatment and initial size class (Trt:Size). Experimental (yellow) and reference (gray); diamond = small, square = medium, triangle = large. Yellow vertical segment (AugRx) indicates August burn; large blue triangle indicates hurricane Irma. Darker hues within a group indicate overlapping records. Reference sites were not visited during the March 2018 survey.

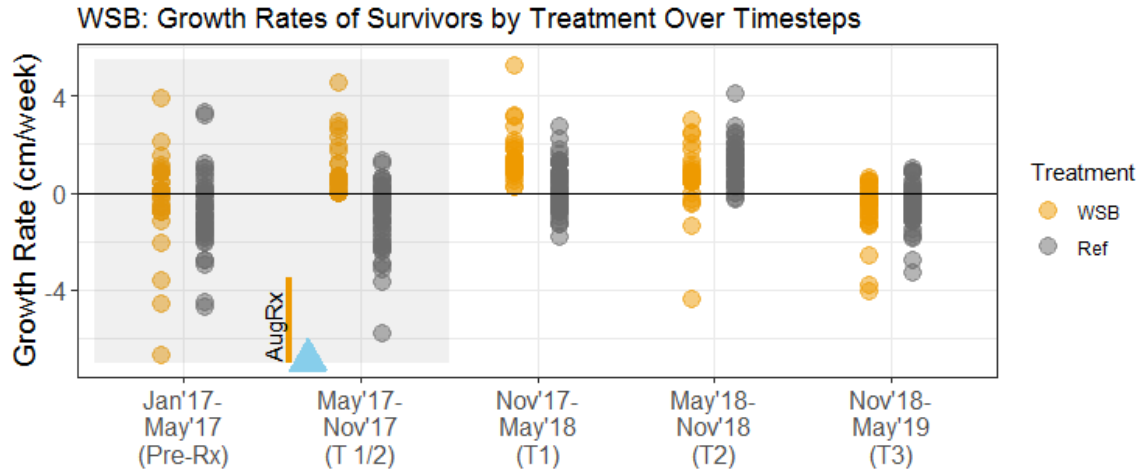

Figure 2.29. WSB growth rates for pooled sizes of burned (yellow) and reference (gray) plants over three timesteps (T). Darker hues within a group indicate overlapping samples. Vertical yellow line (AugRx) indicates August fire, blue triangle indicates hurricane Irma (September 2017). Gray-shaded region denotes shorter time spans: Pre-Rx, the initial survey (Jan 2017) to the WSB initial survey (May 2017); and T½, the early post-fire time period from 0 mo. – 3 mo. post-burn. Growth rates during T ½ for experimental plants were calculated with respect to months-since-burn, while growth rates for reference plants were calculated over the time between survey dates.

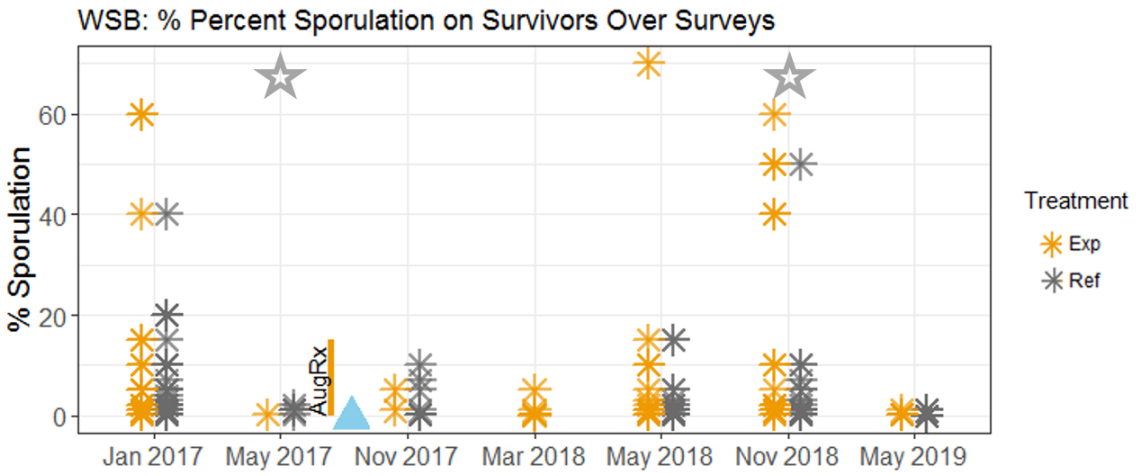

Figure 2.30. WSB percent sporulation on survivor plants over surveys. Experimental (CS = CSA (burned), yellow) and reference (HB, gray). Darker hues indicate overlapping records. Vertical yellow line segment (AugRx) indicates February fire, blue triangle indicates hurricane Irma (September 2017). Significant difference indicated by the gray star at the top of the graph over the relevant survey.

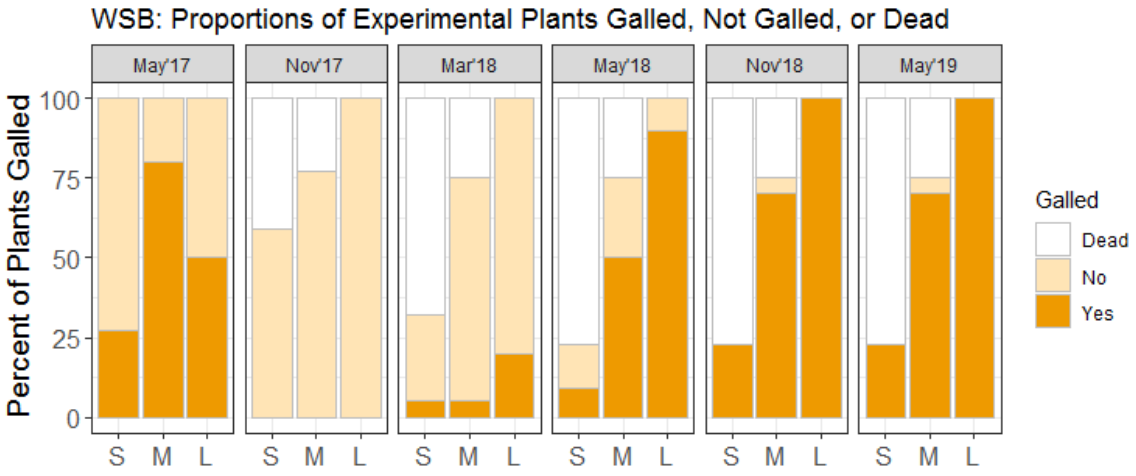

Figure 2.31. WSB proportion of plants at CSA (burned Aug. 2017) with mite galling (yellow), without mite galling (tan), or dead (white) from initial (pre-Aug-burn) survey through 21 months (May 2019). Initial size classes are small (S), medium (M), and large (L). No mites were observed at the reference sites.

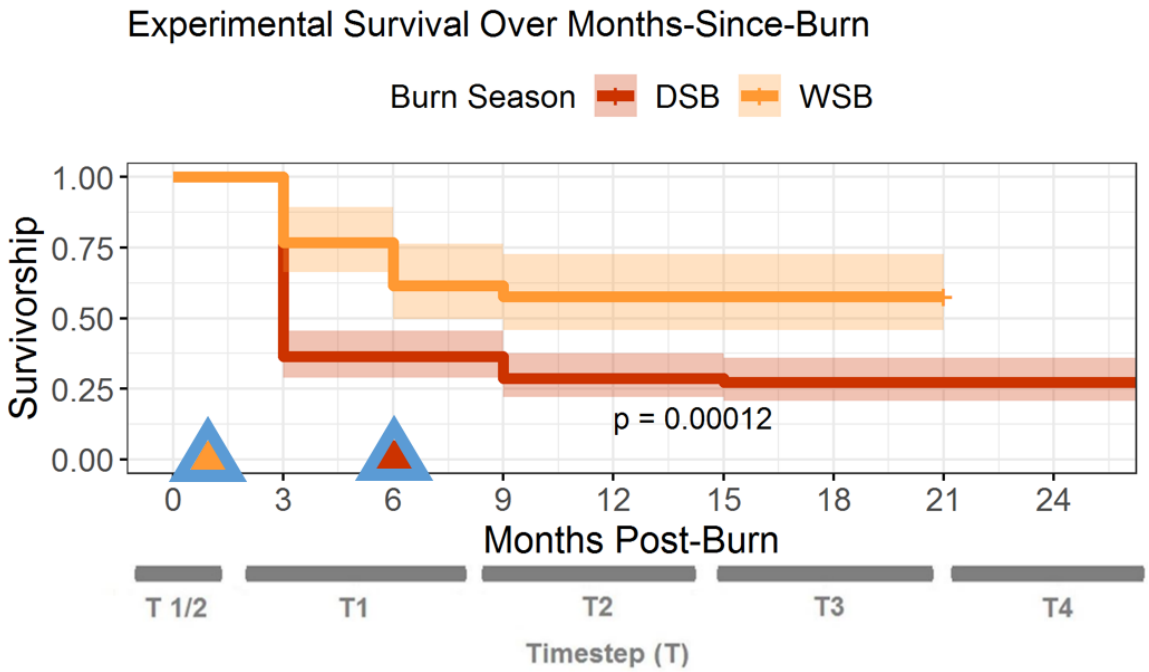

Figure 2.32a. Mortality following February and August burns, aligned by months-since-burn, timesteps (T) denoted below Months Post-Burn. Note, Hurricane Irma occurred at 1-month post-August burn (WSB, blue triangle outline, yellow fill), but this was 7 months post-Feb burn (DSB, blue triangle outline, red fill). Mortality for DSB nd WSB were significantly different.

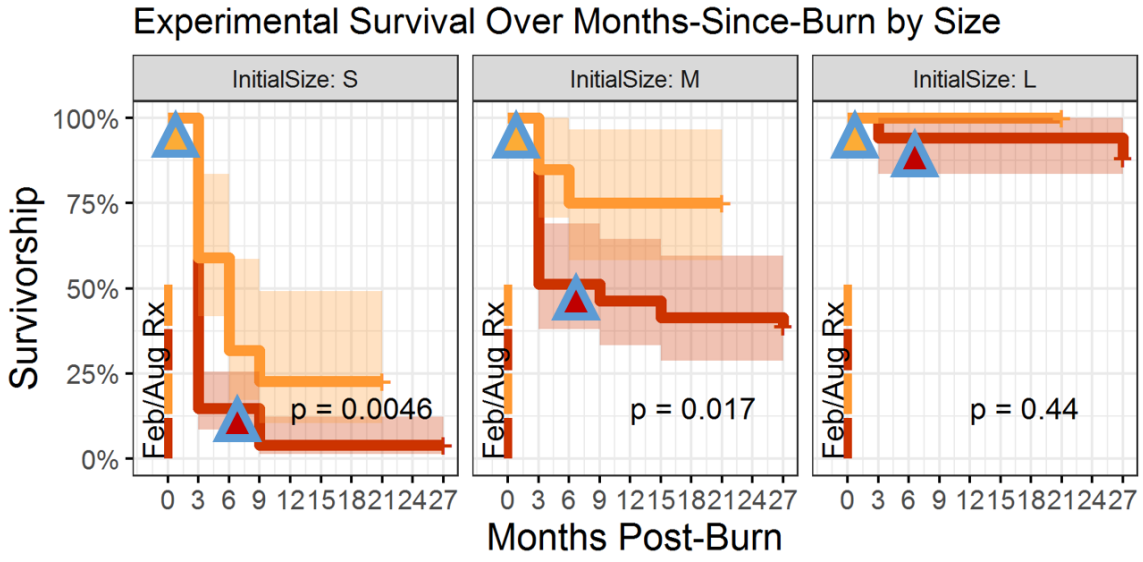

Figure 2.32b. Mortality following February (DSB, red line) and August (WSB, yellow line) burns, aligned by months-since-burn. Note, Hurricane Irma occurred at 1-month post-August burn (WSB, blue triangle outline, yellow fill), but this was 7 months post-Feb burn (DSB, blue triangle outline, red fill).

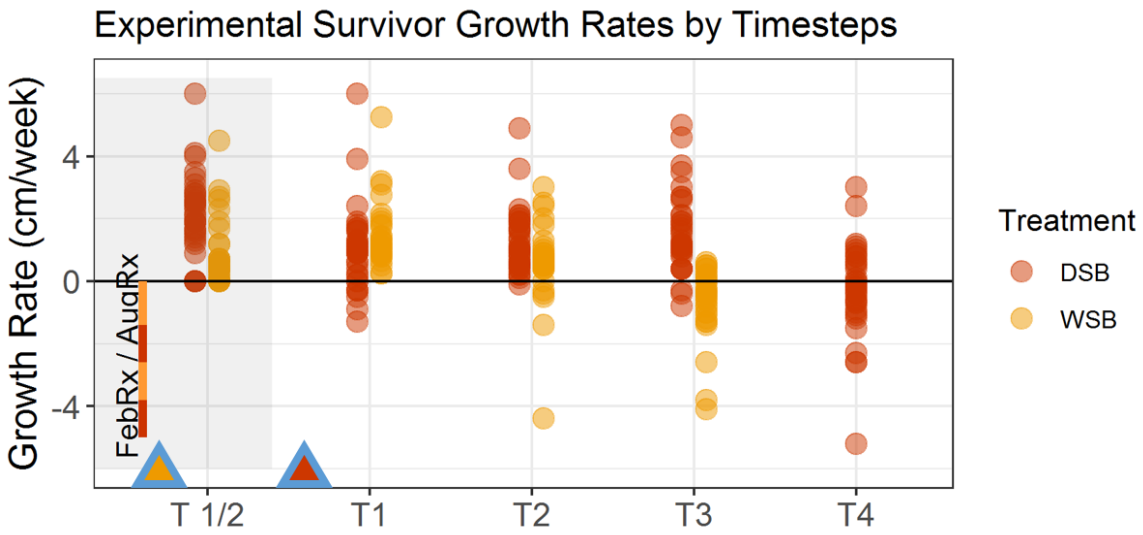

Figure 2.33a. Burned survivors: DSB and WSB experimental plant growth rates by timestep (T). Vertical lines (FebRx/AugRx) indicate fires, Irma occurred in T1 for DSB only (blue triangle outline, red fill)), but in T½ for WSB, immediately following the burn (blue triangle outline, yellow fill).

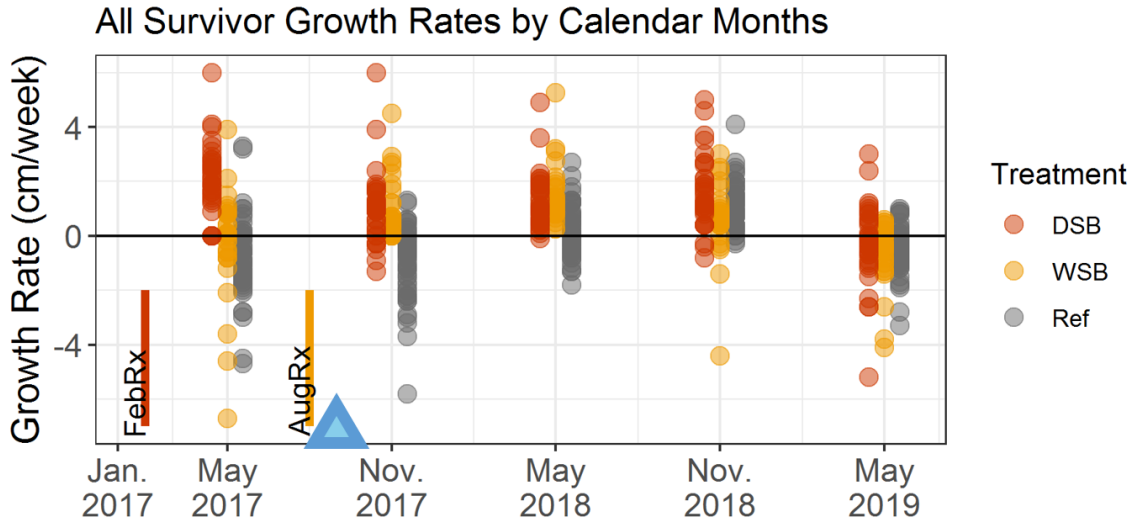

Figure 2.33b. Growth rates of all treatment groups over the study, by actual calendar months. Red points are DSB plant growth rates, yellow points are WSB, and gray points are references. Vertical lines indicate fires (FebRx, DSB (CSB and CSC), red; and AugRx, WSB (CSA), yellow). The blue triangle (at September) indicates hurricane Irma. Differences in growth rates between treatments disappear by the final inter-survey time period ending in May 2019.

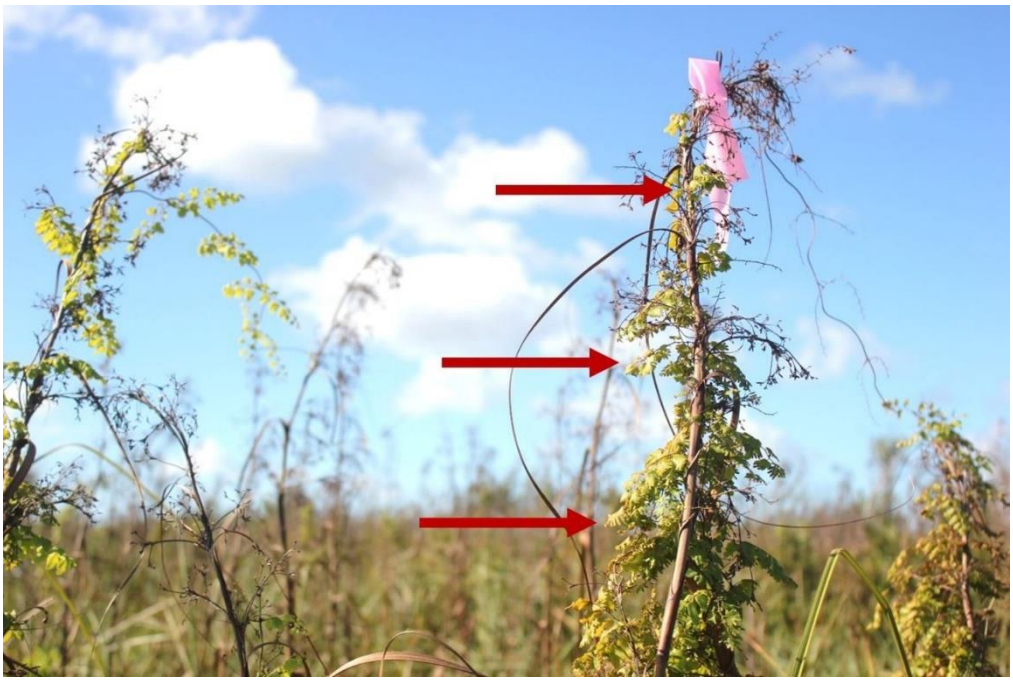

Figure 2.34. Sawgrass inflorescence stalks towering over sawgrass leaves, with *L. microphyllum* fronds overtopping them at CSC (burned). Red arrows indicate fertile pinna.

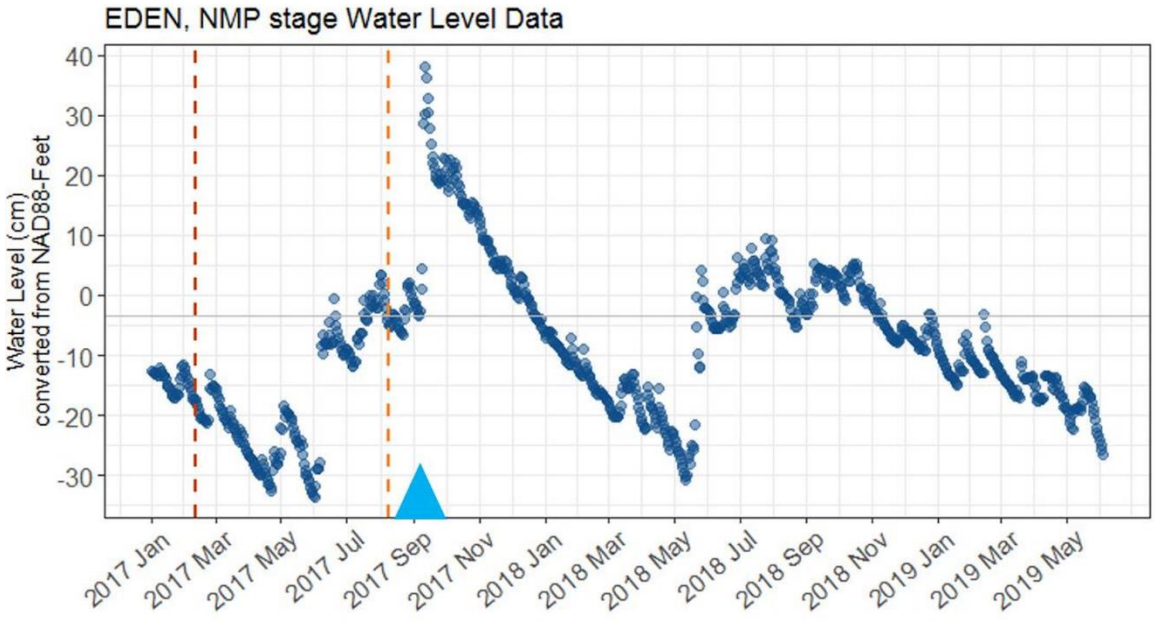

Figure 2.35. Water level recorded by EDEN's NMP stage, 8 km NE from the reference sites. Red vertical line in Feb 2017 marks the dry season burn, yellow line marks the wet season burn (Aug. 2017), blue triangle marks hurricane Irma. Source: EDEN NMP stage data downloaded from www.sofia.usgs.gov/eden. Accessed on April 13, 2020.

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Appendix

Species co-occurring within a 1 m radius of *L. microphyllum* plants followed in this study. CA, CB, and CC = Cape Sable sites, which were burned; HA, HB and HC = Hell's Bay sites, which were reference sites. "x" indicates that species was present during at least one survey. Scientific names follow the Institute for Systematic Botany's on-line "Atlas of Florida Plants" (https://florida.plantatlas.usf.edu/Default.aspx), accessed March 19, 2021.

	CA	**CB**	**CC**	**HA**	**HB**	**HC**
Acrostichum danaeifolium Langsd. & Fisch.	x	x	x		x	x
Ammania coccinea Rottb.	x					
Annona glabra L.			x			
Baccharis sp. c.f. *hamilifolia* L. / *glomeruliflora* Pers.						x
Blechnum serrulatum = *Telmatoblechnum serrulatum* (Rich.) Perrie et al.	x	x	x	x	x	x
Borrichia arborescens (L.) DC.			x			
Cladium jamaicense Crantz	x	x	x	x	x	x
Conocarpus erectus L.	x			x		
Eleocharis cellulosa Torr.	x					
Eleocharis interstincta (Vahl) Roem. & Schult.	x					
Erithalis fruticosa L.			x	x	x	x
Eupatorium capillifolium (Lam.) Small ex Porter & Britton		x				
Ficus aurea Nutt.					x	
Fimbristylis sp.	x					

Funastrum (= *Sarcostemma*) *clausum* (Jacq.) Schltr.						x
Helenium pinnatifidum (Schwein. ex Nutt.)Rydb.	x		x	x		
Hydrocotyle bonariensis Comm. ex Lam. / *umbellata* L.	x					
Ipomoea sagittata Poir.	x	x	x	x	x	
Juncus roemerianis Scheele	x	x			x	
Ludwigia curtissii Chapm.	x					
Lythrum lineare L.	x	x		x		
Mikania scandens (L.)Willd.	x				x	x
Morella (= Myrica) cerifera (L.) Small	x	x	x	x		x
Myrsine cubana (= floridana) A.DC.	x	x		x		
Osmunda regalis L. var. *spectabilis* (Willd.) A.Gray		x				
Pentalinon luteum (L.)B.F.Handsen & Wunderlin				x		
Parthenocissus quinquefolia (L.) Planch.		x		x		
Persea borbonia (L.) Spreng. var. *borbonia*		x	x			
Persicaria (= Polygonum) hydropiperoides (Michx.) Small	x					
Rhabdadenia biflora (Jacq.) Müll.Arg.					x	
Rhizophora mangle L.					x	
Schinus terebinthifolia Raddi				x	x	
Solidago sp. c.f. *semperviens L. / stricta* Aiton				x		
Spartina c.f. bakeri Merr.	x					
Teucrium canadense L.			x			
Toxicodendron radicans (L.) Kuntze			x	x	x	x
Typha domingensis Pers.	x		x		x	
Vitis rotundifolia Michx.			x	x		

CHAPTER III

III. *LYGODIUM MICROPHYLLUM* TOLERANCE TO SALINE CONDITIONS

Introduction

Lygodium microphyllum (Cav.) R. Br., native to subtropical and tropical areas of Austral-Asia and Africa, is typically found in wet or mesic areas, including coastal and subcoastal tree-swamps (Pemberton and Ferriter 1998; Joyce 2010). This fern is a major, invasive, nonnative plant in New World subtropical and tropical habitats, wreaking particular havoc in southern Florida (Volin et al., 2004). Where it is invasive, it alters native communities as its twining leaves grow up and cover woody species, shading out epiphytic and understory plants (Brandt and Black, 2001; Clark, 2002).

Several haplotypes of this species occur across the native range, and one of these—linked to Queensland, Australia—has expanded its range to include sub-tropical North America, where it has invaded a variety of Florida habitats, including cypress swamps, tree islands, pine flatwoods (Goolsby et al., 2006; Pemberton and Ferriter, 1998) and, more recently, freshwater marshes and more coastal habitats. While most of these habitats are freshwater, *L. microphyllum* can also be found in brackish coastal areas. It is unclear whether *L. microphyllum* invaded these brackish areas, or if it invaded when they were freshwater, and they later became salinized. Freshwater areas can become salinized by hurricane events or, as is increasingly common, climate change, sea level rise and salt water intrusion into the water table (Copeland, 1961; Gann, 2015; Roberts et al., 2008). With hurricane-induced changes in salinity, habitats may undergo a shift in species composition, as some plants tolerate or even benefit and increase from the salt pulse (e.g.,

Spartina cynosuroides, Juncus roemerianus) and others decline (e.g., *Eleocharis sp., Osmunda regalis*), depending on initial salinity and the magnitude of salinity increase (Childers et al., 2006; Li and Pennings, 2018; Roberts et al., 2008). Alternatively, species composition may also shift in response to desalinization by a freshwater pulse as a result of heavy rains associated with hurricanes (Sharpe, 2010; Zimmerman et al., 1996).

Although ferns are not usually thought of as halophytes, a number of salt-tolerant ferns exist along the salinity tolerance spectrum. These fern species vary from low-tolerance *Blechnum serrulatum* through the previously mentioned *A. danaeifolium* and its true halophytic congener, *A. aureum*, to the super-halophyte *Asplenium marinum*, which tolerates above-seawater levels of salinity on rocky coastal outcrops (Lloyd and Buckley, 1986; Pangua et al., 2009).

Salinity tolerance for *L. microphyllum* has not been determined but doing so is a management need (Hutchinson et al., 2006). This knowledge will help prioritize management decisions in brackish areas, where the type of treatment required to control this invasive species may differ from management strategies needed in freshwater sites. Furthermore, knowledge of *L. microphyllum*'s salinity tolerance can help focus surveillance efforts to areas within the tolerance range, rather than spending efforts where *L. microphyllum* is unlikely to occur.

While no data exist for *L. microphyllum* specifically, a few references to congeners suggest some salinity tolerance within the genus. In a survey of halophytes in China, two species of *Lygodium* are listed (but not specified) as found growing in at least 70 mM [~ 4 ppt] salinity (Kefu et al., 1995). Indirectly, the congeneric *L. flexuosum* is

reported to have some halophytic potential, as inferred from the species' success in a variety of coastal habitats (Gokhale et al., 2011); however, specific salinity tolerances for this species were not reported. If salinity tolerance for *L. microphyllum* may similarly be inferred, then its occurrence in freshwater to brackish marsh habitats suggests salinity tolerance to several ppt, similar to the unnamed species in the China halophyte survey (Kefu et al., 1995).

In the invaded coastal area, *L. microphyllum* is often found to co-occur with several halophytes, including giant leather fern (*Acrostichum danaeifolium*), white mangrove (*Laguncularia racemosa*), and buttonwood (*Conocarpus erectus*)—all with well-documented salt-tolerances—(Gu et al., 2019; Li and Ong, 1997; Lloyd and Buckley, 1986; Naseer et al., 2017; Sharpe, 2010; Sobrado and Ewe, 2006). Thus, *L. microphyllum* likely shares some level of salt tolerance with these species (Lockhart, 2007; Rayamajhi et al., 2014). Whether or not this tolerance, if legitimate, facilitates invasive spread is another question. The effect of salinity on the long-term survival of *L. microphyllum* is an important consideration with respect to management, as *L. microphyllum* is invading the western coastal prairies in Everglades National Park (ENP), FL, USA. These habitats are estuarine, so salinity could affect *L. microphyllum*'s growth (Lockhart, 2007), as well as its regrowth in response to management efforts such as burning (Chapter 2).

Understanding *L. microphyllum*'s response to salinity will help to interpret results of field experiments, such as those presented in Chap. 2, as well as to predict potentially invasible habitats. Thus, the goal of this study was to investigate growth of *Lygodium*

microphyllum sporophytes when exposed to different salinities under greenhouse conditions. The following hypotheses were tested:

HF_0: *L. microphyllum* sporophytes are unaffected by salinity as high as 10 ppt

HF_1: *L. microphyllum* sporophytes have reduced growth with increasing salinity

Additionally, to determine which saline environments support establishment of *L. microphyllum* from spores, germination success under different salinities was determined in a laboratory experiment, with the following hypotheses tested:

HG_0: *L. microphyllum* spore % germination is unaffected by increasing salinity

HG_1: *L. microphyllum* spore % germination is decreased with increasing salinity

Materials & Methods

Sporophyte Growth in Different Salinities

Lygodium microphyllum plants were grown in a specially constructed greenhouse inside the FIU Research greenhouse on the FIU Modesto Maidique campus (Richards et al. 2020). To isolate this invasive plant species, access to this greenhouse was limited to key personnel.

Dr. Ellen Lake (USDA–ARS Invasive Plant Research Laboratory, Ft. Lauderdale, FL) provided 105 young *L. microphyllum* sporophytes of uniform size (5 cm plugs) and age on November 2, 2018. These were acclimated to the FIU greenhouse conditions and then repotted to 20 d x 15 h cm plastic pots with Sun Gro Horticultural basic potting soil (Agawam, MA). These plants were grown until at least 90 plants reached 40 cm in height (approx. 6 weeks). The salinity experiment was conducted for three months, from December 15, 2018 through March 9, 2019. Three treatments of 0, 5 and 10 parts per

thousand (ppt) salt solutions were prepared using Instant Ocean® (IO) Sea Salt (Spectrum Brands, Inc., Blacksburg, VA) in tap water. Six liters of solution were added to each of 18 85-L plastic bins (Sterilite Corporation, Townsend, MA. USA); under these conditions, when the pots were present, the bottom 5 cm of the pots were submerged. The 90 plants were randomly assigned to each treatment, watered from above with the assigned treatment solution until water ran through the pots, and placed in the bins with 5 pots per bin. The 18 bins (n=6 bins per treatment) were arranged around the greenhouse, alternating treatments to avoid positional effects from light or air currents within the greenhouse (Figure 3.1). Bins were placed in groups of up to four bins to allow access to each plant; all groups had at least one bin of each treatment. Each bin was secured with polypropylene twine (#18, Everbilt, USA) around the midpoint circumference to prevent bowing of the bin. Solution levels were marked with labeling tape and arrows indicating the initial solution level so that these could be easily replenished after evapotranspiration. Salinities were measured 3 times per week with a salinity refractometer (VitalSine, Inc., Model SR-6, Dartmouth, Nova Scotia, Canada) and adjusted back to target treatment salinity levels when necessary. Tap water was added to bins whose solution levels were below the tape. Mosquito pellets (~1/4 tsp, Summit, Baltimore, MD) were added to each bin monthly to reduce mosquito reproduction. Temperatures in the greenhouse throughout the experiment were recorded hourly by i-button thermochron dataloggers (Maxim Integrated, San Jose, CA). Light levels were measured periodically using a quantum meter for photosynthetic photon flux in µmol m^{-2} s^{-1} (Apogee, Logan, UT). Light measurements were taken at the height of the central bulk of each plant.

Lygodium microphyllum grows by producing a series of climbing fronds from the rhizome, each twining up older fronds or other nearby supports. Leaves were trained on a length of vertical polypropylene twine suspended from a horizontal rope lattice attached to the top of the greenhouse walls (Figure 3.1). Initial leaves twined around the twine, while later leaves wrapped around pre-existing leaves and twine. Leaves were continually manually trained onto parent plants in order to keep individual plants separate. Growth of plants was monitored weekly by measuring leaf height as these ascended the support twine and counting the number of leaves at 50 cm, and qualitative observations were made. Height, defined as the height of the tallest green, living frond from its petiole base at the rhizome or soil surface, was measured to the nearest cm with a meter stick. Growth rates were calculated using heights. Qualitative data also recorded each week included subjective overall greenness or yellowing, whether salt was present on plant surfaces, and the occurrence of unusual growth.

After the final survey at 12 weeks, plants were harvested and processed to remove all soil and debris from the plants by successive washes in water. Plants were then stretched out on the lab table along a measuring tape to be photographed from roots to top (Figure 3.2a). Plants were separated into leaves, rhizomes, and roots, and allowed to drain. Fully separated plants were photographed again (Figure 3.2b-d), then the parts were placed in labeled paper bags and dried at 70°C until constant mass, weighed, and weights recorded. Dry weights (DW) were used to calculate whole-plant, leaf, rhizome, and root allocations, as well as root:shoot ratios. Shoot portions included the sum of leaf and rhizome portions. Figure 3.3 shows representative samples for plants from 5 and 0 ppt.

Spore Germination in Different Salinities

Spore germination was tested over a range of salinities that span those seen in the field surveys (Chapter 2). The treatments were 0, 5, 10, and 15 ppt salinity, prepared using Instant Ocean® Sea Salt (Spectrum Brands, Inc., Blacksburg, VA) in 100 mL distilled water. The solutions were mixed, salinity was verified with the salinity refractometer (VitalSine, Inc., Model SR-6, Dartmouth, Nova Scotia, Canada), and then solutions were autoclaved. Salinity was reverified after autoclaving and before sowing the spores.

Spores were germinated in the laboratory in petri dishes (Figure 3.4). After several preliminary trials, the experiment was conducted from November 20, 2019 through Dec. 18, 2019. Fifteen petri plates were prepared with 4 layers of 7 cm Whatman no. 1 filter paper (GE Healthcare UK Ltd., Buckinghamshire, UK). Five mL of treatment solution—either 0, 5, 10, or 15 ppt—were added to each petri dish. Three environmental control plates were prepared similarly, each with 5 mL of distilled water added to the filter paper along with two i-button thermochron dataloggers and one i-button hygrochron programmed to record temperature and humidity hourly. Fresh spores, collected from a *Lygodium microphyllum* population located in Flatford Swamp, Florida, were provided by the USDA-ARS Invasive Plant Research Laboratory, Ft. Lauderdale, Florida, and were applied to the surface filter using small filter wedges. Spores were not sterilized because doing so has been suggested to affect germination rates (Call et al., 2007). Filter wedges (cut previously, approx. 1x2 mm) were dampened by touching them to the wet filter paper, and then scooping into the spores. Spores were distributed across the wet filter paper by dragging the wedge back and forth across the

filter paper until the spores were no longer visibly clumped. Petri plates were sealed with 2-3 layers of Parafilm "M" (American Can Company, Greenwich, CT) and arranged randomly 20 cm below 6 LED grow lights (Monios L, T5 LED array, China). The photosynthetic photon flux, as measured with the quantum meter at the level of the petri dishes, was 138-165 μmol m^{-2} s^{-1}. The lights were put on a timer set to a 13/11 light/dark cycle.

Counts of germinated spores were performed under a compound light microscope at 100x magnification at 2 and 4 weeks after sowing. Plates were observed at 1 week, but no spores had germinated in any plate, which was consistent with previous work (Philippi and Richards, 2007; Sebesta et al., 2016). Germination was surveyed by moving along transects across the plate until at least 300 spores were counted in each plate, recording whether each spore encountered was germinated or ungerminated. Spores that had germinated were identified by emergence of a clear rhizoid or a green prothallus from a crack in the spore wall. Spores that were at an obstructed angle, dented, damaged or abnormally small and dark were not included in the counts.

Statistical Analysis

Sporophyte Growth in Different Salinities – Heights were used to analyze differences in sporophyte responses between treatments. Non-parametric Kruskal-Wallis tests (χ^2) in the R base package (R Core Team, 2020), followed by Dunn post-hoc (z) tests in the R rstatix package (Kassambara, 2020a), were used to test for differences among and between groups, as well as for differences between before/after conditions at the significance level of α=0.05. To examine differences in growth over time, heights were ln-transformed and regressed with a repeated-measures linear mixed model using

the lmer function in the lme4 package (Bates et al., 2015), with salinity and week as fixed effects, plant within week as random effects, and a restricted maximum likelihood estimator. Slopes were compared using lstrends, in the lsmeans package (Lenth, 2016). R-squared values were obtained with r.squaredGLMM in the MuMIn package (Barton, 2020). Test of Equal Proportions (R Core Team, 2020) was used for comparisons of mortality between treatments.

Spore Germination in Different Salinities – Since germination data are binomial with outcomes of either germinated or ungerminated, data were analyzed with a generalized linear model (GLM) assuming a binomial distribution and a logit link. Because no spores germinated in 15 ppt or the first week, data from these were excluded from the glm analyses. Models were evaluated with Akaike Information Criterion (AIC). Differences among mean germination proportions were compared with Scott-Knott clustering using the ScottKnott package in R (Jelihovschi et al., 2014; Scott and Knott, 1974). Data from all treatments, including the 15 ppt, were used in the clustering. Clustering was first on by-week germination means, and then on all germination means factored by the week-treatment combination.

All analyses were performed in R (v.4.0.0, R Core Team, 2020), and additional R packages used included xlsx (Dragulescu and Arendt, 2020) and dplyr (Wickham et al., 2020) for data importation and manipulation. For data formatting, manipulation, visualization, and presentation, the ggplot2 (Wickham et al., 2020) package was used, while summary statistics were obtained using describeBy from the psych package (Revelle, 2020). The package ggpubr (Kassambara, 2020b) was used for plot customization within ggplot2.

To compare the results from this experiment to results from the literature, concentration conversions are included (ppt or mM) in brackets following the form presented in a citation. For example, often salinity is presented as mM, and so is followed by an approximate conversion to ppt: "plants in 70 mM [~4 ppt] survived." When the salts are not specified, NaCl is assumed, as NaCl as the main component of seawater and of the IO mixture (Atkinson and Bingman, 1997).

Results

Sporophyte Growth in Different Salinities

Light levels in the greenhouse were 17% full sunlight (10-20%, most days ranging from115 – 350 µmol m^{-2} s^{-1}), and temperature highs ranged from 20 to 38°C. From December 2018 to March 2019, average daytime temperatures increased 3°C. Daylength at the beginning of the experiment was 10 hr 32 min and increased to 11 hr 52 min by the experiment's conclusion (www.timeanddate.com). Humidity, although not measured, was subjectively very high – always higher than that outside, which exceeded 80% every day during the experiment (www.wunderground.com).

Over the 12 weeks of the experiment, nearly all plants increased in height, but plants in 10 ppt increased significantly less than plants in either 0 or 5 ppt (KW, $\chi^2 = 34$, $p < 0.001$) (Figure 3.5, 3.6). Initial heights ranged from 41-84 cm with no differences between any treatments. For the first month, all plants in all treatments increased in height at similar rates, but by week 7, plants in 10 ppt lagged behind with mean heights of 91 ± 13 cm (Figure 3.5) and differed from the heights of plants in 0 and 5 ppt (Table 3.1). While mean heights of plants in 0 and 5 ppt continued to increase at the same rate

through the final week, those of plants in 10 ppt nearly plateaued (Table 3.1, Figure 3.5). Weekly growth rate over the first 6 weeks, calculated from the change in height per week, was between 5 and 7 cm/week for plants in 0 and 5 ppt, and remained similar throughout the experiment. Growth rates for plants in 10 ppt, however, differed by week 6, and further decreased to 1 ± 7 cm/week by the final week (Table 3.2, Figure 3.7).

In the repeated-measures linear mixed effects model, both week and salinity were significant, as was the interaction for week:salinity (lmer, $p < 0.001$, $R^2 = 0.94$). Slopes differed significantly between 10 ppt and both 0 and 5 ppt (contrast 10:0 and 10:5, both $p < 0.001$), whereas 0 and 5 ppt did not differ from each other (Figure 3.8).

Beginning in week 5, plants in saline treatments (especially those in 10 ppt) began showing signs of stress, including yellowing and browning pinnae and rachises, abscission of tertiary segments, and crozier death. All of these signs worsened during later weeks. Salt deposits were observed on the leaf surfaces of 3 plants in 10 ppt in week 5, and on more plants in both 5 and 10 ppt as the experiment progressed.

During the final three weeks, plants in 10 ppt were dying back—their fronds turned brown and pinnae senesced—as new growth shifted noticeably to resprouting from the rhizomes (Figure 3.9). A drastic drop in height of these plants reflected this shift to resprouting. The new climbing leaves never surpassed 30 cm, and often died within a week or two of emerging. Two plants, both in 10 ppt, died by the final week; one is pictured in Figure 3.2b.

During the harvest, two plants were found to have 1 – 3 pinnae beginning to sporulate (Figure 3.10). Both plants were from 5 ppt treatments. These plants were located in different bins but were both situated in hotter, brighter areas of the greenhouse.

When whole-plant dry weights (DWs) were compared, all treatments differed (KW, $\chi^2 = 24.5$, $p < 0.001$), with plants in 5 ppt having the greatest whole-plant DW and plants in 10 ppt the lowest whole-plant DW (Figure 3.11, Table 3.3). Leaf DW was highest for plants in 5 ppt, differing significantly from both 0 and 10 ppt plants (KW, $\chi^2 = 20.9$, $p < 0.001$). Rhizome DW was lowest for plants in 10 ppt and differed only between 10 ppt and the other treatments (KW, $\chi^2 = 47.4$, $p < 0.001$), but not between 0 and 5 ppt. Root DW, like rhizome DW, was lowest for 10 ppt, and differed significantly from the other treatments (KW, $\chi^2 = 37.9$, $p < 0.001$). Dry weight root:shoot ratios differed among all treatments, with the highest ratio of 0.7 for plants in 0 ppt, followed by those in 5 ppt, and then 10 ppt (KW, $\chi^2 = 53.2$, $p < 0.001$) (Figure 3.12, Table 3.3).

Although final week leaf heights did not differ between plants in 0 and 5 ppt, leaf DW was greater for plants in 5 ppt than for those in 0 ppt (Table 3.3). Resprouting may account for this, as plants in 5 ppt averaged more leaves at 50 cm than the other treatments, although 5 ppt plants only differed significantly from plants in 10 ppt (KW, $\chi^2 = 8$, $p < 0.05$, Table 3.3). Number of leaves at 50 cm varied by individual, with some plants consistently having 7 leaves at 50 cm, and others maintaining just 3. This was unrelated to treatment, and mean number of leaves at 50 cm generally did not differ among treatments other than in two weeks, including the final week (Table 3.4). Although there was as much, if not more, resprouting on plants in 10 ppt, these new climbing leaves never reached 50 cm in height. As the experiment progressed, the resprouts in 10 ppt were noticeably smaller and more numerous, failed to elongate, became chlorotic, and died before reaching 50 cm in height.

Spore Germination in Different Salinities

Temperatures in petri dishes averaged 24.3 ± 2°C (range 19.6 – 28.7°C). One of the three hygrometers failed, so relative humidity data relied on two dataloggers. Humidity in two control plates averaged 99.7% RH (range 93.7 – 100 % RH). Light levels reaching the plates were between 100 and 150 μmol m^{-2} s^{-1}.

Salinity inhibited both the onset and amount of spore germination. Spores in any salinity took longer to germinate than those in freshwater conditions (Table 3.5, Figure 3.13). Additionally, more germination had occurred in all treatments at 4 weeks versus 2 weeks except for those in 15 ppt (Table 3.5, Figure 3.13). The most appropriate GLM (determined using Akaike Information Criterion (AIC)) was the full model. Salinity and weeks of exposure, as well as their interactions, were significant (logistic regression, $p <$ 0.0001). The model predicted that germination success was a function of salinity level (0, 5 or 10 ppt) and weeks of exposure (2 or 4 weeks). When germination proportions were analyzed by the week, the Scott-Knott algorithm separated the treatments into two groups: one for 0 ppt, and one for all the saline treatments. These same clusters were found for both 2-week and 4-week germination (Figure 3.14). When all week-treatment combinations were analyzed together, the Scott-Knott algorithm separated them into three groups: 0 pp at 4 weeks; 0 pp at 2 weeks and 5 ppt at 4 weeks; and the remaining treatment combinations (Figure 3.15, Table 3.5).

At 2 weeks, all gametophytes were small and not yet cordate-shaped. Several of the spores germinating in 5 ppt only had rhizoids and no green prothallus. The three germinating spores in 10 ppt were only just beginning to show an emerging prothallus, and no rhizoids were visible. Mold was present in all the plates except the controls

containing only dataloggers, so presumably the mold arrived on the spores. Fungal colonies were counted and ranged from 5 to 40 per plate, but numbers did not differ among treatments. Although not quantified, spores within pink patches of mold did not germinate and many appeared pink, as if infected as well (Figure 16), but those in other-colored patches of mold did not appear affected either beneficially or detrimentally.

For spores in 0 ppt, approximately half of total germination at 4 weeks had already occurred at two weeks. However, for spores in both 5 and 10 ppt, only 11% and 2% had occurred by two weeks, respectively, with the remaining 89% and 98% occurring between 2 and 4 weeks, suggesting a delay in germination associated with salinity.

Discussion

Salinity affected both sporophyte growth and spore germination of *L. microphyllum*. As salinity increased, both growth and germination decreased. Thus, both null hypotheses are rejected, as increased salinity negatively affected both the growth of sporophytes and the germination of spores.

Plants range in their sensitivity to salt and can be coarsely grouped by their relative salinity tolerances into glycophytes and halophytes. Glycophytes survive only in freshwater conditions, but halophytes have mechanisms for mitigating salt effects to survive in a variety of saline habitats (Acosta-Motos et al., 2017). The most general upper threshold of salinity tolerance for glycophytes is between 100 and 200 mM NaCl [~ 6 - 12 ppt], while plants that can successfully complete their entire life cycle above this are considered halophytes (Flowers et al. 1986). Although *L. microphyllum* was able to survive at least two months in 10 ppt, reproduction was only observed on plants in 5 ppt, and the deteriorating condition of plants in 10 ppt suggests that it is not a holo-

halophyte (Acosta-Motos et al., 2017; Flowers et al., 1986; Pangua et al., 2009), though it is capable of surviving in brackish (1-2 ppt) to moderately saline (3-5 ppt) environments (Hillel, 2000).

Sporophyte Growth

L. microphyllum sporophyte growth was reduced with increased salinity, and effects began to become apparent after only weeks of exposure to low and medium salinities. Munns and Termaat (1986) suggest that in the short-term, plant growth in saline conditions is dependent on how well roots can avoid taking up salt, which strains water relations and reduces growth (Munns 2002). Plants in 10 ppt grew significantly less than plants in either 5 or 0 ppt, and later showed other typical signs of salt stress, including leaves that became chlorotic and senesced (Warwick and Bailey, 1998), which suggests that *L. microphyllum* is not excluding salt at these levels of salinity.

If plants cannot exclude salt at the roots or sequester or excrete it, long-term survival depends on having a rate of leaf expansion high enough to maintain low levels of ions despite their salt uptake (Munns and Termaat 1986). In this experiment, plants in both 5 and 10 ppt, accumulated salty exudates on the pinna segments, indicating salt uptake. One way to mitigate increasing salt concentrations in adult leaves is to produce new leaves (Munns 2002). In *L. microphyllum*, plants in saline water treatments shifted from adult climbing leaf elongation to resprouting new climbing leaves. The ability to shift to producing new leaves in response to salt stress may be what differentiates halophytes from glycophytes (Munns 2002). The resprouting in *L. microphyllum* was similar to that seen following other stressors, including in the field following burning (Chapter 2) and in the greenhouse following burning or clipping (Richards et al., 2020),

and may be a general response to stress. However, resprouting after clipping or burning was usually successful, unlike that in 10 ppt salinity. The inability to successfully expand leaves at 10 ppt indicates that *L. microphyllum* is intolerant to this level of salinity.

In this experiment, plants in 0 and 5 ppt grew at rates similar to those reported for *L. microphyllum* elsewhere (David and Lake, 2020; Volin, 2010). Although light conditions can affect growth rates of some plants (Medina et al., 1990), this was not likely an issue in the experiment, during which light levels were less than 20% of full sun and observed leaf expansion rates of 7 ± 4 cm/week (or ~1 cm/day) were on par with expected rates. Just as Volin (2010) found no differences between growth rates for *L. microphyllum* plants under three different levels of light (20, 50, and 70% full sun, [season not specified]), David and Lake (2020) also found no difference in growth rates between *L. microphyllum* plants in 51% shaded and full sun conditions—but this was only true during winter (December – January). During spring (May) and summer (June – August) experiments, shaded plants' growth rates were 45 and 38% faster, respectively, than growth rates of plants in full sun, with growth rates highest overall during spring (David and Lake, 2020). If this salinity experiment had occurred in the middle to late spring, we would expect the rates to be higher and, perhaps, to see even larger differences between the treatments.

In height, growth rate, and number of leaves at 50 cm, plants in 5 ppt were nearly indistinguishable from those in 0 ppt. Plants in 5 ppt differed from those in 0 ppt only in leaf dry weight. Sexual reproduction occurred only in two plants, both in 5 ppt; and like mortality in 10 ppt, was not statistically significant.

A possible factor in the success of plants in 5 ppt may be humidity. In the greenhouse, humidity was very high—consistently in excess of 80%—since outside ambient humidity reached at least 81% daily (www.wunderground.com historical records) and the greenhouse interior was always more humid. Despite relatively high temperatures (regularly above 35°C), which generally increases transpiration (Sobrado and Ewe, 2006), the increased humidity tends to decrease transpiration and subsequently increase relative growth rate (Salim, 1989). Over constant temperature, increased humidity can increase salinity tolerance, as documented in barley (*Hordeum vulgare*) following an increase in relative humidity (RH) from 45 to 90% (Hoffman and Jobes, 1978). Perhaps at the low (5 ppt) level of salinity, humidity was sufficiently high to ameliorate the mild salinity stress.

An additional variable in the experiment that may have affected plants in 5 ppt is the treatment solution itself, which was composed of tap water and the salt source in the experiment, Instant Ocean® (IO). Tap water in south Florida had negligible salt concentrations (0.031 ppt Na^+, and 0.002 ppt Cl^- ions (www.miamidade.gov, 2019). However, plants in saline treatments, particularly those in 5 ppt, may have benefitted from the salt source, IO, which is a synthetic mix of sodium chloride, magnesium chloride, sodium sulfate, calcium chloride, and potassium chloride in undisclosed proportions (www.carolina.com). Atkinson and Bingman (1997) compared eight artificial seawater products, including IO, and reported their elemental compositions in relation to seawater. Instant Ocean was consistently lower than seawater in most major cations (Na^+, K^+, Mg^{2+}, and Ca^{2+}), as well as major anions (Cl^-, SO_4^{2-}, and PO_4^{3-}) (Atkinson and Bingman, 1997). It is possible that the other components in the salt mix

provided a mild fertilizing effect. For example, IO ingredients $CaCl_2$ and KCl might mitigate Ca^{2+} and K^+ deficiencies resulting from ion toxicity relating to the buildup of Na^+ (Acosta-Motos et al., 2017). Such a fertilization effect could have contributed to the increased leaf growth and sporulation in 5 ppt compared to 0 ppt. These considerations may be particularly important, for example, when considering plant responses in severely nutrient-limited habitats such as the Florida Everglades, in which small additions of phosphorous can have striking effects, even mitigating salt stress in some cases (Wilson et al., 2019).

For plants in 10 ppt, however, high humidity and possible fertilization effects, if helpful, were not sufficient to overcome the salt stress. Both whole-plant dry weights and root:shoot ratios were significantly lower for *L. microphyllum* plants in 10 ppt than for those in 0 or 5 ppt. In glycophytes, salinity usually affects the aboveground portions first, increasing root:shoot ratios, but in halophytes the roots may be affected first (Flowers and Colmer, 2008). Similar effects observed in some brackish-water plants in response to elevated salinity (Janousek and Mayo, 2013; Wilson et al., 2019) further suggest that *L. microphyllum* has some—albeit limited—salt tolerance.

Although exposure to 10 ppt salt for 3 months appears to be very stressful for *L. microphyllum*, it may not exclude this species from mesohaline environments. The very salt-sensitive aquatic fern *Azolla pinnata* does not normally survive in above 30 mM (~1.8 ppt), but can be 'salt-hardened' by temporary exposure to sub-lethal salinity, resulting in salinity tolerance above normally lethal levels – up to 60 mM (~3.5 ppt) (Rai and Rai, 1999). Had *L. microphyllum* been exposed to 5 ppt for some number of weeks and then transferred to 10 ppt, perhaps its tolerance would have changed. It is unknown

if *L. microphyllum* can be 'primed' in this manner, but if so, this phenomenon could explain survival of temporarily salt-stressed plants in the field (Chapter 2). Alternatively, competition, a condition absent from the greenhouse experiment, may provide additional stress that would compound that of increased salinity, reducing overall salinity tolerance under field conditions. How these two possibilities would affect field survival is unknown.

Spore Germination

Spore germination rates in 0 ppt observed in this experiment were similar to those reported for *L. microphyllum* elsewhere (Call, Brandt, and DeAngelis 2007; Hutchinson 2010). Three factors to consider with respect to germination rates are spore genetics, spore age, and humidity. Because the spores used in the experiment were from a single population, the results of the salinity experiments may not reflect the true variability of *L. microphyllum* species. Spore viability decreases over time, but the degree to which this occurs depends on the species (Ballesteros et al., 2012). In *L. microphyllum*, spore viability begins decreasing after 5 months, although some germination may be possible after 7 years, depending on storage conditions (Ballesteros et al., 2012; Hutchinson, 2010; Mikuła et al., 2015). Hutchinson (2010) reported that *Lygodium microphyllum* spores as old as 4 years germinated at over 30%, while spores between the ages of 3.5 – 5 months germinated at 43%, the highest rate found in the experiment. The *L. microphyllum* spores used here were approximately 5 months old at the start of the experiment, but viability was comparable to previous experiments (Sebesta et al., 2016). Two-week germination of spores in 0 ppt was near the rates seen in Hutchinson's (2010) work, and rates were even higher than that by 4 weeks (the time provided in Hutchinson's

work). Higher germination rates could have resulted from their coming from different populations or growing in higher humidity. Relative humidity in the petri plates was extremely high (> 93%), which can also affect germination, and can even be the sole source of moisture for germinating spores as for *Cheilanthes feei*, a xerophytic fern (Nondorf et al., 2003). As high humidity can mitigate some of the effects of salinity for sporophytes (Salim, 1989), it may also be the case that high humidity may aid spore germination under saline conditions. Nonetheless, germination was high in the non-saline treatment and provided a background for comparison of germination rates for spores in saline treatments.

L. microphyllum spore germination decreased with increasing salinity and was entirely prevented at 15 ppt [257 mM], a common trend in salinity response found across other ferns. Even among halophytic ferns like *Asplenium marinum*, *Acrostichum danaeifolium* and *Acrostichum aureum*, whose spores can germinate in 10 ppt, 1.5% NaCl [15 ppt] and 2% NaCl [20 ppt], respectively, all have significantly higher germination rates in lower salinities (Lloyd and Buckley, 1986; Pangua et al., 2009). When salinity is lower, as after a rain, *A. marinum* spore germination, gametangia development, and fertilization are all more successful (Pangua et al., 2009). In the coastal marsh areas invaded by *L. microphyllum*, saline conditions vary both spatially and temporally, likely providing relief from salinity, and facilitating *L. microphyllum*'s successful germination. *Lygodium microphyllum* sporelings are often observed growing just above the water in many habitats (e.g., on cypress knees in some Everglades habitats (S Gandiaga et al., 2009; Lott et al., 2003) and sawgrass culms, where they are somewhat protected from elevated salinity during dry-down events (N. Sebesta, personal

observation). This elevated position of *L. microphyllum* on stumps or sawgrass culms may similarly benefit both gametophyte germination and later sporophyte development, as rainfall could flush out salt.

In addition to differences in total germination of *L. microphyllum* across the salinity treatments, rate of germination also differed between treatments. For spores in 0 ppt, nearly half of the total germination observed had occurred by 2 weeks, but this was not the case for spores in saline treatments. For spores in 5 ppt [86 mM] and 10 ppt [171 mM], less than 11% of the total germination observed had occurred by 2 weeks. The rest of the total germination observed in these two saline treatments (89% and 98%, respectively) occurred between 2 and 4 weeks, suggesting that germination was delayed by saline conditions. A salinity-associated delay was also documented in *Asplenium marinum*, for which germination was delayed 5 days for spores in both 156 mM [~10 ppt] and 330 mM [~20 ppt] compared to freshwater conditions (Pangua et al., 2009).

Germination was followed through 4 weeks of salinity exposure; however, gametophytes were not followed beyond this to determine whether they were able to develop into sporophytes in any of the treatments. Gametophytes that were developing in the saline treatments, especially at 10 ppt, were stunted compared to those growing in 0 ppt—similar to the smaller *Asplenium marinum* gametophytes observed at higher salinities (Pangua et al., 2009). *Lygodium microphyllum* sporophytes are tolerant of temporary stresses, including aboveground biomass removal (Hutchinson, 2010; Richards et al., 2020), and inundation (Philippi and Richards, 2007), and this may also be true of *Lygodium* gametophytes. If, after a period of salinity, the gametophytes had been transferred to freshwater conditions, it is possible that some would have survived, based

on previous observations (N. Sebesta, data not published) and similar trials with other ferns (Lloyd and Buckley, 1986; Pangua et al., 2009).

Beyond recovery, a temporary saline stress can stimulate either time-to-germination or prime spores to survive a more severe salt stress later (Li and Ong 1997; Pangua, Belmonte, and Pajarón 2009). In the case of *A. marinum*, spores germinated in half the freshwater-sown time following 6 weeks of exposure to 32 ppt, with nearly the same success rate as those sown directly into freshwater conditions (Pangua et al., 2009). When spores of *A. aureum* were germinated in either freshwater or weakly saline [2-5 ppt] conditions, only the latter were able to survive a 2-day exposure to high salinity [~30 ppt] (Li and Ong 1997). Of the spores that were "salt-hardened" by the incremental increase in salinity, 40% survived when returned to their original treatment condition (low salinity) (Li and Ong 1997). Subsequent survival likely depends on both the timing of exposure during development and the severity of the stress (e.g., the magnitude of increase in salinity or the duration of exposure). Though *L. microphyllum* germination was reduced at 5 and 10 ppt, it is not known if or how a transfer to freshwater conditions, an initial exposure to higher salinity, or an incremental increase in salinity would affect *L. microphyllum* germination and gametophyte survival. Within the context of spatially and temporally variable saline conditions found in the invaded areas of sawgrass marsh, early exposure to low levels of salinity—and subsequent tolerance to higher salinity later in development—is conceivable, as is recovery following a saline-related period of stress. However, based on field mortality of some *L. microphyllum* plants following hurricane Irma-related salinity increases (Chapter 2), the magnitude of change is likely an important aspect.

Summary

In these experiments, *L. microphyllum* sporophytes were able to grow and reproduce, and spores were able to germinate at near normal rates in up to 5 ppt salinity. Growth was diminished in 10 ppt, and germination reduced as well, suggesting that *L. microphyllum* has some salinity tolerance to between 5 and 10 ppt; this was also apparent in the field (Chap 2). Salinity of 15 ppt prevented germination. Although 15 ppt was not included as a treatment in the greenhouse sporophyte growth experiment, salinities between 8 and 13 ppt were associated with plant death in the field (Chap 2), but exact duration and severity of exposures are not known. While mild, temporary salt stress might have encouraged more growth and even reproduction in *L. microphyllum*, between 5 and 10 ppt the fern's tolerance decreased substantially.

Given *L. microphyllum'*s native habitats, this fern would not be expected to have much tolerance to such a stressor. Yet in the coastal freshwater marshes—which sometimes become saline—*L. microphyllum* can grow and reproduce. In the greenhouse experiments, competition was not a factor, but it would be in the field. If elevated saline conditions, even just those near 10 ppt, persist for several months, it is unlikely that *L. microphyllum* would thrive, given the reduced vigor observed in the greenhouse where there was no competition with which to contend.

Tables

Height	0 ppt	5 ppt	10 ppt	Kruskal-Wallis
Week 0 (Initial)	56 ± 12 [a]	59 ± 9 [a]	58 ± 9 [a]	*ns*
Week 7	104 ± 15 [a]	101 ± 14 [a]	91 ± 13 [b]	$\chi^2 = 12.1$, $p<0.01$
Week 12 (Final)	137 ± 30 [a]	132 ± 24 [a]	100 ± 27 [b]	$\chi^2 = 25.7$, $p<0.001$

Table 3.1. Mean ± sd for *L. microphyllum* leaf heights (cm) within each treatment for selected weeks. Superscripts indicate significant differences among salinities within a row; values with different letters are significantly different.

Growth Rate	0 ppt	5 ppt	10 ppt	Kruskal-Wallis
Week 1	7 ± 4 [a]	6 ± 3 [a]	6 ± 4 [a]	*ns*
Week 6	6 ± 3 [a]	5 ± 3 [a]	3 ± 3 [b]	$\chi^2 = 15.6$, $p<0.001$
Week 12 (Final)	6 ± 4 [a]	5 ± 4 [a]	1 ± 7 [b]	$\chi^2 = 19.7$, $p<0.001$

Table 3.2. Mean ± sd for *L. microphyllum* leaf growth rates (cm/week) within each treatment for selected weeks. Superscripts indicate significant differences among salinities within a row; values with different letters are significantly different.

Final Week (Week 12)	0 ppt	5 ppt	10 ppt	KW *p*-value
Height (cm)	137 ± 30 [a]	132 ± 24 [a]	100 ± 27 [b]	$p < 0.001$
# of Leaves at 50 cm	4.3 ±1.3 [ab]	5.1 ± 1.4 [a]	3.8 ± 1.9 [b]	$p < 0.05$
Total Dry Weight (g)	4.4 ± 1.4 [a]	5.2 ± 1.6 [b]	3.3 ± 1.1 [c]	$p < 0.05$
Leaf Dry Weight (g)	2.2 ± 0.8 [a]	3.3 ± 1.0 [b]	2.3 ± 0.8 [a]	$p < 0.001$
Rhizome Dry Weight (g)	0.4 ± 0.2 [a]	0.4 ± 0.1 [a]	0.1 ± 0.1 [b]	$p < 0.001$
Root Dry Weight (g)	1.8 ± 0.6 [a]	1.6 ± 0.7 [a]	0.8 ± 0.4 [b]	$p < 0.001$
Root:Shoot Ratio	0.7 ± 0.1 [a]	0.4 ± 0.1 [b]	0.4 ± 0.1 [c]	$p < 0.05$

Table 3.3. *Lygodium microphyllum* mean ± sd at week 12 for leaf variables, total dry weight, dry weight allocation to plant parts, and root:shoot ratios. Within a row, differing superscripts denote significantly different values obtained by Dunn post-hoc tests.

Trt	Wk0	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6
0 ppt	0.8 ± 0.8	1.3 ± 0.8	1.9 ± 1.1	2.6 ± 1.3	3.0 ± 1.2	3.3 ± 1.2	3.5 ± 1.2
5 ppt	1.2 ± 0.8	1.6 ± 0.9	2.2 ± 0.9	3.1 ± 1.1	3.3 ± 1.0	3.7 ± 1.4	3.9 ± 1.6
10 ppt	1.3 ± 0.7	1.6 ± 0.8	2.0 ± 0.9	2.4 ± 1.0	2.8 ± 1.0	3.2 ± 1.2	3.5 ± 1.3

Trt	Wk7	Wk8	Wk9	Wk10	Wk11	Wk12
0 ppt	3.5 ± 1.2	3.7 ± 1.1	3.8 ± 1.2	4.0 ± 1.1 [ab]	4.2 ± 1.2	4.3 ± 1.3 [ab]
5 ppt	4.0 ± 1.6	4.2 ± 1.5	4.5 ± 1.6	5.8 ± 1.4 [a]	4.9 ± 1.6	5.1 ± 1.4 [a]
10 ppt	3.6 ± 1.3	3.6 ± 1.3	3.9 ± 1.3	3.8 ± 1.4 [b]	3.9 ± 1.8	3.8 ± 1.9 [b]

Table 3.4. Mean number of *L. microphyllum* leaves at 50 cm per treatment over weeks. Grayed cells and superscripts indicate statistical differences between treatments (Kruskal-Wallis, $p < 0.05$).

Survey	0 ppt	5 ppt	10 ppt	15 ppt
Week 2	27 ± 6 % [b] (n=997)	3 ± 2 % [c] (n=1028)	0 ± 0 % [c] (n=1015)	0 ± 0 % [c] (n=1020)
Week 4	55 ± 7% [a] (n=1186)	24 ± 19% [b] (n=1310)	15 ± 11% [c] (n=1254)	0 ± 0 % [c] (n=1079)

Table 3.5. Mean ± sd germination rates of *L. microphyllum* spores at 2 and 4 weeks post-sowing on saline filter paper (N = 3 plates per treatment, n = total number of spores counted per treatment). Differing superscripts and colors denote different clusters within each week by Scott-Knott clustering ($p<0.05$).

Figures

Figure 3.1. Experimental set-up for *L. microphyllum* salinity experiment. Eighteen bins (not all pictured), each with 5 potted plants sitting in either 0, 5, or 10 ppt solution. Polypropylene twine suspended from a rope lattice supports each plant's twining growth. Several thermochrons suspended from the rope lattice recorded hourly temperature.

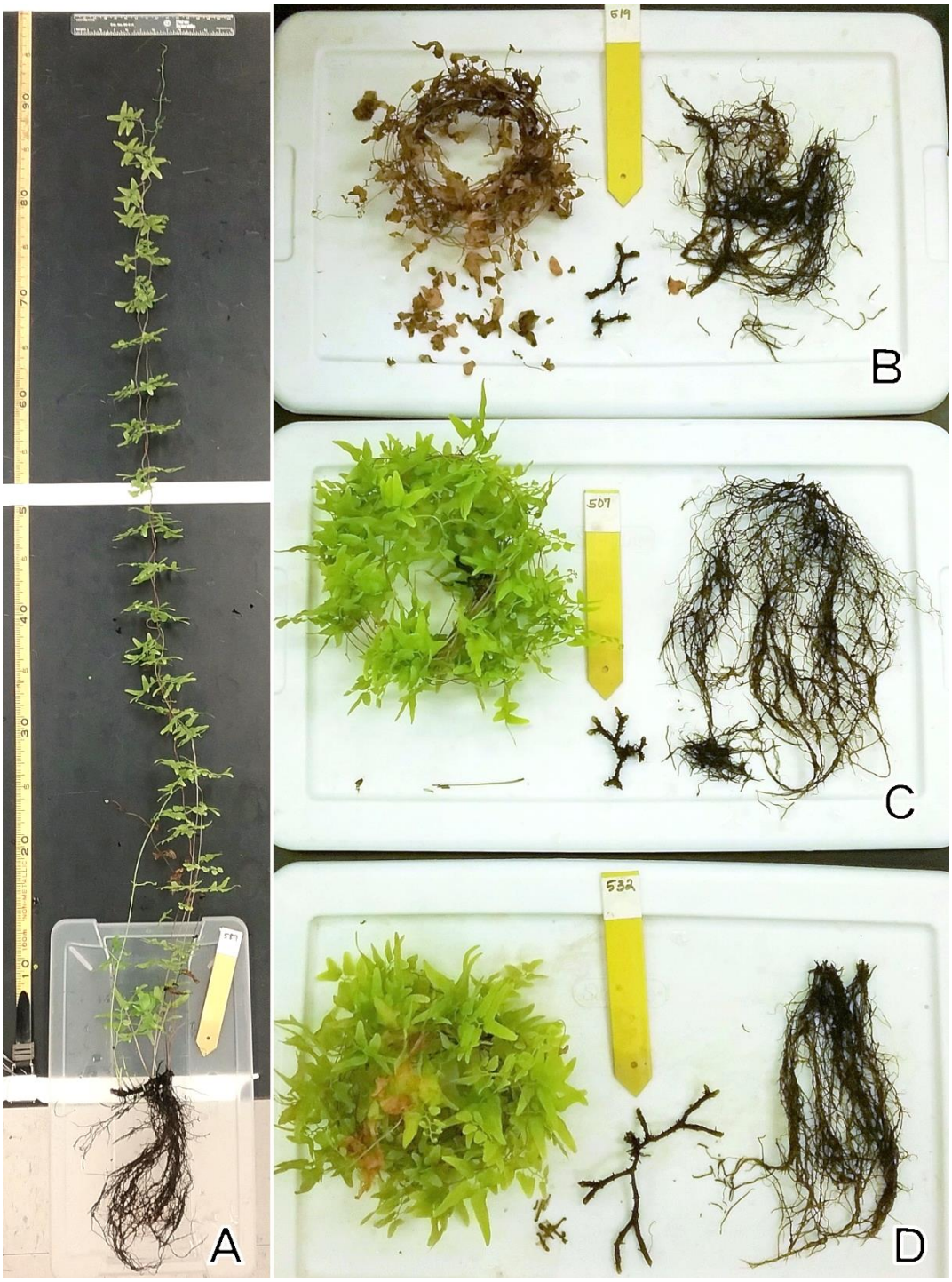

Figure 3.2. During harvest, each *L. microphyllum* plant was washed of soil and stretched out along a measuring tape (A). Once photographed, plants were separated into leaves, rhizomes, and roots for drying. All four examples are plants from 10 ppt, but the top right (B) is one of the two plants that died during the experiment. Plastic label is 11 cm long.

Figure 3.3. Representative samples of *L. microphyllum* plants divided into three parts: leaves, rhizomes, and roots. Upper image from 5 ppt (ID 566); lower image from 0 ppt (ID 503) plants. Plastic label is 11 cm long.

Figure 3.4. Experimental setup for *L. microphyllum* spore germination in response to salinity level. Environmental control plates at left, each with two thermochrons and one hygrochron but no spores. Petri plate columns 2-5 are different salinities at 0, 5, 10 and 15 ppt, respectively. Petri plates were randomly distributed under the lights.

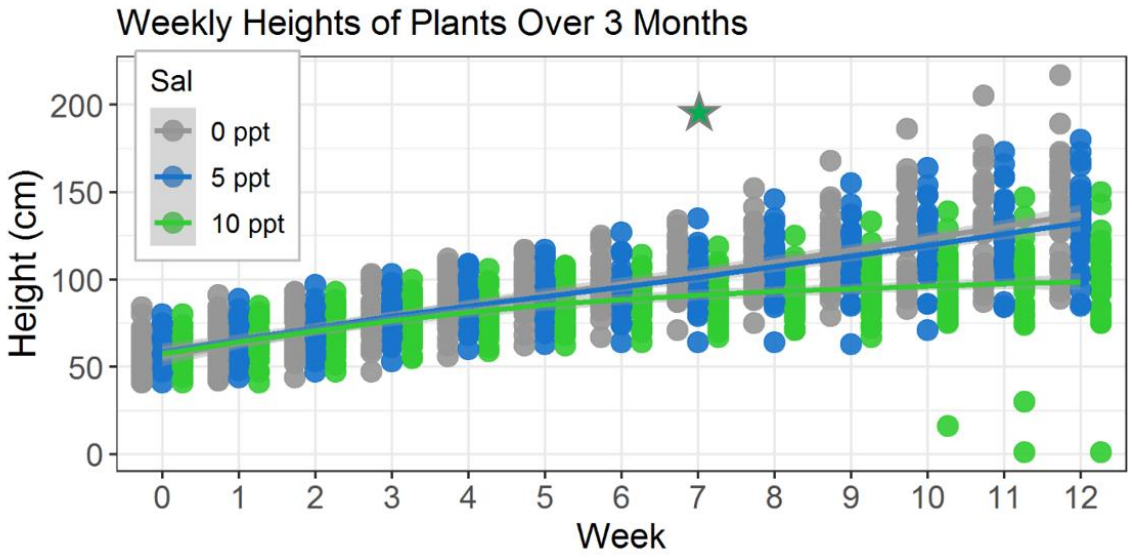

Figure 3.5. *Lygodium microphyllum* leaf heights at weekly surveys. Week 0 shows initial heights on 15 December 2018; week 12 shows final heights on 9 March 2019. Curves fitted curves with loess. Green star indicates first week of significant difference between 10 ppt and the other treatments.

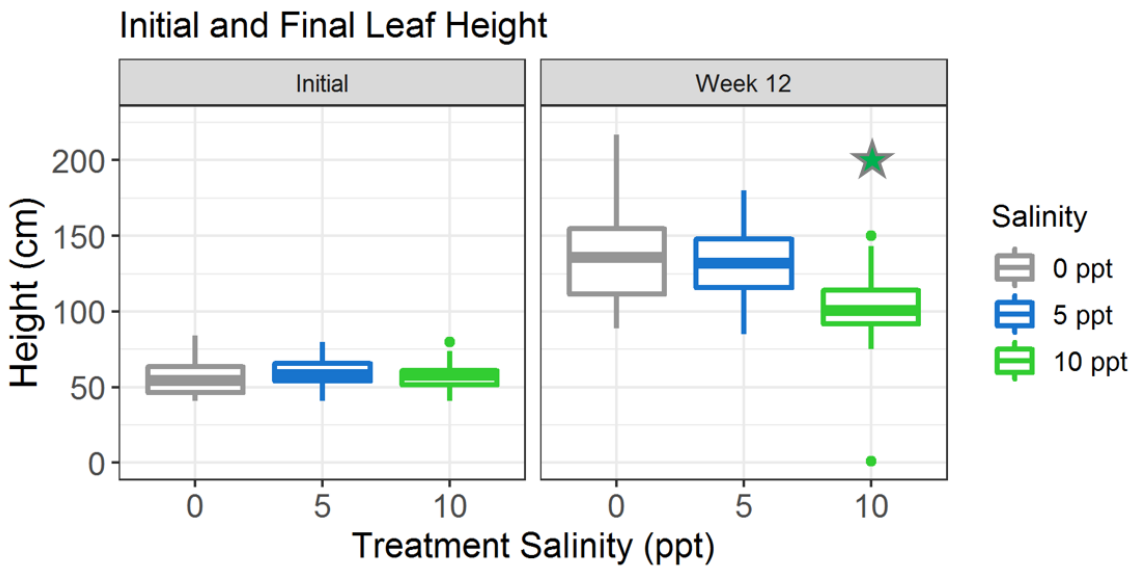

Figure 3.6. *Lygodium microphyllum* leaf heights by treatment initially (week 0) and in the final week (week 12). Green star indicates significant difference between 10 ppt and the other treatments.

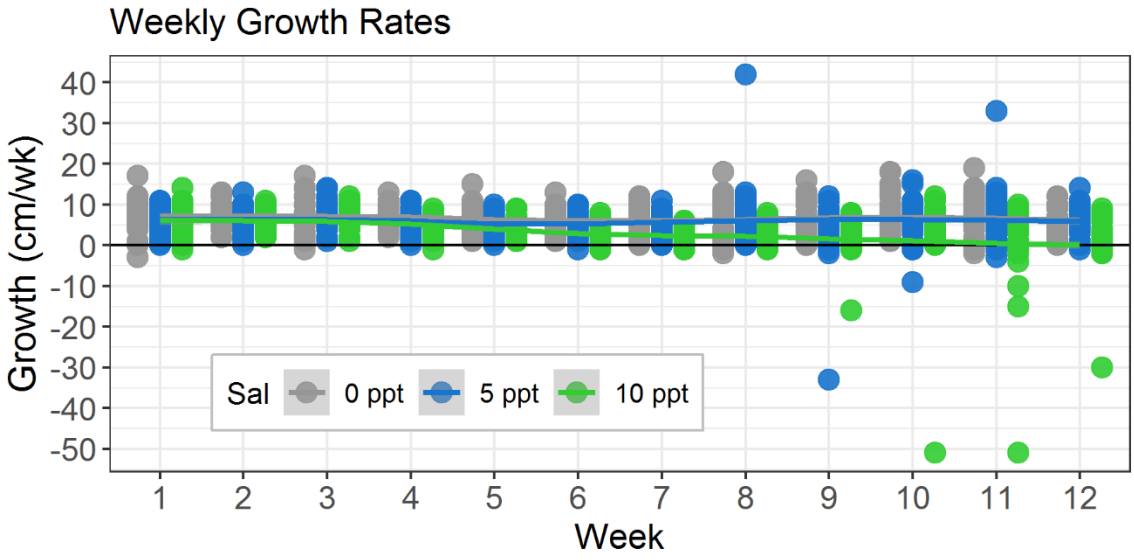

Figure 3.7. *Lygodium microphyllum* mean weekly leaf growth rates (cm/week), calculated from heigh changes per week over the study. Curves fitted with loess.

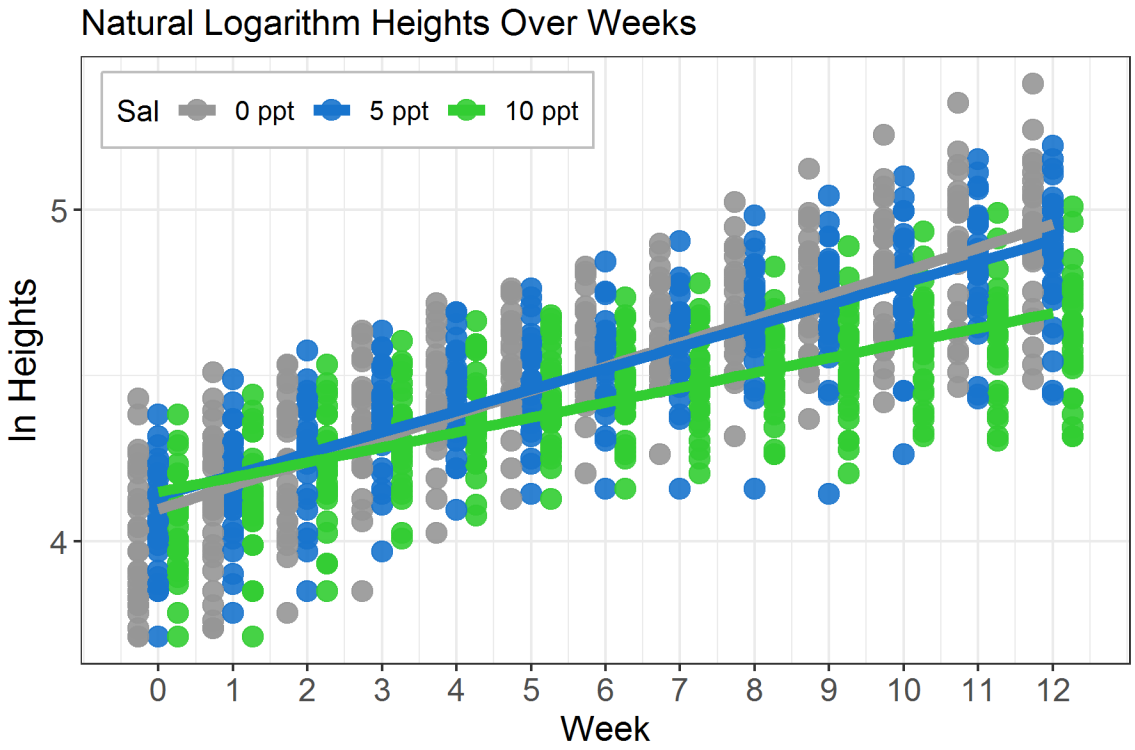

Figure 3.8. Regressions of *L. microphyllum* natural log-transformed leaf heights over weeks. Curves fitted with lm. R^2 for the model is 0.94.

Figure 3.9. *Lygodium microphyllum* resprouting leaves on a plant in 5 ppt (left, blue arrow indicates a resprout) and on a plant in 10 ppt treatment (right, green arrows indicate several of the failed resprouts).

Figure 3.10. *Lygodium microphyllum* developing fertile pinnule segments on one of two reproductive plants, both in 5 ppt. Arrow points to developing sporangia on the lobed margins of an ultimate segment.

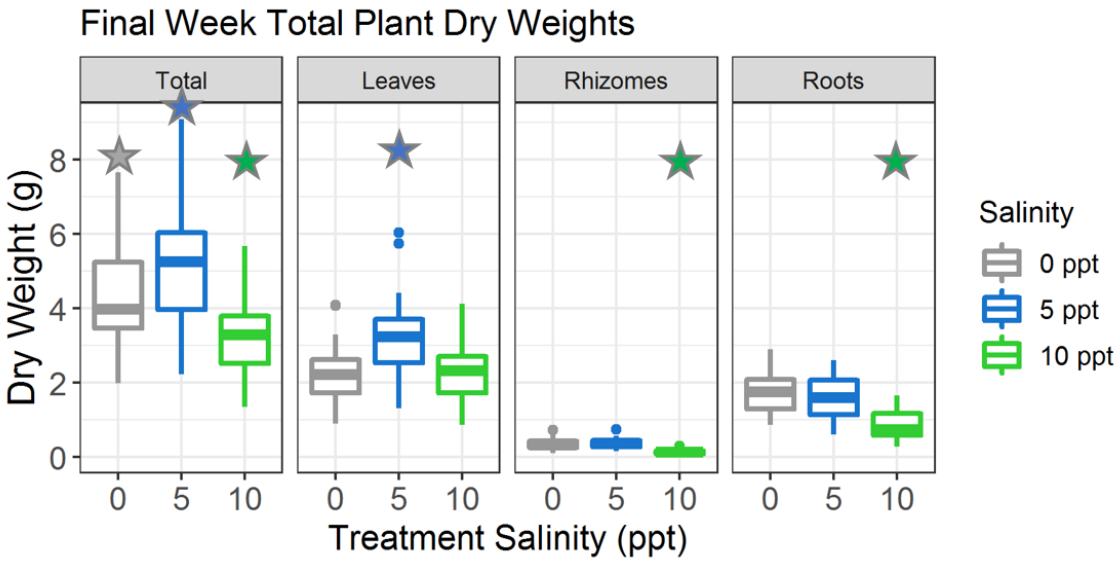

Figure 3.11. Dry weights for *L. microphyllum* whole plants (Total), leaves, rhizomes, and roots. Stars of each treatment color indicate that treatment differs significantly from both the others.

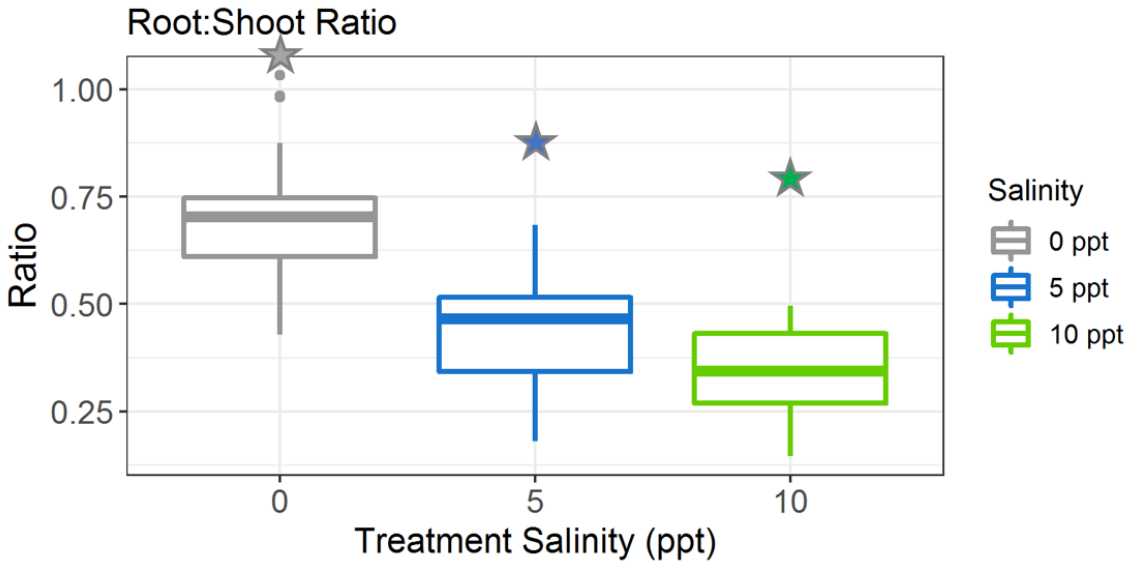

Figure 3.12. Dry weight root:shoot ratios for *L. microphyllum* plants at week 12. Stars of each treatment color indicate that treatment differs significantly from both the others.

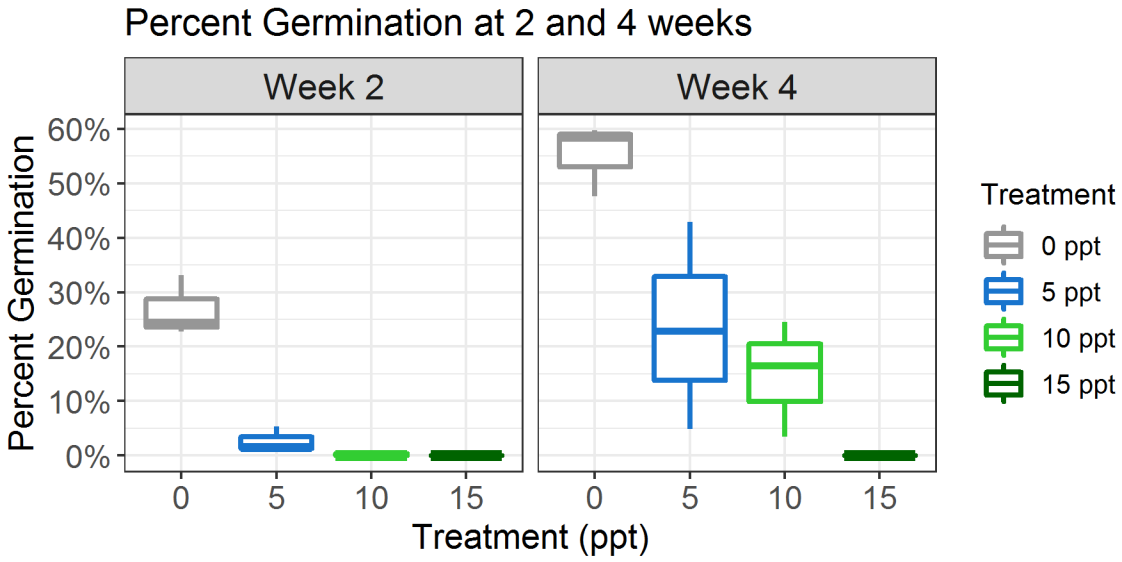

Figure 3.13. Percent germination of *L. microphyllum* spores in 0, 5, 10, 15 ppt at 2 and 4 weeks. N = 3 plates per treatment.

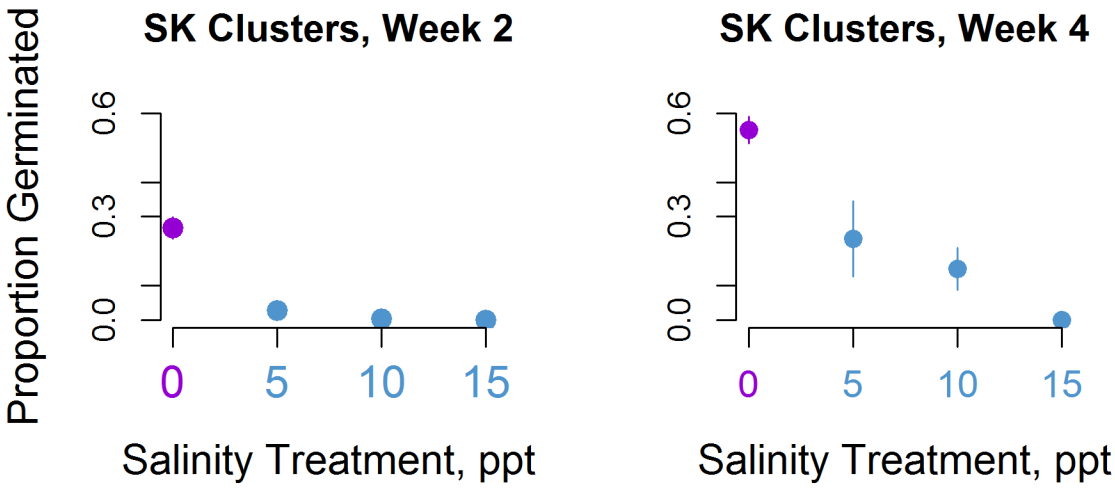

Figure 3.14. *Lygodium microphyllum* spore germination means grouped by color. Scott-Knott (SK) post-hoc test clustered mean germination for salinity treatments within a week into two non-overlapping groups: 0 ppt (purple), and all saline treatments (blue).

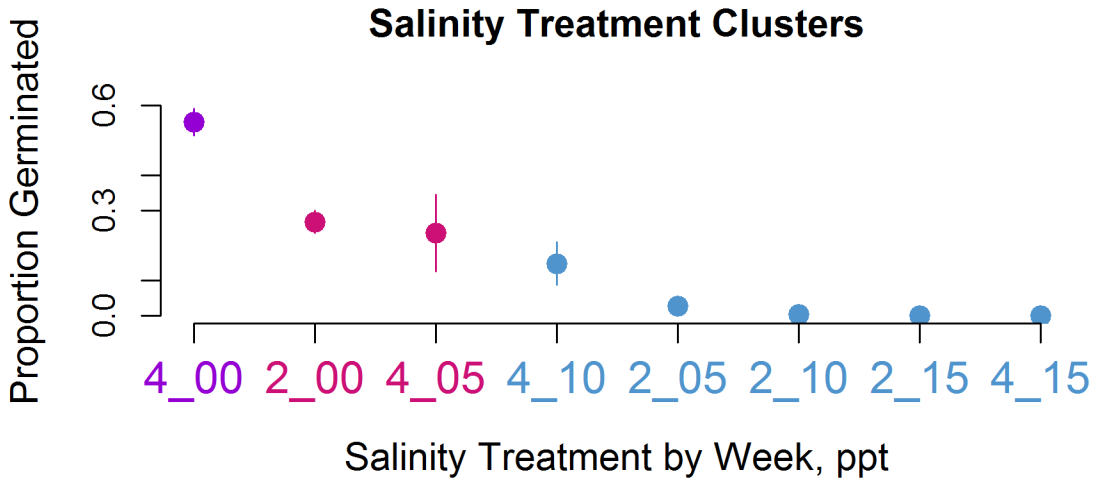

Figure 3.15. *Lygodium microphyllum* spore germination means grouped by color. Scott-Knott post-hoc test clustered treatments into three non-overlapping groups: 4 weeks at 0 ppt (purple), 2 weeks at 0 ppt and 4 weeks at 5 ppt (pink), and the rest of the treatments (blue).

Figure 3.16. *Lygodium microphyllum* spores (approx. 70 µm diameter) observed in patches of pink mold; some of the spores were pink, as if colonized by the mold (left). No germinations were observed on pink mold patches. Left photo, three pink spores; right photo, four normal spores with the left center spore germinating.

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CHAPTER IV

IV. FOLIAR NECTAR PRODUCTION IN SOME *LYGODIUM* SPECIES

Introduction

Over 4,000 angiosperms produce extrafloral nectar in addition to, or instead of, floral nectar (Weber, Porturas, and Keeler, 2015). While floral nectar is usually associated with pollination, extrafloral nectar is often associated with indirect defense against herbivory (De La Barrera and Nobel 2004; Heil 2008). More than three dozen fern species also produce extrafloral nectar (Weber, Porturas, and Keeler, 2015)—properly termed foliar nectar—and some of these have documented associations with one or more arthropods (Douglas, 1983; Koptur et al., 2013; Nepi et al., 2009; Tempel, 1983). Although nonmutual associations exist where the fern garners no benefit from ant presence (Tempel, 1983), foliar nectar can, in other cases, aid plant survival by attracting ants or other predatory insects that then defend the plant against herbivores (Agarwal et al., 2018; Heads, 1986; Nepi et al., 2018). Foliar nectar contains sugars and amino acids that supplement arthropod diets, resulting in repeated visits and patrolling of the sites of nectar drops, as well as the removal or killing of herbivores encountered during the visits (Bentley, 1977a; Blüthgen and Wesenberg, 2001). During previous experiments, several species of ants were observed regularly visiting and consuming nectar droplets on greenhouse-grown *L. microphyllum* plants. The discovery of foliar nectar on *L. microphyllum*

(Sebesta et al., 2018; Appendix A) was unexpected, as nectar production had never been described for any ferns in the Schizaeales.

The Schizaeales—a leptosporangiate group characterized by the arrangement of the annulus surrounding one end of the sporangium (Moran 2004)—includes Lygodiaceae and its sister group comprising Schizaeaceae and Anemiaceae (Schuettpelz et al., 2016). Lygodiaceae is monogeneric, with the single genus *Lygodium* containing at least 26 species, although some reports suggest as many as 40 (Groot and During, 2013; Zhang and Hanks, 2013). *Lygodium* species are pantropical, occurring in areas of Africa, Asia, Australia and the Americas (Wikström et al., 2002). The genus *Lygodium* is one of two groups of 'twining ferns'—the other, *Salpichlaena*, is in the Polypodiales (Moran 2004). Analogous to the stems of some twining angiosperm vines, *Lygodium* fronds (leaves) are indeterminate, and climb by twining (Mueller 1982; Mueller 1983) with the possible exception of *L. hians* E.Fournier (Page et al., 2014). This unusual morphology facilitates easy recognition but also problematic growth in some cases when species become invasive, e.g., the two species invading Florida: *L. microphyllum* (Cav.) R. Br. and *L. japonicum* (Thunb.) Sw. (Hutchinson and Langeland, 2010).

More research on nectar production has been done on angiosperms, many of which produce extrafloral nectar in the morning; however, some angiosperms exhibit continuous diurnal and nocturnal nectar production (Heil et al., 2000; Jones and Koptur, 2015; O'Dowd, 1979). A number of species have been found to produce extrafloral nectar in response to herbivory (damage) in amounts

dependent upon the timing of the damage (Jones and Koptur 2015). Foliar nectar production in ferns has been most often reported as occurring in the early morning (Koptur, Smith, and Baker 1982; Ruffner and Clark 1986; Heil et al. 2000), and generally on younger tissues (e.g., *Pteridium aquilinum*, Temple 1983). During and following herbivory (or mechanical damage), volatile organic compounds are released which can induce foliar (or extra-floral) nectar production in parts of the same plant as well as in neighboring plants via volatile signaling (Heil, 2011; Heil et al., 2001; Heil and Ton, 2008; Radhika et al., 2012). The plant hormone jasmonic acid (JA) is among those compounds released and is commonly used to experimentally induce nectar production in both angiosperms and ferns (Heil 2011).

Given *L. microphyllum*'s success in the invaded range, the prospect of nectar production as a defensive mechanism raised new questions about the fern's invasive ecology. If nectar production could be induced by herbivory damage, and protective ants were subsequently attracted, would this affect the role of biological controls in the management of the fern? Furthermore, as nectar production is a trait that is often conserved within a genus (Weber, Porturas, and Keeler 2015), might any of the more than two-dozen congeners of *L. microphyllum* also produce foliar nectar? This is an important consideration, as defensive mutualisms can have significant effects on invasive plant management efforts (Crider, 2011).

Following preliminary experiments (summarized in Appendix B) that suggested that nectar production in *L. microphyllum* was inducible, two experiments were designed to answer the following questions:

1. Do other *Lygodium* species produce nectar after mechanical damage, and if so, where on the plant body?
2. Do any *Lygodium* species produce nectar after exposure to volatile signaling (jasmonic acid)?
3. What sugars are present and in what concentrations in the foliar nectars?

Materials & Methods

Thirty established plants of six *Lygodium* species in 3- to 11-liter (1-3 gallon) pots were loaned by Drs. Ellen Lake, Aaron David, and Allen Dray (USDA ARS Invasive Plant Research Laboratory, Ft. Lauderdale, FL). Congeners were transported to the FIU greenhouse (and returned) under Allen Dray's transport permit. The species included the North American native, *L. palmatum* (Bernh.) Sw., as well as Florida-invasives *L. microphyllum* and *L. japonicum*, which are both Old World species (Figure 4.1). The three other species were two Central American / Caribbean (*L. venustum* Sw., *L. oligostachyum* (Willd.) Desv.) and one South American (*L. volubile* Sw.) species (Mehltreter 2006; Madeira, Pemberton, and Center 2008; NYBG; The Plant List) (Figure 4.1). Twenty-five of the 30 plants (n = 5 for each species) were randomly assigned to positions in the greenhouse to reduce bias from local variations in light and air flow. However, the five plants of *L. palmatum*, which is more sensitive to light, were grouped in the shadier center of the greenhouse.

Plants were watered daily, adding water directly onto the soil surface until run-through. All plants except *L. palmatum* were fertilized biweekly with 430 mL Miracle Grow solution (concentration 3.33 mg/L tap water, irrigated until run-through). On days with fertilization, this replaced that day's watering. *Lygodium palmatum* was not fertilized because USDA staff advised against it, as they found that fertilization damaged rather than benefited this species, so this species was watered as usual.

Temperatures were recorded hourly using 10 i-button dataloggers (Maxim Integrated, San Jose, CA) suspended at heights proximal to the greatest aerial biomass of plants. The i-buttons included seven thermochrons, recording temperature (°C), and three hygrochrons, recording temperature and relative humidity (%RH). Light levels were measured using a quantum meter for photosynthetic photon flux in µmol m^{-2} s^{-1} (Apogee, Logan, UT), placing the meter on top of the bulk of each plant.

All analyses were performed in R (v.4.0.0, R Core Team, 2020), and additional R packages used for data importation, formatting, manipulation, and summary statistics included xlsx (Dragulescu and Arendt, 2020), dplyr (Wickham et al., 2020) psych (Revelle, 2020) packages. For visualization, customization, and presentation, the ggplot2 (Wickham et al., 2020) and ggpubr (Kassambara, 2020) packages were used.

Nectar Production by *Lygodium* species

During preliminary observations made on *L. microphyllum*, nectar production was monitored across the entirety of each plant at both dawn and dusk,

but nectar was only observed during the mornings, and usually just on the younger fronds. Because of this, congener nectar production monitoring focused on mornings, but included two evening surveys to capture differences should any nectar production occur in the evening. During nectar production surveys, all individuals of each species were examined for nectar production, and, if present, the location of production was recorded. Terminology used to describe these locations on a pinna are illustrated in Figure 4.2. All leaf areas were monitored for nectar production.

Ant Presence vs. Nectar Presence

High ant presence is often associated with nectar production (Koptur, 1992a, 1992b), and during this experiment, many plants were observed to have ants. Ant presence/absence within the experimental area was recorded for all plants during all surveys. The correlation of ant presence/absence to nectar presence/absence was explored by looking at the distribution of four conditions of ants and nectar observations: ants and nectar, ants but no nectar, no ants but nectar, or no ants and no nectar. If the presence of ants is unrelated to the presence of nectar, the proportions of the four conditions would be expected to be similar. We used Chi-square tests for Independence (χ^2) in the R base package (R Core Team, 2020) to compare expected and observed instances of ants and nectar for each species.

Nectar Production After Mechanical Damage

To determine whether mechanical damage could induce nectar production, four individuals of each species were randomly selected to be damaged, while the

remaining individual served as an undamaged control. Plants were damaged by cutting the tips of ultimate segments with scissors to mimic chewing herbivore damage and to avoid reducing the areas of biomass previously associated with nectar production. Approximately 30% of leaflet material was removed from the experimental area of each plant (from 100 cm to the top). Given *L. japonicum*'s invasive status, immature sporulating pinnae required frequent additional trimming to prevent spore maturation and release by this species. Tanglefoot® (TF) insect barrier was applied to leaf rachises at approximately 1 m height and to support-strings (above the croziers) to exclude nectar-harvesting ants from the leaves. Damage and TF were applied concurrently because several species were so foliose that additional trimming of pinnae was required to eliminate leaflet bridges past the TF barrier.

For four days prior to the first damage application, all plants were monitored for baseline nectar production. Data recorded before and during the experiment included presence/absence of ants within the experimental area of each plant and presence/absence of nectar and of the droplets' locations on each individual of all six species. When present, nectar was collected for % sugar testing and stored for later constituent analysis. During the experiment, one or two species at a time were damaged at dawn on a Monday (Day 1). Damaged species were monitored for nectar production for the first hour after damage, and then assessed at 12, 24, 48, 72, 84, and 96 hours post-damage (Table 4.1); these 5 days are referred to as the "damage week." Morning damage was administered because in other studies, this timing elicited a larger extrafloral nectar response

than evening damage (Jones and Koptur, 2015). Morning monitoring occurred during the first two hours after dawn. Two evenings (12 and 84 hr) were included to sample evening production in the hour preceding dusk.

The damage schedule spanned one month from May 9 to June 8, 2019 (Table 4.2). Following the 4 days of initial observation, Week 1 (May 13) began by dawn application of Tanglefoot to all 5 *L. venustum* plants, immediately followed by mechanical damage to 4 individuals. Week 2 (May 20) began with damage to *L. volubile* (TF was applied the previous week). In Week 3 (May 27), two species, *L. japonicum* and *L. oligostachyum* had TF applied and were damaged. In Week 4 (June 3), *L. microphyllum* and *L. palmatum* had TF applied and were damaged.

Throughout the month, all individuals of all species were also monitored for non-damage nectar (ND), defined as nectar production occurring on any plant without damage. Any nectar observed in the 4 days preceding Week 1 damage was also recorded as ND nectar, as was any nectar encountered on weekends between damage weeks.

During each damage week, when the species of interest produced nectar, droplets were collected from each individual plant to obtain 1 µL for % sugar testing. Collections were made using 1 µL micropipettes (Drummond Scientific Company, USA), and expressed onto the stage of a pocket refractometer (wt./total wt. type, Bellingham & Stanley, Tunbridge Wells, UK) for % sugar measurement. To obtain sufficient nectar for subsequent analyses when nectar from one individual was insufficient, collections were made from additional individuals of

the same species (including the undamaged control) and pooled. The date, individual(s) from which the nectar sample was collected, and % sugar was recorded. If additional nectar was available, it was collected the same way but expressed into a glass vial containing 100 µL isopropyl for storage and later constituent analysis. Date, source individual(s), and volume of nectar deposited into the vial (to the 1/32nd µL and tallied over the day) were recorded. Vials were stored in a freezer at 17°C until constituent analyses could be conducted.

Nectar Production After by Jasmonic Acid Application

To investigate whether *Lygodium* species may be induced to produce nectar by volatile chemical signaling, a second experiment was done using the plant hormone jasmonic acid (JA). This experiment followed the mechanical damage experiment and used the same 30 individuals, which were allowed to reacclimate for one month, continuing the fertilizer schedule. A week before this experiment, all plants were repositioned (coiled upon themselves) to make space for growth on the twines, and fresh tanglefoot was applied to exclude ants. A JA treatment solution was prepared by dissolving pure jasmonic acid (Tokyo Chemical Company, CSA 221-682-41-3, Toshima, Kitu-ku, Tokyo, Japan) into isopropyl alcohol and then diluting to a 1 mM solution (Radhika *et al.* 2012). Just after dawn on July 5, 2019, two spray-pumps (approximately 2 mL) with the nozzle directed at the bulk of each plant were used to administer the JA solution to all 30 plants in the greenhouse (no unsprayed controls); treatment of all plants was completed within 2 minutes. All plants were monitored for nectar production for the first 30 minutes and then hourly for 3 hours. Following this, morning

monitoring continued for 4 additional days (Table 4.3). Nectar was collected for measurement of sugar concentration, as described above.

Nectar Concentration and Constituent Analysis

Simple % sugars obtained using the pocket refractometer were compared across species and production type (Damage/Non-Damage/JA) with Kruskal-Wallis tests. As sugar concentration measurements from refractometers include all sugars, sugar identification and ratios were determined using High Performance Liquid Chromatography (HPLC). Standards of fructose and glucose (Tokyo Chemical Industry Co., Japan) and sucrose (Acros Organics, USA) were prepared at seven concentrations (0, 2, 5, 10, 15, 20, and 35%) to encompass the range of % sugars measured via refractometer, which generally varied from 6 to 30%. These standards were used to construct calibration curves from which to calculate sugar concentrations in the samples.

Nectar samples were prepared for HPLC by evaporating off the original solvent (isopropyl) and then adding 75% ethanol to each vial, transferring through successive rinses to Eppendorf tubes (Eppendorf, Germany). These were then vacuum-centrifuged for 80 minutes at 40-46°C. Samples were standardized and diluted by a factor of 15, with 15 µL HPLC water added for every 1 µL of original nectar volume.

Sugar identification and specific concentrations were determined by Dr. Diego Salazar (Dept. of Biological Sciences, Florida International University). Sugars were analyzed with an Agilent 1100 HPLC system coupled with an Agilent DAD and a Sederex ELSD detector. The ULTRA Amino column from

Restek (USA) used was 150 mm long with an inner diameter of 4.6 mm and 3 µm particle size. No precolumn was used, and the main column was kept at 35°C. Samples were run using an isocratic program with a 92:8 acetonitrile:water mixture at a flow of 1.4 mL/min. The DAD detector was set to detect in the lower UV range (350 nm). The ELSD detector used nitrogen for the nebulizer at 3.4 bar, temperature of 80°C, and a gain of 6. Integration of the chromatograms was performed in Agilent Chemstation CDS.

Results

Daytime temperatures in the greenhouse, from May 9 to July 9, ranged from 30 to 45°C, with an average daily high of 38.8°C. Nighttime temperature never dipped below 23°C. Relative humidity ranged from 45% to 99% RH, with a mean of 82%, but humidity exceeded 90% daily. Over the two experiments, 24-hr temperatures increased only slightly (average daily high for first 6 days, 39.5°C and last 6 days, 43.6°C). Light levels were 25 – 114 µmol/m^2s (<15% full sun).

Foliar Nectar Production Patterns by *Lygodium* species

Four species produced foliar nectar during these experiments: *Lygodium microphyllum*, *L. volubile*, *L. oligostachyum*, and *L. japonicum*. Neither *L. palmatum* nor *L. venustum* produced obvious nectar at any point during the experiments.

Nectar was observed on the petiolules and pinna buds of all four nectar-producing species, but additional locations differed among species (Figure 4.3). On *L. microphyllum* (Figures 4.3, 4.4), nectar droplets appeared along the rachis,

primary and secondary petiolules, the pinnule-rachis, and as a single large droplet at the base of the tertiary segments, as described previously (Sebesta *et al.* 2018; see Appendix A). On *L. japonicum*, the droplets appeared on the petiolule and on the nearest portions of rachis, as well as along the pinnule-rachises and even in the distal angles of quaternary segments and segment stalks (Figures 4.3, 4.5, 4.6). In contrast to *L. microphyllum*, the most noticeable nectar droplets on *L. japonicum* appeared predominantly on the abaxial leaflet surface, along and between venation, as well as on both sterile and fertile quaternary segment stalks (Figures 4.5, 4.6). These droplets were smaller but more numerous than those seen on *L. microphyllum*. On *L. volubile*, nectar accumulated in comparatively large droplets along the petiolule and surrounding its larger pinna buds, as well as along a section of secondary petiolule, but not on the pinnule-rachis or the tertiary segments themselves (Figures 4.3, 4.7). On *L. oligostachyum*, nectar was observed in minute amounts on the petiolule, surrounding the pinna bud, as well as in the distal angles of the pinnule rachis (Figures 4.3, 4.8).

Ant Presence vs. Nectar Presence

The plants that had the highest ant presence before tanglefoot application (and few observations of nectar) produced the most observable amounts of nectar after tanglefoot application (because the ants were prevented from consuming it). Analysis of ant presence/absence vs. nectar presence/absence revealed significant differences for *L. japonicum* and *L. volubile*, (χ^2 = 51.2, and 7.3, respectively, $p <$ 0.01 both), suggesting a relationship between ant presence and nectar production. Although there were instances of ants and nectar for both *L. microphyllum* and *L.*

oligostachyum, the difference between expected and observed proportions were not significant, probably as a result of comparatively fewer observations. However, when ant-nectar incidences for all four nectar-producing species were combined, the observed distribution was again significantly different from expected ($\chi^2 = 32.5$, $p < 0.001$). The relative contribution to this difference of each condition was highest for those involving ants: the condition of ant presence but nectar absence and the condition of presence of both ants and nectar. However, ant presence was strongly positively correlated with nectar absence, contributing 30% to the difference between observed and expected values, while ant presence was most strongly, and negatively, correlated with nectar presence, contributing 56% to the difference. This relationship can be explained by the ants' consumption of the nectar.

The analysis above was not applicable for *L. venustum* and *L. palmatum*, as they had only very few incidences of ants, and no incidences of nectar.

Nectar Production After Mechanical Damage

Of each of the four nectar-producing species, at least one of the damaged individuals produced nectar following damage application (Figure 4.9 a-f, 4.10). No species produced nectar within the first 12 hours post damage, or during either of the evenings surveyed (Table 4.1). Nectar production was observed on one or more individuals of all four species the next morning (Day 2), between 24 and 26 hr after damage (within the first 1-2 hours of daylight). Depending on the species, nectar was observed on one additional day during the damage week (*L. oligostachyum*, Figure 4.9c) or on all days of the damage week: *L. japonicum*:

(4.9a), *L. microphyllum* (4.9b), *L. volubile* (4.9f). Nectar was also observed on one or more individuals of the four species beyond their respective damage weeks (Figure 4.9 a-c, f).

Two species did not produce obvious nectar following damage: *L. palmatum* (4.9d) and *L. venustum* (4.9e). *Lygodium palmatum* produced no nectar but on the final day of its damage week several disorganized droplets were observed on two pinnae segments. These were likely honeydew, as a few scale insects were also present on this species. *Lygodium venustum* also produced no obvious nectar at any point during the experiment; however, this species produced a sweet exudate from all wounds immediately after damage, which continued to collect for over an hour following damage (Table 4.1, Figure 4.11). This was interpreted as phloem exudate and was collected for sugar content analysis. None of the other 5 species produced such an exudate from their wounds.

Non-Damage Nectar Production

All plants were monitored daily for nectar production throughout the damage experiment, revealing that the four nectar-producing species also produced nectar without the application of direct damage. All undamaged control plants produced nectar during their species' damage week, and one or more individuals of each species produced nectar at times outside their respective damage weeks (Table 4.2, Figure 4.9 a-f). Although not quantified, damaged and control plants within a species produced visually similar amounts of nectar.

Since ants were present on all the nectar-producing species before the application of TF, it was difficult to assess the presence of nectar, especially when

ants were abundant. Nevertheless, sometimes it was possible to observe both ants and nectar and these instances were documented. Most notably, *L. japonicum*, *L. microphyllum,* and *L. volubile* produced some nectar during the initial observation days, following final rachis adjustments (to maintain separation) of plants but preceding any experimental damage (Table 4.2). Because of the timing of TF application, the observations of nectar production on undamaged plants occurred primarily after each species' damage week ended (because ants were then excluded). The only exception was *L. volubile,* to which TF was applied one week before mechanical damage (Figure 4.9f) and concurrent to the damage week for *L. venustum*. During this ant-free week, nectar was observed on all five undamaged *L. volubile* individuals on one or more days.

Nectar Production After Jasmonic Acid Application

Only the four species that produced nectar during the mechanical damage experiment also produced nectar following JA application (Table 4.3, Figure 4.12). *Lygodium microphyllum*, *L. volubile* and *L. japonicum* responded most strongly to the JA application, producing enough nectar to test sugar content, although insufficient amounts for collection for constituent analyses. Several individuals of these three species began producing nectar within the first 2 hours after JA application; additional individuals began producing nectar between 24 – 48 hours and continued for the duration of the experiment (through Day 5 (96 hr)). On *L. oligostachyum*, in contrast, nectar production was seen only on two individuals at 72 or 96 hours after JA application, and amounts were insufficient for % sugar measurement.

Timing of Nectar Production After Damage versus JA

Although both damage and jasmonic acid application were followed by the appearance of foliar nectar, the timing of nectar production differed between the two experiments (Figure 4.13). Following mechanical damage, no nectar production was observed in the first hour, nor at 12 hours post damage, but all 4 nectar-producing species were producing nectar by 24 hours post-damage. Except for *L. oligostachyum*, which produced very little nectar, nectar production continued every morning for at least three more days. Following JA application, in contrast, a few plants began producing nectar within the first hour, but by 3 hours post JA application, this nectar was drying up, and no more nectar was observed until the following morning. Three of the four species (not *L. oligostachyum*) were producing nectar again by 24 hours post-JA and continued to do so in the mornings for the next three days, as observed after damage (Figure 4.13). Foliar nectar was not observed on *L. oligostachyum* until Day 4, 72 hours after JA application (Figure 4.13), and then only on one of the five individuals (Figure 4.12).

For a subset of days after damage and JA application, enough nectar was collected for % sugar testing with the refractometer (Figure 4.14). Between 48 and 72 hours after both damage and JA, % sugars increased for both *L. microphyllum* and *L. japonicum*. While the % sugars seemed to stabilize for both species by 96 hr (Day 5) in the damage experiment, % sugars decreased in the JA experiment over the same time period (Figure 4.14).

Nectar Concentrations and Constituents

Total percent sugars obtained by refractometer from nectar samples during damage weeks, as non-damage nectar (ND), or following JA application did not differ statistically among types (damage, ND, or JA) within a species, or among species. During respective damage weeks, percent sugars for the four species averaged 15% to 24% and did not differ between species (Table 4.4). The wound secretion from *L. venustum* wounds had sugar content of 25%. Following JA application, enough nectar was produced for testing on *L. japonicum*, *L. microphyllum*, and *L. volubile*, which were 14%, 16%, and 20%, respectively; these did not differ from post-damage percentages. When all three types of collected nectar were pooled, total percent sugars averaged 15% for *L. japonicum*, 17% for *L. microphyllum*, 20% for *L. oligostachyum*, and 20% for *L. volubile* (Table 4.4).

Of the three sugars tested in HPLC analysis, glucose, fructose, and sucrose, only glucose was detected in any of the nectars (Table 4.5). None of these were detected in the nectar of *L. oligostachyum*. The wound secretions from *L. venustum* were also assayed, and in these, glucose was detected—as well as some larger, unidentified oligosaccharides. The glucose concentrations for each sample were plausible given the estimates obtained by the refractometer. Other sugars, although unidentified, were present in all samples and would have contributed to the overall % sugar estimates, as might have amino acids, if present. Mean glucose concentration for the pooled species tested was 10.8 ± 9.0%, but concentrations ranged from 2.4% to 28.3% and did not differ between

species (Table 4.5). *Lygodium volubile* nectar samples contained additional sugars that eluted both before and after the glucose, suggesting the possible presence of some 5-carbon sugars as well as some larger oligosaccharides. Although no glucose (or fructose or sucrose) was detected in the pooled sample for *L. oligostachyum*, some early peaks suggest the presence of 5-carbon sugars, though these were not identified.

Discussion

Lygodium microphyllum (Sebesta et al., 2018) and three additional *Lygodium* species (*L. japonicum, L. volubile,* and *L. oligostachyum*) produced foliar nectar, supporting the idea that these traits are shared among several of the members within the genus, as is the case for some other fern genera (Weber, Porturas, and Keeler 2015). In a molecular phylogeny of the genus (Madeira et al., 2008), *L. palmatum* is basal to the majority of the 14 species considered (and all 5 congeners in this study) (Figure 4.15). Of the other 5 species considered in the nectar experiments, *L. microphyllum* is sister to the remaining 4 species. *Lygodium japonicum* and *L. venustum* are more closely related to each other than to the other species, as are *L. volubile* and *L. oligostachyum* (Figure 4.15). Since nectar production is not found across the species in any obvious grouping, it may be that nectar production is an ancestral character in the genus and was lost for *L. palmatum* and *L. venustum*. Alternatively, nectar production could have been gained after the split from *L. palmatum* and lost by *L. venustum*. A third possibility is that the trait could have evolved multiple times in the genus. It is also possible that *L. palmatum* and/or *L. venustum* can produce foliar nectar but

require different conditions than those in the experiment. As there are no data on the remaining 20 species regarding nectar production, more work is needed to determine which other species of *Lygodium*, if any, produce foliar nectar.

The locations in which nectar was observed on *L. microphyllum* were consistent with those seen in previous experiments, and ants, when present, were observed visiting these same locations. This ant behavior was similar to that observed of ants defending *Pteridium aquilinum*, which spent 2-15 minutes visiting nectaries on each frond (Douglas, 1983). The *Lygodium* congeners exhibited nectar in similar locations to *L. microphyllum*, but also in some unexpected locations, with ants also visiting these species-specific locations (Figure 4.3). Ant presence provided some indication of which species might produce nectar, as their heaviest activity matched plants and locations where nectar was later seen after ant-exclusion. During the experiment, ants were observed collecting nectar from these same areas after barrier failures.

Ants were not expected to be found in such abundance, and because TF application was planned to coincide with damage application, four species (including *L. japonicum* and *L. oligostachyum*) had no TF ant barriers for the first two weeks of the experiment. During this time, only minute amounts of nectar were occasionally seen on these species, but the ants were ubiquitous, especially on *L. japonicum*. Tempel (1983) suggested that in *Pteridium aquilinum*, increased ant activity is seen on younger fronds, which also tend to produce more nectar, and this association has been used as evidence of nectar production in a number of studies (Koptur, 1992a, 1992b). Both preliminary studies and the full

experiment reported here showed similar results, and when these younger fronds (top 2-40 cm of rachis, newly expanding pinnae, and crozier) on *Lygodium microphyllum* were removed, both nectar production and associated ant activity were diminished (N. Sebesta, pers. obs.). When ants were present, their consumption of the nectar most often precluded observations of nectar. When ants were excluded, nectar observations could then be made. With over 85% of the difference between observed and expected incidence of nectar attributable to ant presence, it is likely that ant presence can be indicative of nectar production. The lack of obvious nectaries to suggest the production of nectar and of visible accumulation of nectar if ants are present could explain why nectar production had not previously been reported for *Lygodium* species.

It is possible that the few ants attracted to *L. venustum* wounds were purely opportunistic and would not necessarily play a defensive role, as is common in many forest tree species, for example, of Fagaceae (Staab et al., 2017). However, that the exudate on *L. venustum* continued for nearly half a day suggests that this might be a defensive strategy, and could attract a variety of defensive insects including ants and parasitoids (Blüthgen and Wesenberg, 2001; Naganuma and Hespenheide, 1988). In *Solanum dulcamara*, wounds leaked a nectar, and plants were consequently more protected from two herbivores by ants (Lortzing et al., 2016). Nectar production at these *S. dulcamara* wound sites was also JA inducible, and not associated with any nectar-producing structure (Lortzing et al., 2016). The wound exudate from *L. venustum* was 25% sugars, continued to collect for more than an hour, and was still present during the

evening assessment at 8 PM, whereas the 5 other *Lygodium* species sealed their wounds almost immediately. Exudate from wounds has been documented in *Lygodium* previously on *L. venustum* and *L. heterodoxum* at the cut site on adult frond stipes but these were not tested for sugars (Fisher et al., 1997). In our experiment, no additional exudate was observed in this species following JA application; however, by then the wounds were 4 weeks old and had healed over. Perhaps if JA were applied shortly after damage, exudate amount might change.

Sometimes guttation is confused for nectar production. Guttation via hydathodes is common among herbaceous plants in mesic environments and some plants release sugars this way (Goatley and Lewis, 1966). Hydathode exudates tend to leave behind a whitish residue when they evaporate, usually the result of calcium salts (Koptur et al., 1982); this was not observed on any of the *Lygodium* plants, on which only clear sticky dots were found, if anything at all. Additionally, hydathodes are typically located at the laminar margins in both fern and angiosperm examples (Cantamessa et al., 2016; Feild et al., 2005; Husby et al., 2011; Sperry, 1983), while the locations of nectar droplets on the four species of *Lygodium* were never on the pinna segment margins, but rather they occurred along more proximal regions of segment surfaces and along rachis/petiolules (Figure 4.3), supporting that observed exudates are not the result of guttation.

The amount of time between the application of mechanical damage or JA and the first observations of nectar production differed between stimuli. Following mechanical damage, all four nectar-producing species began doing so no sooner than by the next morning (24+ hours), consistent with similar

experiments inducing foliar nectar production via damage (Heil et al. 2001; Jones and Koptur 2015). In contrast to mechanical damage, following the application of jasmonic acid, nectar production was observed much more quickly in three of the species—within just 2 hours. Herbivory (or mechanical) damage is known to release volatile compounds, including JA, which diffuse to proximal tissues and neighboring plants, inducing nectar production (Heil and Ton 2008). The fact that nectar was induced by both mechanical damage and JA application suggests that the JA is ultimately responsible for inducing nectar production. However, following mechanical damage, the natural release and diffusion of JA to neighboring tissues or plants may take some time, resulting in a lag between the application of damage and nectar production. In contrast, by applying JA directly to the plant tissues, the time-lag associated with damage may be bypassed. This may partially explain the difference in timing of nectar production following the application of damage versus JA.

Volatile signaling could also explain the production of nectar by undamaged control and congener plants during the damage experiment (Farmer and Ryan 1990; Heil and Ton 2008; Baldwin 2010). For example, *L. volubile* was afforded one week with ants excluded (TF applied) before damage was applied, and during this week all five individuals were observed to produce nectar (Figure 4.9f). During this same week, *L. venustum* plants were damaged, perhaps releasing jasmonic acid and inducing *L. volubile* to produce nectar. However, another possibility is that *L. volubile* was producing nectar constitutively and was unaffected by volatile signaling. The presence of ants preceding damage on the

four nectar-producing species suggests that they were already producing nectar and ants were collecting it. To determine whether nectar production is constitutive or induced, a period of observation is needed during which ants are completely excluded. Quantification of nectar amounts produced during this damage- and ant-free time will provide the baseline (constitutive) production against which to compare nectar production following the application of mechanical damage or jasmonic acid (induced, if above constitutive rates). It is likely that certain *Lygodium* species produce some foliar nectar constitutively, and that this production may be upregulated by damage and/or jasmonic acid exposure. More data are needed to answer this question.

The % sugars measured in nectar produced after damage and JA seemed to change over the course of a week. While % sugars increased between 48 and 72 hours for both treatments, they appeared to stabilize in nectar post-damage, but decreased drastically for nectar post-JA (Figure 4.14). There are three possible explanations for these changes: First, changes in humidity (and temperature) can affect evaporation rates (or deliquescence), thereby concentrating or diluting the nectar. Humidity in the greenhouse was consistently very high, and temperatures were stable, but this remains a likely factor. Second, new nectar may be secreted into old nectar that had been evaporating from a previous morning, increasing the sugar concentration. Although possible, when any viscous-looking or dried droplets were encountered, these were excluded from sampling—but old nectar was only occasionally obvious on *L. japonicum*, so this accumulation over time is still a possibility, especially on species whose nectar dries inconspicuously.

However, the fact that sugars concentration decreased after the increase suggests that a buildup of nectar was not occurring. Third, a breakdown of sugars (e.g., microbial, chemical) may result in changing % sugar readings; for example, the breakdown of sucrose via hydrolysis into glucose and fructose (Heil, Rattke, and Boland 2005). In our analyses, glucose predominated, no fructose or sucrose was detected in any of the nectar samples, and % glucose did not change in a consistent direction among the samples collected. Additionally, nectar samples were collected shortly after nectar production, leaving little time for these processes to occur.

Percent sugars by refractometer in nectar ranged from 2 to 41% (mean 18%) during both damage and JA exposure experiments, similar to levels found in the foliar nectar of several species of *Polypodium* (18-54%, Koptur et al. 1982), and in the extra-floral nectar of some angiosperms, like two species of *Inga* (29-47%) and *Ferocactus acanthodes* (28-38%) (Koptur, 1984; Ruffner and Clark, 1986). The % sugar data from the refractometer only gives total sugars, and may be less accurate if there are large amounts of amino acids present, which can alter the refractive index of nectar (Inouye et al., 1980). Constituent analyses using HPLC were used to determine concentrations of specific sugars. The HPLC data revealed that the *Lygodium* spp. foliar nectars were sucrose-poor, as noted in most of the *Polypodium* species analyzed by Koptur, Smith and Baker (1982), *Pteridium aquilinum* (Lawton and Heads, 1984), and some angiosperm extrafloral nectars (Butler Jr et al., 1972; Douglas, 1983). Fern foliar nectar, similar to defensive extrafloral nectar of angiosperms, can be expected to have similar

constituents that could attract/support defenders, most commonly ants (Bentley, 1977b; Douglas, 1983; Nepi et al., 2009). For example, *Pteridium aquilinum*, with glucose-rich axillary nectar during frond expansion, was defended by *Camponotus pennsylvanicus* and two *Formica* species against both herbivores and nectar-attracted arthropods (Douglas, 1983). Most of the *Lygodium* nectars analyzed contained mainly glucose, but since ants were consistently attracted, it is possible that the nectars contain other constituents, as well. Further work is needed to identify the additional oligosaccharides and other constituents of *Lygodium* foliar nectars.

The production of nectar on both invasive exotic *Lygodium* species in Florida is concerning in light of management efforts utilizing biological control agents. While there are still no approved biocontrol agents in use for *L. japonicum* (Minogue et al., 2019), two—a moth and a mite—are in current use for *L. microphyllum*. Although ants may not interfere with the tiny leaf-galling mites, some ants (e.g., *Pseudomyrmex gracilis* Fabricius) have been documented removing moth larvae (pers. obs. Dr. Ellen Lake, USDA-ARS Invasive Plant Research Laboratory, Davie, FL) during releases. Similar interference has been documented by Crider (2011) for a *Senecio jacobaea* biological control agent, *Tyria jacobaeae*, whose presence was reduced by several species of *Camponotus* ants under certain site conditions. *Austromusotima camptozonale*, a previous biocontrol agent used on *L. microphyllum*, failed in part as a result of predation by *Pseudomyrmex gracilis* in the field sites (Boughton and Pemberton, 2008; Rayamajhi et al., 2014). Another biological control agent under review for

Lygodium is a sawfly, and this group may also be sensitive to ant presence, as they have been documented as having reduced oviposition rates on bracken fronds in the mere presence of ants (Jones and Paine 2012). Furthermore, invasive and native populations of plants can differ in their defensive nectar production, particularly when subjected to specialist herbivores (Wang et al., 2013), which in some cases elicit more extrafloral nectar than generalist herbivores. This may be problematic because specialist herbivores are typically preferred for biological control agents, as they pose lower risk to native congeners or other related species (Müller-Schärer and Schaffner, 2008). Higher production of defensive nectar could inhibit biocontrol efforts by attracting predators that may dispatch the biological control agents.

Tables

Species	Pre-damage Nectar/Ants	Date of damage	Damage Week							Week Mean % Sugar
			Day 1 AM	Day 1 PM	Day 2 AM	Day 3 AM	Day 4 AM	Day 4 PM	Day 5 AM	
			1 hr	12 hr	24 hr	48 hr	72 hr	84 hr	96 hr	
*L. venustum**	- / -	13 May	25%	-	-	-	1 ant	-	-	-
L. volubile	FN / ant	20 May	-	-	23% (2)	25% (1)	FN	-	FN	24% (3)
L. japonicum	FN / ant	27 May	-	-	10% (1)	17% (1)	26% (1)	-	21% (1)	19% (4)
L. oligostachyum	- / ant	27 May	-	-	23% (1)	-	FN	-	-	23% (1)
L. microphyllum	FN / ant	3 June	-	-	12% (2)	10% (4)	24% (2)	-	26% (1)	15% (9)
L. palmatum	- / ant	3 June	-	-	-	-	-	-	-	-

Table 4.1. Average % sugars in nectar produced over 96 hr after damage for six *Lygodium* species. Average % sugars are given as *mean% (no. samples)* when available. If only % is given, it is from a single sample. Week Mean % Sugar is the average for the 96 hours as mean% (no. samples). "FN" denotes nectar observed but insufficient amounts for collection, while "ant" indicates presence of one or more ants, despite efforts to exclude them. Pre-damage refers to the 4 days preceding the experiment, "-" denotes absence of nectar/ants during this period. *Lygodium venustum** is wound exudate.

Species	Pre-Damage May 9-12	Week 1 May 13-17	wknd	Week 2 May 20-24	wknd	Week 3 May 27-31	wknd	Week 4 June 3-7	wknd
L. venustum	-	TF+dmg	-	-	-	-	-	-	-
L. volubile	FN, ant	TF, 10.1% (5), ant	FN, ant	dmg	32% (1), ant	41% (1), ant	26.5% (2), ant	19% (2), ant	17% (1)
L. japonicum	FN, ant	ant	ant	ant	ant	TF+dmg	15.4% (4)	13.5% (4)	FN
L. oligostachyum	ant	ant	ant	ant	ant	TF+dmg	FN	17 (1)	FN
L. microphyllum	9.5% (2), ant	FN, ant	ant	FN, ant	FN, ant	31% (2)	FN	TF+dmg	FN
L. palmatum	ant	-	-	-	-	-	-	TF+dmg	21%* (1)

Table 4.2. Mean percent sugar (no. samples) for non-damage (ND) nectar tested by refractometer. *Lygodium palmatum* * maybe be honeydew. Tanglefoot (TF) and damage (dmg, orange shading) were applied simultaneously on each species except for *L. volubile* for which TF was applied the week preceding its damage (yellow shading). "FN" indicates foliar nectar was present but insufficient for testing. "ant" indicates presence of one or more ants, despite attempts to exclude them, usually on several of the individuals of the species. Pre-damage and weekend (wknd) dates are grayed columns.

Species	Day1			Day 2	Day 3	Day 4	Day 5	
	30 min	2 hr	3 hr	24 hr	48 hr	72 hr	96 hr	Means
L. japonicum	-	FN	-	FN, ant	12% (1), ant	17% (1), ant	13% (2), ant	14%
L. microphyllum	FN, ant	FN	-	FN, ant	17% (1), ant	23% (3), ant	7% (2), ant	16%
L. oligostachyum	-	-	-	-	-	FN	FN	n/a
L. volubile	FN, ant	FN	-	FN, ant	18% (1) ant	19% (6), ant	FN, ant	19%

Table 4.3. Nectar production and % sugars (no. samples) over 96 hr after jasmonic acid (JA) application for four species of *Lygodium*. The % sugars are given as mean% (range) when available. "FN" indicates at least one individual was producing nectar at a given survey, but there was not enough to test for % sugars; "-" indicates no nectar observed. Ants were observed on several individuals of all the above species (except *L. oligostachyum* by 24 hours post-application) and are denoted by "ant."

Species	Damage week only	Non-damage	JA only	$KW_{dmg:ND:JA}$	$Overall_{dmg+ND+JA}$
L. japonicum	18.5 ± 6.8 (4)	14.4 ± 4.1 (8)	13.5 ± 5.8 (4)	χ^2 = 1.6, p=0.46	15.2 ± 5.3 (16)
L. microphyllum	15.2 ± 7.8 (9)	20.3 ± 14.6 (4)	16.3 ± 9.5 (6)	χ^2 = 0.14, p=0.93	16.6 ± 9.6 (19)
L. oligostachyum	23 (1)	17 (1)	n/a	χ^2 = 1, p=0.32	20.0 ± 4.2 (2)
L. volubile	24 ± 1.4 (2)	19.6 ± 10 (13)	19.7 ± 4.1 (9)	χ^2 = 1.8, p=0.41	20.1 ± 7.6 (24)
$KW_{species}$	χ^2 = 2.6, p=0.47	χ^2 = 1.8, p=0.62	χ^2 = 3.3, p=0.19		χ^2 = 5.1, p=0.16

Table 4.4. Total percent sugars mean + sd (no. samples) for foliar nectar-producing species. Damage (dmg) week includes all nectar collected from that species (damaged and control plants) during that species' week. Non-damage (ND) is any nectar produced by the species outside of the damage week. JA only are samples collected following JA application to all plants, pooled by species. Overall mean percent sugar includes all samples (Damage/Non-damage/JA) for each species. Kruskal-Wallis tests for differences between species ($KW_{species}$) or within a species between damage types ($KW_{dmg:ND:JA}$).

Species	Fructose	Glucose	Sucrose	Other Unidentified Oligosaccharides
L. japonicum	-	10.3 ± 8.4 % (n=4)	-	
L. microphyllum	-	13.9 ± 12.4 % (n=4)	-	
L. oligostachyum	-	-	-	possibly 5-carbon
L. volubile	-	3.7 ± 1.3 % (n=3)	-	possibly 5-carbon and larger
L. venustum *	-	16.0 ± 7.22 % (n=2)	-	larger

Table 4.5. Sugar type and concentration (mean ± sd, no. samples) identified from HPLC constituent analysis of foliar nectar for 4 *Lygodium* species, and of wound secretion for *L. venustum** (samples pooled by species). *Lygodium palmatum* did not produce foliar nectar or enough other exudate for analysis and so is excluded.

Figures

Figure 4.1. *Lygodium* congeners used in this study: *L. microphyllum* (A), *L. venustum* (B), *L. volubile* (C), *L. oligostachyum* (D), *L. japonicum* (E), and *L. palmatum* (F).

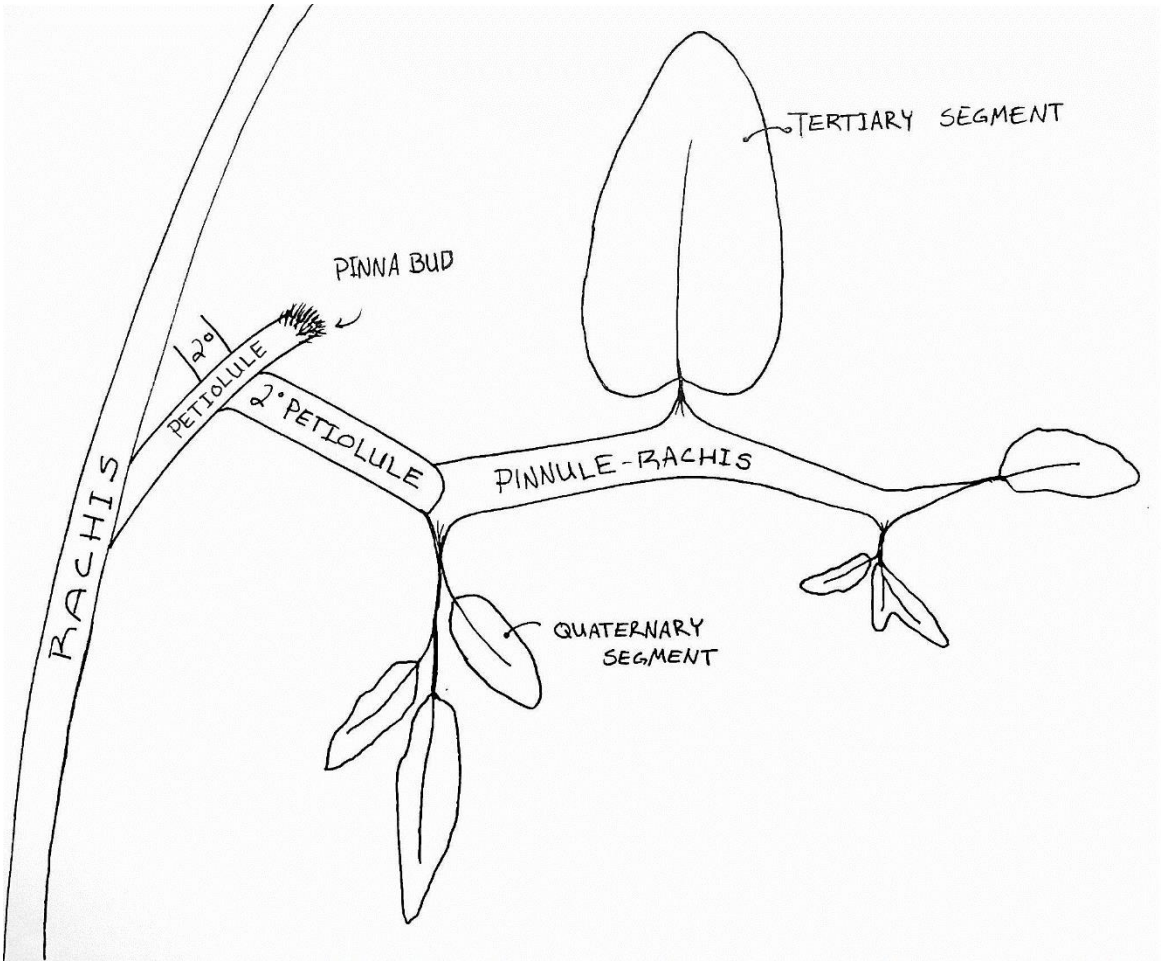

Figure 4.2. Stylized diagram of rachis, petiolule and one pinnule of the bipinnate compound pinna. Pinna and pinnule parts are labeled with terms used in the text; both tertiary segments (as on *L. microphyllum*, *L. volubile*, and *L. palmatum*) and quaternary segments (once more divided, as on *L. japonicum*, and sometimes on *L. oligostachyum* and *L. venustum* (3-4 pinnate)) are illustrated.

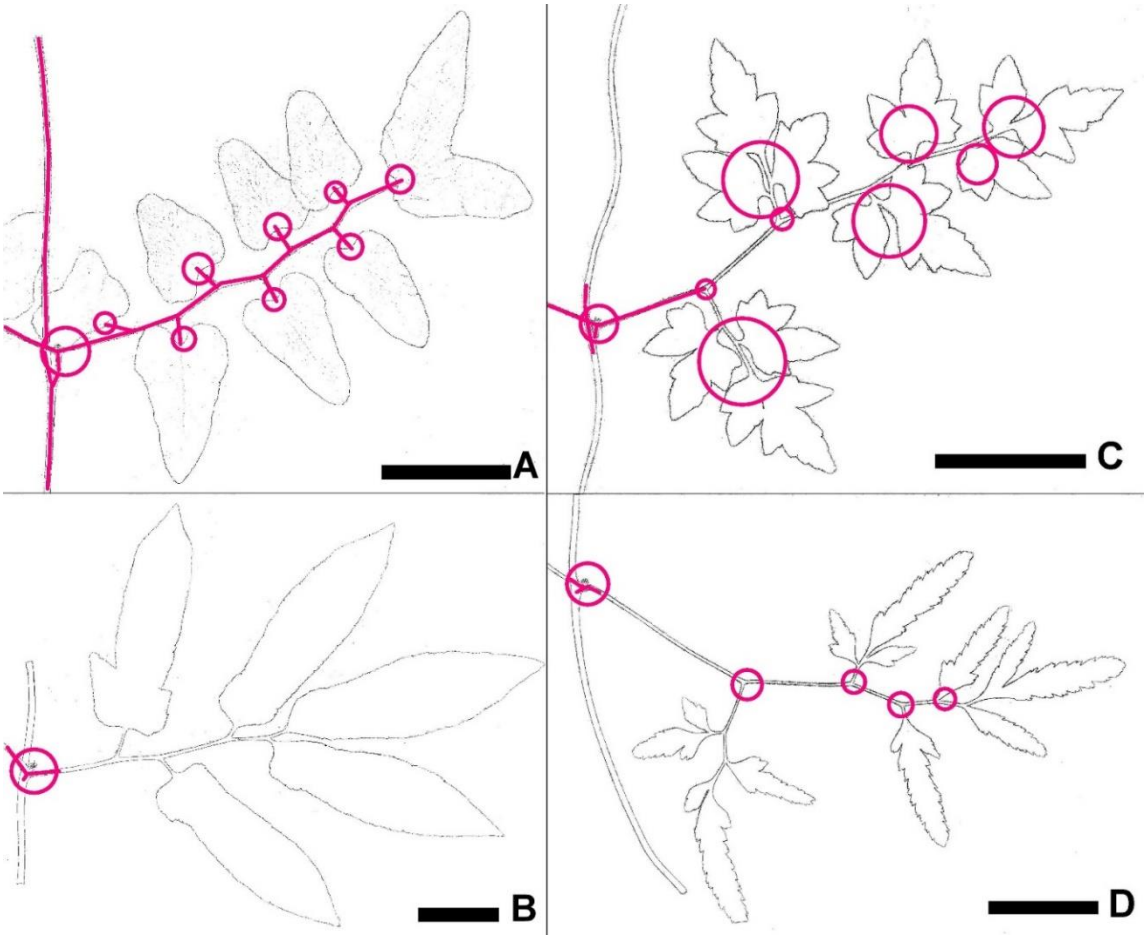

Figure 4.3. Locations of nectar production in diagrams of one pinnule from each nectar-producing species. Pinnae depicted are from younger fronds, where nectar was most often observed. Locations where nectar droplets were observed are traced or circled in hot pink. A, *L. microphyllum*; B, *L. volubile*; C, *L. japonicum*, D, *L. oligostachyum*. Scale bars = 2 cm.

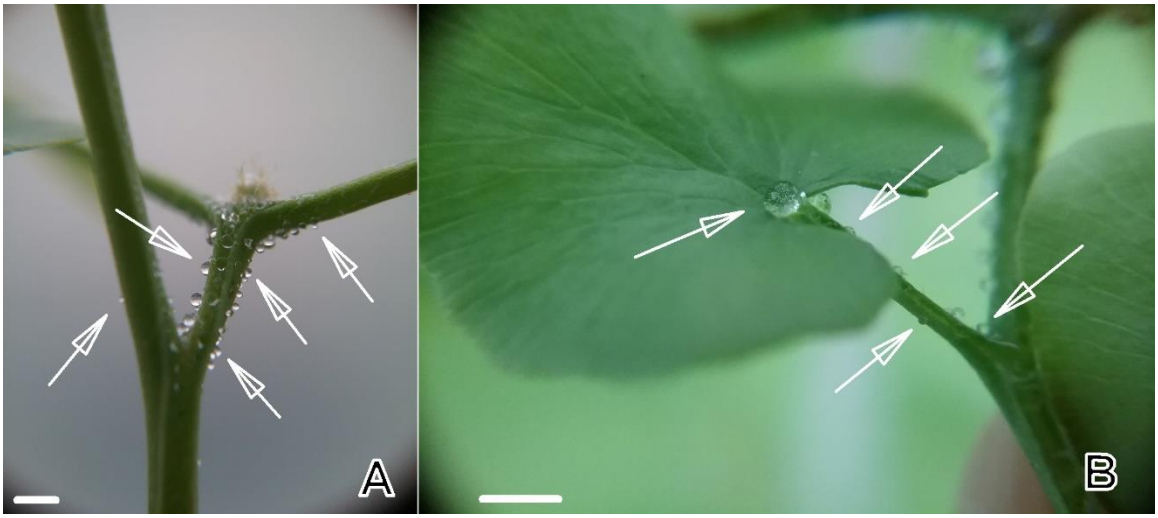

Figure 4.4. Nectar droplets on *L. microphyllum*, indicated by white arrows. In A, nectar appears in some droplets on the main rachis, many on the petiolule and along the transition to the secondary petiolule. In B, nectar collects into a single large droplet at the base of the tertiary segment, while smaller drops can be seen on the segment stalk (pinnule-rachis). Scale bars = 1 mm.

Figure 4.5. *Lygodium japonicum* nectar droplets on sporulating pinnules. White arrows indicate some droplets along and between segment veins on the abaxial surface of fertile segments. Scale bar = 1 cm. Red oval indicates the developing sporangia on one tertiary segment tip.

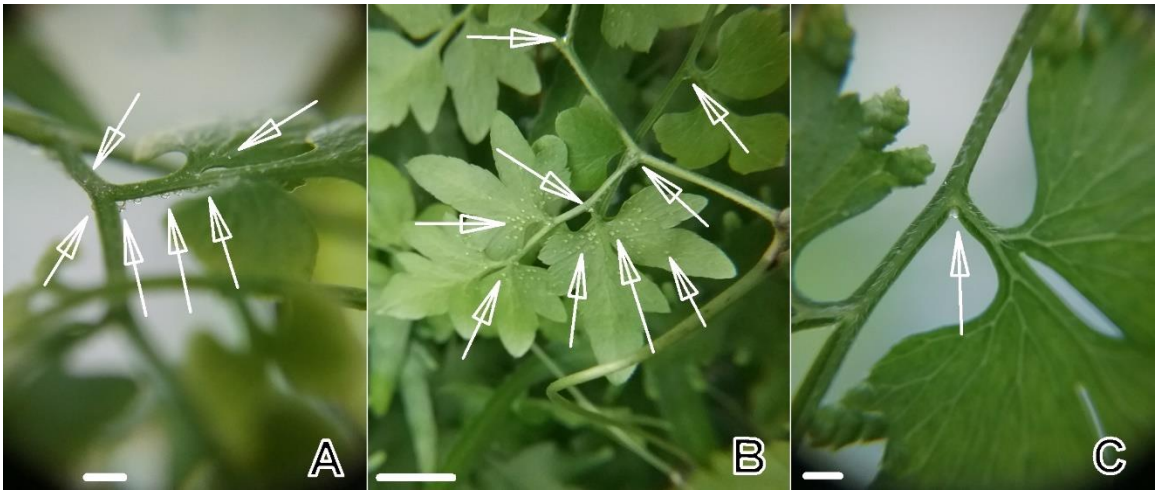

Figure 4.6. *Lygodium japonicum* quaternary segments and pinnule-rachis (A, B) with nectar droplets; pinnule-rachis axil with nectar (C). White arrows indicate nectar droplets. Scale bars a, c = 1 mm; b = 1 cm.

Figure 4.7. Nectar droplets of *L. volubile* indicated by white arrows on the short petiolule and a portion of the secondary petiolules. Scale bar = 2 mm.

Figure 4.8. Nectar droplets on *L. oligostachyum* indicated by white arrows at the ultimate segment axil (A), and on the petiolule and surrounding the pinna bud (B). Scale bars = 2 mm.

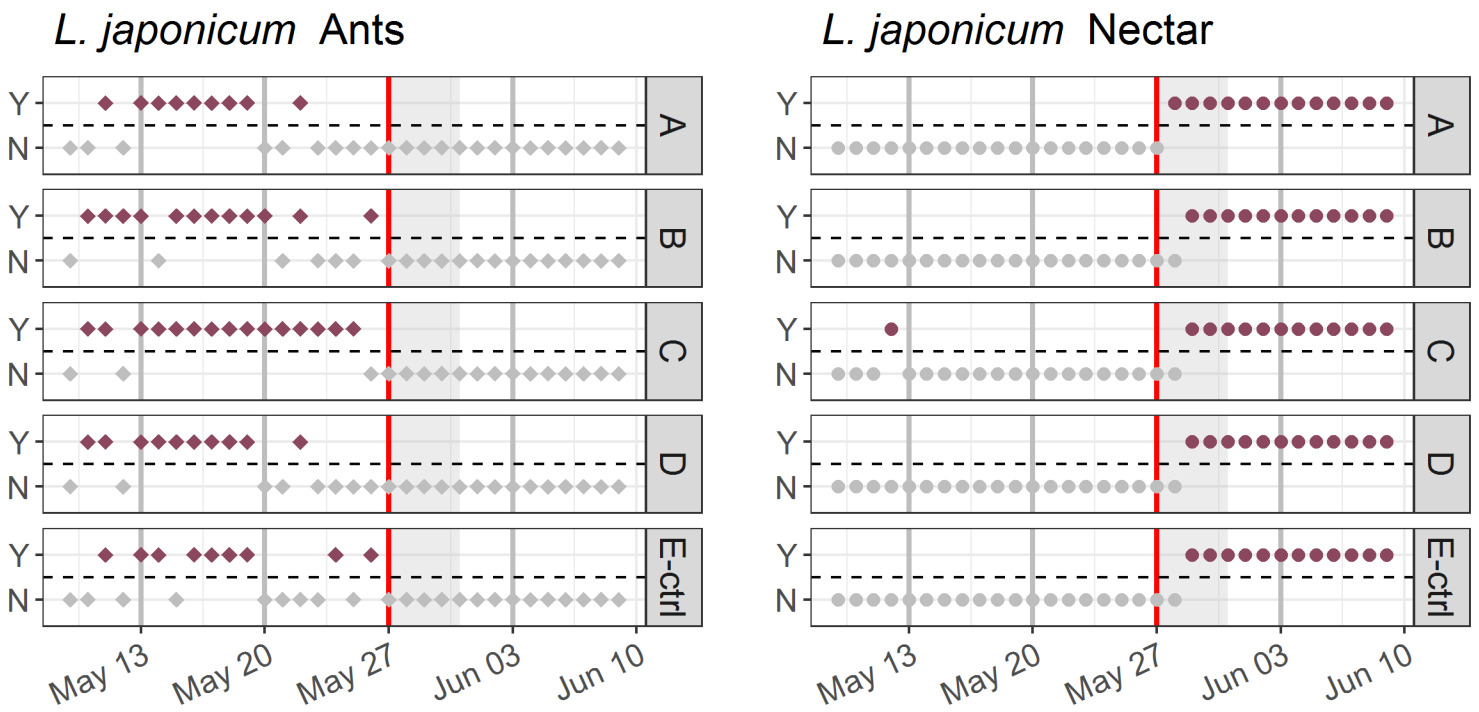

Figure 4.9a. Observations of ants (left, diamonds) and nectar (right, circles) per *L. japonicum* individual (A to E) within species over the whole month of the damage experiment. Undamaged control plant is labeled "E-ctrl." Presence (Y) or absence (N). Red vertical line indicates the application of Tanglefoot (TF) and mechanical damage. Grayed-in area to right of red line encompasses the damage week.

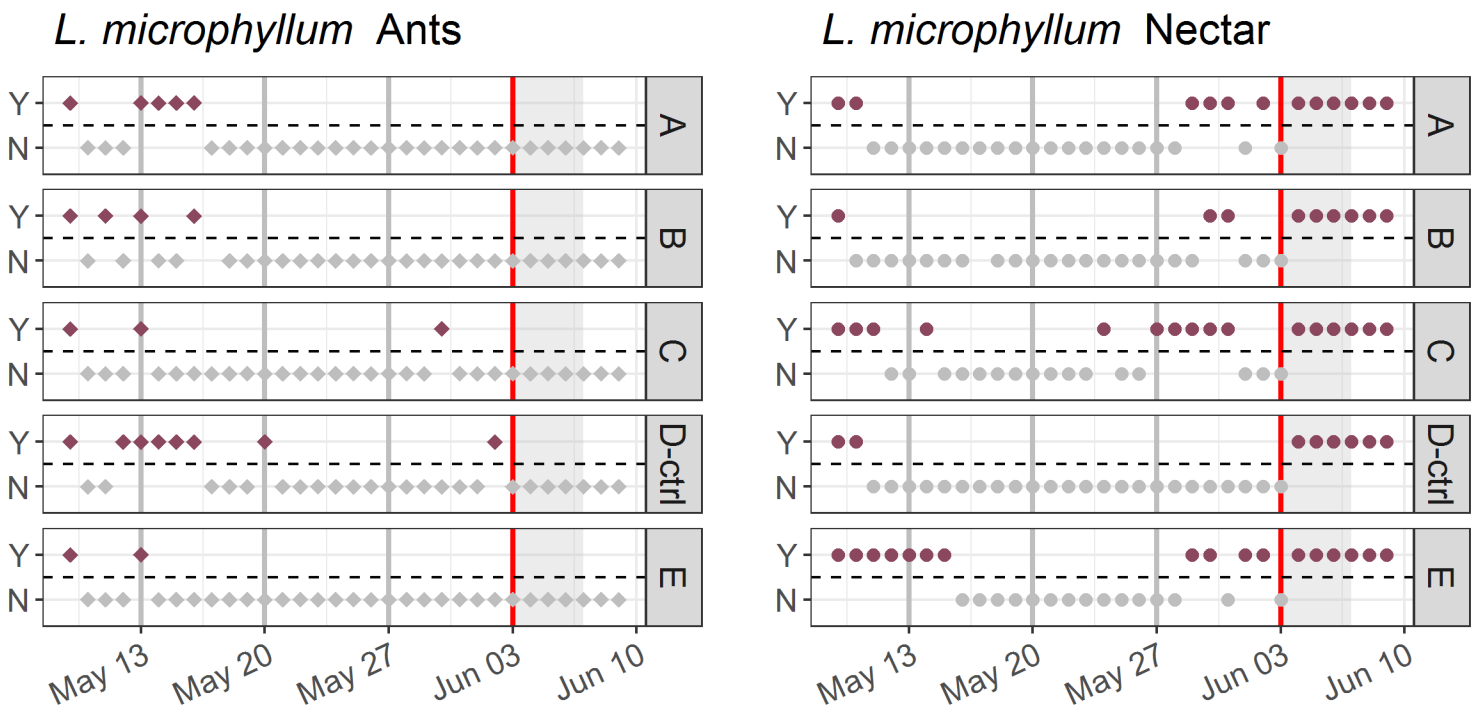

Figure 4.9b. Observations of ants (left, diamonds) and nectar (right, circles) per *L. microphyllum* individual (A to E) within species over the whole month of the damage experiment. Undamaged control plant is labeled "D-ctrl." Presence (Y) or absence (N). Red vertical line indicates the application of Tanglefoot (TF) and mechanical damage. Grayed-in area to right of red line encompasses the damage week.

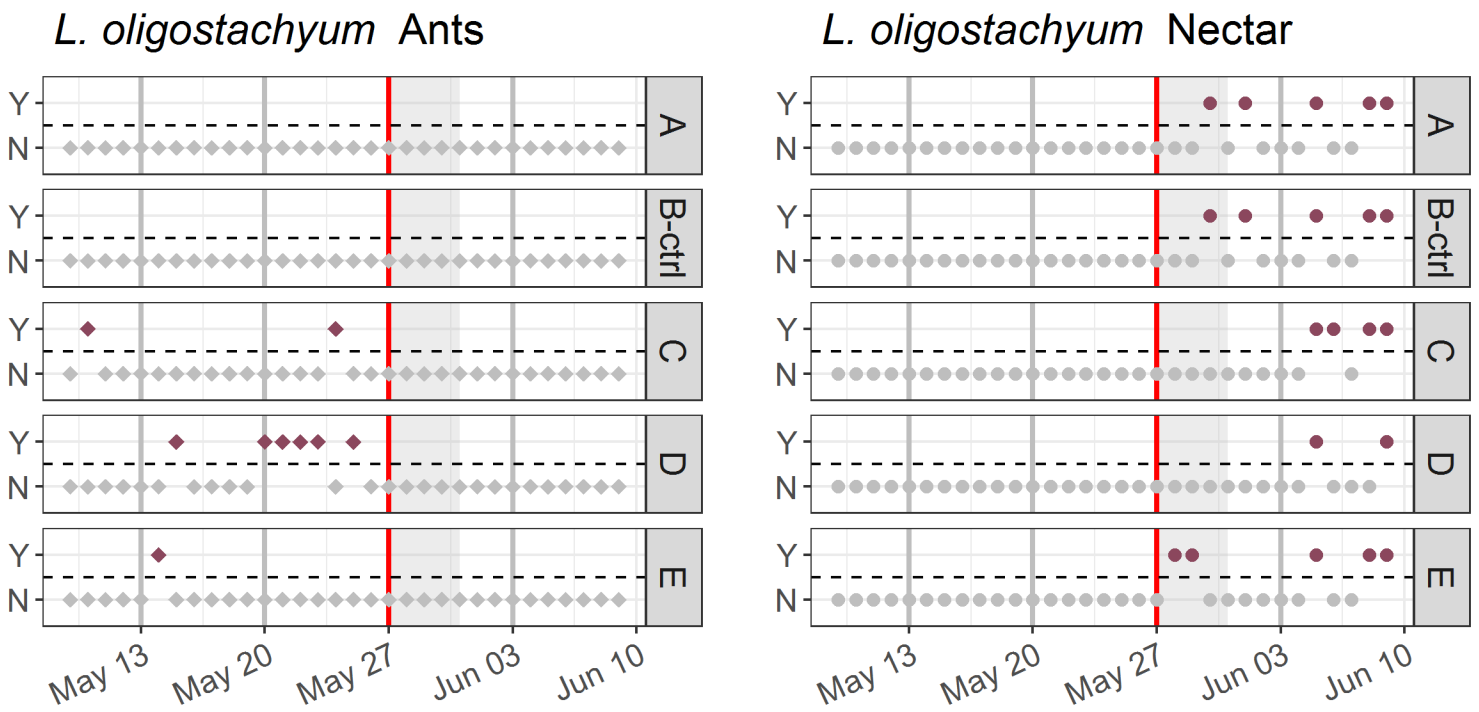

Figure 4.9c. Observations of ants (left, diamonds) and nectar (right, circles) per *L. oligostachyum* individual (A to E) within species over the whole month of the damage experiment. Undamaged control plant is labeled "B-ctrl." Presence (Y) or absence (N). Red vertical line indicates the application of Tanglefoot (TF) and mechanical damage. Grayed-in area to right of red line encompasses the damage week.

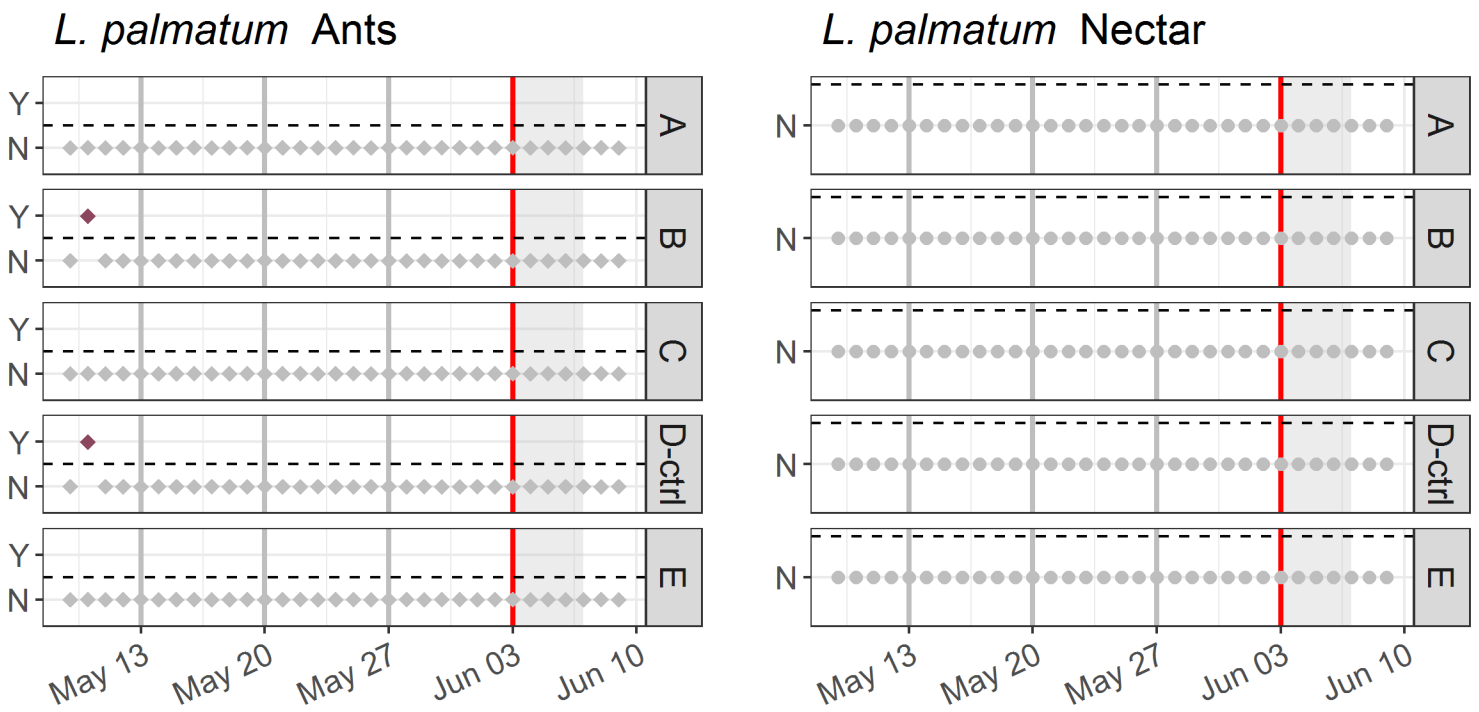

Figure 4.9d. Observations of ants (left, diamonds) and nectar (right, circles) per *L. palmatum* individual (A to E) within species over the whole month of the damage experiment. Undamaged control plant is labeled "D-ctrl." Presence (Y) or absence (N). Red vertical line indicates the application of Tanglefoot (TF) and mechanical damage. Grayed-in area to right of red line encompasses the damage week.

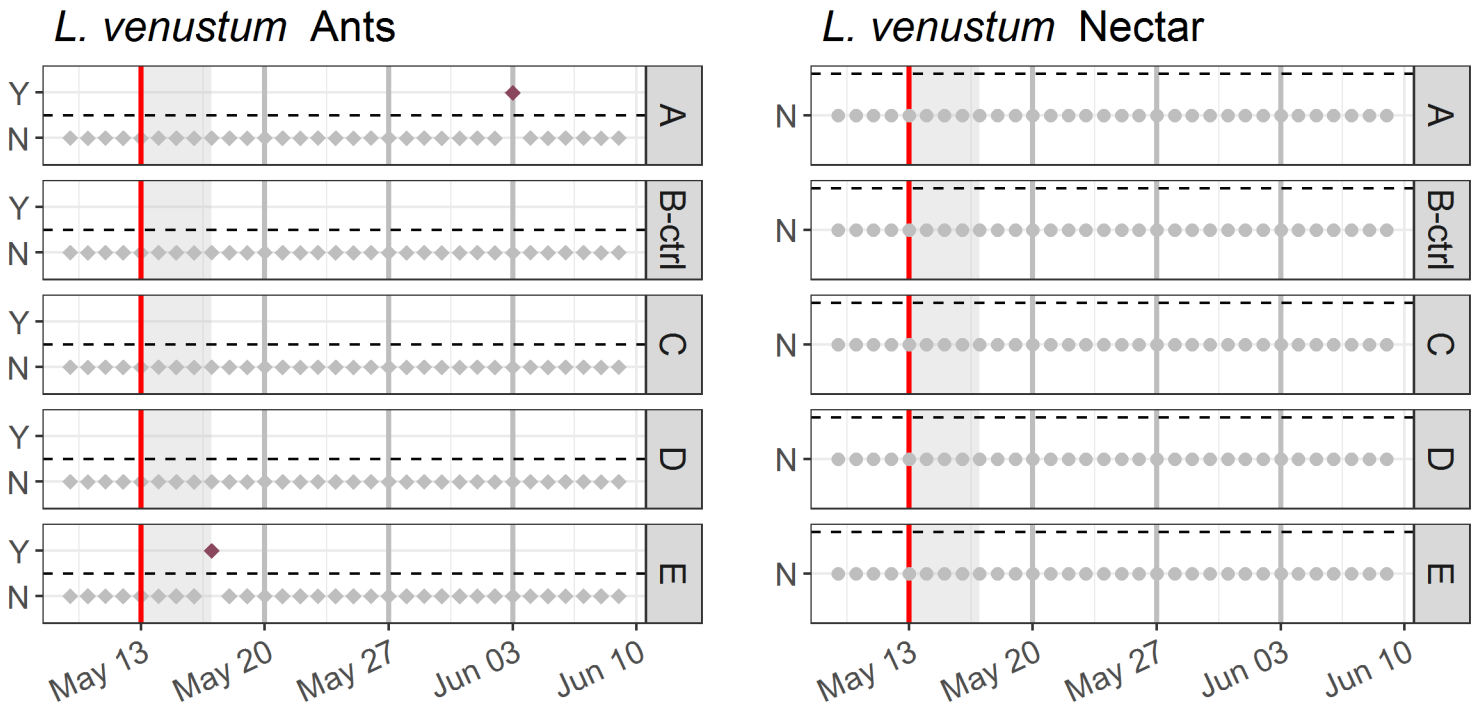

Figure 4.9e. Observations of ants (left, diamonds) and nectar (right, circles) per *L. venustum* individual (A to E) within species over the whole month of the damage experiment. Undamaged control plant is labeled "B-ctrl." Presence (Y) or absence (N). Red vertical line indicates the application of Tanglefoot (TF) and mechanical damage. Grayed-in area to right of red line encompasses the damage week.

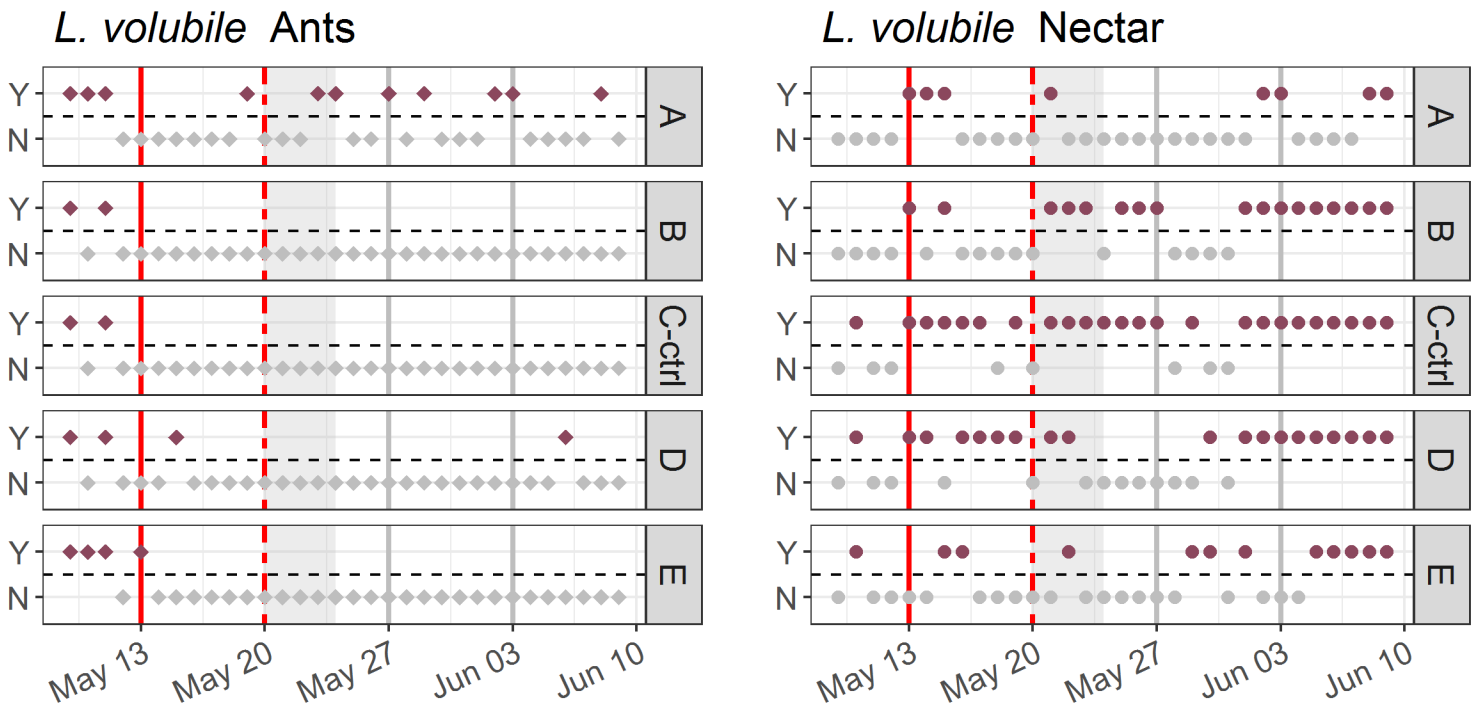

Figure 4.9f. Observations of ants (left, diamonds) and nectar (right, circles) per *L. volubile* individual (A to E) within species over the whole month of the damage experiment. Undamaged control plant is labeled "C-ctrl." Presence (Y) or absence (N). Red vertical lines indicate Tanglefoot (TF) application (solid), and mechanical damage (dashed). Grayed-in area to right of dashed red line encompasses the damage week.

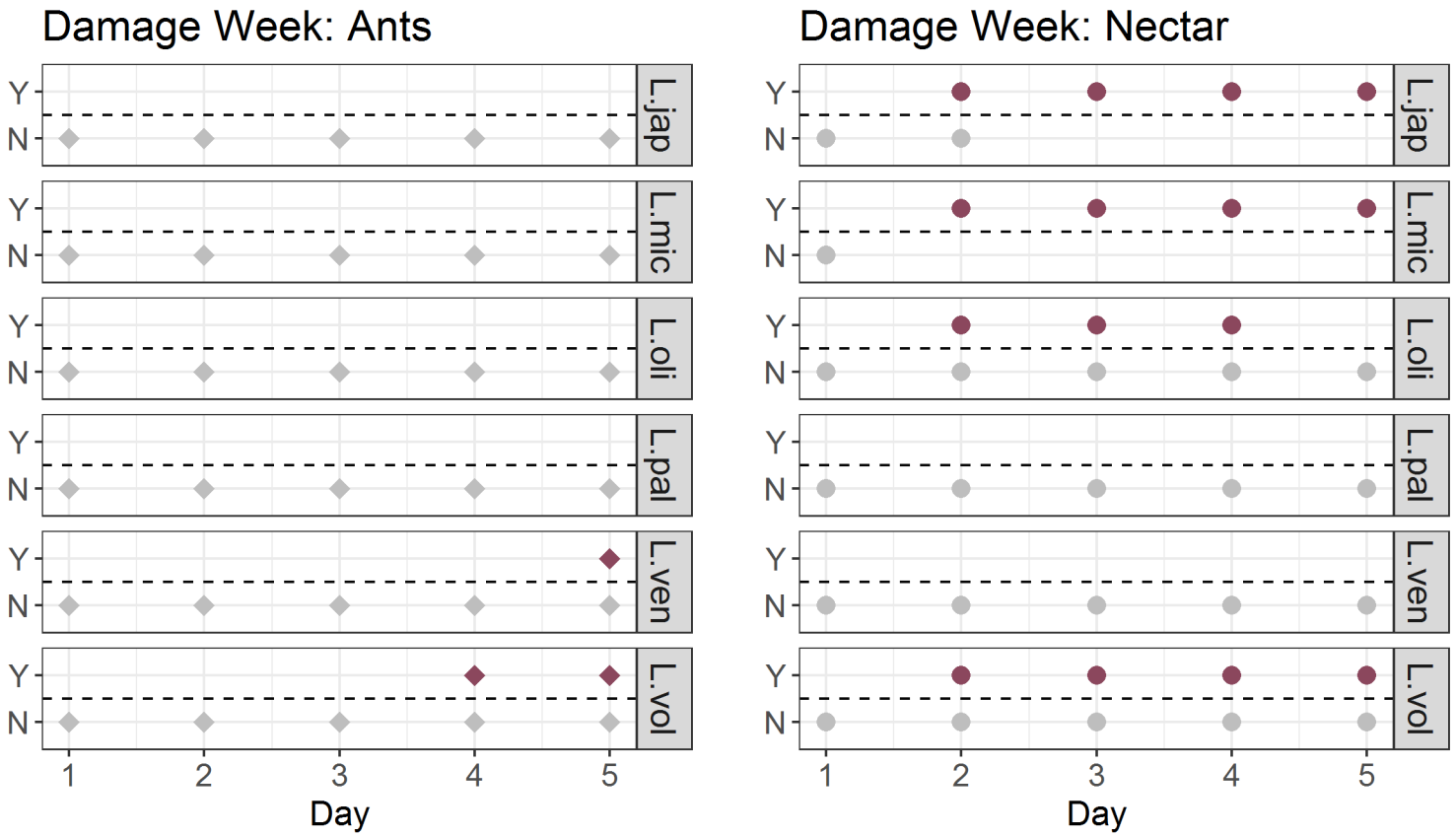

Figure 4.10. Presence (Y, purple) or absence (N, gray) of ants (left, diamonds) or nectar (right, circles) for each species during its damage week. L.jap, *L. japonicum*; L.mic, *L. microphyllum*; L.oli, *L. oligostachyum*; L.pal, *L. palmatum*; L.ven, *L. venustum*; L.vol, *L. volubile*. Each symbol represents at least one (max 5) individual. Ant presence coincided with TF barrier failures.

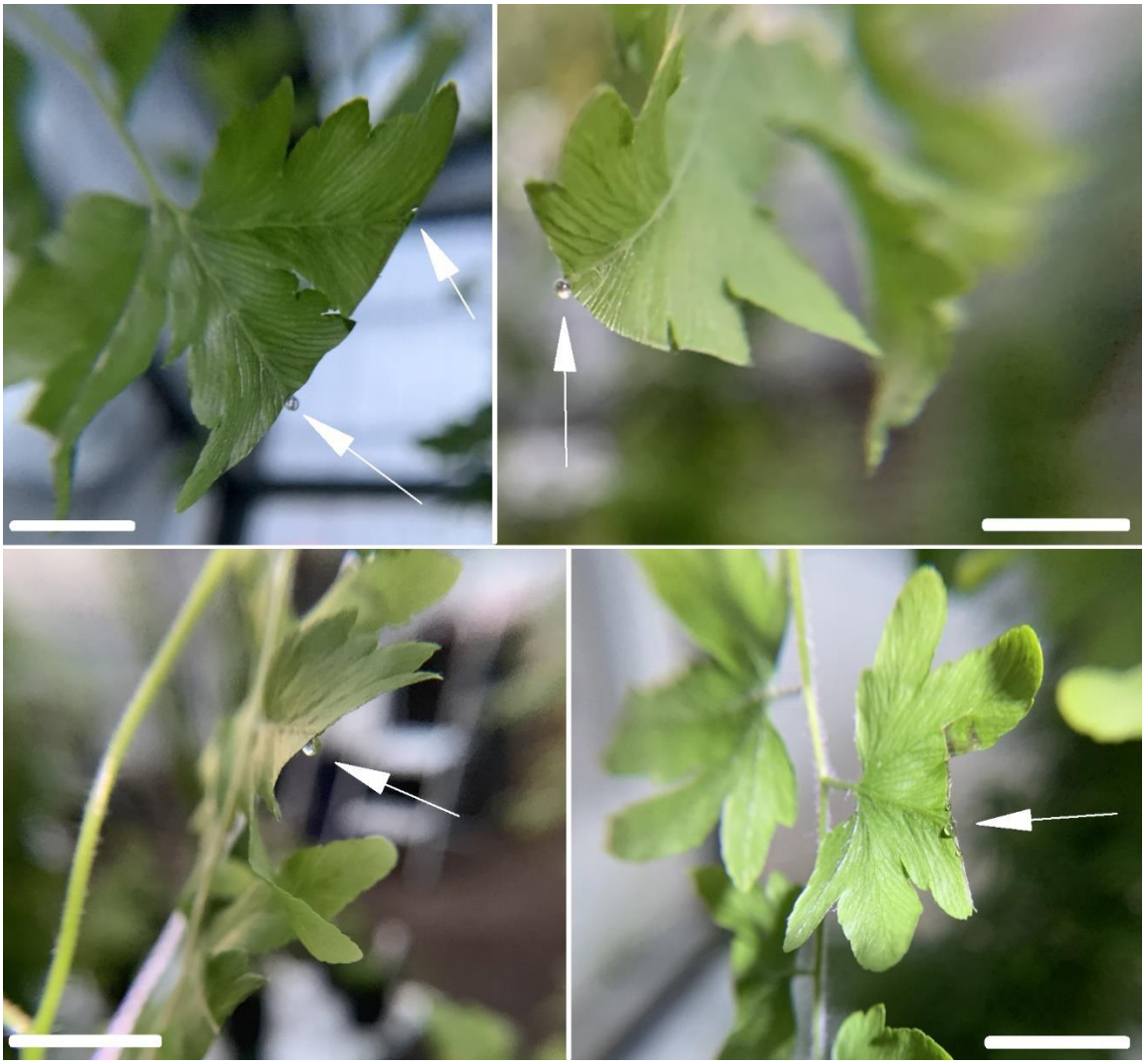

Figure 4.11. Cut wound exudate, indicated by white arrows, on the wounds of *L. venustum* pinnule segments (photos by Ana Flores, FIU Biological Sciences undergraduate). Scale bars = 1 cm.

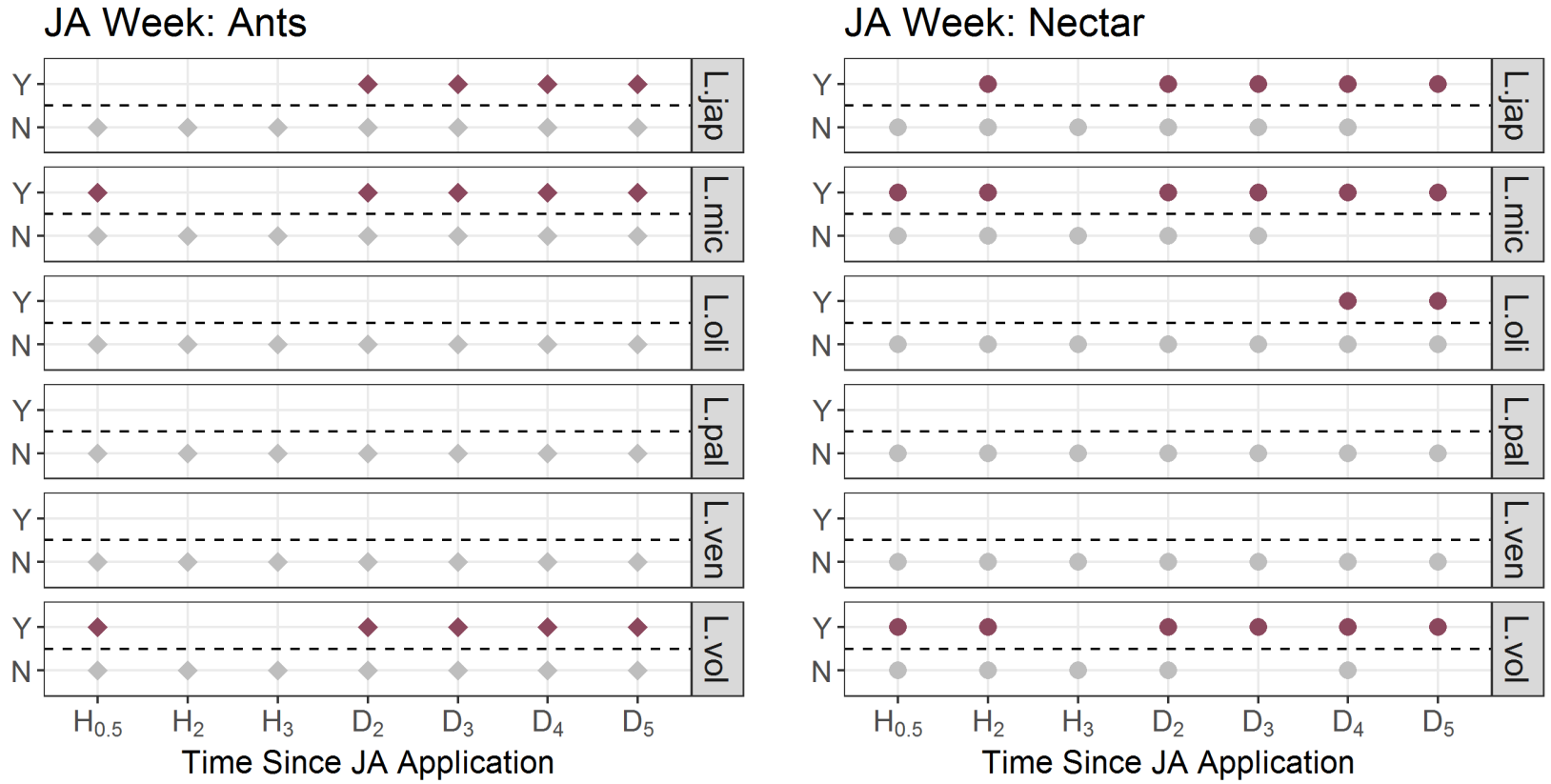

Figure 4.12. Presence (Y, purple) or absence (N, gray) of ants (left, diamonds) or nectar (right, circles) for all species following jasmonic acid (JA) application. L.jap, *L. japonicum*; L.mic, *L. microphyllum*; L.oli, *L. oligostachyum*; L.pal, *L. palmatum*; L.ven, *L. venustum*; L.vol, *L. volubile*. Times within Day 1 are in hours ($H_{0.5}$, H_2, H_3), followed by D_2 (24 hr), D_3 (48 hr), D_4 (72 hr), and D_5 (96 hr). Each symbol represents at least one (max 5) individual. Ant presence coincided with TF barrier failures.

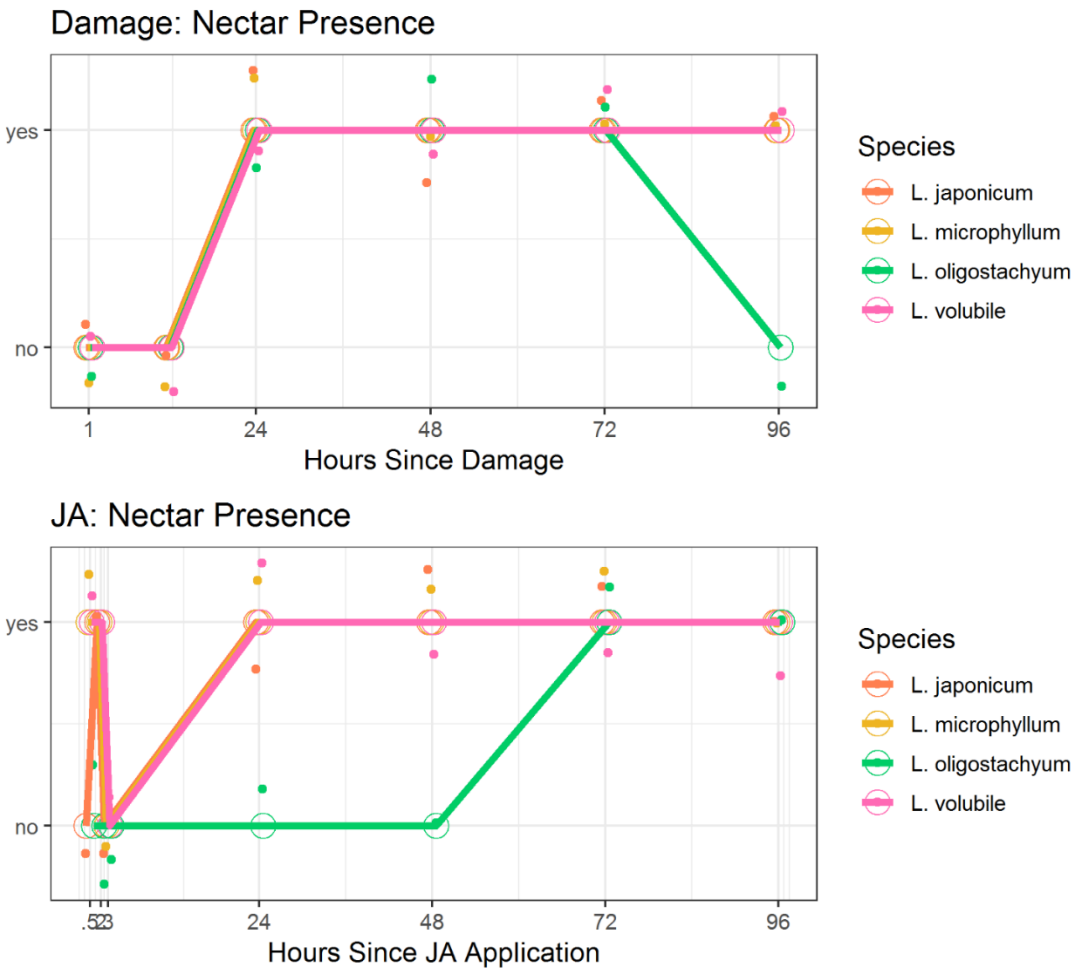

Figure 4.13. Presence of nectar over time following damage (top) or jasmonic acid (JA) (bottom) application. '.523' is 0.5 hr, 2 hr, and 3 hr post JA.

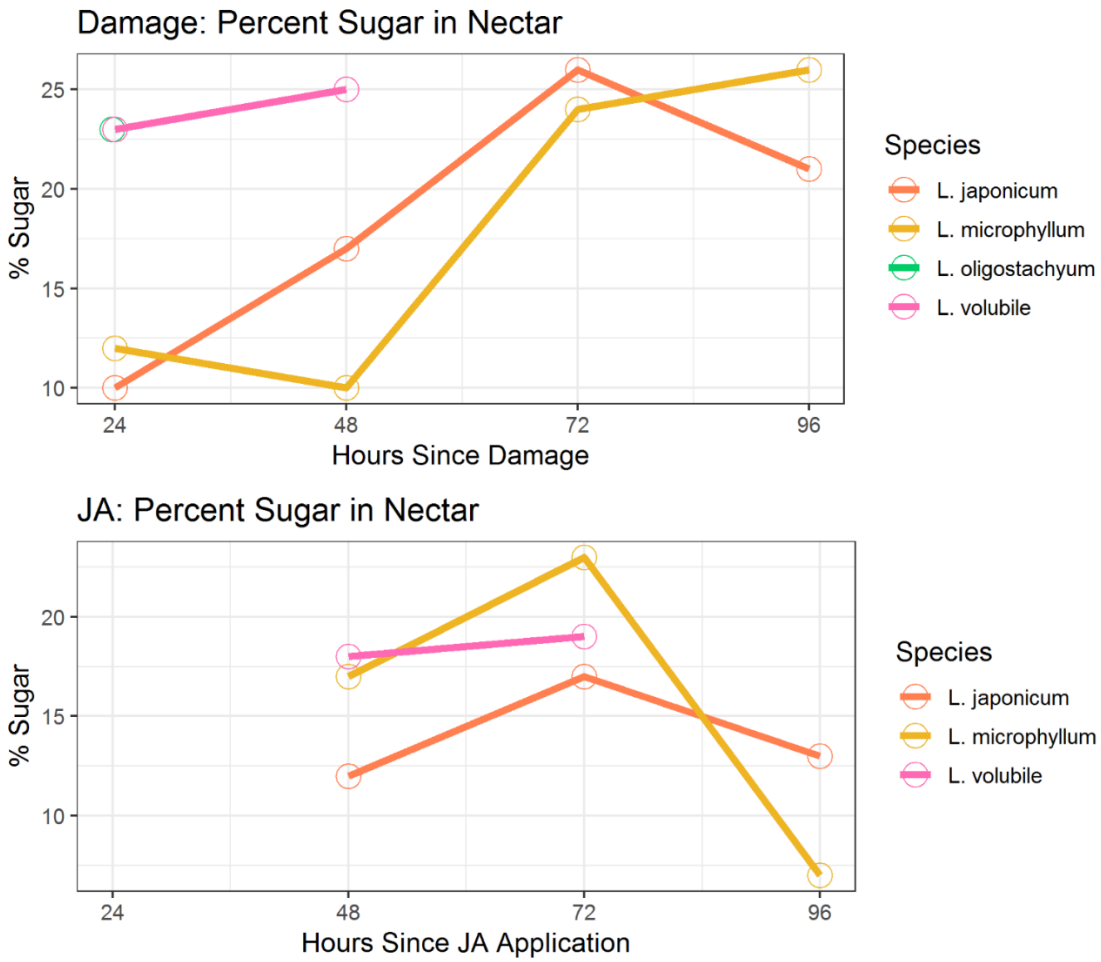

Figure 4.14. Percent sugars in nectar samples from Day 2 (24 hr) to Day 5 (96 hr) during damage (top) and jasmonic acid (JA) application (bottom) weeks.

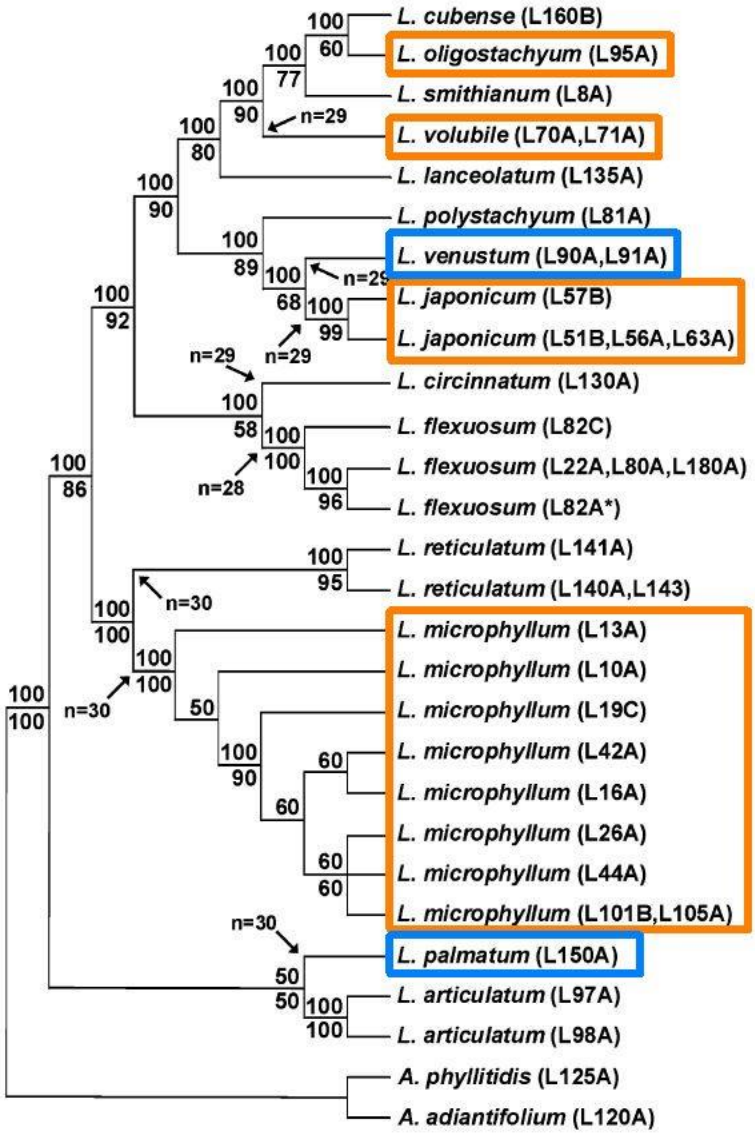

Figure 4.15. Partial phylogeny of *Lygodium* genus based on the parsimony analysis of chloroplast sequence data by Madeira et al. 2008. The six species used in this chapter's nectar experiments are highlighted in color boxes. Orange boxes indicate species on which nectar was observed; blue boxes indicate species on which nectar was not observed. Reprinted from Biological Control, 45(3), Paul T. Madeira, Robert W. Pemberton, and Ted D. Center, "A Molecular Phylogeny of the Genus Lygodium (Schizaeaceae) with Special Reference to the Biological Control and Host Range Testing of *Lygodium microphyllum*" 308–318, 2008, with permission from Elsevier.

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Appendices

Appendix A: First Report of Foliar Nectar Production by *Lygodium microphyllum* (Lygodiaceae), an Invasive Fern in Florida.

Foliar nectar has been documented on 43 extant fern species belonging to nine genera in five families (Koptur *et al*., Annals of Botany 111:1277–1283. 2013; Nepi *et al*., Annals of Botany 104:205–219. 2009). Here we report the first observation of foliar nectar production by Old World climbing fern, *Lygodium microphyllum* (Cav.) R. Br., which is a member of a sixth family, Lygodiaceae.

The sole genus in the Lygodiaceae, *Lygodium*, comprises 40 species of climbing ferns with members native to southeast Asia, subtropical Africa, Australia, North and Central America, and northern South America (Kramer 1990, Schizaeaceae, 258–262 *in*

The Families and Genera of Vascular Plants Vol. I. Pteridophytes and Gymnosperms, Springer-Verlag; Pemberton, American Fern Journal 88:176–182. 1998; Wikström, Kenrick, and Vogel, Review of Palaeobotany and Palynology 119:35–50. 2002). *Lygodium microphyllum* is the first species in the genus—indeed, in the order (Schizaeales)—for which foliar nectar has been reported. *Lygodium microphyllum* is native to tropical and subtropical areas of Asia, Africa, and Australasia (Pemberton, 1998). First cultivated in the USA as an ornamental, the species was reported as naturalized in Florida in 1965 (Beckner, American Fern Journal 58:93–94. 1968; Nauman and Austin, American Fern Journal 68:65–66. 1978). *Lygodium microphyllum* has since become widespread in wet and mesic environments of south and central Florida, infesting both natural areas and highly disturbed sites (Rodgers, Pernas, and Hill, Invasive Plant Science and Management 7:360–374. 2014). The invasive fern degrades habitats both by smothering native vegetation and by acting as a fire ladder, facilitating the spread of fire into tree canopies that would not normally burn (Pemberton and Ferriter, American Fern Journal 88:165–175. 1998; Schmitz et al. 1997, The Ecological Impact of Non-indigenous Plants. 29–74 *in* Strangers in Paradise, Island Press).

Lygodium microphyllum plant architecture and nectar production

For clarity, morphological descriptions follow terminology of Tryon (Taxon 9:104–109. 1960) and Vasco, Moran, and Ambrose (Plant Evolution and Development 4:1–13. 2013). The fronds of *L. microphyllum*, which are unusual in being indeterminate, are tripinnate, with alternating pinnae. Each pinna (Fig. 1) consists of a stalk (petiolule) with one set of opposite pinnules; the pinna terminates in a dormant pinna apex protected by numerous trichomes. Each pinnule has a secondary petiolule and is alternately divided

into 4–13 tertiary segments. Each tertiary segment is also petiolulate. The pinnule-rachis refers to the midrib of the pinnule, including tertiary segment petiolules. Uniseriate trichomes are present on all surfaces but are especially abundant on the adaxial surface of the rachis, petiolules, and pinnule-rachis. Presence of uniseriate hairs on the rachis has been previously reported in three genera of the order Schizaeales: *Schizaea, Anemia,* and *Lygodium* (Clarke, doctoral thesis U. Wisconsin. 1935)

Small beads of nectar were first observed in June 2017 on *L. microphyllum* plants between 2 and 3 m in height housed in the greenhouse at Florida International University. The nectar beads appeared on the petiolule, pinnule-rachises, and tertiary segments of several sterile and fertile pinnae of the *L. microphyllum* plants. Copious nectar was observed a few days later in numerous droplets present on the slightly concave adaxial surface of main rachises, along the petiolules, secondary petiolules, pinnule-rachises, and on the basal area of tertiary segments. Nectar was found on newly matured fronds with green fleshy rachises, on middle-aged fronds with gray adaxial rachis surfaces, and occasionally on very mature fronds with brown rachises and petiolules. The nectar appeared most abundant, however, on petiolules, around newly expanding leafbud apices, and along intermediate-aged rachises. The structures from which the nectar originates are not obvious and the rather uniform droplets along multiple fern parts do not appear to be associated with the uniseriate hairs previously mentioned.

Nectar collections performed throughout the day determined peak volume production to occur just before dawn. Subsequent collections of nectar for sugar concentration analyses were performed in the early mornings using a micropipette (Drummond Scientific Company, Broomall, P.A. USA), and sugar concentration of

nectar was measured using a pocket sugar refractometer (0-50%, wt./total wt. type, Bellingham & Stanley LTD. England). The sugar concentration measured ranged from 6% to 21%, but mean concentration found on mature sporophyte pinnae was 14.0% (SE = 0.9, n = 7).

The average nectar volume collected from a single pinna (from petiolule, secondary petiolules, pinnule-rachises, and tertiary segments) with active nectar secretion was 4.3 µL (max. 8.1 µL, SE = 0.5, n = 11). Nectar secretion along the rachis was also extensive, frequently occurring along its entire length between the oldest pinna and the most recently expanded, nectar-secreting pinna. At the time of these observations, no pests (scales, mites, ants, etc.) were present on any of the plants, so we are confident that the nectar present did not originate from an external source. The most noticeable nectar was visible a few days after inflicting damage by cutting back the fronds (for example, from 2 m in height down to 1 m in height) and appeared along all the surfaces previously mentioned.

Humidity in the greenhouse was high, though not measured directly. Temperatures ranged from 19°C to 36°C during the winter, and 25°C to 45°C during the summer, as logged hourly by eight thermochrons (DS1922L, Maxim Integrated, San Jose, CA) suspended around the greenhouse at mid-plant height. Light levels were measured at approximately 1 m height, at the bulk of each plant's biomass, using an Apogee quantum meter (Apogee Instruments, Logan UT). Average peak light was 297 $\mu molm^{-2}s^{-1}$ but reached as high as 600 $\mu molm^{-2}s^{-1}$. Plants were watered daily for the first year in the greenhouse, and then were transitioned into bins of water with roots partially submerged for another six months. These conditions are potentially warmer and more

humid than both the native and invaded regions where *L. microphyllum* is thriving, and may contribute to this abundance of nectar production.

Previous work on the role of nectar in ferns has produced mixed results. Koptur *et al.* (2013) showed that nectar-producing fronds of an epiphytic fern, *Pleopeltis crassinervata* (Feé) T. Moore, suffered less herbivore damage than fronds on which nectaries had been covered with nail polish, which decreased activity by predatory ants. Conversely, Tempel (Ecology 64:1411–1422. 1983) observed that ants feeding on the nectaries of the bracken fern, *Pteridium aquilinum* (L.) Kuhn, provided no defensive benefits for the plant. Future work should seek to determine the composition of nectar on *L. microphyllum*, as the relative concentrations of sugars, amino acids, and other relevant compounds can provide insight into the kinds of arthropods that might be attracted (Nepi *et al.* 2009) and may indicate whether nectar production is a defensive trait in *L. microphyllum*.

The source and mode of nectar secretion must be determined, as well as whether nectar production is inducible. Future work should also consider alternative explanations for the function, or lack thereof, of nectar in *L. microphyllum*. One such explanation is the 'leaky phloem hypothesis' (De la Barrera and Nobel, Trends in Plant Science 9:65–69. 2004), which suggests that phloem solution may 'leak' as a result of increased hydrostatic pressure within the phloem, and the structural weakness of developing plant tissues. Although nectar was abundant on newly expanding leafbud apices, which could support this theory for *L. microphyllum*, it was also present on much older parts of the leaves.

Finally, it is not uncommon for multiple members of a genus to share nectar-producing traits (Weber, Porturas, and Keeler, World List of Plants with Extrafloral Nectaries. www.extrafloralnectaries.org. Accessed 9 June 2017. 2015). Efforts should be made to determine if foliar nectar is also present on congeners of *L. microphyllum*, in particular, *Lygodium japonicum* (Thunb.) Sw., another major invasive exotic species in the southeastern USA.

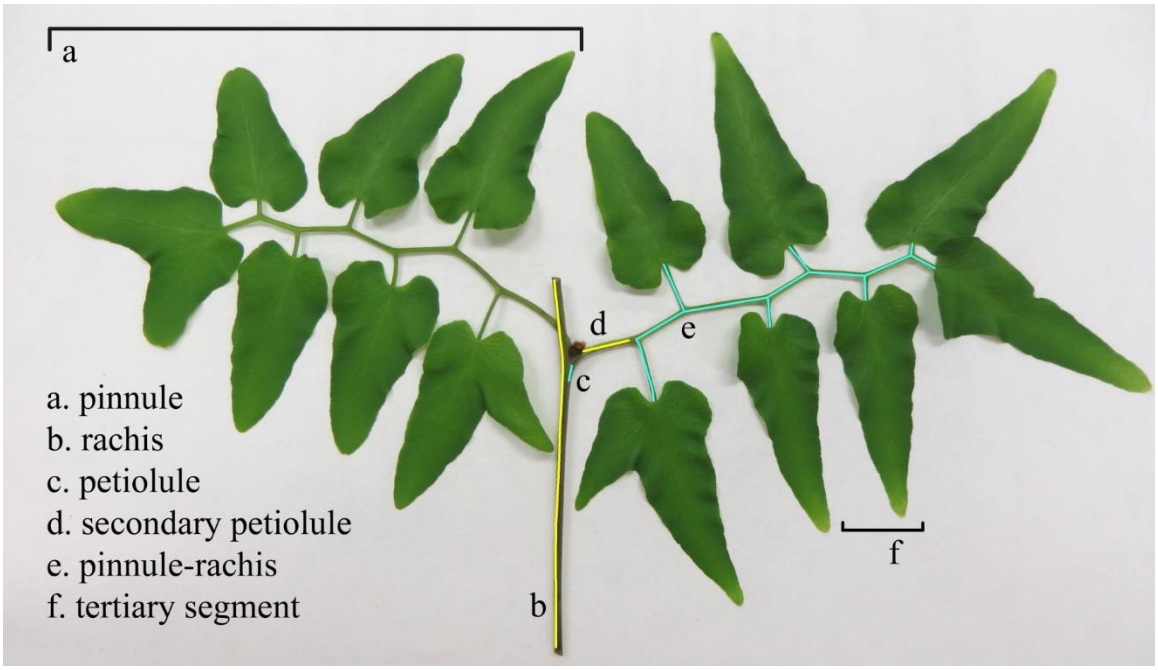

Figure 1: Structure of a single pinna on the leaf of *Lygodium microphyllum*; pinnae alternate along the climbing, indeterminate leaf. Yellow and blue lines designate the rachis and parts of the pinna: long yellow (rachis, b), short blue (petiolule, c), short yellow (secondary petiolule, d), and long, branched blue (pinnule-rachis, e).

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Appendix B: **Preliminary Experiments**

Two preliminary studies were designed to determine how damage was related to the type and timing of nectar production by *Lygodium microphyllum.*

Type of Damage

The first study (November 2018) explored damage types on nectar production. To approximate leaf loss by herbivory, 50% of leaf biomass on each of four *L. microphyllum* plants was damaged by cutting with scissors. Two types of damage, rachis or leaflet, that may approximate different herbivores, were administered: tertiary segment tips were removed from two plants, while rachises were severed on the other two plants. Damage was administered at dawn, and plants were monitored for nectar production (presence/absence and location) throughout the first hour, then surveyed every 6 hours for 2 days. All accessible rachises and pinnae were inspected for nectar, which appeared first as a shine, then becoming droplets of up to 1 µL. Nectar presence was most pronounced at dawn, 24 hours post-damage. There were numerous ants present collecting the nectar, however, so quantification was not possible. Damage type did not appear to affect nectar production (locations and presence of at least some nectar), although plants with rachises removed had inadvertently lost substantially more young

green vegetative material where nectar was more reliably found. New growth, especially on well-established adult plants, supported the most noticeable amounts of nectar, although amounts were still insufficient for collection.

Timing of Damage

A second study (March 2019) addressed the effect of time of damage. Tanglefoot® (TF) insect barrier was used to exclude ants from the test plants by applying the TF to the rachises near the soil surface, shaking the plant to remove ants, and then applying more TF to the support twines above the height of each plant. Five plants were randomly assigned to dusk damage (2 plants), dawn damage (2 plants), or control (1 plant). Damage was administered by cutting 25% of the rachises and trimming 25% of tertiary segments for approximately 50% damage to the leaves. Monitoring for nectar production began with the first (dusk) group's damage application, followed 12 hours later with morning damage to the dawn damage group. Monitoring then occurred every 12 hours for four more days. Regardless of time of damage, all plants, including the control, produced nectar in the mornings, with subjective peaks between 24-48 hours after damage (again insufficient amounts of nectar present for collection). Plants were not investigated for nectar prior to damage application, leaving open the possibility that all of them were producing some baseline amounts of nectar prior to damage and may have produced nectar regardless of damage. Alternatively, production of nectar by the undamaged control may indicate volatile signaling between plants. For example, jasmonic acid (JA) released from damaged tissues induced nectar production in a proximal, but physically undamaged plant (Heil 2011). Again, rachis cuts reduced

somewhat the areas more prone to nectar production, indicating that damage to tertiary segments is preferable for monitoring nectar production.

Although timing and type of damage did not appear to greatly affect nectar production, the knowledge that nectar production occurs in the morning and is generally more abundant on younger growth informed subsequent experiments.

CHAPTER V

V. CONCLUSIONS

Management of invasive plant species depends on a thorough understanding of the biology and ecology of the species within the invaded range. As knowledge about *L. microphyllum* (Cav.) R. Br. is gained, management efforts are adapted to best apply the new information (Enloe, 2015; Ferriter et al., 2001; Hutchinson et al., 2006). Extensive research on *L. microphyllum*'s growth habits and susceptibility to various stressors have helped inform management efforts, yet questions remained regarding the responses of individual plants to these management methods. One major question was whether fire is effective for *L. microphyllum* management (Hutchinson et al., 2006). The previous knowledge that *L. microphyllum* resprouts after fire is important, as prescribed fire is one of the main management strategies in use for this invasive species. But more importantly, what the response of individual plants is to burning, and what this response is in relation to co-occurring species, will enable burning schedules to be improved. Building on previous greenhouse work on burned *L. microphyllum* (Richards et al., 2020), I addressed some aspects of this question in Chapters 2-4.

The field experiment in Chapter 2 showed that burning in the dry season yielded higher rates of mortality and longer recovery times for surviving plants than burning in the wet season. Mortality was size-dependent and decreased with increased plant size. Additionally, sexual reproduction resumed later in dry-season burned plants than those burned in the wet season. Although many surviving plants eventually recovered to pre-burn size, overall cover was reduced because of high rates of mortality in smaller

individuals, suggesting that fire is especially effective in reducing early/smaller infestations. Unburned plants were stable in leaf height over the experiment, and this was largely the result of *L. microphyllum*'s growth habit, as its vertical reach completely depends on host height. When burning reduced trees or tall shrubs, thereby removing tall structures, *L. microphyllum* height was limited to the height of the host that regrew most quickly—here, sawgrass.

Burning is prescribed every few years to maintain the sawgrass marshes in ENP and is dependent on sufficient fuel buildup of persistent sawgrass leaves. Since the surviving *L. microphyllum* plants recovered to their pre-burn sizes in under two years, a 3+ year fire return interval may not be enough, alone, to suppress *L. microphyllum* growth/spread. However, through integrated pest management (IPM), combining fire and biological controls may be an efficient method to stymie the infestations while providing appropriate fire return intervals for the habitat. Although biocontrol mite populations in the field sites crashed with burning, they began rebounding within a year, suggesting that prescribed fire is not at odds with biocontrol methods, and has potential for combined use in fire-maintained habitats. Mites, in high enough densities, exert a crippling effect on *L. microphyllum* growth—especially new growth—(David and Lake, 2020), suggesting that the optimum combination of these treatments may be a matter of timing. Although the mites are wind-dispersed and began returning within months, planned releases could accelerate their return and maximize damage to regrowing plants. Alternatively, as mite populations continue to establish throughout the invaded regions, they may recolonize more quickly following subsequent fires, obviating the need for additional releases—an ideal outcome of integrated pest management.

In chapter 3, salinity tolerance of *L. microphyllum* was investigated, revealing some tolerance to brackish levels. After 2 months at 10 ppt, sporophytes had reduced growth rates, suggesting that growth in this level of salinity is not sustainable in the long term. This agrees with mortality observed in the field, some of which coincided with exposure to salinities between 8 and 13 ppt after Hurricane Irma. Similarly, spores germinated in brackish conditions, but at decreasing rates with increasing salinity, and germination was delayed for spores in all saline treatments compared to those in freshwater conditions. This suggests that although *L. microphyllum* can tolerate mildly brackish conditions for short periods of time, salinization events may thwart their survival, especially as other stressors like competition or increasing temperatures continue. Additionally, because plants were stressed and spore germination was greatly reduced in 10 ppt, and because no spores germinated in 15 ppt, it is unlikely *L. microphyllum* will be able to establish in areas with prolonged exposure to these higher salinities. Thus, management efforts may be better spent on infestations in freshwater to brackish areas.

These findings are relevant to other habitats, as moderate salinity seems to be a potentially limiting factor in *L. microphyllum* spread. Saline conditions can be stressful for *L. microphyllum* sporophytes, as was fire, but their interaction has not yet been investigated. If the combined stresses of burning and salinity could be applied in succession, this combination might be enough to suppress infestations in brackish areas. While spores were limited to 10 ppt for germination, a short exposure to higher salinity, followed by a period of freshwater conditions was not investigated. In some ferns, a period of saline stress can affect subsequent germination when the salinity is decreased

(Groot and During, 2013; Pangua et al., 2009). If *L. microphyllum* spores can tolerate a short exposure to saline stress, how does this affect either the rates of germination or the speed of gametophyte maturation?

In chapter 4, foliar nectar production was examined in six species of *Lygodium* and documented to occur in four of them. *Lygodium* nectar production was seen following ant exclusion and mechanical damage or jasmonic acid application. Initial ant presence predicted nectar production by species, with no nectar production observed on species that had little to no ant presence before ant exclusion and treatment. Foliar nectar was produced on the petiolule and pinna bud on all four nectar-producing species, but additional locations of nectar production differed among species, with the most pronounced patterns observed on *L. japonicum* and *L. microphyllum*. Observations of foliar nectar on *L. volubile* after ant exclusion (without damage) may suggest constitutive nectar production, although additional/continued production after both damage and jasmonic acid on this and the other three species may also indicate inducible nectar production. A fifth species, *L. venustum*, exuded a sugary wound exudate, which may be another defensive strategy, but this needs further study.

Presence of foliar nectar suggests a defensive role that may be relevant to the management of the two serious invasive species, *L. microphyllum* and *L. japonicum*, as nectar's attraction of ants (or other predatory insects) could reduce the efficacy of some biological control agents. This may apply to other invaded habitat types as well, particularly those with high ant diversity, as such habitats are more likely to harbor ants that will provide protective services. Mixed results have been reported for a previous biocontrol agent (Boughton and Pemberton, 2008) and the discovery of foliar nectar on *L.*

microphyllum may help explain some of the variation in biological control success following field releases. For the two invasive *Lygodium* species, foliar nectar production should be further investigated to determine whether it is constitutive or induced (or both), and whether this foliar nectar is successfully attracting defensive arthropods. Potential impacts on current and future biological control agents could be sizeable and should also be investigated.

This dissertation contributes to the knowledge of *L. microphyllum* biology and invasive ecology in the sawgrass marshes of southwestern Everglades National Park. With respect to prescribed burning, the high mortality of small *L. microphyllum* plants suggests that fire can be an effective management strategy, particularly in the integrated pest management framework.

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PUBLICATIONS AND PRESENTATIONS

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